CONVERSION OF PRENYL DERIVATIVES TO ISOPRENE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This applications claims priority to U.S. Provisional Patent Application 61/097,204, filed on September 15, 2008, the entire contents of which are hereby incorporated by reference in its entirety.

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. 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.

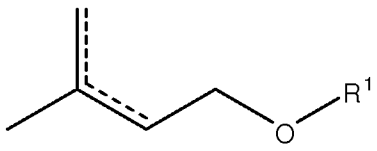
[0003] Currently, the tire and rubber industry is based on the use of natural and synthetic rubber. Natural rubber is obtained from the milky juice of rubber trees or plants found in the rainforests of Africa. Synthetic rubber is based primarily on butadiene polymers. For these polymers, butadiene is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

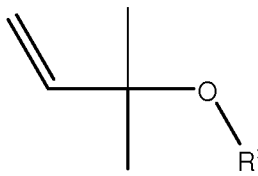
[0005] The present invention also provides, *inter alia*, compositions, cells, systems and methods of producing isoprene. In one aspect, the invention provides for methods of

producing isoprene comprising (a) culturing cells under suitable culture conditions for the production of a prenyl derivative, and wherein the cells comprise a heterologous nucleic acid that encodes an isoprene synthase polypeptide in operable combination with a promoter; (b) recovering said prenyl derivative; and (c) dehydrating or decarboxylating said prenyl derivative to produce isoprene. In some embodiments, the prenyl derivative is a compound of Formula (I):



(I);

wherein R^1 is hydrogen or $-C(O)R^2$; and R^2 is C_1-C_5 linear or branched alkyl. In some embodiments, the prenyl derivative is a compound of Formula (II):

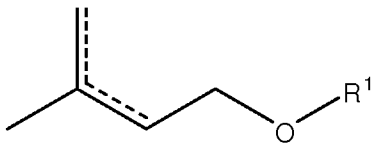


(II);

wherein R^3 is hydrogen or $-C(O)R^4$; and R^4 is C_1-C_5 linear or branched alkyl. In some embodiments, the prenyl derivative is a C5 unsaturated alcohol or a C_1-C_5 linear or branched acyl ester thereof. In some embodiments, the method comprises comprises contacting said prenyl derivative with a catalyst such that isoprene is produced by dehydration or decarboxylation of said prenyl derivative. In some embodiments, the catalyst is an inorganic acid catalyst (e.g. HCl, HBr, H_2SO_4, or H_3PO_4), a solid acid catalyst (e.g. activated alumina, a zeolite, or an inorganic acid on an inert carrier), an organic acid catalyst (e.g. p-toluenesulfonic acid or trifluoromethane sulfonic acid), or an organic acid resin (e.g. Nafion or other fluorosulfonic acid resin). In some embodiments, the catalyst is on a solid support. In some embodiments, the catalyst is in solution. In a preferred embodiment, the catalyst is an acidic salt solution. In some embodiments, the catalyst is optionally buffered with additional salts. In some embodiments, the prenyl derivative is recovered from fermentation off-gas of the cultured cells using a process selected from the group consisting of distillation, gas-stripping, two-phase recovery, and pervaporation. In some embodiments, the prenyl derivative is a prenyl alcohol. In some embodiments, the prenyl alcohol is converted to an acetyl ester prior to gas stripping. In some preferred embodiments, the prenyl alcohol comprises one or both of prenol and isoprenol. In some embodiments, the prenyl derivative is an acetyl ester. In some embodiments, the prenyl derivative is 3-methyl-3-buten-1-ol, 3-

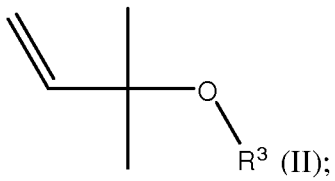
methyl-2-butene-1-ol, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, or 2-methyl-3-buten-2-ol. In some embodiments, the cells further comprise one or both of an IDI polypeptide and an MVA pathway enzyme.

[0006] In addition, the present invention provides methods of producing isoprene, the method comprising (a) culturing cells in culture medum under suitable culture conditions for the production of isoprene, wherein said culture medium comprises a prenyl derivative, and wherein said cells comprise a heterologous nucleic acid that encodes an isoprene synthase polypeptide in operable combination with a promoter; and (b) producing said isoprene. In some embodiments, the prenyl derivative is a compound of Formula (I):



(I);

wherein R^1 is hydrogen or $-C(O)R^2$; and R^2 is C_1-C_5 linear or branched alkyl. In some embodiments, the prenyl derivative is a compound of Formula (II):



(II);

wherein R^3 is hydrogen or $-C(O)R^4$; and R^4 is C_1-C_5 linear or branched alkyl. In some embodiments, the prenyl derivative is a C5 unsaturated alcohol or a C_1-C_5 linear or branched acyl ester thereof. In some embodiments, the cells are bacterial cells or yeast cells. In some embodiments, the cells are selected from the group consisting of *E. coli, P. citrea, B. subtilis, T. reesei, Y. lipolytica,* and *S. cerevisiae.* In some embodiments, the prenyl derivative is a prenyl alcohol. In some embodiments, the prenyl alcohol comprises one or both of prenol and isoprenol. In some embodiments, the prenyl derivative is an acetyl ester. In some embodiments, the prenyl derivative is 3-methyl-3-buten-1-ol, 3-methyl-2-butene-1-ol, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, or 2-methyl-3-buten-2-ol. In some embodiments, the cells further comprise one or both of an IDI polypeptide and an MVA pathway enzyme. In some embodiments, the cells further comprise prenol kinase activity. In some embodiments, the cells further comprise isoprene synthase.

[0007] 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 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 a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, alcohol, 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, component from a yeast extract, or any combination of two or more of the foregoing. In some embodiments, the cells are cultured under limited glucose conditions.

[0008] 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 a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, alcohol, 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, component from a yeast extract, or any combination of two or more of the foregoing. In some embodiments, the cells are cultured under limited glucose conditions.

[0009] 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 a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, alcohol, 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, component from a yeast extract, or any combination of two or more of the foregoing. In some embodiments, the cells are cultured under limited glucose conditions.

[0010] 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 a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, alcohol, 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, component from a yeast extract, or any combination of two or more of the foregoing. 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.

[0011] 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 a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, alcohol, 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, component from a yeast extract, or any combination of two

or more of the foregoing. In some embodiments, the cells are cultured under limited glucose conditions.

[0012] 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.

[0013] 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.

[0014] 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, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, 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) 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, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, 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) 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.

[0015] 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.

[0016] 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.

[0017] 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).

[0018] 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, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, 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) 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, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, 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) 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.

[0019] 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.

[0020] 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.

[0021] 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.

[0022] 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.

[0023] 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.

[0024] 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. In another aspect, the invention provides methods of manufacturing a tire wherein the improvement comprises using any one or more the compositions, cells, systems and/or methods described herein to produce isoprene for the manufacture of the tire.

[0025] 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.

[0026] 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.

[0027] 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.

[0028] 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.

[0029] 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.

[0030] 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.

[0031] 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).

[0032] 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*).

[0033] 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 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).

[0034] 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.

[0035] In one aspect, the invention features a product produced by any of the compositions or methods of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] 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.

[0037] Figure 2 is a map of pTrcKudzu.

[0038] Figure 3 is 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, capitol, italics letters. The vector backbone is pTrcHis2B.

[0039] Figure 4 is a map of pETNHisKudzu.

[0040] Figure 5 is the nucleotide sequence of pETNHisKudzu (SEQ ID NO:5).

[0041] Figure 6 is a map of pCL-lac-Kudzu.

[0042] Figure 7 is the nucleotide sequence of pCL-lac-Kudzu (SEQ ID NO:7).

[0043] Figure 8A is a graph showing the production of isoprene in *E. coli* BL21 cells with no vector.

[0044] Figure 8B is a graph showing the production of isoprene in *E. coli* BL21 cells with pCL-lac-Kudzu

[0045] Figure 8C is a graph showing the production of isoprene in *E. coli* BL21 cells with pTrcKudzu.

[0046] Figure 8D is a graph showing the production of isoprene in *E. coli* BL21 cells with pETN-HisKudzu.

[0047] Figure 9A is a graph showing OD over time of fermentation of *E. coli* BL21/pTrcKudzu in a 14 liter fed batch fermentation.

[0048] Figure 9B is a graph showing isoprene production over time of fermentation of *E. coli* BL21/pTrcKudzu in a 14 liter fed batch fermentation.

[0049] 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}.

[0050] 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}.

[0051] 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}.

[0052] 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-BG3594comK is a *B. subtilis* strain with pBSKudzu (recombinant isoprene production). IS on the y-axis indicates isoprene.

[0053] Figure 12 is the nucleotide sequence of pBS Kudzu #2 (SEQ ID NO:57).

[0054] Figure 13 is the nucleotide sequence of kudzu isoprene synthase codon-optimized for expression in *Yarrowia* (SEQ ID NO:8).

[0055] Figure 14 is a map of pTrex3g comprising a kudzu isoprene synthase gene codon-optimized for expression in *Yarrowia*.

[0056] Figure 15 is the nucleotide sequence of vector pSPZ1(MAP29Spb) (SEQ ID NO:11).

[0057] Figure 16 is the nucleotide sequence of the synthetic kudzu (*Pueraria montana*) isoprene gene codon-optimized for expression in *Yarrowia* (SEQ ID NO:12).

[0058] Figure 17 is the nucleotide sequence of the synthetic hybrid poplar (*Populus alba x Populus tremula*) isoprene synthase gene (SEQ ID NO:13). The ATG start codon is in bold and the stop codon is underlined.

[0059] Figure 18A shows a schematic outlining construction of vectors pYLA 1, pYL1 and pYL2.

[0060] Figure 18B shows a schematic outlining construction of the vector pYLA(POP1).

[0061] Figure 18C shows a schematic outlining construction of the vector pYLA(KZ1)

[0062] Figure 18D shows a schematic outlining construction of the vector pYLI(KZ1)

[0063] Figure 18E shows a schematic outlining construction of the vector pYLI(MAP29)

[0064] Figure 18F shows a schematic outlining construction of the vector pYLA(MAP29)

[0065] 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.

[0066] 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 fo the modified MVA pathway. The modified MVA pathway is present, for example, in some archaeal organisms, such as *Methanosarcina mazei.*

[0067] 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.

[0068] Figure 21 is a map of pTrcKudzu yIDI DXS Kan.

[0069] Figure 22 is the nucleotide sequence of pTrcKudzu yIDI DXS Kan (SEQ ID NO:20).

[0070] 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).

[0071] 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).

[0072] 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).

[0073] 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).

[0074] 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).

[0075] 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).

[0076] 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).

[0077] 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 inoculation; the y-axis is OD600 and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD600, triangles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0078] Figure 24 is a map of pTrcKKDyIkIS kan.

[0079] Figure 25 is a nucleotide sequence of pTrcKKDyIkIS kan (SEQ ID NO:33).

[0080] Figure 26 is a map of pCL PtrcUpperPathway.

[0081] Figures 27A-27D is a nucleotide sequence of pCL PtrcUpperPathway (SEQ ID NO:46).

[0082] 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.

[0083] Figure 29 is 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:47).

[0084] Figure 30 is a map of p9796-poplar.

[0085] Figure 31 is a nucleotide sequence of p9796-poplar (SEQ ID NO:48).

[0086] Figure 32 is a map of pTrcPoplar.

[0087] Figure 33 is a nucleotide sequence of pTrcPoplar (SEQ ID NO:49).

[0088] Figure 34 is a map of pTrcKudzu yIDI Kan.

[0089] Figure 35 is a nucleotide sequence of pTrcKudzu yIDI Kan (SEQ ID NO:50).

[0090] Figure 36 is a map of pTrcKudzuDXS Kan.

[0091] Figure 37 is a nucleotide sequence of pTrcKudzuDXS Kan (SEQ ID NO:51).

[0092] Figure 38 is a map of pCL PtrcKudzu.

[0093] Figure 39 is a nucleotide sequence of pCL PtrcKudzu (SEQ ID NO:52).

[0094] Figure 40 is a map of pCL PtrcKudzu A3.

[0095] Figure 41 is a nucleotide sequence of pCL PtrcKudzu A3 (SEQ ID NO:53).

[0096] Figure 42 is a map of pCL PtrcKudzu yIDI.

[0097] Figure 43 is a nucleotide sequence of pCL PtrcKudzu yIDI (SEQ ID NO:54).

[0098] Figure 44 is a map of pCL PtrcKudzu DXS.

[0099] Figure 45 is a nucleotide sequence of pCL PtrcKudzu DXS (SEQ ID NO:55).

[0100] Figure 46 shows 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.

[0101] 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).

[0102] 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).

[0103] 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).

[0104] 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).

[0105] Figure 48 shows 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.

[0106] Figure 49 shows 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.

[0107] Figure 50 is a map of pJMupperpathway2.

[0108] Figure 51 is the nucleotide sequence of pJMupperpathway2 (SEQ ID NO:56).

[0109] Figure 52 is a map of pBS Kudzu #2.

[0110] 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 *Bacillus* with pBSKudzu (recombinant isoprene production).

[0111] 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 *Bacillus* with pBSKudzu (recombinant isoprene production).

[0112] Figure 54 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0113] 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.

[0114] Figure 56 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0115] Figure 57 is a time course of optical density within the 15-L bioreactor fed with glycerol.

[0116] 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.

[0117] Figure 59 is a time course of total isoprene produced from the 15-L bioreactor fed with glycerol.

[0118] Figures 60A-60C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 150-L bioreactor fed with glucose.

[0119] Figures 61A-61C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0120] Figures 62A-62C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0121] Figure 63A-63C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0122] Figures 64A-64C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0123] Figures 65A-65C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0124] Figures 66A-66C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0125] Figure 67A-67C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0126] 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.

[0127] 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.

[0128] 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.

[0129] 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.

[0130] 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.

[0131] 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.

[0132] 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.

[0133] Figure 75A is a table of the conversion of the CAFT Model results from weight percent to volume percent for series A.

[0134] Figure 75B is a graph of the flammability results from the CAFT model for Series A in Figure 68 plotted as volume percent.

[0135] Figure 76A is a table of the conversion of the CAFT Model results from weight percent to volume percent for series B.

[0136] Figure 76B is a graph of the flammability results from the CAFT model for Series B in Figure 69 plotted as volume percent.

[0137] Figure 77 is a figure of the flammability test vessel.

[0138] Figure 78A is a graph of the flammability Curve for Test Series 1: 0% Steam, 0 psig, and 40°C.

[0139] Figure 78B is a table summarizing the explosion and non-explosion data points for Test Series 1.

[0140] Figure 78C is a graph of the flammability curve for Test Series 1 compared with the CAFT Model.

[0141] Figure 79A is a graph of the flammability curve for Test Series 2: 4% Steam, 0 psig, and 40°C.

[0142] Figure 79B is a table summarizing the explosion and non-explosion data points for Test Series 2.

[0143] Figure 79C is a graph of the flammability curve for Test Series 2 compared with the CAFT Model.

[0144] Figures 80A and 80B are a table of the detailed experimental conditions and results for Test Series 1.

[0145] Figure 81 is a table of the detailed experimental conditions and results for Test Series 2.

[0146] 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.

[0147] 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.

[0148] Figure 84 is a graph of the flammability envelope constructed using data from Figure 82 and following the methodology described in Example 12. The experimental data points (circles) are from tests described herein that were conducted at 1 atmosphere initial system pressure.

[0149] Figure 85 is a graph of the flammability envelope constructed using data from Figure 83 and following the methodology described in Example 12. The experimental data

points (circles) are from tests described herein that were conducted at 1 atmosphere initial system pressure.

[0150] Figure 86A is a GC/MS chromatogram of fermentation off-gas.

[0151] Figure 86B is an expansion of Fig 86A to show minor volatiles present in fermentation off-gas.

[0152] Figure 87A is a GC/MS chromatogram of trace volatiles present in off-gas following cryo-trapping at -78 °C.

[0153] Figure 87B is a GC/MS chromatogram of trace volatiles present in off-gas following cryo-trapping at -196 °C.

[0154] Figure 87C is an expansion of Fig. 87B.

[0155] Figure 87D is an expansion of Fig. 87C.

[0156] Figures 88A and 88B are GC/MS chromatogram comparing C5 hydrocarbons from petroleum-derived isoprene (Fig. 88A) and biologically produced isoprene (Fig. 88B). The standard contains three C5 hydrocarbon impurities eluting around the main isoprene peak (Fig. 88A). In contrast, biologically produced isoprene contains amounts of ethanol and acetone (run time of 3.41 minutes) (Fig. 88A).

[0157] 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.

[0158] Figure 90 shows the structures of several impurities that are structurally similar to isoprene and may also act as polymerization catalyst poisons.

[0159] Figure 91 is a map of pTrcHis2AUpperPathway (also called pTrcUpperMVA).

[0160] Figures 92A-92C are the nucleotide sequence of pTrcHis2AUpperPathway (also called pTrcUpperMVA) (SEQ ID NO:86).

[0161] Figure 93 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0162] 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.

[0163] Figure 95 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0164] Figure 96 is a time course of optical density within the 15-L bioreactor fed with invert sugar.

[0165] 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.

[0166] Figure 98 is a time course of total isoprene produced from the 15-L bioreactor fed with invert sugar.

[0167] Figure 99 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0168] 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.

[0169] Figure 101 is a time course of isoprene specific activity from the 15-L bioreactor fed with glucose.

[0170] Figure 102 is a map of pCLPtrcUpperPathwayHGS2 (also referred to as pCL UpperHGS2).

[0171] Figures 103A-103C are the nucleotide sequence of pCLPtrcUpperPathwayHGS2 (SEQ ID NO:87).

[0172] Figure 104 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0173] 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.

[0174] Figure 106 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0175] Figure 107 is a map of plasmid MCM330.

[0176] Figures 108A-108C are the nucleotide sequence of plasmid MCM330 (SEQ ID NO:90).

[0177] Figure 109 is a map of pET24D-Kudzu.

[0178] Figures 110A and 110B are the nucleotide sequence of pET24D-Kudzu (SEQ ID NO:101).

[0179] Figure 111A is a time course of optical density within the 15-L bioreactor fed with glucose. 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. Figure 111C is a time course of specific productivity of isoprene in the 15-L bioreactor fed with glucose.

[0180] Figure 112A provides a map of the *M. mazei* archeal lower pathway operon. Figures 112B and 112C provide the nucleotide sequence of the *M. mazei* archeal lower pathway operon (SEQ ID NO:102).

[0181] Figure 113A provides a map of MCM382 – pTrcKudzuMVK(mazei). Figures 113B and 113C provide the nucleotide sequence of MCM382 – pTrcKudzuMVK(mazei) (SEQ ID NO:103).

[0182] Figure 114A provides a map of MCM376 - MVK from *M. mazei* archeal Lowerin pET200D. Figures 114B and 114C provide the nucleotide sequence of MCM376 - MVK from *M. mazei* archeal Lowerin pET200D (SEQ ID NO:104).

[0183] Figure 115A provides a time course of optical density within the 15-L bioreactor fed with glucose. Figure 115B provides a time course of isoprene titer within the 15-L bioreactor fed with glucose, in which titer is defined as the amount of isoprene produced per

liter of fermentation broth. Figure 115C provides a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0184] Figure 116 provides a graph showing the production of isoprene from dimethyl allyl alcohol by recombinant *E. coli* engineered to express kudzu isoprene synthase.

[0185] Figure 117A provides a map of plasmid pDW14. Figure 117B provides the nucleotide sequence of kudzu isoprene synthase that has been codon-optimized for expression in yeast (SEQ ID NO:112). Figures 117C and 117D provide the nucleotide sequence of plasmid pDW14 (SEQ ID NO:113).

[0186] Figures 118A and 118B provide an SDS-PAGE gel and a western blot respectively, demonstrating production of isoprene synthase by recombinant yeast. A 4-12% bis tris gel (Novex, Invitrogen) of lysates obtained from yeast cell strains harboring pDW14 or pYES-DEST52 prepared after induction with galactose was stained with SimplyBlue SafeStain (Invitrogen). The western blot was prepared using the WesternBreeze kit (Invitrogen). Lanes are as follows: 1) INVSc-1 + pYES-DEST52; 2) INVSc-1 + pDW14 (isolate 1); and 3) INVSc-1 + pDW14 (isolate 2). The MW (kDa) of the SeeBlue Plus 2 molecular weight standards is shown.

[0187] Figures 119A and 119B provide graphs showing the growth of galactose-induced INVSc-1 yeast cells harboring pDW14 or pYES-DEST52and their production of isoprene in the headspace as determined by the DMAPP assay, respectively. Specific activity was calculated as ug isoprene/L/OD. Samples are as follows: Control, INVSc-1 + pYES-DEST52; HGS-1, INVSc-1 + pDW14 (isolate 1); and HGS-2, INVSc-1 + pDW14 (isolate 2).

[0188] Figure 120 provides a graph showing the production of isoprene from dimethyl allyl alcohol by recombinant *S. cerevisiae* engineered to express kudzu isoprene synthase.

DETAILED DESCRIPTION OF THE INVENTION

[0189] In one aspect, the invention features compositions and methods for the production of isoprene in increased amounts and/or purity. 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.

[0190] 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-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne (Fig. 90) and 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). 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-1-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.

[0191] 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.

[0192] 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)
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 (ug/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 pathway	3815	3044	992.5 (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 100 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).

[0193] 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.

[0194] 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.

[0195] 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).

[0196] 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.

[0197] In some embodiments, the production of isoprene by cells 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.

[0198] For example, *E. coli* cells containing a nucleic acid encoding a kudzu isoprene synthase polypeptide and nucleic acids encoding *Saccharomyces cerevisia* 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).

[0199] 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.

[0200] 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.

[0201] 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.

[0202] 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.

[0203] 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 a lot 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.

[0204] 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(λDE3), BL21(λDE3) 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

[0205] Various isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids can be used in the compositions and methods of the invention.

[0206] As used herein, "polypeptides" includes polypeptides, proteins, peptides, fragments of polypeptides, and fusion polypeptides. In some embodiments, the fusion polypeptide includes part or all of a first polypeptide (*e.g.*, an isoprene synthase, DXS, IDI, or MVA pathway 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.

[0207] 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, or MVA pathway 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, or MVA pathway polypeptide.

[0208] In some embodiments, the polypeptide is an isolated polypeptide. 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.

[0209] In some embodiments, the polypeptide is a heterologous polypeptide. 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 nucleic acid that is found in the same host cell in nature.

[0210] As used herein, a "nucleic acid" refers to two or more deoxyribonucleotides and/or ribonucleotides in either single or double-stranded form. In some embodiments, the nucleic acid is a recombinant nucleic acid. By "recombinant nucleic acid" means 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. In various embodiments, a nucleic acid is a recombinant nucleic acid. In some embodiments, an isoprene synthase, DXS, IDI, or MVA pathway 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, or MVA pathway 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. It is to be understood that mutations, including single nucleotide mutations, can occur within a nucleic acid as defined herein.

[0211] In some embodiments, the nucleic acid is a heterologous nucleic acid. 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.

[0212] In particular embodiments the nucleic acid includes a segment of or the entire nucleic acid sequence of any naturally-occurring isoprene synthase, DXS, IDI, or MVA pathway 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, or MVA pathway 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, or MVA pathway 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, or MVA pathway nucleic acid. In some embodiments, the nucleic acid is a degenerate variant of any nucleic acid encoding an isoprene synthase, DXS, IDI, or MVA pathway polypeptide.

[0213] "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.

[0214] 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

[0215] 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 -80°C.

[0216] 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.

[0217] 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.

[0218] 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

[0219] 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

[0220] 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

[0221] 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.

[0222] 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*.

[0223] 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*.

[0224] 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*.

[0225] 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*.

[0226] 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*.

[0227] 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*.

[0228] 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*.

[0229] 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*.

[0230] Exemplary IDI polypeptides and nucleic acids are described above.

Exemplary Methods for Isolating Nucleic Acids

[0231] Isoprene synthase, DXS, IDI, and/or MVA pathway 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.

[0232] Alternatively, isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acids (such as any isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acids with a known nucleic acid sequence) can be chemically synthesized using standard methods.

[0233] Additional isoprene synthase, DXS, IDI, or MVA pathway 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.

[0234] 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.

[0235] Additional methods for obtaining isoprene synthase, DXS, IDI, and/or MVA pathway 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, and/or MVA pathway 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).

[0236] Additionally, standard sequence alignment and/or structure prediction programs can be used to identify additional DXS, IDI, or MVA pathway polypeptides and nucleic acids based on the similarity of their primary and/or predicted polypeptide secondary structure with that of known DXS, IDI, or MVA pathway 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, or MVA pathway polypeptides and nucleic acids. The secondary and/or tertiary structure of an isoprene synthase, DXS, IDI, or MVA pathway 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, or MVA pathway polypeptide can be determined

using standard methods. Additional isoprene synthase, DXS, IDI, or MVA pathway nucleic acids can also be identified by hybridization to probes generated from known isoprene synthase, DXS, IDI, or MVA pathway nucleic acids.

Exemplary Promoters and Vectors

[0237] Any of the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid 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, or MVA pathway polypeptides that are described herein. 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. In some embodiments, the vector contains a nucleic acid under the control of an expression control sequence.

[0238] 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.

[0239] In some embodiments, the vector contains a selective marker. The term "selective 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*. 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, or MVA pathway nucleic acid integrates into a chromosome of the cells without a selective marker.

[0240] 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).

[0241] 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, or MVA pathway nucleic acid in the host cell. Initiation control regions or promoters, which are useful to drive expression of isoprene synthase, DXS, IDI, or MVA pathway 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*).

[0242] 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).

[0243] In various embodiments, an isoprene synthase, DXS, IDI, and/or MVA pathway 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, or MVA pathway nucleic acid is operably linked to a T7 promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway 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, or MVA pathway nucleic acid is operably linked to a Trc promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway 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, or MVA pathway nucleic acid is operably linked to a Lac promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway 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, or MVA pathway 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, or MVA pathway promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway 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.

[0244] 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.

[0245] In some embodiments, an isoprene synthase, DXS, IDI, or MVA pathway 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).

[0246] 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.

[0247] In some embodiments, the promoter, coding, region, and terminator all originate from the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid to be expressed. In some embodiments, the coding region for an isoprene synthase, DXS, IDI, or MVA pathway 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.

[0248] An isoprene synthase, DXS, IDI, or MVA pathway 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, or MVA pathway 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, or MVA pathway nucleic acid and the vector. Then, the compatible ends of the cleaved isoprene synthase, DXS, IDI, or MVA pathway 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).

[0249] In some embodiments, it may be desirable to over-express isoprene synthase, DXS, IDI, or MVA pathway 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.

[0250] 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

[0251] Isoprene synthase, DXS, IDI, or MVA pathway nucleic acids (and their encoded polypeptides) can be obtained from any organism that naturally contains isoprene synthase, DXS, IDI, and/or MVA pathway 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.

[0252] In some embodiments, the nucleic acid sequence of the isoprene synthase, DXS, IDI, or MVA pathway 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, or MVA pathway 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, or MVA pathway 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.

[0253] 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.*

[0254] 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.

[0255] In some embodiments, the source organism is a yeast, such as *Saccharomyces sp., Schizosaccharomyces sp., Pichia sp.,* or *Candida sp.*

[0256] 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,* strains of *Streptomyces* such as *S. lividans* or *S. rubiginosus,* or strains of *Escherichia* such as *E. coli.*

[0257] 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.*

[0258] 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* or *Pseudomonas sp.*

[0259] 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*.

[0260] In some embodiments, the source organism is an algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates.

[0261] 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

[0262] A variety of host cells can be used to express isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and to produce isoprene 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.

Exemplary Transformation Methods

[0263] Isoprene synthase, DXS, IDI, and/or MVA pathway 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, and/or MVA pathway 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.

[0264] 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.

[0265] 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.

[0266] 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.

[0267] 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).

[0268] 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.

[0269] 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

[0270] The invention also includes a cell or a population of cells in culture that produce isoprene. By "cells in culture" is meant two or more cells in a solution (*e.g.*, a cell 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.

[0271] 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.

[0272] In some embodiments, the carbon source is a carbohydrate (such as monosaccharide, disaccharide, oligosaccharide, or polysaccharids), 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.

[0273] 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 a carbon source 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.

[0274] 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.

[0275] 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.

[0276] 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.

[0277] Exemplary lipids are any substance containing one or more fatty acids that are C4 and above fatty acids that are saturated, unsaturated, or branched.

[0278] 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.

[0279] 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, palmitelaidic 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 triglycerols are fatty acid esters of glycerol.

[0280] 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.

[0281] 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.

[0282] 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.

[0283] 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.

[0284] 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.

[0285] 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.

[0286] 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).

[0287] 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.

[0288] 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).

[0289] 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 media). 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.

[0290] 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

[0291] 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.

[0292] 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 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 desired amount of isoprene 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. 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.

[0293] 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.

[0294] 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.

[0295] 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.

[0296] 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.

[0297] In some embodiments, cells are immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isoprene production.

[0298] 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.

[0299] 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).

[0300] 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

[0301] 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.

[0302] 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.

[0303] 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}.

[0304] 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%.

[0305] 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.

[0306] 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.

[0307] *Production of Isoprene within Safe Operating Ranges*

[0308] 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).

[0309] 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.

[0310] 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

[0311] 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.

[0312] 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.

[0313] 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.

[0314] 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.

[0315] 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).

[0316] 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.

[0317] 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.

[0318] 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.%).

[0319] 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.

[0320] Exemplary Production of Isoprene

[0321] In some embodiments, the cells are cultured in a culture medium under conditions permitting the production of isoprene by the cells. 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.

[0322] 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 specific productivity is determined by dividing the total productivity by the amount of cells, as determined by optical density at 600nm (OD600). 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.

[0323] 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.

[0324] 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.

[0325] 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.

[0326] 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.

[0327] 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).

[0328] 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}.

[0329] 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.

[0330] 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}.

[0331] 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%.

[0332] 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

[0333] 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%

[0334] 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.

[0335] 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)

[0336] 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

[0337] If desired, Equation 11 can be used to convert between units of ppm and ug/L. In particular, "ppm" means parts per million defined in terms of ug/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 uL/L (vol/vol). Conversion of ug/L to ppm (*e.g.*, ug 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 (ug/g) equals 1.29 ug/L at STP (equation 11). The conversion of ppm (ug/g) to ug/L is a function of pressure, temperature, and overall composition of the off-gas.

Equation 11

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

[0338] 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

[0339] 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.

[0340] 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.

[0341] Equation 13

[0342] 1 $ug/L = 1\ mg/m^3$

[0343] 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.

[0344] 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.

[0345] 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.

[0346] 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. .

[0347] 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).

[0348] 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 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 dimmers (*e.g.*, cyclic C10 compounds derived from the dimerization of two isoprene units).

[0349] 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.

[0350] 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).

[0351] In some embodiments, the isoprene composition includes one or more of the following: an alcohol, an aldehyde, or a ketone (such as any of the alcohols, aldehyes, 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.

[0352] 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).

[0353] 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.

[0354] 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.

[0355] 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 Isoprene Purification Methods

[0356] In some embodiments, any of the methods described herein further include recovering the isoprene. For example, the isoprene 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 and 4,570,029, which are each hereby incorporated by reference in their entireties, particularly with respect to isoprene recovery and purification methods). 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.

[0357] 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.

[0358] 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

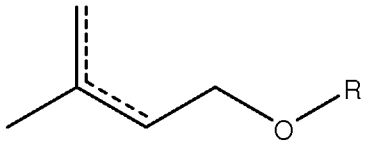
various embodiments, the isoprene is at least or about 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, or 99%, by weight, pure. Purity can be assayed by any appropriate method, *e.g.*, by column chromatography, HPLC analysis, or GC-MS analysis.

[0359] 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.

[0360] 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.

Exemplary Methods for Recovery of Prenyl Derivatives

[0361] In some embodiments, the methods of the present invention include recovering the prenyl derivatives of Formula (I):



(I);

wherein R is $-C(O)R^1$; and

R^1 is C_1-C_5 linear or branched alkyl;

from fermentation off-gas of recombinant host cells. In some embodiments, the prenyl derivative is a prenyl alcohol or acetyl ester thereof. Production of prenyl alcohols is achieved by the methods and compositions of Example 10. However, the compositions and methods of the present invention are not limited to the use of this particular cell line or prenyl derivative. Other suitable compositions and methods for producing prenyl alcohols are provided by U.S. Application No. 61/134,094, filed July 2, 2008 (Attorney Docket No. 24625-30003200, now 64384-3000400) herein incorporated by reference in its entirety. Additional compositions and methods for producing prenyl alcohols are provided by U.S. Patent No. 6, 689,593, and EP 1 354 956.

[0362] Prenyl derivatives, such as prenyl alcohols, may be removed from the fermentation broth by a number of techniques well known in the art including but not limited to distillation,

gas-stripping, two-phase recovery, and pervaporation. A description and comparison of several of these techniques as applied to butanol recovery from fermentations are provided in Groot et al., *Process Biochemistry,* 27:61-75 (1992). As used herein the term "prenyl alcohol" refers to alcohols derived from C5 isoprenyl units, including C5, C10, C15 and higher alcohols. The term "C5 prenyl alcohol" refers to the unsaturated C5 alcohols 3-methyl-2-buten-1-ol (prenol, DMAPP-ol) and 3-methyl-3-buten-1-ol (isoprenol, IPP-ol). The term "prenol" refers specifically to 3-methyl-2-buten-1-ol, also refered to a 3,3-dimethylallyl alcohol or DMAPP-ol, while the term "isoprenol" refers specifically to 3-methyl-3-buten-1-ol, also refered to as IPP-ol.

[0363] Distillation may be used to purify the prenyl derivatives, such as prenyl alcohols, from the fermentation broth. An entrainer may be optionally added to the distillation to enhance separation of the desired product from the bulk medium and may include benzene, toluene, cyclohexane, iso-octane, pentane, carbon tetrachloride, trichloroethylene, diethyl ether, 1-butanol, ethyl acetate, and other organic solvents or mixtures of solvents. The term "entrainer" refers to a component that is added to a distillation mixture to modify the separation characteristics of the material to be separated from the bulk medium. A successful entrainer may form one or more binary and/or ternary azeotropes with water and may have a very low solubility with water. The term "azeotrope" refers to a liquid mixture that is characterized by a constant minimum or maximum boiling point that is lower or higher than that of any of the pure components and that distills without change in composition. Distillation is generally described in Doherty and Malone, *Conceptual Design of Distillation Systems,* McGraw-Hill, New York (2001).

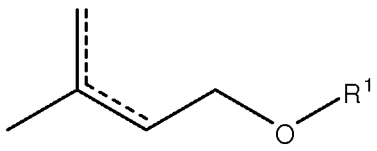
[0364] Gas stripping removes volatile compounds from fermentation broth by passing a flow of stripping gas through the fermentor culture or through an external stripping column to form an enriched stripping gas. Examples of stripping gases include helium, argon, carbon dioxide, hydrogen, nitrogen, or mixture of such gases in any desired ratio. The desired product may be condensed from the enriched stripping gas, and the stripping gas may be recycled. The flow rate of the gas may be dependent on such factors such as configuration of the system, volatility of the desired product, and solvent concentration in the fermentor. The enriched stripping gas stream may also be further treated with distillation to produce the product in the desired purity.

[0365] Two-phase partitioning bioreactors have been developed to separate hydrophobic products or reactants from growing cultures in the aqueous phase. Generally, an organic solvent such as dodecane is added to the culture medium and is present during fermentation. The solubility of the organic solvent is preferably low, so as to not adversely affect the microorganisms in the fermentation broth. The hydrophobic product extracts into the organic layer during fermentation which is attractive in systems where the product inhibits further production. The organic layer may be separated from the fermentation broth and the product may be isolated by distillation or evaporation of the organic solvent. Alternatively, an ionic liquid may be used in place of the organic solvent. Use of such bioreactors is described in Newman et al., *Biotech and Bioeng,* 95:684-691 (2006).

[0366] Pervaporation refers to a process in which a mixture of miscible components is placed in contact with one side of a membrane. A concentration gradient is created by applying a vacuum use or purge gas on the other side of the membrane. The components permeate through the membrane and evaporate into the vapor phase. The vapor or "permeate" is then condensed and the desired product collected. Different species in the fermentation broth will have different affinities for the membrane and diffusion rates through the membrane; therefore, components at low concentration in the feed may be highly enriched in the permeate. The membrane may be a non-porous polymeric membrane or a molecularly porous inorganic membrane. A review of the technique is provided in Vane, *J Chem Technol Biotechnol,* 80:603-629 (2005).

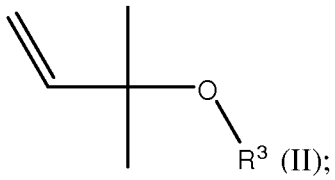
Exemplary Chemical Methods for Conversion of Prenyl Derivatives to Isoprene

[0367] In some embodiments, the methods of the present invention further include chemically synthesizing isoprene from biologically-obtained prenyl derivatives. In some embodiments, the prenyl derivative is a compound of Formula (I):



(I);

wherein R^1 is hydrogen or $-C(O)R^2$; and R^2 is C_1-C_5 linear or branched alkyl. In some embodiments, the prenyl derivative is a compound of Formula (II):



(II);

wherein R^3 is hydrogen or $-C(O)R^4$, and R^4 is C_1-C_5 linear or branched alkyl. In somce embodiements, the prenyl derivative is a C5 unsaturated alcohol or a C_1-C_5 linear or branched acyl ester thereof. In some embodiments, the prenyl derivative is a prenyl alcohol (e.g., 3-methyl-3-buten-1-ol, CAS Registry No. 763-32-6, isoprenol; and 3-methyl-2-buten-1-ol, CAS Registry No. 556-82-1, prenol). In some embodiments, the prenyl derivative is an acyl ester (e.g. 3-methyl-3-buten-1-yl acetate and 3-methyl-2-buten-1-yl acetate). In some embodiments, the prenyl derivative is 2-methyl-3-buten-2-ol. In some embodiments, chemical synthesis of isoprene from one or more biologically-obtained prenyl derivatives is achieved by use of a catalyst. In some embodiments, the catalyst is an inorganic acid catalyst (e.g. HCl, HBr, H_2SO_4, or H_3PO_4), a solid acid catalyst (e.g. activated alumina, a zeolite, or an inorganic acid on an inert carrier), an organic acid catalyst (e.g. p-toluenesulfonic acid or trifluoromethane sulfonic acid), or an organic acid resin (e.g. Nafion or other fluorosulfonic acid resin). In some embodiments, the catalyst is on a solid support. In some embodiments, the catalyst is in solution. In a preferred embodiment, the catalyst is an acidic salt solution. In some embodiments, the catalyst is optionally buffered with additional salts.

[0368] For instance, production of isoprene from isoprenol is accomplished by dehydration of isoprenol in an acidic salt (e.g., HCl/NaCl) solution as known in the art (Weitz and Loser, "Isoprene," in Ullmann's Encyclopedia of Industrial Chemistry, 7th edition, electronic release, Wiley-VCH Verlag GMBH, Weinheim, pp. 1-20, 2005; and U.S. Patent No. 3,792,104 to Mueller). Briefly, a solution containing sodium chloride, water and hydrochloric acid is placed in a reaction (e.g., dehydration) vessel equipped with a reflux condenser (operated at 45°C), which is connected to a descending condenser (operated at -10°C). The descending condenser causes condensation of the isoprene product and the isopentenols. The prenyl alcohol(s) are metered to the stirred dehydration vessel (heated at 95°C) to produce a condensate comprising isoprene. The aqueous phase is continuously recycled to the dehydration vessel, while the organic phase is neutralized with a concentrated aqueous sodium carbonate solution and separated to pure isoprene and isopentonols in a continuous fractionating column. The aqueous phase is removed from the dehydrator at intervals of one hour to keep the liquid at its original level. Common salt and hydrochloric acid are

replenished. At one hour intervals, the higher-boiling oligomers are removed from the dehydrator.

Exemplary Biological Methods for Conversion of Prenyl Derivatives to Isoprene

[0369] In some embodiments, the methods of the present invention further include producing isoprene from prenyl derivatives, such as prenyl alcohols, of petrochemical or biological origin by feeding the prenyl derivatives to host cells having isoprene synthase and prenol kinase activity. The prenyl derivatives may be as defined in any of the embodiments herein described. In some embodiments, the prenol kinase activity results in an increase in isoprene production by at least about 10, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100% compared to host cells lacking prenol kinase activity. In some embodiments, the host cells are capable of converting a prenyl derivative, such as a prenyl alcohol, to a diphosphate derivative (e.g. IPP or DMAPP) in one or more sequential steps. In some particularly preferred embodiments, the cells further comprise one or both of an IDI polypeptide and an MVA pathway enzyme. In some embodiments, the IDI polypeptide is overexpressed. In some embodiments the host cells are recombinant host cells comprising a heterologous isoprene synthase enzyme. In further embodiments, the host cells overexpress an enzyme having prenol kinase activity. In some embodiments, the host cells comprise prenol kinase activity, an IDI polypeptide, and an isoprene synthase. In some embodiments, the host cells are capable of converting a prenyl derivative, such as a prenyl alcohol, to a diphosphate derivative (e.g. IPP or DMAPP), optionally converting IPP to DMAPP, and converting DMAPP to isoprene in one or more sequential steps. Suitable host cells include but are not limited to bacterial cells and yeast cells.

[0370] In one embodiment, the host cells are recombinant *E. coli* that express heterologous integrated lower and upper MVA pathway enzymes as well as a plant isoprene synthase. Briefly, the bacterial host cells are grown in culture medium that is supplemented with dimethyl allyl alcohol. Isoprene produced by the recombinant bacteria is recoverable from the fermentation off-gas (See, Fig. 116).

[0371] In another embodiment, the host cells are recombinant *S. cerevisiae* that express a plant isoprene synthase. Briefly, the yeast host cells are grown in culture medium containing raffinose and galactose, as well as dimethyl allyl alcohol. Isoprene produced by the recombinant yeast is recoverable from the fermentation off-gas (See, Fig. 120).

[0372] In another embodiment, the methods of the present invention further include producing isoprene from prenyl derivatives, such as prenyl alcohols, of biological origin, wherein host cells having isoprene synthase and prenol kinase activity a) release a prenyl derivative; b) reuptake the released prenyl derivative, and c) produce isoprene. The prenyl derivatives may be as defined in any of the embodiments herein described. In some embodiments, the prenol kinase activity results in an increase in isoprene production by at least about 10, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100% compared to host cells lacking prenol kinase activity. In some embodiments, the host cells are capable of converting a prenyl derivative, such as a prenyl alcohol, to a diphosphate derivative (e.g. IPP or DMAPP). In some particularly preferred embodiments, the cells further comprise one or both of an IDI polypeptide and an MVA pathway enzyme. In some embodiments, the IDI polypeptide is overexpressed. In some embodiments the host cells are recombinant host cells comprising a heterologous isoprene synthase enzyme. In further embodiments, the host cells overexpress an enzyme having prenol kinase activity. In some embodiments, the host cells comprise prenol kinase activity, an IDI polypeptide, and an isoprene synthase. Suitable host cells include but are not limited to bacterial cells and yeast cells.

EXAMPLES

[0373] 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. 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 *Escherichia coli* Expressing Recombinant Kudzu Isoprene Synthase

I. Construction of vectors for expression of the kudzu isoprene synthase in *E. coli*

[0374] The protein sequence for the kudzu (*Pueraria montana*) isoprene synthase gene (IspS) is obtained from GenBank (AAQ84170). A kudzu isoprene synthase gene, optimized for *E. coli* codon usage, is purchased from DNA2.0 (SEQ ID NO:1). The isoprene synthase gene is 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 is designed such that the stop codon in the isoprene synthase gene 5' to the *Pst*I site. As a result, when the construct is expressed the His-Tag is not attached to the isoprene synthase protein. The resulting plasmid, pTrcKudzu, is verified by sequencing (Figures 2 and 3).

[0375] The isoprene synthase gene is also cloned into pET16b (Novagen). In this case, the isoprene synthase gene is inserted into pET16b such that the recombinant isoprene synthase protein contains the N-terminal His tag. The isoprene synthase gene is amplified from pTrcKudzu by PCR using the primer set pET-His-Kudzu-2F: 5'-CGTGAGATCATATGTGTGCGACCTCTTCTCAATTTAC (SEQ ID NO:3) and pET-His-Kudzu-R: 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:4). These primers add 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, is used as template DNA, Herculase polymerase (Stratagene) iss used according to manufacture's directions, and primers are added at a concentration of 10 pMols. The PCR is carried out in a total volume of 25 µl. The PCR product is digested with *Nde*I/*Bam*H1 and cloned into pET16b digested with the same enzymes. The ligation mix is transformed into *E. coli* Top10 (Invitrogen) and the correct clone selected by sequencing. The resulting plasmid, in which the kudzu isoprene synthase gene is expressed from the T7 promoter, is designated pETNHisKudzu (Figures 4 and 5).

[0376] The kudzu isoprene synthase gene is also cloned into the low copy number plasmid pCL1920. Primers are used to amplify the kudzu isoprene synthase gene from pTrcKudzu described above. The forward primer adds a *Hin*dIII site and an *E. coli* consensus RBS to the 5' end. The *Pst*I cloning site is already present in pTrcKudzu just 3' of the stop codon so the

reverse primer is constructed such that the final PCR product includes the *Pst*I site. The sequences of the primers are: HindIII-rbs-Kudzu F: 5'-CATATGAAAGCTTGTATCGATTAAATAAGGAGGAATAAACC (SEQ ID NO:6) and BamH1-Kudzu R:

[0377] 5'- CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:4). The PCR product is amplified using Herculase polymerase with primers at a concentration of 10 pmol and with 1 ng of template DNA (pTrcKudzu). The amplification protocol includes 30 cycles of (95° C for 1 minute, 60° C for 1 minute, 72° C for 2 minutes). The product is digested with *Hin*dIII and *Pst*I and ligated into pCL1920 which has also been digested with *Hin*dIII and *Pst*I. The ligation mix is transformed into *E. coli* Top10. Several transformants are checked by sequencing. The resulting plasmid is designated pCL-lac-Kudzu (Figures 6 and 7).

II. Determination of isoprene production

[0378] For the shake flask cultures, one ml of a culture is transferred from shake flasks to 20 ml CTC headspace vials (Agilent vial cat# 5188 2753; cap cat# 5188 2759). The cap is screwed on tightly and the vials incubated at the equivalent temperature with shaking at 250 rpm. After 30 minutes the vials are removed from the incubator and analyzed as described below (*see* Table 1 for some experimental values from this assay).

[0379] In cases where isoprene production in fermentors is determined, samples are taken from the off-gas of the fermentor and analyzed directly as described below (*see* Table 2 for some experimental values from this assay).

[0380] The analysis is 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) is used for separation of analytes. The sampler is set up to inject 500 μL of headspace gas. The GC/MS method utilizes helium as the carrier gas at a flow of 1 ml/min. The injection port is held at 250° C with a split ratio of 50:1. The oven temperature is held at 37° C for the 2 minute duration of the analysis. The Agilent 5793N mass selective detector is run in single ion monitoring (SIM) mode on m/z 67. The detector is 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) is observed to elute at 1.78 minutes. A calibration table is used to quantify the

absolute amount of isoprene and is found to be linear from 1 μg/L to 2000 μg/L. The limit of detection is 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

[0381] The vectors described above are introduced to *E. coli* strain BL21 (Novagen) to produce strains BL21/ptrcKudzu, BL21/pCL-lac-Kudzu and BL21/pETHisKudzu. The strains are spread for isolation onto LA (Luria agar) + carbenicillin (50 μg/ml) and incubated overnight at 37° C. Single colonies are inoculated into 250 ml baffled shake flasks containing 20 ml Luria Bertani broth (LB) and carbenicillin (100 μg/ml). Cultures are grown overnight at 20° C with shaking at 200 rpm. The OD_{600} of the overnight cultures are measured and the cultures are 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 is incubated at 30° C with shaking at 200 rpm. When the OD_{600} ~ 0.5 - 0.8, 400 μM IPTG is added and the cells are incubated for a further 6 hours at 30° C with shaking at 200 rpm. At 0, 2, 4 and 6 hours after induction with IPTG, 1 ml aliquots of the cultures are collected, the OD_{600} is determined and the amount of isoprene produced is measured as described above. Results are shown in Figure 8.

IV. Production of Isoprene from BL21/ptrcKudzu in 14 liter fermentation

[0382] Large scale production of isoprene from *E. coli* containing the recombinant kudzu isoprene synthase gene is determined from a fed-batch culture. The recipe for the fermentation media (TM2) per liter of fermentation medium is 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 are added together and dissolved in diH_2O. The pH is adjusted to 6.8 with potassium hydroxide (KOH) and q.s. to volume. The final product is filter sterilized with 0.22 μ filter (only, do not autoclave).

[0383] The recipe for 1000X Modified Trace Metal Solution is as follows: citric acid * 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 μ filter.

[0384] This experiment is carried out in 14 L bioreactor to monitor isoprene formation from glucose at the desired fermentation, pH 6.7 and temperature 34° C. An inoculum of *E. coli* strain BL21/ptrcKudzu taken from a frozen vial is prepared in soytone-yeast extract-glucose medium. After the inoculum grows to OD_{550} = 0.6, two 600 ml flasks are 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 are removed and the amount of isoprene produced is determined as described above. Results are shown in Figure 9.

Example 2: Production of Isoprene in *Escherichia coli* Expressing Recombinant Poplar Isoprene Synthase

[0385] The protein sequence for the poplar (*Populus alba* x *Populus tremula*) isoprene synthase (Schnitzler, J-P, *et al.* (2005) *Planta* 222:777-786) is obtained from GenBank (CAC35696). A gene, codon optimized for *E. coli*, is purchased from DNA2.0 (p9796-poplar, Figures 30 and 31). The isoprene synthase gene is 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), is verified by sequencing.

Example 3: Production of Isoprene in *Panteoa citrea* Expressing Recombinant Kudzu Isoprene Synthase

[0386] The pTrcKudzu and pCL-lac Kudzu plasmids described in Example 1 are electroporated into *P. citrea* (U.S. Pat. No. 7,241,587). Transformants are 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 is 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

[0387] The kudzu isoprene synthase gene is 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 are amplified separately and fused using PCR. The construct is then cloned into pBS19 and transformed into *B. subtilis*.

a) Amplification of the *aprE* promoter

[0388] The *aprE* promoter is amplified from chromosomal DNA from *Bacillus subtilis* using the following primers:

CF 797 (+) Start aprE promoter MfeI
5'- GACATCAATTGCTCCATTTTCTTCTGCTATC (SEQ ID NO:58)

CF 07-43 (-) Fuse aprE promoter to Kudzu ispS
5'- ATTGAGAAGAGGTCGCACACACTCTTTACCCTCTCCTTTTA (SEQ ID NO:59)

b) Amplification of the isoprene synthase gene

[0389] The kudzu isoprene synthase gene is amplified from plasmid pTrcKudzu (SEQ ID NO:2). The gene is codon optimized for *E. coli* and synthesized by DNA 2.0. The following primers are used:

CF 07-42 (+) Fuse the aprE promoter to kudzu isoprene synthase gene (GTG start codon)
5'- TAAAAGGAGAGGGTAAAGAGTGTGTGCGACCTCTTCTCAAT (SEQ ID NO:60)

CF 07-45 (-) Fuse the 3' end of kudzu isoprene synthase gene to the terminator
5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:61)

c) Amplification of the transcription terminator

[0390] The terminator from the alkaline serine protease of *Bacillus amyliquefaciens* is 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:62)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:63)

[0391] The kudzu fragment is 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:61)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:63)

[0392] The kudzu-terminator fragment is fused to the promoter fragment using PCR with the following primers:

CF 797 (+) Start *aprE* promoter MfeI
5'- GACATCAATTGCTCCATTTTCTTCTGCTATC (SEQ ID NO:64)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:63)

[0393] The fusion PCR fragment is purified using a Qiagen kit and digested with the restriction enzymes *Mfe*I and *Bam*HI. This digested DNA fragment is gel purified using a Qiagen kit and ligated to a vector known as pBS19, which has been digested with *Eco*RI and *Bam*HI and gel purified.

[0394] The ligation mix is transformed into *E. coli* Top 10 cells and colonies are selected on LA+50 carbenicillin plates. A total of six colonies are chosen and grown overnight in LB+50 carbenicillin and then plasmids are isolated using a Qiagen kit. The plasmids are digested with *Eco*RI and *Bam*HI to check for inserts and three of the correct plasmids are sent in for sequencing with the following primers:

CF 149 (+) EcoRI start of *aprE* promoter
5'- GACATGAATTCCTCCATTTTCTTCTGC (SEQ ID NO:65)

CF 847 (+) Sequence in pXX 049 (end of *aprE* promoter)
5'- AGGAGAGGGTAAAGAGTGAG (SEQ ID NO:66)

CF 07-45 (-) Fuse the 3' end of kudzu isoprene synthase to the terminator
5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:61)

CF 07-48 (+) Sequencing primer for kudzu isoprene synthase
5'- CTTTTCCATCACCCACCTGAAG (SEQ ID NO:67)

CF 07-49 (+) Sequencing in kudzu isoprene synthase
5'- GGCGAAATGGTCCAACAACAAAATTATC (SEQ ID NO:68)

[0395] The plasmid designated pBS Kudzu #2 (Figures 52 and 12) is correct by sequencing and is transformed into BG 3594 comK, a *Bacillus subtilis* host strain. Selection is done on LA + 5 chloramphenicol plates. A transformant is chosen and struck to single colonies on LA + 5 chloramphenicol, then grown in LB+5 chloramphenicol until it reaches an OD_{600} of 1.5. It is stored frozen in a vial at -80° C in the presence of glycerol. The resulting strain is designated CF 443.

II. Production of isoprene in shake flasks containing *B. subtilis* cells expressing recombinant isoprene synthase

[0396] Overnight cultures are inoculated with a single colony of CF 443 from a LA + Chloramphenicol (Cm, 25 μg/ml). Cultures are grown in LB + Cm at 37° C with shaking at 200 rpm. These overnight cultures (1 ml) are 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 is 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 is 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 are incubated at 37° C and samples are taken at 18, 24, and 44 hours. At 18 hours the headspaces of CF443 and the control strain are sampled. This represents 18 hours of accumulation of isoprene. The amount of isoprene is determined by gas chromatography as described in Example 1. Production of isoprene is enhanced significantly by expressing recombinant isoprene synthase (Figure 11).

III. Production of isoprene by CF443 in 14 L fermentation

[0397] Large scale production of isoprene from *B. subtilis* containing the recombinant kudzu isoprene synthase gene on a replication plasmid is 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 are 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 is started when glucose in the batch is non-detectable. The feed rate is ramped over several hours and is adjusted to add oil on an equal carbon basis. The pH is controlled at 6.8 – 7.4 using 28% w/v ammonium hydroxide. In case of foaming, antifoam agent is added to the media. The fermentation temperature is controlled at 37°C and the fermentation culture is agitated at 750 rpm. Various other parameters such as pH, DO%, airflow, and pressure are monitored throughout the entire process. The DO% is maintained above 20. Samples are 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*.

[0398] The kudzu isoprene synthase gene is cloned in an integrating plasmid (pJH101-cmpR) under the control of the *aprE* promoter. Under the conditions tested, no isoprene is detected.

Example 5: Production of Isoprene in *Trichoderma reesei* Expressing Recombinant Kudzu Isoprene Synthase

I. Construction of vectors for expression of the kudzu isoprene synthase in *T. reesei*

[0399] The *Yarrowia lipolytica* codon-optimized kudzu IS gene is synthesized by DNA 2.0 (SEQ ID NO:8) (Figure 13). This plasmid serves 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:9), 1 µl Primer EL-965 (10uM) 5'-CACCATGTGTGCAACCTCCTCCCAGTTTAC (SEQ ID NO:10), 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 contains an additional 4 nucleotides at the 5'-end that do not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but is required for cloning into the pENTR/D-TOPO vector. The reverse primer contains an additional 21 nucleotides at the 5'-end that do not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but are inserted for cloning into other vector backbones. Using the MJ Research PTC-200 Thermocycler, the PCR reaction is 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 is analyzed on a 1.2% E-gel to confirm successful amplification of the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene.

[0400] The PCR product is 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 is incubated at room temperature for 5 minutes. One microliter of TOPO reaction is transformed into TOP10 chemically competent *E. coli* cells. The transformants are selected on LA + 50 µg/ml kanamycin plates. Several colonies are picked and each is 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 are isolated from the overnight culture tubes using QIAprep Spin Miniprep Kit, following manufacturer's protocol. Several plasmids are sequenced to verify that the DNA sequence is correct.

[0401] A single pENTR/D-TOPO plasmid encoding a *Y. lipolytica* codon-optimized kudzu isoprene synthase gene is used for Gateway Cloning into a custom-made pTrex3g vector. Construction of pTrex3g is described in WO 2005/001036 A2. The reaction is 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 is incubated at room temperature for 1 hour and then 1 µl proteinase K solution is added and the incubation continued at 37° C for 10 minutes. Then 1 µl of reaction is transformed into TOP10 chemically competent *E. coli* cells. The transformants are selected on LA + 50 µg/ml carbenicillin plates. Several colonies are picked and each is inoculated into a 5 ml tube containing LB + 50 µlg/ml carbenicillin and the cultures are grown overnight at 37° C with shaking at 200 rpm. Plasmids are isolated from the overnight culture tubes using QIAprep Spin Miniprep Kit (Qiagen, Inc.), following manufacturer's protocol. Several plasmids are sequenced to verify that the DNA sequence was correct.

[0402] Biolistic transformation of *Y. lipolytica* codon-optimized kudzu isoprene synthase pTrex3g plasmid (Figure 14) into a quad delete *Trichoderma reesei* strain is performed using the Biolistic PDS-1000/HE Particle Delivery System (see WO 2005/001036 A2). Isolation of stable transformants and shake flask evaluation is performed using protocol listed in Example 11 of patent publication WO 2005/001036 A2.

II. Production of isoprene in recombinant strains of *T. reesei*

[0403] One ml of 15 and 36 hour old cultures of isoprene synthase transformants described above are transferred to head space vials. The vials are sealed and incubated for 5 hours at 30° C. Head space gas is measured and isoprene is identified by the method described in Example 1. Two of the transformants show traces of isoprene. The amount of isoprene can be increased by a 14 hour incubation. The two positive samples show isoprene at levels of about 0.5 µg/L for the 14 hour incubation. The untransformed control shows 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 lipolytica* Expressing Recombinant Kudzu Isoprene Synthase

I. Construction of vectors for expression of the kudzu isoprene synthase in *Y. lipolytica*.

[0404] The starting point for the construction of vectors for the expression of the kudzu isoprene synthase gene in *Yarrowia lipolytica* is the vector pSPZ1(MAP29Spb). The complete sequence of this vector (SEQ ID No:11) is shown in Figure 15.

[0405] The following fragments are 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 are used:

ICL1 3
5'- GGTGAATTCAGTCTACTGGGGATTCCCAAATCTATATATACTGCAGGTGAC (SEQ ID NO:69)

ICL1 5
5'- GCAGGTGGGAAACTATGCACTCC (SEQ ID NO:70)

XPR 3
5'- CCTGAATTCTGTTGGATTGGAGGATTGGATAGTGGG (SEQ ID NO:71)

XPR 5
5'- GGTGTCGACGTACGGTCGAGCTTATTGACC (SEQ ID NO:72)

XPRT3
5'- GGTGGGCCCGCATTTTGCCACCTACAAGCCAG (SEQ ID NO:73)

XPRT 5
5'- GGTGAATTCTAGAGGATCCCAACGCTGTTGCCTACAACGG (SEQ ID NO:74)

Y18S3
5'- GGTGCGGCCGCTGTCTGGACCTGGTGAGTTTCCCCG (SEQ ID NO:75)

Y18S 5

5'- GGTGGGCCCATTAAATCAGTTATCGTTTATTTGATAG (SEQ ID NO:76)

YURA3

5'- GGTGACCAGCAAGTCCATGGGTGGTTTGATCATGG (SEQ ID NO:77)

YURA 50

5'- GGTGCGGCCGCCTTTGGAGTACGACTCCAACTATG (SEQ ID NO:78)

YURA 51

5'- GCGGCCGCAGACTAAATTTATTTCAGTCTCC (SEQ ID NO:79)

[0406] For PCR amplification the PfuUltraII polymerase (Stratagene), supplier-provided buffer and dNTPs, 2.5 μM primers and the indicated template DNA are used as per the manufacturer's instructions. The amplification is 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.

[0407] Synthetic DNA molecules encoding the kudzu isoprene synthase gene, codon-optimized for expression in *Yarrowia,* are obtained from DNA 2.0 (Figure 16; SEQ ID NO:12). 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 are also constructed (Figure 18E and 18F).

[0408] 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:13). 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.*

[0409] Vectors pYLA(KZ1), pYLI(KZ1), pYLA(MAP29) and pYLI(MAP29) are 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, are 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 are 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 are further incubated for 1 hour at room temperature followed by a 2 minutes heat shock at 42° C. Cells are then plated on SC his leu plates (0.67% yeast nitrogen base, 2% glucose, 100 mg/L each of leucine and histidine). Transformants appear after 3-4 days of incubation at 30° C.

[0410] 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 are 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 are collected by centrifugation, resuspended in 3 ml of fresh YEP7 and placed into 15 ml screw cap vials. The vials are incubated overnight at room temperature with gentle (60 rpm) shaking. Isoprene content in the headspace of these vials is analyzed by gas chromatography using mass-spectrometric detector as described in Example 1. All transformants obtained with pYLA(KZ1) and pYLI(KZ1) produce readily detectable amounts of isoprene (0.5 µg/L to 1 µg/L, Figure 20). No isoprene is 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

[0411] The *bla* gene of pTrcKudzu (described in Example 1) is replaced with the gene conferring kanamycin resistance. To remove the *bla* gene, pTrcKudzu is digested with *Bsp*HI, treated with Shrimp Alkaline Phosphatase (SAP), heat killed at 65° C, then end-filled with Klenow fragment and dNTPs. The 5 kbp large fragment is purified from an agarose gel

and ligated to the kan^r gene which has been PCR amplified from pCR-Blunt-II-TOPO using primers MCM22 5'- GATCAAGCTTAACCGGAATTGCCAGCTG (SEQ ID NO:14) and MCM23 5'- GATCCGATCGTCAGAAGAACTCGTCAAGAAGGC (SEQ ID NO:15), digested with *Hin*dIII and *Pvu*I, and end-filled. A transformant carrying a plasmid conferring kanamycin resistance (pTrcKudzuKan) is selected on LA containing kanamycin 50 μg/ml.

ii) Construction of pTrcKudzu yIDI Kan

[0412] pTrcKudzuKan is digested with *Pst*I, treated with SAP, heat killed and gel purified. It is ligated to a PCR product encoding *idi* from *S. cerevisiae* with a synthetic RBS. The primers for PCR are NsiI-YIDI 1 F 5'- CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC (SEQ ID NO:16) and PstI-YIDI 1 R 5'- CCTTCTGCAGGACGCGTTGTTATAGC (SEQ ID NO:17); and the template is *S. cerevisiae* genomic DNA. The PCR product is digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The ligation mixture is transformed into chemically competent TOP10 cells and selected on LA containing 50 μg/ml kanamycin. Several transformants are isolated and sequenced and the resulting plasmid is called pTrcKudzu-yIDI(kan) (Figures 34 and 35).

iii) Construction of pTrcKudzu DXS Kan

[0413] Plasmid pTrcKudzuKan is digested with *Pst*I, treated with SAP, heat killed and gel purified. It is ligated to a PCR product encoding *dxs* from *E. coli* with a synthetic RBS. The primers for PCR are MCM13 5'- GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAAT ACCCG (SEQ ID NO:18) and MCM14 5'- CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:19); and the template is *E. coli* genomic DNA. The PCR product is digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The resulting transformation reaction is transformed into TOP10 cells and selected on LA with kanamycin 50 μg/ml. Several transformants are isolated and sequenced and the resulting plasmid is called pTrcKudzu-DXS(kan) (Figures 36 and 37).

iv) Construction of pTrcKudzu-yIDI-dxs (kan)

[0414] pTrcKudzu-yIDI(kan) is digested with *Pst*I, treated with SAP, heat killed and gel purified. It is ligated to a PCR product encoding *E. coli dxs* with a synthetic RBS (primers

MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAATACCCG (SEQ ID NO:18) and MCM14 5'-CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:19); template TOP10 cells) which have been digested with *Nsi*I and *Pst*I and gel purified. The final plasmid is called pTrcKudzu-yIDI-dxs (kan) (Figures 21 and 22).

v) Construction of pCL PtrcKudzu

[0415] A fragment of DNA containing the promoter, structural gene and terminator from Example 1 above is digested from pTrcKudzu using *Ssp*I and gel purified. It is ligated to pCL1920 which has 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 are isolated and sequenced and two are selected. pCL PtrcKudzu and pCL PtrcKudzu (A3) have the insert in opposite orientations (Figures 38-41).

vi) Construction of pCL PtrcKudzu yIDI

[0416] The *Nsi*I-*Pst*I digested, gel purified, IDI PCR amplicon from (ii) above is ligated into pCL PtrcKudzu which has been digested with *Pst*I, treated with SAP, and heat killed. The ligation mixture is transformed into TOP10 cells and selected in LA containing spectinomycin 50 μg/ml. Several clones are isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu yIDI (Figures 42 and 43).

vii) Construction of pCL PtrcKudzu DXS

[0417] The *Nsi*I-*Pst*I digested, gel purified, DXS PCR amplicon from (iii) above is ligated into pCL PtrcKudzu (A3) which has been digested with *Pst*I, treated with SAP, and heat killed. The ligation mixture is transformed into TOP10 cells and selected in LA containing spectinomycin 50 μg/ml. Several clones are isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu DXS (Figures 44 and 45).

II. Measurement of isoprene in headspace from cultures expressing kudzu isoprene synthase, *idi*, and/or *dxs* at different copy numbers.

[0418] 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) are grown in LB kanamycin 50 μg/mL. Cultures of pCL PtrcKudzu (E), pCL PtrcKudzu, pCL PtrcKudzu-yIDI (F) and pCL PtrcKudzu-DXS (G) are grown in LB spectinomycin 50 μg/mL. Cultures are 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.

[0419] Plasmid pTrcKudzu-yIDI-dxs (kan) was introduced into *E. coli* strain BL21 by transformation. The resulting strain BL21/pTrc Kudzu IDI DXS is 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 are incubated at 30° C until an OD_{600} of 0.8 is reached, and then induced with 400 μM IPTG. Samples are taken at various times after induction and the amount of isoprene in the head space is measured as described in Example 1. Results are shown in Figure 23H.

III. Production of isoprene from biomass in *E. coli*/pTrcKudzu yIDI DXS

[0420] The strain BL21 pTrcKudzuIDIDXS is 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 are 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 are equal to 1% glucose. A single colony from a plate freshly transformed cells of BL21(DE3) pTrcKudzu yIDI DXS (kan) is used to inoculate 5 ml of LB plus kanamycin (50 μg/ml). The culture is incubated overnight at 25° C with shaking. The following day the overnight culture is diluted to an OD_{600} of 0.05 in 25 ml of TM3 + 0.2% YE + 1% feedstock. The feedstock is corn stover, bagasse, or softwood pulp. Glucose is used as a positive control and no glucose is used as a negative control. Cultures are incubated at 30° C with shaking at 180 rpm. The culture is monitored for OD_{600} and when it reaches an OD_{600} of ~0.8, cultures are 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 are done in duplicate and are shown in Figure 46.

IV. Production of isoprene from invert sugar in *E. coli*/pTrcKudzuIDIDXS

[0421] A single colony from a plate freshly transformed cells of BL21(λDE3)/pTrcKudzu yIDI DXS (kan) is used to inoculate 5 mL of LB + kanamycin (50 μg/ml). The culture is incubated overnight at 25° C with shaking. The following day the overnight culture is diluted to an OD_{600} of 0.05 in 25 ml of TM3 + 0.2% YE + 1% feedstock. Feedstock is glucose, inverted glucose or corn stover. The invert sugar feedstock (Danisco Invert Sugar) is prepared by enzymatically treating sucrose syrup. AFEX corn stover is prepared as described below (Part V). The cells are grown at 30° C and the first sample is measured when the cultures reach an OD_{600} ~0.8-1.0 (0 hour). The cultures are 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

[0422] AFEX pretreated corn stover is obtained from Michigan Biotechnology Institute. The pretreatment conditions are 60% moisture, 1:1 ammonia loading, and 90 °C for 30 minutes, then air dried. The moisture content in the AFEX pretreated corn stover is 21.27%. The contents of glucan and xylan in the AFEX pretreated corn stover are 31.7% and 19.1% (dry basis), respectively. The saccharification process is as follows; 20 g of AFEX pretreated corn stover is 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 is put in an orbital shaker and incubated at 50° C for 96 hours. One sample is 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

[0423] Fermentation is 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) is fed at an exponential rate. The total amount of yeast

extract delivered to the fermentor is varied between 70-830 g during the 40 hour fermentation. Optical density of the fermentation broth is measured at a wavelength of 550 nm. The final optical density within the fermentors is proportional to the amount of yeast extract added (Figure 48A). The isoprene level in the off-gas from the fermentor is determined as previously described. The isoprene titer increases over the course of the fermentation (Figure 48B). The amount of isoprene produced is linearly proportional to the amount of fed yeast extract (Figure 48C).

VII. Production of isoprene in 500 L fermentation of pTrcKudzu DXS yIDI

[0424] 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) is 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 is calculated to be approximately 17 g.

VIII. Production of isoprene in 500 L fermentation of E. coli grown in fed-batch culture

[0425] 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 are added together and dissolved in diH_2O. This solution is autoclaved. The pH is adjusted to 7.0 with ammonium gas (NH_3) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotic are added after sterilization and pH adjustment.

[0426] 1000X Modified Trace Metal Solution: citric acid * 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 DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0427] Fermentation is performed in a 500-L bioreactor with *E. coli* cells containing the pTrcKudzu yIDI DXS plasmid. This experiment is 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 is prepared in soytone-yeast extract-

glucose medium. After the inoculum grows to OD 0.15, measured at 550 nm, 20 ml is used to inoculate a bioreactor containing 2.5-L soytone-yeast extract-glucose medium. The 2.5-L bioreactor is grown at 30° C to OD 1.0 and 2.0-L is transferred to the 500-L bioreactor.

[0428] Yeast extract (Bio Springer, Montreal, Quebec, Canada) and glucose are fed at exponential rates. The total amount of glucose and yeast extract delivered to the bioreactor during the 50 hour fermentation is 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 is determined as previously described. The isoprene titer increases over the course of the fermentation (Figure 49B). The total amount of isoprene produced during the 50 hour fermentation is 55.1 g and the time course of production is shown in Figure 49C.

Example 8: Production of Isoprene in *E. coli* Expressing Heterologous Isoprene Synthase and Mevalonic Acid Pathway Genes

I. Cloning the lower MVA pathway

[0429] The strategy for cloning the lower mevalonic pathway is as follows. Four genes of the mevalonic acid biosynthesis pathway; mevalonate kinase (MVK), phosphomevalonate kinase (PMK), diphosphomevalonte decarboxylase (MVD) and isopentenyl diphosphate isomerase genes are amplified by PCR from *S. cerevisiae* chromosomal DNA and cloned individually into the pCR BluntII TOPO plasmid (Invitrogen). In some cases, the *idi* gene is amplified from *E. coli* chromosomal DNA. The primers are designed such that an *E. coli* consensus RBS (AGGAGGT (SEQ ID NO:80) or AAGGAGG (SEQ ID NO:81)) is inserted at the 5' end, 8 bp upstream of the start codon and a *Pst*I site is added at the 3' end. The genes are then cloned one by one into the pTrcHis2B vector until the entire pathway is assembled.

[0430] Chromosomal DNA from *S. cerevisiae* S288C is obtained from ATCC (ATCC 204508D). The MVK gene is amplified from the chromosome of *S. cerevisiae* using primers MVKF (5'-AGGAGGTAAAAAAACATGTCATTACCGTTCTTAACTTCTGC, SEQ ID NO:21) and MVK-Pst1-R (5'-ATGGCTGCAGGCCTATCGCAAATTAGCTTATGAAGTCCATGGTAAATTCGTG, SEQ ID NO:22) using PfuTurbo as per manufacturer's instructions. The correct sized PCR product (1370 bp) is identified by electrophoresis through a 1.2% E-gel (Invitrogen) and cloned into pZeroBLUNT TOPO. The resulting plasmid is designated pMVK1. The plasmid

pMVK1 is digested with *Sac*I and *Taq*1 restriction endonucleases and the fragment is gel purified and ligated into pTrcHis2B digested with *Sac*I and *Bst*BI. The resulting plasmid is named pTrcMVK1.

[0431] The second gene in the mevalonic acid biosynthesis pathway, PMK, is amplified by PCR using primers: PstI-PMK1 R (5'-GAATTCGCCCTTCTGCAGCTACC, SEQ ID NO:23) and BsiHKA I-PMK1 F (5'-CGACTGGTGCACCCTTAAGGAGGAAAAAAACATGTCAG, SEQ ID NO:24). The PCR reaction is performed using Pfu Turbo polymerase (Stratagene) as per manufacturer's instructions. The correct sized product (1387 bp) is digested with *Pst*I and *Bsi*HKI and ligated into pTrcMVK1 digested with *Pst*I. The resulting plasmid is named pTrcKK. The MVD and the *idi* genes are cloned in the same manner. PCR is carried out using the primer pairs PstI-MVD 1 R (5'-GTGCTGGAATTCGCCCTTCTGCAGC, SEQ ID NO:25) and NsiI-MVD 1 F (5'-GTAGATGCATGCAGAATTCGCCCTTAAGGAGG, SEQ ID NO:26) to amplify the MVD gene and PstI-YIDI 1 R (5'-CCTTCTGCAGGACGCGTTGTTATAGC, SEQ ID NO:27) and NsiI-YIDI 1 F (5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC, SEQ ID NO:28) to amplify the yIDI gene. In some cases the IPP isomerase gene, *idi* from *E. coli* is used. To amplify *idi* from *E. coli* chromosomal DNA, the following primer set is used: PstI-CIDI 1 R (5'-GTGTGATGGATATCTGCAGAATTCG, SEQ ID NO:29) and NsiI-CIDI 1 F (5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAACATG, SEQ ID NO:30). Template DNA is 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 are named pKKDIy for the construct encoding the yeast *idi* gene or pKKDIc for the construct encoding the *E. coli idi* gene. The plasmids are transformed into *E. coli* hosts BL21 for subsequent analysis. In some cases the isoprene synthase from kudzu is cloned into pKKDIy yielding plasmid pKKDIyIS.

[0432] The lower MVA pathway is also cloned into pTrc containing a kanamycin antibiotic resistance marker. The plasmid pTrcKKDIy is digested with restriction endonucleases *Apa*I and *Pst*I, the 5930 bp fragment is 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, is digested with restriction endonucleases *Apa*I and

*Pst*I, and the 3338 bp fragment containing the vector is 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 are ligated using the Roche Quick Ligation kit. The ligation mix is 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 are verified by restriction enzyme digestion and one is frozen as a stock. The plasmid is designated pTrcKanKKDIy.

II. Cloning a kudzu isoprene synthase gene into pTrcKanKKDIy

[0433] The kudzu isoprene synthase gene is amplified by PCR from pTrcKudzu, described in Example 1, using primers MCM50 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGTGTGCGACCTCTTCTCAATTTACT (SEQ ID NO:31) and MCM53 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:32). The resulting PCR fragment is 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 are incubated overnight at 37° C with selection on LA containing carbenicillin (50 μg/ml). The correct insertion of the fragment is verified by sequencing and this strain is designated MCM93.

[0434] The plasmid from strain MCM93 is 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 is separated on a 1.2% agarose E-gel and purified using the Qiagen Gel Purification kit according to the manufacturer's instructions. Plasmid pTrcKanKKDIy is 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 are ligated using the Roche Quick Ligation kit. The ligation mix is transformed into *E. coli* TOP10 cells and transformants are grown overnight at 37° C with selection on LA containing Kanamycin at 50 μg/ml. The correct transformant is verified by restriction digestion and the plasmid is designated pTrcKKDyIkISKan (Figures 24 and 25). This plasmid is 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.

[0435] Strain BL21/pTrcKKDyIkISKan is 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 is also set up using identical conditions but without the addition of 0.5% mevalonic acid. The culture is started from an overnight seed culture with a 1% inoculum and induced with 500 μM IPTG when the culture has reached an OD_{600} of 0.3 to 0.5. The cultures are grown at 30° C with shaking at 250 rpm. The production of isoprene is analyzed 3 hours after induction by using the head space assay described in Example 1. Maximum production of isoprene is 6.67 x 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 does not produce measurable isoprene.

IV. Cloning the upper MVA pathway

[0436] The upper mevalonate biosynthetic pathway, comprising two genes encoding three enzymatic activities, is 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 the second enzyme in the pathway, HMG-CoA synthase. The *mvaE* gene is 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:34)

CF 07-62 (-) Fuse *mvaE* to *mvaS* with RBS in between
5' - TTTATCAATCCCAATTGTCATGTTTTTTTACCTCCTTTATTGTTTTCTTAAATC
(SEQ ID NO:35)

[0437] The *mvaS* gene is 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:36)

CF 07-102 (-) End of *mvaS* gene *Bgl*II
5' -GACATGACATAGATCTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:37)

[0438] The PCR fragments are 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:34)

CF 07-102 (-) End of *mvaS* gene *Bgl*II
5'-GACATGACATAGATCTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:37)

[0439] The fusion PCR fragment is purified using a Qiagen kit and digested with the restriction enzymes *Sac*I and *Bgl*II. This digested DNA fragment is gel purified using a Qiagen kit and ligated into the commercially available vector pTrcHis2A, which has been digested with *Sac*I and *Bgl*II and gel purified.

[0440] The ligation mix is transformed into *E. coli* Top 10 cells and colonies are selected on LA+50 μg/ml carbenicillin plates. A total of six colonies are chosen and grown overnight in LB+50 μg/ml carbenicillin and plasmids are isolated using a Qiagen kit. The plasmids are digested with *Sac*I and *Bgl*II to check for inserts and one correct plasmid is sequenced with the following primers:

CF 07-58 (+) Start of *mvaE* gene
5' – ATGAAAACAGTAGTTATTATTGATGC (SEQ ID NO:38)

CF 07-59 (-) End of *mvaE* gene
5' – ATGTTATTGTTTTCTTAAATCATTTAAAATAGC (SEQ ID NO:39)

CF 07-82 (+) Start of *mvaS* gene
5' – ATGACAATTGGGATTGATAAAATTAG (SEQ ID NO:40)

CF 07-83 (-) End of *mvaS* gene
5' – TTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:41)

CF 07-86 (+) Sequence in *mvaE*
5' – GAAATAGCCCCATTAGAAGTATC (SEQ ID NO:42)

CF 07-87 (+) Sequence in *mvaE*
5' – TTGCCAATCATATGATTGAAAATC (SEQ ID NO:43)

CF 07-88 (+) Sequence in *mvaE*
5' – GCTATGCTTCATTAGATCCTTATCG (SEQ ID NO:44)

CF 07-89 (+) Sequence *mvaS*
5' – GAAACCTACATCCAATCTTTTGCCC (SEQ ID NO:45)

[0441] The plasmid called pTrcHis2AUpperPathway#1 is correct by sequencing and is transformed into the commercially available *E. coli* strain BL21. Selection is done on LA+ 50 μg/ml carbenicillin. Two transformants are chosen and grown in LB+ 50 μg/ml carbenicillin until they reach an OD_{600} of 1.5. Both strains are frozen in a vial at -80° C in the presence of glycerol. Strains are designated CF 449 for pTrcHis2AUpperPathway#1 in BL21, isolate #1 and CF 450 for pTrcHis2AUpperPathway#1 in BL21, isolate #2. Both clones are found to behave identically when analyzed.

V. Cloning of UpperMVA Pathway into pCL1920

[0442] The plasmid pTrcHis2AUpperPathway is digested with the restriction endonuclease *Ssp*I to release a fragment containing pTrc-*mvaE-mvaS*-(His tag)-terminator. In this fragment, the His-tag is not translated. This blunt ended 4.5 kbp fragment is purified from a 1.2% E-gel using the Qiagen Gel Purification kit. A dephosphorylated, blunt ended 4.2 kbp fragment from pCL1920 is 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 are ligated using the Roche Quick Ligation Kit and transformed into TOP10 chemically competent cells. Transformants are selected on LA containing

spectinomycin (50 μg/ml). A correct colony is identified by screening for the presence of the insert by PCR. The plasmid is designated pCL PtrcUpperPathway (Figures 26 and 27A-27D).

VI. Strains expressing the combined Upper and Lower Mevalonic Acid Pathways

[0443] To obtain a strain with a complete mevalonic acid pathway plus kudzu isoprene synthase, plasmids pTrcKKDyIkISkan and pCLpTrcUpperPathway are both transformed into BL21(λDE3) competent cells (Invitrogen) and transformants are selected on LA containing kanamycin (50 μg/ml) and Spectinomycin (50 μg/ml). The transformants are checked by plasmid prep to ensure that both plasmids are retained in the host. The strain is designated MCM127.

VII. Production of mevalonic acid from glucose in *E. coli*/pUpperpathway

[0444] 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 are diluted into 50 ml medium in 250 ml baffled flasks to an OD_{600} of 0.1. The medium is 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 are grown at 30° C with shaking at 200 rpm for approximately 2-3 hours until an OD_{600} of 0.4 is reached. At this point the expression from the *mvaE mvaS* construct is induced by the addition of IPTG (400 μM). Cultures are 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 is 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.

[0445] 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 produce 22 grams of mevalonic acid with TM3 medium and 2% glucose as the cell medium. A shake flask of these cells produces 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 indicates 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.

[0446] The following strains are 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 are chemically competent BL21(λDE3) and the transformations are done by standard methods. Transformants are 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 are 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)

[0447] The above strains are streaked from freezer stocks to LA + appropriate antibiotic and grown overnight at 37° C. A single colony from each plate is used to inoculate shake flasks (25 ml LB + the appropriate antibiotic). The flasks are incubated at 22° C overnight with shaking at 200 rpm. The next morning the flasks are transferred to a 37° C incubator and grown for a further 4.5 hours with shaking at 200 rpm. The 25 ml cultures are centrifuged to pellet the cells and the cells are resuspended in 5 ml LB + the appropriate antibiotic. The cultures are then diluted into 25 ml LB+1% glucose + the appropriate antibiotic to an OD_{600} of 0.1. Two flasks for each strain are set up, one set for induction with IPTG (800 μM) the second set is not induced. The cultures are incubated at 37° C with shaking at 250 rpm. One set of the cultures are induced after 1.50 hours (immediately following sampling time point 1). At each sampling time point, the OD_{600} is measured and the amount of isoprene determined as described in Example 1. Results are presented in Table 8-1. The amount of isoprene made is presented as the amount at the peak production for the particular strain.

Table 8-1. 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

[0448] Mevalonolactone (1.0 g, 7.7 mmol) (CAS# 503-48-0) is supplied from Sigma-Aldrich (WI, USA) as a syrup that is dissolved in water (7.7 mL) and is treated with potassium hydroxide (7.7 mmol) in order to generate the potassium salt of mevalonic acid. The conversion to mevalonic acid is confirmed by ^{1}H NMR analysis. Samples for HPLC analysis are 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) is then added followed by mixing and cooling on ice for 5 minutes. The samples are 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) are prepared in the same fashion. Analysis of mevalonic acid (20 uL injection volume) is 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 elutes 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

[0449] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase is used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions results in the production of 2.2 g/L of isoprene.

[0450] Medium Recipe (per liter fermentation medium): The medium is 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 are added together and dissolved in diH_2O. This solution is autoclaved. The pH is adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics are added after sterilization and pH adjustment.

[0451] 1000X Modified Trace Metal Solution: The 1000X modified trace metal solution is generated using the following components: citric acid * 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 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.

[0452] Fermentation is performed in a 15-L bioreactor with BL21(DE3) *E. coli* cells containing the pCL PtrcUpperPathway (Figure 26) and pTrcKKDyIkIS plasmids. This experiment is 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 is streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into soytone-yeast extract-glucose medium. After the inoculum grows to OD 1.0 when measured at 550 nm, 500 mL is used to inoculate a 15-L bioreactor containing an initial working volume of 5 L.

[0453] Glucose is fed at an exponential rate until cells reach the stationary phase. After this time the glucose feed is decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 54 hour fermentation is 3.7 kg. Induction is achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration is brought to 25 uM when the optical density at 550 nm (OD_{550}) reaches a value of 10. The IPTG concentration is raised to 50 uM when OD_{550} reached 190. IPTG concentration is raised to 100 uM at 38 hours of fermentation. The OD_{550} profile within the bioreactor over time is shown in Fig. 54. The isoprene level in the off gas from the bioreactor is determined as described herein. The isoprene titer increases over the course of the fermentation to a final value of 2.2 g/L (Fig. 55). The total amount of isoprene produced during the 54 hour fermentation is 15.9 g, and the time course of production is shown in Fig. 56.

[0454] XI. Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale

[0455] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase is used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions results in the production of 3.0 g/L of isoprene.

[0456] Medium Recipe (per liter fermentation medium): The medium is 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 sre added together and dissolved in diH_2O. This solution is autoclaved. The pH is adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics are added after sterilization and pH adjustment.

[0457] 1000X Modified Trace Metal Solution: The 1000X modified trace metal solution is generated using the following components: citric acid * 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 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.

[0458] Fermentation is performed in a 15-L bioreactor with BL21(DE3) *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment is 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 is streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into tryptone-yeast extract medium. After the inoculum grows to OD 1.0, measured at 550 nm, 500 mL is used to inoculate a 15-L bioreactor containing an initial working volume of 5 L.

[0459] Glucose is fed at an exponential rate until cells reach the stationary phase. After this time, the glucose feed is decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 59 hour fermentation is 2.2 kg. Induction is achieved by adding IPTG. The IPTG concentration is brought to 25 uM when the optical

density at 550 nm (OD_{550}) reaches a value of 10. The IPTG concentration is raised to 50 uM when OD_{550} reaches 190. The OD_{550} profile within the bioreactor over time is shown in Fig. 93. The isoprene level in the off gas from the bioreactor is determined as described herein. The isoprene titer increases over the course of the fermentation to a final value of 3.0 g/L (Fig. 94). The total amount of isoprene produced during the 59 hour fermentation is 22.8 g, and the time course of production is shown in FIG 95. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 2.2%. The weight percent yield of isoprene from glucose is 1.0%.

[0460] XII. Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale

[0461] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides, *Pueraria lobata* isoprene synthase, and Kudzu isoprene synthase is used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions results in the production of 3.3 g/L of isoprene.

[0462] i) Construction of pCLPtrcUpperPathwayHGS2

[0463] The gene encoding isoprene synthase from *Pueraria lobata* is PCR-amplified using primers NsiI-RBS-HGS F (CTTGATGCATCCTGCATTCGCCCTTAGGAGG, SEQ ID NO:88) and pTrcR (CCAGGCAAATTCTGTTTTATCAG, SEQ ID NO:89), and pTrcKKDyIkIS as a template. The PCR product thus obtained is restriction-digested with *Nsi*I and *Pst*I and gel-purified. The plasmid pCL PtrcUpperPathway is restriction-digested with *Pst*I and dephosphorylated using rAPid alkaline phosphatase (Roche) according to manufacturer's instructions.

[0464] These DNA fragments are ligated together and the ligation reaction is transformed into *E. coli* Top10 chemcially competent cells (Invitrogen), plated on L agar containing spectinomycin (50 μg/ml) and incubated overnight at 37°C. Plasmid DNA is prepared from 6 clones using the Qiaquick Spin Mini-prep kit. The plasmid DNA is digested with restriction enzymes *Eco*RV and *Mlu*I to identify a clone in which the insert has the right orientation (*i.e.*, the gene oriented in the same way as the pTrc promoter).

[0465] The resulting correct plasmid is designated pCLPtrcUpperPathwayHGS2. This plasmid is 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 is transformed into BL21(λDE3) 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 has 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

[0466] Medium Recipe (per liter fermentation medium): The medium is 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 are added together and dissolved in diH_2O. This solution is autoclaved. The pH is 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.

[0467] 1000X Modified Trace Metal Solution: The 1000X modified trace metal solution is generated using the following components: citric acid * 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 is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0468] Fermentation is performed in a 15-L bioreactor with BL21(DE3) *E. coli* cells containing the pCLPtrcUpperPathwayHGS2 and pTrc KKDyIkIS plasmids. This experiment is 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 is streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into tryptone-yeast extract medium. After the inoculum grows to OD 1.0 measured at 550 nm, 500 mL is used to inoculate a 15-L bioreactor containing an initial working volume of 5 L.

[0469] Glucose is fed at an exponential rate until cells reach the stationary phase. After this time the glucose feed is decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 58 hour fermentation is 2.1 kg. Induction is achieved by adding IPTG. The IPTG concentration is brought to 25 uM when the optical density at 550 nm (OD_{550}) reach a value of 9. The IPTG concentration is raised to 50 uM when OD_{550} reached 170. The OD_{550} profile within the bioreactor over time is shown in Fig. 104. The isoprene level in the off gas from the bioreactor is determined as described herein. The isoprene titer increases over the course of the fermentation to a final value of 3.3 g/L (Fig. 105). The total amount of isoprene produced during the 58 hour fermentation is 24.5 g and the time course of production is shown in Fig. 106. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 2.5%. The weight percent yield of isoprene from glucose is 1.2%. Analysis shows that the activity of the isoprene synthase is increased by approximately 3-4 times that compared to BL21 expressing CL PtrcUpperMVA and pTrc KKDyIkIS plasmids (data not shown).

[0470] XIII. Chromosomal Integration of the Lower Mevalonate Pathway in *E. coli.*

[0471] A synthetic operon containing mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and the IPP isomerase is integerated into the chromosome of *E. coli.* If desired, expression may be altered by integrating different promoters 5' of the operon. Table 8-2 lists primers used for this experiment.

TABLE 8-2. PRIMERS

Primer (Description)	Sequence
MCM78 (attTn7 up rev for integration construct)	gcatgctcgagcggccgcTTTTAATCAAACATCCTGCCAA CTC (SEQ ID NO:91)
MCM79 (attTn7 down rev for integration construct)	gatcgaagggcgatcgTGTCACAGTCTGGCGAAACCG (SEQ ID NO:92)
MCM88 (attTn7 up forw for integration construct)	ctgaattctgcagatatcTGTTTTTCCACTCTTCGTTCACTT T (SEQ ID NO:93)
MCM89 (attTn7 down forw for integration construct)	tctagagggcccAAGAAAAATGCCCCGCTTACG (SEQ ID NO:94)
MCM104 (GI1.2 promoter – MVK)	Gatcgcggccgcgcccttgacgatgccacatcctgagcaaataattcaaccac taattgtgagcggataacacaaggaggaaacagctatgtcattaccgttcttaact tc (SEQ ID NO:95)
MCM105 (aspA terminator – yIDI)	Gatcgggccccaagaaaaaaggcacgtcatctgacgtgccttttttatttgtaga cgcgttgttatagcattcta (SEQ ID NO:96)
MCM120 (forw of attTn7: attTn7 homology, GB marker homology)	aaagtagccgaagatgacggtttgtcacatggagttggcaggatgtttgattaaa agcAATTAACCCTCACTAAAGGGCGG (SEQ ID NO:97)
MCM127 (Rev complement of 1.2 GI: GB marker homology (extra long), promoter, RBS, ATG)	AGAGTGTTCACCAAAAATAATAACCTTTCCCGG TGCAgaagttaagaacggtaatgacatagctgtttcctccttgtgttatccgct cacaattagtggttgaattatttgctcaggatgtggcatcgtcaagggcTAAT ACGACTCACTATAGGGCTCG (SEQ ID NO:98)

i) Target vector construction

[0472] The attTn7 site is selected for integration. Regions of homology upstream (attTn7 up) (primers MCM78 and MCM79) and downstream (attTn7 down) (primers MCM88 and MCM89) are 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 is 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 is 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 is 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 is cloned into *Pst*I-*Not*I digested and gel purified MCM276, creating plasmid MCM281.

[0473] ii) Cloning of lower pathway and promoter

[0474] MVK-PMK-MVD-IDI genes are amplified from pTrcKKDyIkIS with primers MCM104 and MCM105 using Roche Expand Long PCR System according to the manufacturer's instructions. This product is digested with *Not*I and *Apa*I and cloned into MCM281 which has been digested with *Not*I and *Apa*I and gel purified. Primers MCM120 and MCM127 are 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 is used with four 50uL PCR reactions containing 1uL ~10ng/uL template, 1uL each primer, 1.25 uL 10mM dNTPs, 5uL 10x buffer, 1uL enzyme, and 39.75uL ddH20. Reactions are pooled, purified on a Qiagen PCR cleanup column, and used to electroporate water-washed Pir1 cells containing plasmid MCM296. Electroporation is carried out in 2mM cuvettes at 2.5V and 200 ohms. Electroporation reactions are recovered in LB for 3hr at 30°C. Transformant MCM330 is selected on LA with CMP5, Kan50 (Figures 107 and 108A-108C).

[0475] iii) Integration into *E. coli* chromosome

[0476] Miniprepped DNA (Qiaquick Spin kit) from MCM330 is digested with *Sna*BI and used to electroporate BL21(DE3) (Novagen) or MG1655 containing GeneBridges plasmid pRedET Carb. Cells are grown at 30°C to ~OD1 then induced with 0.4% L-arabinose at 37°C for 1.5 hours. These cells are washed three times in 4°C ddH2O before electroporation with 2uL of DNA. Integrants are selected on L agar containing chloramphenicol (5 ug/ml) and subsequently confirmed to not grow on L agar + Kanamycin (50 ug/ml). BL21 integrant MCM331 and MG1655 integrant MCM333 are frozen.

[0477] iv) Construction of pET24D-Kudzu encoding Kudzu isoprene synthase

[0478] The kudzu isoprene synthase gene is subcloned into the pET24d vector (Novagen) from the pCR2.1 vector (Invitrogen). In particular, the kudzu isoprene synthase gene is amplified from the pTrcKudzu template DNA using primers MCM50 5'-GATCATGCAT TCGCCCTTAG GAGGTAAAAA AACATGTGTG CGACCTCTTC TCAATTTACT (SEQ ID NO:99) and MCM53 5'-CGGTCGACGG ATCCCTGCAG TTAGACATAC ATCAGCTG (SEQ ID NO:100). PCR reactions are carried out using Taq DNA Polymerase (Invitrogen), and the resulting PCR product is cloned into pCR2.1-TOPO TA cloning vector (Invitrogen), and transformed into *E. coli* Top10 chemically competent cells (Invitrogen).

Transformants are 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 are inoculated with single transformants and grown overnight at 37°C. Five colonies are 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.

[0479] The kudzu coding sequence is 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 is 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 is 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) is transformed into *E. coli* Top 10 chemically competent cells and plated on L agar containing kanamycin (50 μg/ml). The correct transformant is confirmed by sequencing and transformed into chemically competent BL21(λDE3)pLysS cells (Novagen). A single colony is 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:101) is shown in Figures 110A and 110B. Isoprene synthase activity is confirmed using a headspace assay.

[0480] v) Production strains

[0481] Strains MCM331 and MCM333 are cotransformed with plasmids pCLPtrcupperpathway and either pTrcKudzu or pETKudzu, resulting in the strains shown in Table 8-3.

Table 8-3. Production Strains

Background	Integrated Lower	Upper MVA plasmid	IpS 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.

[0482] Medium Recipe (per liter fermentation medium): The medium is 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 are added together and dissolved in diH2O. This solution is autoclaved. The pH is adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics are added after sterilization and pH adjustment.

[0483] 1000X Modified Trace Metal Solution: The 1000X modified trace metal solution is generated using the following components: citric acid $* H_2O$ 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 is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0484] Fermentation is 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 is 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 is streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into tryptone-yeast

extract medium. After the inoculum grows to OD 1.0, measured at 550 nm, 500 mL is used to inoculate a 15-L bioreactor containing an initial working volume of 5 L.

[0485] Glucose is fed at an exponential rate until cells reach the stationary phase. After this time, the glucose feed is decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 57 hour fermentation is 3.9 kg. Induction is achieved by adding IPTG. The IPTG concentration is brought to 100 uM when the carbon dioxide evolution rate reaches 100 mmol/L/hr. The OD_{550} profile within the bioreactor over time is shown in Fig. 111A. The isoprene level in the off gas from the bioreactor is determined as described herein. The isoprene titer increases over the course of the fermentation to a final value of 1.6 g/L (Fig. 111B). The specific productivity of isoprene over the course of the fermentation is shown in Fig. 111C and peaks at 1.2 mg/OD/hr. The total amount of isoprene produced during the 57 hour fermentation is 16.2 g. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 0.9%. The weight percent yield of isoprene from glucose is 0.4%.

XIV. Production of isoprene from *E. coli* BL21 containing the kudzu isoprene synthase using glycerol as a carbon source

[0486] A 15-L scale fermentation of *E. coli* expressing Kudzu isoprene synthase is 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) results in the production of 2.2 mg/L of isoprene.

[0487] Medium Recipe (per liter fermentation medium): The medium is 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 are added together and dissolved in diH_2O. This solution is autoclaved. The pH is adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glycerol 5.1 g, thiamine * HCl 0.1 g, and antibiotics are added after sterilization and pH adjustment.

[0488] 1000X Modified Trace Metal Solution: The medium is generated using the following components per liter fermentation medium: citric acid * 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 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.

[0489] Fermentation is performed in a 15-L bioreactor with BL21(DE3) *E. coli* cells containing the pTrcKudzu plasmid. This experiment is 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 is streaked onto an LA broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into soytone-yeast extract-glucose medium and grown at 35°C. After the inoculum grows to OD 1.0, measured at 550 nm, 600 mL is used to inoculate a 7.5-L bioreactor.

[0490] Glycerol is fed at an exponential rate until cells reach an optical density at 550 nm (OD_{550}) of 153. The total amount of glycerol delivered to the bioreactor during the 36 hour fermentation is 1.7 kg. Other than the glucose in the inoculum, no glucose is added to the bioreactor. Induction is achieved by adding IPTG. The IPTG concentration is 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 Fig. 57. The isoprene level in the off gas from the bioreactor is determined as described herein. The isoprene titer increases over the course of the fermentation to a final value of 2.2 mg/L (Fig. 58). The total amount of isoprene produced during the 54 hour fermentation is 20.9 mg, and the time course of production is shown in Fig. 59.

[0491] 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

[0492] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase is 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 results in the production of 2.4 g/L of isoprene.

[0493] Medium Recipe (per liter fermentation medium): The medium is 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 are added together and dissolved in diH_2O. This solution is autoclaved. The pH is adjusted to 7.0 with ammonium hydroxide

(30%) and q.s. to volume. Invert sugar 10 g, thiamine * HCl 0.1 g, and antibiotics are added after sterilization and pH adjustment.

[0494] 1000X Modified Trace Metal Solution: The 1000X modified trace metal solution is generated using the following components: citric acid * 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 is dissolved one at a time in Di H2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0495] Fermentation is performed in a 15-L bioreactor with BL21(DE3) *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment is 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 is streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into tryptone-yeast extract medium. After the inoculum grows to OD 1.0, measured at 550 nm, 500 mL is used to inoculate a 15-L bioreactor containing an initial working volume of 5 L.

[0496] Invert sugar is fed at an exponential rate until cells reached the stationary phase. After this time the invert sugar feed is decreased to meet metabolic demands. The total amount of invert sugar delivered to the bioreactor during the 44 hour fermentation is 2.4 kg. Induction is achieved by adding IPTG. The IPTG concentration is brought to 25 uM when the optical density at 550 nm (OD_{550}) reaches a value of 9. The IPTG concentration is raised to 50 uM when OD_{550} reaches 200. The OD_{550} profile within the bioreactor over time is shown in Fig. 96. The isoprene level in the off gas from the bioreactor is determined as described herein. The isoprene titer increases over the course of the fermentation to a final value of 2.4 g/L (Fig. 97). The total amount of isoprene produced during the 44 hour fermentation is 18.4 g and the time course of production is shown in Fig. 98. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 1.7%. The weight percent yield of isoprene from glucose is 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 *B. subtilis*

[0497] 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.

[0498] Four DNA fragments are amplified by PCR such that they contain overhangs that will allow 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:82)

CF 07-94 (-) Fuse PaprE to *mvaE*

5'- CAATAATAACTACTGTTTTCACTCTTTACCCTCTCCTTTTAA (SEQ ID NO:83)

Template: *Bacillus subtilis* chromosomal DNA

2. *mvaE*

CF 07-93 (+) fuse *mvaE* to the *aprE* promoter (GTG start codon)

5'- TTAAAAGGAGAGGGTAAAGAGTGAAAACAGTAGTTATTATTG (SEQ ID NO:84)

CF 07-62 (-) Fuse *mvaE* to *mvaS* with RBS in between

5'- TTTATCAATCCCAATTGTCATGTTTTTTTACCTCCTTTATTGTTTTCTTAAATC (SEQ ID NO:35)

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:36)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator
5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:85)
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:86)

CF 07-46 (-) End of *B. amyliquefaciens* terminator BamHI
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:63)

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:84)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator
5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:85)

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:82)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator
5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:85)

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:82)

CF 07-46 (-) End of *B. amyliquefaciens* terminator BamHI
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:63)

Template: #4 and #6

[0499] 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, DC) 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). 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.

[0500] 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. This strain is used subsequently as a host for the integration of the lower mevalonic acid pathway.

[0501] 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:82)

CF 07-58 (+) Start of *mvaE* gene

5'- ATGAAAACAGTAGTTATTATTGATGC (SEQ ID NO:38)

CF 07-59 (-) End of *mvaE* gene

5'- ATGTTATTGTTTTCTTAAATCATTTAAAATAGC (SEQ ID NO:39)

CF 07-82 (+) Start of *mvaS* gene

5'- ATGACAATTGGGATTGATAAAATTAG (SEQ ID NO:40)

CF 07-83 (-) End of *mvaS* gene

5'- TTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:41)

CF 07-86 (+) Sequence in *mvaE*

5'- GAAATAGCCCCATTAGAAGTATC (SEQ ID NO:42)

CF 07-87 (+) Sequence in *mvaE*

5'- TTGCCAATCATATGATTGAAAATC (SEQ ID NO:43)

CF 07-88 (+) Sequence in *mvaE*

5'- GCTATGCTTCATTAGATCCTTATCG (SEQ ID NO:44)

CF 07-89 (+) Sequence *mvaS*

5'- GAAACCTACATCCAATCTTTTGCCC (SEQ ID NO:45)

[0502] 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*

[0503] 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). 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

[0504] A 14 L scale fermentation is 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 is 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.

[0505] Headspace analysis is 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 is used for sampling 500 uL aliquots from 20 mL headspace vials. The GC/MS method utilizes helium as the carrier gas at a flow of 1 mL/min. The injection port is held at 250°C with a split ratio of 50:1. The oven temperature is held at 37°C for an initial 2 minute period, followed by an increase to 237°C at a rate of 25°C/min for a total method time of 10

minutes. The Agilent 5793N mass selective detector scans 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.

[0506] The off-gas consists 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 is not determined but is estimated to be equal to the equilibrium vapor pressure at 0°C. The composition of the volatile organic fraction is determined by integration of the area under the peaks in the GC/MS chromatogram (Figs. 86A and 86B) and is listed in Table 10-1. Calibration curves for ethanol and acetone standards enable the conversion of GC area to gas phase concentration in units of ug/L using standard methods.

Table 10-1. Composition of volatile organic components in fermentation off-gas. The off-gas is 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

[0507] A 14 L scale fermentation is 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.

[0508] Fermentation off-gas is passed through cooled headspace vials in order to concentrate and identify trace volatile organic components. The off-gas from this fermentation is 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°C with dry ice. The vial is 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°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°C.

[0509] 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 10-2 and 10-3.

Table 10-2: 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 Area*[1]	*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-buten-1-yl_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 10-3. 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 Area*[1]	*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
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.

[0510] Cryo-trapping of isoprene present in fermentation off-gas is performed using a 2 mL headspace vial cooled in liquid nitrogen. The off-gas (1 L/min) is 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°C). Approximately 10L of off-gas is passed through the

vial, after which it is allowed to warm to -78°C with venting, followed by resealing with a fresh vial cap and analysis by GC/MS.

[0511] GC/MS headspace analysis is 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) is used for separation of analytes. The GC autoinjector is fitted with a gas-tight 100 uL syringe, and the needle height is adjusted to allow the injection of a 50 uL headspace sample from a 2 mL GC vial. The GC/MS method utilizes helium as the carrier gas at a flow of 1 mL/min. The injection port is held at 200°C with a split ratio of 20:1. The oven temperature is held at 37°C for the 5 minute duration of the analysis. The Agilent 5793N mass selective detector is run in single ion monitoring (SIM) mode on m/z 55, 66, 67 and 70. Under these conditions, isoprene is observed to elute at 2.966 minutes (Fig. 88B). A standard of petroleum derived isoprene (Sigma-Aldrich) is also analyzed using this method and is found to contain additional C5 hydrocarbon isomers, which elute shortly before or after the main peak and are quantified based on corrected GC area (Fig. 88A).

Table 10-4: GC/MS analysis of petroleum-derived isoprene

Compound	*RT (min)*	*GC area*	*Area % of total C5 hydrocarbons*
2-methyl-1-butene	2.689	18.2×10^3	0.017%
(Z)-2-pentene	2.835	10.6×10^4	0.101%
Isoprene	2.966	10.4×10^7	99.869%
1,3-cyclopentadiene (CPD)	3.297	12.8×10^3	0.012%

Table 10-5: GC/MS analysis of fermentation-derived isoprene (% total C5 hydrocarbons)

Compound	*RT (min)*	*Corrected GC Area*	*% of total C5 hydrocarbons*
Isoprene	2.966	8.1×10^7	100%

[0512] In a separate experiment, a standard mixture of C5 hydocarbons is analyzed to determine if the detector response is the same for each of the compounds. The compounds are 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 is 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 utilizes helium as the carrier gas at a flow of 1 mL/min. The injection port is held at 200°C with a

split ratio of 50:1. The Agilent 5793N mass selective detector is run in full scan mode from m/z 19 to m/z 250. Under these conditions, a 100 ug/L concentration of each standard produces the same detector response within experimental error.

IV. Compositions comprising isoprene adsorbed to a solid phase.

[0513] Biologically-produced isoprene is 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.

[0514] Fermentation off-gas is run through a copper condensation coil held at 0°C, followed by a granulated silica desiccant filter in order to remove water vapor. The dehumidified off-gas is then run through carbon containing filters (Koby Jr, Koby Filters, MA) to the point at which breakthrough of isoprene is 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.

[0515] V. Collection and analysis of condensed isoprene.

[0516] 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.

[0517] VI. Production of prenyl alcohols by fermentation

[0518] Analysis of off-gas from an *E. coli* BL21(DE3) strain expressing a kudzu isoprene synthase reveals 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 Fig. 89 as determined by headspace GC/MS. Levels of isoprenol (3-methyl-3-buten-1-ol, 3-MBA) attained are nearly 10 ug/L_{offgas} in this experiment. Additional experiments produce levels of approximately 20 ug/L_{offgas} in the fermentation off-gas.

[0519] Example 11: De-Coupling of Growth and Production of Isoprene in *E. coli* Expressing Genes of the Mevalonic Acid Pathway and Fermented in a Fed-Batch Culture

[0520] This example illustrates the de-coupling of cell growth from mevalonic acid and isoprene production.

I. Fermentation Conditions

[0521] Medium Recipe (per liter fermentation medium): The medium is 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 are added together and dissolved in diH2O. This solution is autoclaved. The pH is adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics are added after sterilization and pH adjustment.

[0522] 1000X Modified Trace Metal Solution: The 1000X modified trace metal solution is generated using the following components: citric acid * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, CoCl2 * $6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0523] Fermentation is performed with *E. coli* cells containing the pTrcHis2AUpperPathway (also called pTrcUpperMVA, FIGs. 91 and 92A-92C) (50 μg/ml carbenicillin) or the pCL PtrcUpperMVA (also called pCL PtrcUpperPathway (Fig. 26)) (50 μg/ml spectinomycin) plasmids. For experiments in which isoprene is produced, the *E. coli* cells also contain the pTrc KKDyIkIS (50 μg/ml kanamycin) plasmid. These experiments are 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 is streaked onto an LA broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into tryptone-yeast extract medium. After the inoculum grows to optical density 1.0 when measured at 550 nm, it is used to inoculate the bioreactor.

[0524] Glucose is fed at an exponential rate until cells reach the stationary phase. After this time the glucose feed is decreased to meet metabolic demands. Induction is achieved by

adding IPTG. The mevalonic acid concentration in fermentation broth is 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 is 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 is 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

[0525] BL21(DE3) cells that are grown on a plate as explained above in part I are 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 is transferred to a 5-L bioreactor of tryptone-yeast extract medium, and the cells are grown at 30 °C and 27.5 rpm until the culture reaches an OD_{550} of 1.0. The 5 L of inoculum is seeded into a 150-L bioreactor containing 45-kg of medium. The IPTG concentration is brought to 1.1 mM when the OD_{550} reaches a value of 10. The OD_{550} profile within the bioreactor over time is shown in Fig. 60A. The mevalonic acid titer increases over the course of the fermentation to a final value of 61.3 g/L (Fig. 60B). The specific productivity profile throughout the fermentation is shown in Fig. 60C and a comparison to Fig. 60A illustrates the de-coupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 52.5 hour fermentation is 4.0 kg from 14.1 kg of utilized glucose. The molar yield of utilized carbon that goes into producing mevalonic acid during fermentation is 34.2%.

III. Mevalonic acid production from *E. coli* BL21(DE3) cells expressing the pTrcUpperMVA plasmid at a 15-L scale

[0526] BL21(DE3) cells that are grown on a plate as explained above in part I are 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 is seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration is brought to 1.0 mM when the OD_{550} reaches a value of 10. The OD_{550} profile within the bioreactor over time is shown in Fig. 61A. The mevalonic acid titer increases over the course of the fermentation to a final value of 53.9 g/L

(Fig. 61B). The specific productivity profile throughout the fermentation is shown in Fig. 61C and a comparison to Fig. 61A illustrates the de-coupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 46.6 hour fermentation is 491 g from 2.1 kg of utilized glucose. The molar yield of utilized carbon that goes into producing mevalonic acid during fermentation is 28.8%.

IV. Mevalonic acid production from *E. coli* FM5 cells expressing the pTrcUpperMVA plasmid at a 15-L scale

[0527] FM5 cells that are grown on a plate as explained above in part I are 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 is seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration is 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 Fig. 62A. The mevalonic acid titer increases over the course of the fermentation to a final value of 23.7 g/L (Fig. 62B). The specific productivity profile throughout the fermentation is shown in Fig. 62C and a comparison to Fig. 62A illustrates the de-coupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 51.2 hour fermentation is 140 g from 1.1 kg of utilized glucose. The molar yield of utilized carbon that went into producing mevalonic acid during fermentation is 15.2%.

V. Isoprene production from *E. coli* BL21(DE3) cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0528] BL21(DE3) cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that are grown on a plate as explained above in part I are 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 is seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration is brought to 25 μM when the OD_{550} reaches a value of 10. The IPTG concentration is raised to 50 uM when OD_{550} reaches 190. The IPTG concentration is raised to 100 uM at 38 hours of fermentation. The OD_{550} profile within the bioreactor over time is shown in Fig. 63A. The isoprene titer increases over the course of the fermentation to a final value of 2.2 g/L broth (Fig. 63B). The specific productivity profile throughout the fermentation is shown in Fig. 63C and a comparison to Fig. 63A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 54.4 hour

fermentation is 15.9 g from 2.3 kg of utilized glucose. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 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

[0529] BL21(DE3) tuner cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that are grown on a plate as explained above in part I are 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 is seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration is brought to 26 μM when the OD_{550} reaches a value of 10. The IPTG concentration is raised to 50 uM when OD_{550} reaches 175. The OD_{550} profile within the bioreactor over time is shown in Fig. 64A. The isoprene titer increases over the course of the fermentation to a final value of 1.3 g/L broth (Fig. 64B). The specific productivity profile throughout the fermentation is shown in Fig. 64C and a comparison to Fig. 64A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 48.6 hour fermentation is 9.9 g from 1.6 kg of utilized glucose. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 1.34%.

VII. Isoprene production from *E. coli* MG1655 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0530] MG1655 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that are grown on a plate as explained above in part I are 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 is seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration is brought to 24 μM when the OD_{550} reaches a value of 45. The OD_{550} profile within the bioreactor over time is shown in Fig. 65A. The isoprene titer increases over the course of the fermentation to a final value of 393 mg/L broth (Fig. 65B). The specific productivity profile throughout the fermentation is shown in Fig. 65C and a comparison to Fig. 65A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 67.4 hour fermentation is 2.2 g from 520 g of utilized glucose. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 0.92%.

VIII. Isoprene production from *E. coli* MG1655ack-pta cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0531] MG1655ack-pta cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that are grown on a plate as explained above in part I are 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 is seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration is brought to 30 μM when the OD_{550} reaches a value of 10. The OD_{550} profile within the bioreactor over time is shown in Fig. 66A. The isoprene titer increases over the course of the fermentation to a final value of 368 mg/L broth (Fig. 66B). The specific productivity profile throughout the fermentation is shown in Fig. 66C and a comparison to Fig. 66A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 56.7 hour fermentation is 1.8 g from 531 g of utilized glucose. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 0.73%.

IX. Isoprene production from *E. coli* FM5 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0532] FM5 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that are grown on a plate as explained above in part I are 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 is seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration is brought to 27 μM when the OD_{550} reaches a value of 15. The OD_{550} profile within the bioreactor over time is shown in Fig. 67A. The isoprene titer increases over the course of the fermentation to a final value of 235 mg/L broth (Fig. 67B). The specific productivity profile throughout the fermentation is shown in Fig. 67C and a comparison to Fig. 67A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 52.3 hour fermentation is 1.4 g from 948 g of utilized glucose. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 0.32%.

Example 12: Production of Isoprene during the Exponential Growth Phase of *E. coli* Expressing Genes of the Mevalonic Acid Pathway and Fermented in a Fed-Batch Culture

[0533] This example illustrates the production of isoprene during the exponential growth phase of cells.

[0534] Medium Recipe (per liter fermentation medium): The medium is 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 are added together and dissolved in diH_2O. This solution is autoclaved. The pH is adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics are added after sterilization and pH adjustment.

[0535] 1000X Modified Trace Metal Solution: The 1000X modified trace metal solution is generated using the following components: citric acid * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, CoCl2 * $6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in Di H2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0536] Fermentation is performed in a 15-L bioreactor with ATCC11303 *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment is 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 is streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into tryptone-yeast extract medium. After the inoculum grows to OD 1.0, measured at 550 nm, 500 mL is used to inoculate a 15-L bioreactor containing an initial working volume of 5 L.

[0537] Glucose is fed at an exponential rate until cells reach the stationary phase. After this time the glucose feed is decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 50 hour fermentation is 2.0 kg. Induction is achieved by adding IPTG. The IPTG concentration is brought to 25 uM when the optical density at 550 nm (OD_{550}) reaches a value of 10. The IPTG concentration is raised to 50 uM when OD_{550} reaches 190. The OD_{550} profile within the bioreactor over time is shown in Fig. 99. The isoprene level in the off gas from the bioreactor is determined as described herein. The isoprene titer increases over the course of the fermentation to a final value of 1.4 g/L (Fig.

100). The total amount of isoprene produced during the 50 hour fermentation is 10.0 g. The profile of the isoprene specific productivity over time within the bioreactor is shown in Fig. 101. The molar yield of utilized carbon that contributes to producing isoprene during fermentation is 1.1%. The weight percent yield of isoprene from glucose is 0.5%.

Example 13: Flammability Modeling and Testing of Isoprene

I. Summary of flammability modeling and testing of isoprene

[0538] Flammability modeling and experiments are performed for various hydrocarbon/oxygen/nitrogen/water/carbon dioxide mixtures. This modeling and experimental tested is 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 13-1, and a matrix of the experiments performed is shown in Table 13-2.

Table 13-1. Summary of Modeled Isoprene Flammability

Series	Temperature (°C)	Pressure (psig)	Steam Concentration (wt %)	CO_2 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 13-2. 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

[0539] Calculated adiabatic flame temperatures (CAFT) along with a selected limit flame temperature for combustion propagation are 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.

[0540] 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.

[0541] 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 are present in the program database. The program database does not have isoprene as a species; therefore the thermodynamic properties are incorporated manually.

[0542] 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 is 1 atmosphere (absolute) and the temperature is 40°C, the boiling point of isoprene.

[0543] The limit flame temperature for combustion can be either selected based on theoretical principles or determined experimentally. Each method has its own limitations.

[0544] 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 is 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.

[0545] 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.

[0546] 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

[0547] 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.

[0548] Using Series A as an example the data in Fig. 68 can be plotted in the form of a traditional flammability envelope. Using Fig. 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 (Fig. 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.

[0549] 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 (Fig. 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 Fig. 69. A similar approach can be used on data presented in Figs. 70-74.

IV. Flammability testing experimental equipment and procedure

[0550] Flammability testing is conducted in a 4 liter high pressure vessel. The vessel is 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) is maintained using external heaters that are controlled by a PID controller. To prevent heat losses, ceramic wool and reflective insulation are wrapped around the pressure vessel. Type K thermocouples are used the measure the temperature of the gas space as well as the temperature of the vessel itself. Fig. 77 illustrates the test vessel.

[0551] Before a test is ran, the vessel is evacuated and purged with nitrogen to ensure that any gases from previous tests are removed. A vacuum is then pulled on the vessel. The pressure after this had been done is typically around 0.06 bar(a). Due to the nitrogen purging, the gas responsible for this initial pressure is assumed to be nitrogen. Using partial pressures, water, isoprene, nitrogen, and oxygen are then added in the appropriate amounts to achieve the test conditions in question. A magnetically driven mixing fan within the vessel ensures mixing of the gaseous contents. The gases are allowed to mix for about 2 minutes with the fan being turned off approximately 1 minute prior to ignition.

[0552] The igniter is comprised of a 1.5 ohm nicrome coil and an AC voltage source on a timer circuit. Using an oscilloscope, it is determined that 34.4 VAC are delivered to the igniter for 3.2 seconds. A maximum current of 3.8 amps occurs approximately halfway into the ignition cycle. Thus, the maximum power is 131 W and the total energy provided over the ignition cycle is approximately 210 J.

[0553] Deflagration data is acquired using a variable reluctance Validyne DP215 pressure transducer connected to a data acquisition system. A gas mixture is considered to have deflagrated if the pressure rise was greater than or equal to 5%.

V. Results of flammability testing

[0554] The first experimental series (Series 1) is run at 40°C and 0 psig with no steam. Running tests at varying concentrations of isoprene and oxygen produces the flammability curve shown in Fig. 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.

[0555] Fig. 78B summarizes the explosibility data points shown in Fig. 78A. Fig. 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.

[0556] 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.

[0557] The second experimental series (Series 2) is run at 40°C and 0 psig with a fixed steam concentration of 4%. Running tests at varying concentrations of isoprene and oxygen produces the flammability curve shown in Fig. 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 Fig. 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 are tested. The addition of 4% steam to the test mixture does 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.

[0558] Fig. 79B summarizes the explosibility data points shown in Fig. 79A. Fig. 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

[0559] The methods described in Example 12, parts I to IV are also used to calculate the flammability limits of isoprene at an absolute system pressure of 3 atmospheres and 40°C. These results are compared to those of Example 12, parts I to IV at an absolute system pressure of 1 atmosphere and 40°C. This higher pressure is 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, in particular with respect to the calculation of flammability limits).

[0560] 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 (Fig. 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

[0561] A calculated adiabatic temperature model is developed for the flammability envelope of the isoprene/oxygen/nitrogen/water/ carbon dioxide system at 40°C and 0 psig. The CAFT model that is developed agrees well with the experimental data generated by the tests conducted in this work. The experimental results from Series 1 and 2 validate the model results from Series A and B.

Example 14: Archaeal Mevalonate Kinase Expression Constructs and Recombinant Bacterial Host Strains

I. Construction of plasmids encoding mevalonate kinase.

[0562] 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) is synthesized with codon optimization for expression in *E. coli*. This construct is named *M. mazei* archaeal Lower Pathway operon (Figures 112A-112C) and encodes *M. mazei* MVK, decarboxylase, IPK, and IDI enzymes.

The gene encoding MVK (Accession number NC_003901.1) is PCR amplified using primers MCM165 and MCM177 (Table 14-1) 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 is 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 are added for an additional hour at 37°C. The digested DNA is purified over a Qiagen PCR column and ligated to a similarly digested and purified plasmid MCM29 (MCM29 is also called pTrcKudzuKan) in an 11uL 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). The ligation reaction is 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 is sequenced (Figures 113A-113C).

Table 14-1. Oligonucleotides

Primer (Description)	**Sequence**
MCM161 (*M. mazei* MVK for)	CACCATGGTATCCTGTTCTGCG (SEQ ID NO:105)
MCM162 (*M. mazei* MVK rev)	TTAATCTACTTTCAGACCTTGC (SEQ ID NO:106)
MCM165 (*M. mazei* MVK for w/ RBS)	gcgaacgATGCATaaaggaggtaaaaaaacATGGTATCC TGTTCTGCGCCGGGTAAGATTTACCTG (SEQ ID NO:107)
MCM177 (*M. mazei* MVK rev Pst)	gggcccgtttaaactttaactagactTTAATCTACTTTCAGA CCTTGC (SEQ ID NO:108)

II. Creation of strains overexpressing mevalonate kinase and isoprene synthase.

[0563] Plasmid MCM382 is transformed into MCM331 cells (which contain 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. 1 μL of DNA is added to 50 μL of cell suspension, and this mixture is 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 is 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).

Table 14-2. Strains Overexpressing Mevalonate Kinase and Isoprene Synthase

Strain	**Genotype**
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.

[0564] The MVK ORF from the *M. mazei* archeal Lower Pathway operon (Figures 112A-112C) is PCR amplified using primers MCM161 and MCM162 (Table 14-1) using the Invitrogen Platinum HiFi PCR mix. 45 uL of PCR mix is 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 is ligated to Invitrogen pET200D plasmid according to the manufacturer's protocol. 3 uL of this ligation is introduced into Invitrogen TOP10 cells, and transformants are selected on LA/kan50. A plasmid from a transformant is isolated and the insert sequenced, resulting in MCM376 (Figures 114A-114C).

V. Creation of expression strain MCM378.

[0565] Plasmid MCM376 is transformed into Invitrogen BL21(DE3) pLysS cells according to the manufacturer's protocol. Transformant MCM378 is selected on LA/kan50.

Example 15: Production of Isoprene by *E. coli* expressing the Upper and Integrated Lower MVA Pathway, Mevalonate Kinase from *M. mazei,* and Isoprene Synthase from Kudzu

[0566] Medium Recipe (per liter fermentation medium): Each liter of fermentation medium contains 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 are added together and dissolved in diH_2O. This solution is autoclaved. The pH is 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.

[0567] 1000X Modified Trace Metal Solution: 1000X Modified Trace Metal Solution contains citric acid * 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 is dissolved one at a time in DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0568] Fermentation is 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 is 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 is streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony is inoculated into tryptone-yeast extract medium. After the inoculum grows to OD 1.0, measured at 550 nm, 500 mL is used to innoculate 5-L of cell medium in the 15-L bioreactor. The liquid volume increases throughout the fermentation (such as to approximately 10 liters).

[0569] Glucose is fed at an exponential rate until cells reach the stationary phase. After this time the glucose feed is decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 55 hour fermentation is 2.2 kg. Induction is achieved by adding IPTG. The IPTG concentration is brought to 51 uM when the optical density at 550 nm (OD_{550}) reaches a value of 10. In addition to the IPTG spike, at $OD_{550} = 10$ a constant feed begins and delivers 164 mg of IPTG over 18 hours. The OD_{550} profile within the bioreactor over time is shown in Figure 115A. The isoprene level in the off gas from the bioreactor is determined using a Hiden mass spectrometer. The isoprene titer increases over the course of the fermentation to a final value of 22.0 g/L as shown in Figure 115B. The total

amount of isoprene produced during the 55 hour fermentation is 170.5 g and the time course of production is shown in Figure 115C. The molar yield of utilized carbon that goes into producing isoprene during fermentation is 16.6%. The weight percent yield of isoprene from glucose over the entire fermentation is 7.7%.

Example 16: Recovery of Prenyl Alcohols from Fermentation Off-Gas

[0570] This example provides several suitable methods for recovery of prenyl alcohols for subsequent conversion to isoprene by chemical or biological catalysis. In addition to the gas-stripping, two-phase, and pervaporation recovery methods described below, the prenyl alcohols can be recovered by distillation as known in the art for commercial recovery of ethanol and butanol.

I. Prenyl Alcohol Recovery via Gas Stripping

[0571] A fermentation reaction is carried out in a reactor. When the prenyl alcohol concentration reaches the desired level, gas stripping is applied by introducing a stripping gas into the reactor. After passing through the reaction medium, the stripping gas enriched in prenyl alcohols is passed through a condenser where prenyl alcohol vapors are collected. The stripping gases are recycled through the system using a pump. Distilled water is added into the reactor at intervals to compensate for water loss due to gas-stripping and to maintain a constant liquid level inside the reactor. Continuous operation is maintained at optimum flow rates to give the desired level of prenyl alcohol removal.

[0572] Alternatively, the prenyl alcohols are recovered as the carboxylic acid ester derivatives, for example as prenyl acetate and isoprenyl acetate. Such ester derivatives are formed biologically, as detailed above in Example 10 (See, e.g., Table 10-2), by the action of an acyltransferase and an acyl donor on the prenyl alcohols). Both prenyl acetate and isoprenyl acetate are collected from the fermentation off-gas by gas stripping.

II. Prenyl Alcohol Recovery via Two-Phase Recovery

[0573] A fermentation reaction is carried out in a two-phase partitioning bioreactor. An organic solvent is added to 20% (v/v) of the media volume. Suitable organic solvents are high boiling solvents with low water solubility and low toxicity including but not limited to dodecane, paraffin oil, silicone oils and fluorocarbons. Alternatively, a triglyceride is used to

serve a dual purpose as a both a carbon source and a second organic phase. After the prenyl alcohol concentration reaches the desired level in the organic phase, the organic and aqueous layers are separated, and the prenyl alcohols are isolated by evaporation of the organic solvent.

III. Prenyl Alcohol Recovery via Pervaporation

[0574] A fermentation reaction is carried out in a reactor. The liquid stream from the fermentation reactor is first passed through a heating module. The heated liquid is then passed into the pervaporation module equipped with a commercial polydimethylsiloxane membrane. A vacuum is applied to the other side of the membrane. The prenyl alcohols permeate through the membrane and evaporate into the vapor phase on the other side of the membrane. The prenyl alcohol enriched vapor phase is passed through a condenser to collect the liquid prenyl alcohols.

Example 17: Chemical Conversion of Biologically-Produced Isoprenol to Isoprene

[0575] This example describes the production of isoprene from prenyl alcohols recovered from recombinant host cells. Briefly as described above in Example 10, 3-methyl-3-buten-1-ol (CAS Registry No. 763-32-6, isoprenol) and 3-methyl-2-buten-1-ol (CAS Registry No. 556-82-1, prenol) are produced by recombinant *E. coli* engineered to express enzymes of the full mevalonate pathway (MVAE and MVAS from *E. faecalis;* and MVK, PMK, and MVD from *S. cerevisia*), an isoprenyl pyrophosphate isomerase (IDI from *S. cerevisia*) and an isoprene synthase (IS from *P. montana*). In particular, a significant amount of isoprenol is found in the trace volatile fraction of the bacterial fermentation off-gas following cryo-trapping at -78°C (See, Table 10-2). However, the compositions and methods of the present invention for chemical conversion of prenyl alcohol to isoprene are not limited to the use of this particular cell line. Other suitable compositions and methods for producing isoprene and for producing prenyl alcohols are provided by U.S. Application No. 61/134,094, filed July 2, 2008 (Attorney Docket No. 24625-30003200, now 64384-3000400) herein incorporated by reference in its entirety.

[0576] In alternative embodiments, isoprenol and/or prenol are produced by recombinant *E. coli* containing plasmids encoding the lower mevalonate pathway (MK, PMK, and MVD from *S. cerevisiae*), in the presence or absence of an isoprenyl pyrophosphate isomerase (IDI from *S. cerevisia*), and in the presence of either an ADP-ribose pyrophosphatase (nudF from

B. subtilis) or another enzyme that utilizes IPP as a substrate (yhfR from *B. subtilis* having homology to a phosphoglycerase mutase). In particular, 3-methyl butenol is found in the organic phase of the liquid culture after extraction with ethyl acetate. However, the compositions and methods of the present invention for chemical conversion of prenyl alcohol to isoprene are not limited to the use of this particular cell line. Other suitable compositions and methods for producing isoprene and for producing prenyl alcohols are provided by U.S. Publication No. 2008/0092829 of Renninger et al., herein incorporated by reference in its entirety.

[0577] The C5 prenyl alcohols, are produced by recombinant bacterial cells engineered to express at least the lower portion of the mevalonate pathway, is recovered from the fermentation off-gas or from the liquid culture of a shake flask. In preferred embodiments the recombinant bacterials cells are further engineered to express a phosphatase for removal of the pyrophosphate group from IPP or DMAPP for subsequent conversion into isoprenol to prenol respectively. Suitable enzymes for catalyzing conversion of IPP or DMAPP into a C5 prenyl alcohol include but are not limited to an allyl diphosphatase, an ADP-sugar pyrophosphatase, an ADP-sugar phophorylase, a nucleoside triphosphate pyrophosphatase, a FAD pyrophosphatase, a monoterpenyl pyrophosphastase, an alkaline phosphatase, an acid phosphatase, and other phosphatases classified under enzyme commission classes 3.6.1, 3.1.7 or 3.1.3.

[0578] Production of isoprene from isoprenol is accomplished by dehydration of isoprenol in an acidic salt (e.g., HCl/NaCl) solution as known in the art (Weitz and Loser, "Isoprene," in Ullmann's Encyclopedia of Industrial Chemistry, 7th edition, electronic release, Wiley-VCH Verlag GMBH, Weinheim, pp. 1-20, 2005; and U.S. Patent No. 3,792,104 to Mueller). Briefly, a 400 parts sodium chloride, 600 parts water and 40 parts of a 35% hydrochloric acid solution is placed in a reaction (e.g., dehydration) vessel equipped with a reflux condenser (operated at 45°C), which is connected to a descending condenser (operated at -10°C). The descending condenser causes condensation of the isoprene product and the isopentenols.

[0579] About 0.45 part/hr (dehydration rate of the system) of 3-methyl-3-buten-1-ol is metered to the stirred dehydration vessel, which is heated at 95°C. The hourly output is about 0.350 part of condensate, which consists of 0.33 part isoprene, 0.01 part dimethyvinyl carbinol and 0.01 part water. The aqueous phase is continuously recycled to the dehydration vessel, while the organic phase is neutralized with a concentrated aqueous sodium carbonate

solution and the separated to pure isoprene and isopentonols in a continuous fractionating column. The aqueous phase is removed from the dehydrator at intervals of one hour to keep the liquid at its original level. Common salt and hydrochloric acid are replenished. At one hour intervals, the higher-boiling oligomers are removed from the dehydrator. Alternatively, prenol in the off-gas is continuously converted to isoprene by passing the off-gas over a suitable heterogenous catalyst.

Example 18: Biological Conversion of Dimethyl Allyl Alcohol to Isoprene by Recombinant Bacteria

[0580] This example describes the conversion of dimethyl allyl alcohol to isoprene by recombinant *E. coli.*

[0581] The bacterial host cells used in this example are of the MCM343 strain, which is a production strain having a BL21(DE3) background that expresses heterologous integrated lower and upper MVA pathway enzymes as well as kudzu isoprene synthase. The MCM343 strain is grown in TM3 containing spectinomycin (50 μg/ml) and kanamycin (50 μg/ml) at 37°C with shaking at 200 rpm to an OD_{600} of 0.6 and then induced with 400 μM IPTG. The cultures are allowed to grow for a further 5 hours at 30°C. An 800 μL aliquot is placed in a GC headspace vial and dimethyl allyl alcohol (DMAPP-OL) is added (12 mM). The vial is sealed and incubated at 30°C for a further 14 hours. The isoprene is then measured using the Headspace assay.

[0582] Results are shown as compared to no alcohol (control) added and to DMAPP added to the culture (Fig. 116). Interestingly addition of DMAPP and DMAPP-OL increase the amount of isoprene obtained from the culture. The control cells produce 142 μg/L of isoprene compared to 202 μg/L produced by the cells to which DMAPP-OL was added. This demonstrates a 25% increase in isoprene in cultures supplemented with dimethyl allyl alcohol.

Example 19: Biological Conversion of Dimethyl Allyl Alcohol to Isoprene by Recombinant Yeast

[0583] This example describes the conversion of dimethyl allyl alcohol to isoprene by recombinant *S. cerevisiae.*

I. Expression of isoprene synthase and production of isoprene in *S. cerevisiae*

[0584] The kudzu isoprene synthase enzyme is optimized for expression according to a hybrid *Saccharomyces cerevisia/Pichia pastoris* codon usage table, synthesized, and cloned into pDONR221:19430 by DNA 2.0. A GATEWAY® Cloning (Invitrogen) reaction is performed according to the manufacturer's protocol. Since pDONR221:19430 is an "entry" vector, the LR Clonase II enzyme (the LR Reaction) is used to introduce the codon-optimized isoprene synthase into the "destination" vector pYES-DEST52 (Invitrogen). The LR Reaction is 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 are selected for on LA plates containing 50 μg/ml carbenicillin. Individual positive transformants are tested by colony PCR using illustra PuReTaq READY-TO-GO™ PCR Beads (GE Healthcare) with the T7 forward primer and the YeastHGS-Rev2 primer 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 is added to each tube containing the PCR mix. PCR cycling parameters were as follows: 1) 95°C – 4 min; 2) 95°C – 20 sec; 3) 52°C – 20 sec; 4) 72°C – 30 sec; 5 cycles of steps 2 through 4; 5) 95°C – 20 sec; 6) 55°C – 20 sec; 7) 72°C – 30 sec; 25 cycles of steps 5 through 7; 72°C – 10 min; and 4°C – end. Plasmids that yield a PCR fragment of the correct size (1354 bp) are purified by miniprep (Qiagen) and sent for sequencing (Quintara Biosciences, Berkeley, CA) with the T7 Forward and YeastHGS-For2 primers. The primers used are as follows: YeastHGS-For2: 5'-CACCAAAGAC TTCATAGACT-3' (SEQ ID NO:109); YeastHGSRev2: 5'-AGAGATATCT TCCTGCTGCT-3' (SEQ ID NO:110); and T7 forward, 5'-TAATACGACT CACTATAGGG-3' (SEQ ID NO:111). Results from sequencing runs are compared to the known sequence of pDONR221:19430 (using Vector NTI software, Invitrogen), and a single plasmid designated as pDW14 (Fig. 117A) is selected for further study. The kudzu isoprene synthase sequence of pDW14 diverges from that of pDONR221:19430 by a single nucleotide, which is marked in bold in the sequences of Fig. 117B (SEQ ID NO:112), and Fig. 117C-D (SEQ ID NO:113), respectively. The single nucleotide change (G to A) does not result in a change in the ORF, since it is in the third position of a lysine-encoding codon.

[0585] Purified pDW14 is transformed into *S. 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) are 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 are chosen for further analysis. To induce isoprene synthase expression, cultures are grown overnight in liquid SC minimal medium (as described above). The cultures are then diluted to an OD_{600} of approximately 0.2 and grown for 2-3 hours. Cultures are 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 is determined and strains are harvested by centrifugation and resuspended in 2 ml of lysis buffer (1:1 mix of 50% glycerol and PEB pH 7.4: tris base 2.423g/L, MgCl2 (Anhydrous) 1.904g/L, KCl 14.910g/L, DTT 0.154 g/L, glycerol 50 mL/L).

[0586] The lysis mixtures are passed through a French press three times, and lysates are analyzed by SDS-PAGE. For Coomassie gel analysis (Fig. 1118A), samples are 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 (Invitrogen Novex system). The WesternBreeze kit (Invitrogen) is used for transfer and chromogenic detection of isoprene synthase on a nitrocellulose membrane. The primary antibody is 1799A 10 week diluted 1:1000 in Invitrogen antibody diluent. All subsequent steps for Western detection are performed according to the manufacturer's protocol. Isoprene synthase is found to be present in the induced INVSc-1 strains harboring pDW14 (Fig. 118B, lanes 2 and 3) in comparison to the control harboring pYES-DEST52 (Fig. 1118B, lane 1). The DMAPP assay for HG headspace is performed on 25 µl of the lysate from each strain. DMAPP Assay Reagents (total volume of 100 µL) included: 25 µL lysate mixture; 5 µL $MgCl_2$ (1 M); 5 µL DMAPP (100mM); and 65 µL 50 mM Tris pH 8. The reaction was performed at 30°C for 15 minutes in a gas tight 1.8 mL GC tube. Reactions are terminated by the addition of 100 µL 250 mM EDTA (pH 8). The OD_{600} and the specific activity values (in µg isoprene/L/OD) of the induced strains harboring pDW14 are shown in comparison to the control (Fig. 119A and 119B). Induced strains harboring pDW14 display approximately 20X higher activity than the control lacking isoprene synthase.

II. Growth Conditions

[0587] INVSc-1 strains harboring pDW14 or the parent plasmid pYES-DEST52 (which contains an intact URA3 gene) are streaked from frozen glycerol stocks and grown for two

days at 30°C on SC minimal medium plates with 2% glucose without uracil, as described in the pYES-DEST52 Gateway Vector manual (Invitrogen). To induce isoprene synthase expression, cultures are inoculated from single colonies and grown overnight in liquid SC minimal medium with 2% glucose without uracil at 30°C. The cultures are then diluted to an OD_{600} of approximately 0.2 and grown for 4 hours. Cultures are spun by centrifugation, washed once, resuspended in an equal volume (10 ml) of SC minimal medium with 1% raffinose, 2% galactose without uracil (induction medium), and grown overnight to induce the expression of isoprene synthase. The following day, the culture of INVSc-1 harboring pYES-DEST52 is back-diluted in induction medium to the same OD_{600} as INVSc-1 harboring pDW14 (since induction of isoprene synthase at low initial OD_{600} prohibits growth compared to the control). Thus the OD_{600} of both strains prior to addition of isoprenol or dimethyl allyl alcohol in sealed 2 ml GC vials is approximately 2.0.

III. Assay

[0588] In the appropriate media, 200 μL of 5 mg/mL dimethyl allyl alcohol or isoprenol is prepared. Then, 800 μl of culture is added to a headspace vial along with 200 μL of media, media supplemented with prenol, or media supplemented with isoprenol. This is then incubated for 17 hours at 37°C and the isoprene (ug/L) is measured on the GC/MS as described for the Headspace Assay. Results are shown as compared to no alcohol (no alc) added (Fig. 120).

Example 20: Production of Isoprene using Cell Culture System for Overflow

[0589] This example describes the use of cells as an overflow system for producing isoprene. Isoprene is made by using a cell culture system which includes one or more substrates, such as isoprenyl derivatives. The cells used are any one of the cells described *supra*. The cells process the substrates to produce isoprene biologically using the MVA pathway and/or the DXP pathway. In some cases, such as when the isoprene production through the biological pathways is maxed out, the cells also produce other derivatives, including the isoprenyl derivatives as described herein. These isoprenyl derivatives are then chemically converted to isoprene using the chemical reactions known to one of skill in the art and/or as described herein. The isoprenyl derivatives produced by the cells are also recycled as substrates for the cells.

[0590] 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.

[0591] 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.

[0592] For use herein, unless clearly indicated otherwise, use of the terms "a", "an," and the like refers to one or more.

[0593] 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.

[0594] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments.

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 gi|2621082

Methanococcus jannaschii DSM 2661 gi|1590842 ;

Methanocaldococcus jannaschii gi|1590842

Methanothermobacter thermautotrophicus gi|2621082

Picrophilus torridus DSM9790 (IG-57) gi|48477569

Pyrococcus abyssi gi|14520758

Pyrococcus horikoshii OT3 gi|3258052

Archaeoglobus fulgidus DSM4304 gi|2648231

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

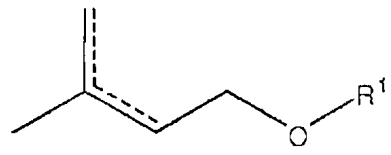
AJ457070

AY182241

CLAIMS:

1. A method of producing isoprene, the method comprising

(a) culturing cells for the production of a prenyl derivative of Formula (I):



(I);

wherein R^1 is hydrogen or $-C(O)R^2$; and R^2 is C_1-C_5 linear or branched alkyl; and wherein the cells comprise a gene that encodes a phosphatase and/or a pyrophosphatase in operable combination with a promoter;

(b) recovering said prenyl derivative; and

(c) dehydrating or decarboxylating said prenyl derivative to produce isoprene.

2. The method of claim 1, wherein the gene that encodes a phosphatase and/or pyrophosphatase is heterologous.

3. The method of claim 1, wherein the prenyl derivative is prenyl alcohol.

4. The method of claim 1, wherein the prenyl derivative is recovered from a fermentation of the cultured cells using one or more processes selected from the group consisting of distillation, gas- stripping, two-phase recovery, and pervaporation.

5. The method of claim 3, wherein the process for recovering prenyl alcohol from a fermentation broth of the cultured cells comprises gas- stripping.

6. The method of claim 3, wherein process for recovering prenyl alcohol from a fermentation of the cultured cells comprises two-phase recovery.

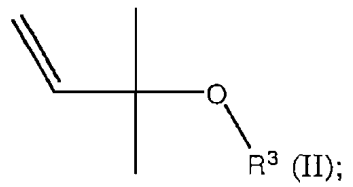
7. The method of claim 1, wherein the prenyl derivative comprises one or more of prenol, isoprenol, 3-methyl-3-buten-1-yl acetate, and 3-methyl-2-buten-1-yl acetate.

8. The method of claim 1, wherein the cells further comprise one or more of an IDI polypeptide, an MVA pathway enzyme, an isoprene synthase polypeptide, or a DXP pathway enzyme.

9. The method of claim 1, wherein a catalyst selected from the group consisting of an inorganic acid catalyst, HCl, HBr, H2SO4, H3PO4, a solid acid catalyst, and inorganic acid on an inert carrier, activated alumina, a zeolite, and organic acid catalyst, p-toluenesulfonic acid, trifluoromethane sulfonic acid, an organic acid resin, Nafion, and a fluoro sulfonic acid resin is used for dehydrating or decarboxylating said prenyl derivative to produce isoprene.

10. A method of producing isoprene, the method comprising

(a) culturing cells for the production of a prenyl derivative of Formula (II):



(II);

wherein R^3 is hydrogen or -C(O)R^4; and R^4 is C_1-C_5 linear or branched alkyl; and wherein the cells comprise a gene that encodes a phosphatase and/or a pyrophosphatase in operable combination with a promoter;

(b) recovering said prenyl derivative; and

(c) dehydrating or decarboxylating said prenyl derivative to produce isoprene.

11. The method of claim 1, wherein the gene that encodes a phosphatase and/or pyrophosphatase is heterologous.

12. The method of claim 10, wherein the prenyl derivative is recovered from a fermentation of the cultured cells using one or more processes selected from the group consisting of distillation, gas- stripping, two-phase recovery, and pervaporation.

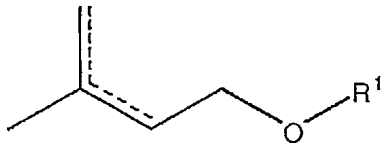
13. The method of claim 10, wherein the prenyl derivative comprises 2-methyl-3-buten-2- ol.

14. The method of claim 10, wherein the cells further comprise one or more of an IDI polypeptide, an MVA pathway enzyme, an isoprene synthase polypeptide, or a DXP pathway enzyme.

15. The method of claim 10, wherein a catalyst selected from the group consisting of an inorganic acid catalyst, HCl, HBr, H2SO4, H_3PO_4, a solid acid catalyst, and inorganic acid on an inert carrier, activated alumina, a zeolite, and organic acid catalyst, p-toluenesulfonic acid, trifluoromethane sulfonic acid, an organic acid resin, Nafion, and a fluoro sulfonic acid resin is used for dehydrating or decarboxylating said prenyl derivative to produce isoprene.

16. A method of producing isoprene, the method comprising

(a) culturing cells in culture medium under suitable culture conditions for the production of isoprene; wherein said culture medium comprises a prenyl derivative of Formula (I):



(I);

wherein R^1 is hydrogen or $-C(O)R^2$; and R^2 is C_1-C_5 linear or branched alkyl; and wherein said cells comprise

(i) a heterologous nucleic acid that encodes an isoprene synthase polypeptide in operable combination with a promoter,

(ii) nucleic acids encoding one or more polypeptides of the mevalonate (MVA) pathway, and

(iii) a heterologous gene encoding a polypeptide having prenol kinase activity; and

(b) producing said isoprene.

17. The method of claim 16, wherein the cells are bacterial cells or yeast cells.

18. The method of claim 16, wherein the cells are selected from the group consisting of *E. coli, P. citrea, B. subtilis, T. reesei, Y. lipolytica,* and *S. cerevisiae.*

19. The method of Claim 16, wherein the prenyl derivative comprises one or more of prenol, isoprenol, 3-methyl-3-buten-1-yl acetate, and 3-methyl-2-buten-1-yl acetate.

20. The method of Claim 16, wherein the cells further comprise one or more of an IDI polypeptide, an MVA pathway enzyme, and a DXP pathway enzyme.

21. The method of claim 16, wherein the cells further comprise an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide.

22. The method of claim 16, wherein the cells produce isoprene at a rate of 0.1 – 10 g/L/hour.

23. The method of claim 16, wherein said nucleic acids encoding said one or more polypeptides of the mevalonate (MVA) pathway are encoded by heterologous nucleic acids.

24. The method of claim 16, wherein said nucleic acids encoding said one or more polypeptides of the mevalonate (MVA) pathway are encoded by endogenous nucleic acids.

25. The method of claim 16, wherein R^1 is hydrogen.

26. The method of claim 16, wherein the cells are selected from the group consisting of *Escherichia coli, Trichoderma reesei, Yarrowia lipolytica, and Saccharomyces cerevisiae.*

27. The method of claim 16, wherein the polypeptides of the MVA pathway comprise acetyl-CoA acetyltransferase (AACT), HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and diphosphomevalonate decarboxylase (MVD).

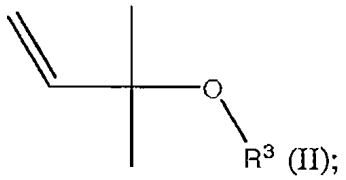
28. The method of claim 16, wherein the polypeptides of the mevalonate (MVA) pathway comprise acetyl-CoA acetyltransferase (AACT), HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate decarboxylase (PMDC), and isopentenyl phosphate kinase (IPK).

29. The method of claim 16, wherein one or more vectors comprise said nucleic acids encoding one or more polypeptides of the MVA pathway.

30. The method of claim 16, wherein the cells further comprise an endogenous gene encoding a polypeptide having prenol kinase activity.

31. A method of producing isoprene, the method comprising

(a) culturing cells in culture medium for the production of isoprene; wherein said culture medium comprises a prenyl derivative of Formula (II):



(II);

wherein R^3 is hydrogen or $-C(O)R^4$; and R^4 is C_1-C_5 linear or branched alkyl; and wherein said cells comprise

(i) a heterologous nucleic acid that encodes an isoprene synthase polypeptide in operable combination with a promoter,

(ii) nucleic acids encoding one or more polypeptides of the mevalonate (MVA) pathway, and

(iii) a heterologous gene encoding a polypeptide having prenol kinase activity; and

(b) producing said isoprene.

32. The method of claim 31, wherein the cells are bacterial cells or yeast cells.

33. The method of claim 31, wherein the cells are selected from the group consisting of *E. coli, P. citrea, B. subtilis, T. reesei, Y. lipolytica,* and *S. cerevisiae.*

34. The method of Claim 31, wherein the prenyl derivative comprises 2-methyl-3-buten- 2-ol.

35. The method of claim 31, wherein the cells further comprise one or more of an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide or a non-mevalonate (DXP) pathway enzyme.

36. The method of claim 31, wherein the cells further comprise one or more of an IDI polypeptide, an MVA pathway enzyme, and a DXP pathway enzyme.

37. The method of claim 31, wherein the cells further comprise an endogenous gene encoding a polypeptide having prenol kinase activity.

38. The method of claim 31, wherein the cells produce isoprene at a rate of 0.1 - 10 g/L/hour.

39. The method of claim 31, wherein said nucleic acids encoding said one or more polypeptides of the mevalonate (MVA) pathway are encoded by heterologous nucleic acids.

40. The method of claim 31, wherein said nucleic acids encoding said one or more polypeptides of the mevalonate (MVA) pathway are encoded by endogenous nucleic acids.

41. The method of claim 31, wherein R^3 is hydrogen.

42. The method of claim 31, wherein the cells are selected from the group consisting of *Escherichia coli, Trichoderma reesei, Yarrowia lipolytica,* and *Saccharomyces cerevisiae.*

43. The method of claim 31, wherein the polypeptides of the MVA pathway comprise acetyl-CoA acetyltransferase (AACT), HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and diphosphomevalonate decarboxylase (MVD).

44. The method of claim 31, wherein the polypeptides of the mevalonate (MVA) pathway comprise acetyl-CoA acetyltransferase (AACT), HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate decarboxylase (PMDC), and isopentenyl phosphate kinase (IPK).

45. The method of claim 31, wherein one or more vectors comprise said nucleic acids encoding one or more polypeptides of the MVA pathway.

46. The method of any one of claims 1, 10, 16, or 31, further comprising the step of recovering the isoprene.