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DESCRIPTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority benefit of United States Provisional patent application No. 61/013,574, filed on December 13, 2007.

FIELD OF THE INVENTION

[0002] The present invention relates generally to methods for producing isoprene from cultured cells and compositions that include these cultured cells.

BACKGROUND OF THE INVENTION

[0003] Isoprene (2-methyl-1,3-butadiene) is the critical starting material for a variety of synthetic polymers, most notably synthetic rubbers. Isoprene is naturally produced by a variety of microbial, plant, and animal species. In particular, two pathways have been identified for the biosynthesis of isoprene: the mevalonate (MVA) pathway and the non-mevalonate (DXP) pathway (Figures 19A and 19B). However, the yield of isoprene from naturally-occurring organisms is commercially unattractive. About 800,000 tons per year of *cis*-polyisoprene are produced from the polymerization of isoprene; most of this polyisoprene is used in the tire and rubber industry. Isoprene is also copolymerized for use as a synthetic elastomer in other products such as footwear, mechanical products, medical products, sporting goods, and latex.

[0004] 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.

[0005] 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

[0006] The present invention provides:

1. [1] Bacterial, fungal, yeast or plant cells comprising one or more nucleic acids encoding:
    1. (a) an isoprene synthase polypeptide, wherein the isoprene synthase polypeptide is encoded by a heterologous nucleic acid, and

2. (b) an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide, wherein the IDI polypeptide is encoded by a heterologous nucleic acid; and/or wherein the IDI polypeptide is encoded by an insertion of a copy of an endogenous nucleic acid encoding an IDI polypeptide and
3. (c)
    1. (i) a 1-Deoxyxylulose-5-phosphate synthase (DXS) polypeptide wherein the DXS polypeptide is encoded by a heterologous nucleic acid; and/or wherein the DXS polypeptide is encoded by
    an insertion of a copy of an endogenous nucleic acid encoding an DXS polypeptide
    and/or
    2. (ii) one or more mevalonate (MVA) pathway polypeptides, wherein said one or more MVA pathway polypeptides is encoded by a heterologous nucleic acid; and/or wherein said one or more MVA pathway polypeptides is encoded by an insertion of a copy of an endogenous nucleic acid encoding one or more MVA pathway polypeptides,

wherein the cells produce greater than about 400 nmole/g_{wcm}/hr of isoprene.

2. [2] Bacterial, fungal, yeast or plant cells comprising one or more nucleic acids encoding:
    1. (a) an isoprene synthase polypeptide, wherein the isoprene synthase polypeptide is encoded by a heterologous nucleic acid, and
    2. (b) an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide, wherein the IDI polypeptide is encoded by a heterologous nucleic acid; and/or wherein the IDI polypeptide is encoded by an insertion of a copy of an endogenous nucleic acid encoding an IDI polypeptide and
    3. (c)
        1. (i) a 1-Deoxyxylulose-5-phosphate synthase (DXS) polypeptide wherein the DXS polypeptide is encoded by a heterologous nucleic acid; and/or wherein the DXS polypeptide is encoded by an insertion of a copy of an endogenous nucleic acid encoding an DXS polypeptide
        and/or
        2. (ii) one or more mevalonate (MVA) pathway polypeptides, wherein said one or more MVA pathway polypeptides is encoded by a heterologous nucleic acid; and/or wherein said one or more MVA pathway polypeptides is encoded by an insertion of a copy of an endogenous nucleic acid encoding one or more MVA pathway polypeptides,

    wherein the cells convert more than about 0.2 molar percent of the carbon that the cells consume from a cell culture medium into isoprene.
3. [3] The cells of [1] or [2], wherein the heterologous nucleic acid is operably linked to a promoter.
4. [4] The cells of any one of [1]-[3], wherein the isoprene synthase polypeptide is a plant isoprene synthase polypeptide.
5. [5] The cells of any one of [1]-[4], wherein the MVA pathway polypeptide is a mevalonate kinase (MVK), the MVK optionally being a polypeptide from *Methamosarcina.*
6. [6] The cells of any one of [1]-[5], wherein the isoprene synthase polypeptide is a naturally-occurring polypeptide from *Pueraria* or *Populus.*
7. [7] The cells of any one of [1]-[6],
    wherein the cells are bacterial cells, optionally Bacillus, Escherichia or Pantoea cells, or
    wherein the cells are fungal cells, optionally *Trichoderma* cells, or
    wherein the cells are yeast cells, optionally *Yarrowia* or *Saccharomyces* cells.
8. [8] A method for producing isoprene, the method comprising culturing cells of any one of [1]-[7]

under conditions sufficient for producing isoprene.

9. [9] The method of [8], the method comprising
    1. (a) culturing cells comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide under suitable culture conditions for the production of isoprene, wherein the cells produce greater than about 400 nmole/g_{wcm}/hr of isoprene, and
    2. (b) producing isoprene.
10. [10] The method of [8], the method comprising
    1. (a) culturing cells comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide under suitable culture conditions for the production of isoprene, wherein the cells convert more than about 0.2 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and
    2. (b) producing isoprene.
11. [11] The method of any one of [8]-[10], further comprising recovering the isoprene produced by the cells.
12. [12] Use of cells of any one of [1]-[7] in the production of isoprene.
13. [13] The method of any one of [8]-[10], further comprising the step of purifying the isoprene produced by the cells, and/or comprising the step of polymerizing the isoprene produced by the cells.

[0007] In one aspect, the invention features cell as claimed in culture that produce isoprene. The invention provides cells in culture that produce greater than about 400 nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr) of isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes a carbon source, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.*, a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing.

[0008] In some embodiments, the invention provides cells as claimed in culture that have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the invention provides cells in culture that have a peak volumetric productivity of isoprene greater than about 0.5 mg/L_{broth}/hr. 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 (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.*, a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing.

[0009] In some embodiments, the invention provides cells as claimed 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 (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source *(e.g.,* a hydrolyzed biomass carbon source), polypeptide *(e.g.,* a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing.

[0010] In some embodiments, the invention provides cells as claimed 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 (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source *(e.g.,* a hydrolyzed biomass carbon source), polypeptide *(e.g.,* a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing.

[0011] In one aspect, the invention features methods of producing isoprene, such as methods of using any of the cells claimed 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 (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.*, a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing.

[0012] In one aspect, the invention features methods of producing isoprene, such as methods of using any of the cells claimed herein to produce isoprene. In some embodiments, the method involves culturing cells under conditions resulting in an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the method involves culturing cells under conditions resulting in a peak volumetric productivity of isoprene greater than about 0.5 mg/L_{broth}/hr. 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 (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.*, a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing.

[0013] In some embodiments, the method includes culturing cells under conditions sufficient to convert

more than about 0.002% of the carbon 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 (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source *(e.g.,* a hydrolyzed biomass carbon source), polypeptide *(e.g.,* a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing.

[0014] 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, 10,000, 12,500, 20,000, 30,000, 40,000, 50,000, 75,000, 100,000, 125,000, 150,000, 188,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments, the cells in culture have an average volumetric productivity of isoprene at greater than or about 0.1, 1.0, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1100, 1200, 1300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400, 2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, or more mg of isoprene/L of broth/hr (mg/L_{broth}/hr, wherein the volume of broth includes the volume of the cells and the cell medium). In some embodiments, the cells in culture have a peak volumetric productivity of isoprene at greater than or about 0.5, 1.0, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1100, 1200, 1300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400, 2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, 3,750, 4,000,4,250, 4,500, 4,750, 5,000, 5,250, 5,500, 5,750, 6,000, 6,250, 6,500, 6,750, 7,000, 7,250, 7,500, 7,750, 8,000, 8,250, 8,500, 8,750, 9,000, 9,250, 9,500, 9,750, 10,000, 12,500, 15,000, or more mg of isoprene/L of broth/hr (mg/L_{broth}/hr, wherein the volume of broth includes the volume of the cells and the cell medium). In some embodiments of any of the aspects of the disclosure, 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, 2.0, 2.2, 2.4, 2.6, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0, 15.0, 16.0, 17.0, 18.0, 19.0, 20.0, 21.0, 22.0, 23.0, 23.2, 23.4, 23.6, 23.8, 24.0, 25.0, 30.0, 31.0, 32.0, 33.0, 35.0, 37.5, 40.0, 45.0, 47.5, 50.0, 55.0, 60.0, 65.0, 70.0, 75.0, 80.0, 85.0, 90.0 molar %, 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.

[0015] 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.

[0016] 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.

[0017] 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.

[0018] 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 cerevisi, Methanosarcina mazei,* 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, Methanosarcina mazei,* 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).

[0019] In some embodiments of any of the aspects of the invention, the isoprene synthase polypeptide is a naturally-occurring polypeptide from a plant such as *Pueraria* (*e.g., Pueraria montana*) or *Populus* (*e.g., Populus tremuloides, Populus alba* (*P. alba*), *Populus nigra, Populus trichocarpa,* or the hybrid, *Populus alba x Populus tremula*).

[0020] 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, Streptomyces coelicolor, Streptomyces albus,* 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, the *E. coli* cells are *E. coli FadR atoC* mutant cells. In some embodiments, the *E. coli* cells express (such as constitutively express) *ybhE* (also known as *pgl*). In some embodiments of any of the aspects of the invention, the cells are fungal, cells such as filamentous funal 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).

[0021] 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.

[0022] In one aspect, the disclosure provides a tire comprising polyisoprene. In some embodiments, the polyisoprene is produced by (i) polymerizing isoprene from any of the compositions or methods described herein or (ii) polymerizing isoprene recovered from any of the compositions or methods described herein. In some embodiments, the polyisoprene comprises *cis*-1,4-polyisoprene.

[0023] In one aspect, the disclosure features a product (such as a tire) produced by any of the compositions or methods of the invention.

[0024] It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention. These and other aspects of the invention will become apparent to one of skill in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025]

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.

Figure 2 is a map of pTrcKudzu.

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.

Figure 4 is a map of pETNHisKudzu.

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

Figure 6 is a map of pCL-lac-Kudzu.

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

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

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

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

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

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

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

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}.

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}.

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}.

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.

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

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

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

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

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

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.

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

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

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

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

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

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

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 referred to 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.

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 referred to particularly with respect to nucleic acids and polypeptides of the modified MVA pathway. The modified MVA pathway is present, for example, in some archaeal organisms, such as *Methanosarcina mazei.*

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.

Figure 21 is a map of pTrcKudzu yIDI DXS Kan.

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

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).

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).

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).

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).

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).

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).

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).

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

Figure 24 is a map of pTrcKKDyIkIS kan.

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

Figure 26 is a map of pCL PtrcUpperPathway.

Figure 27 is a nucleotide sequence of pCL PtrcUpperPathway (SEQ ID NO:46).

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.

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).

Figure 30 is a map of p9796-poplar.

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

Figure 32 is a map of pTrcPoplar.

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

Figure 34 is a map of pTrcKudzu yIDI Kan.

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

Figure 36 is a map of pTrcKudzuDXS Kan.

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

Figure 38 is a map of pCL PtrcKudzu.

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

Figure 40 is a map of pCL PtrcKudzu A3.

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

Figure 42 is a map of pCL PtrcKudzu yIDI.

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

Figure 44 is a map of pCL PtrcKudzu DXS.

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

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.

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).

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).

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).

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).

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.

Figure 49 shows graphs demonstrating isoprene production from a 500 L bioreactor with *E. coli* cells containing the pTrcKudzu and yIDI and 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.

Figure 50 is a map of pJMupperpathway2.

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

Figure 52 is a map of pBS Kudzu #2.

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).

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).

Figures 54 is a map of plasmid pET24 P.alba HGS.

Figures 55A and 55B are the nucleotide sequence of plasmid pET24 P.alba HGS (SEQ ID NO:87).

Figure 56 is a schematic diagram showing restriction sites used for endonuclease digestion to construct plasmid EWL230 and compatible cohesive ends between BspHI and NcoI sites.

Figure 57 is a map of plasmid EWL230.

Figures 58A and 58B are the nucleotide sequence of plasmid EWL230 (SEQ ID NO:88).

Figure 59 is a schematic diagram showing restriction sites used for endonuclease digestion to construct plasmid EWL244 and compatible cohesive ends between NsiI and PstI sites.

Figure 60 is a map of EWL244.

Figures 61A and 61B are the nucleotide sequence of plasmid EWL244 (SEQ ID NO:89).

Figure 62 is a map of plasmids MCM484-487.

Figures 63A-63C are the nucleotide sequence of plasmid MCM484 (SEQ ID NO:90).

Figures 64A-64C are the nucleotide sequence of plasmid MCM485 (SEQ ID NO:91).

Figures 65A-65C are the nucleotide sequence of plasmid MCM486 (SEQ ID NO:92).

Figures 66A-66C are the nucleotide sequence of plasmid MCM487 (SEQ ID NO:93).

Figures 67A-67D are graphs of isoprene production by *E. coli* strain (EWL256) expressing genes from the MVA pathway and grown in fed-batch culture at the 15-L scale without yeast extract feeding. Figure 67A shows the time course of optical density within the 15-L bioreactor fed with glucose. Figure 67B shows the time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Figure 67C shows the time course of total isoprene produced from the 15-L bioreactor fed with glucose. Figure 67D shows the total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

Figures 68A-68E are graphs of isoprene production by *E. coli* strain (EWL256) expressing genes from the MVA pathway and grown in fed-batch culture at the 15-L scale with yeast extract feeding. Figure 68A shows the time course of optical density within the 15-L bioreactor fed with glucose. Figure 68B shows the time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Figure 68C shows the time course of total isoprene produced from the 15-L bioreactor fed with glucose. Figure 68D shows the volumetric productivity within the 15-L bioreactor fed with glucose. An average value of 1.1 g/L/hr was maintained for a 40-hour period (23-63 hours) with yeast extract feeding. Figure 68E shows the carbon dioxide evolution rate (CER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

Figures 69A-69D shows production of isoprene from different carbon sources via the MVA (pathway). Figure 69A shows growth *of E. coli* EWL256, which contains both the MVA pathway and isoprene synthase, on either glucose, biomass hydrolysate, glycerol, or acetate as the only carbon source. The different carbon sources were added to a concentration of 1% in the media. A negative control with no added carbon source was included. Growth was measured as optical density at 600 nM. Figure 69B shows specific productivity of isoprene from *E. coli* EWL256 containing both the MVA pathway and isoprene synthase when grown on either glucose, biomass hydrolysate, glycerol, or acetate as only carbon source. The different carbon sources were added to a concentration of 1% in the media. A negative control with no added carbon source was included. Samples were taken 190 minutes, 255 minutes and 317 minutes after inoculation and isoprene produced by the bacteria was measured using GC-MS. Figure 69C shows growth *of E. coli* EWL256 on either glucose or xylose as the only carbon source. The different carbon sources were added to a concentration of 1% in the media. A negative

control with no added carbon source was included. Growth was measured as optical density at 600nM. Figure 69D shows specific productivity of isoprene from *E. coli* EWL256 when grown on either glucose or xylose as only carbon source. The carbon sources were added to a concentration of 1% in the media. A negative control with no added carbon source was included. Samples were taken 260 minutes, 322 minutes and 383 minutes after inoculation and isoprene produced by the bacteria was measured using GC-MS.

Figures 70A and 70B show the production of isoprene by *E. coli* strains from glucose and from fatty acid, respectively. For Figure 70A, eleven colonies from the transformation of WW4 with pMCM1 18, the plasmid bearing the lower mevalonic acid pathway, were picked to verify the presence of the lower pathway. Cell from the colonies were cultured in TM3 medium containing 0.1% yeast extract and 2% glucose. Aliquots of induced culture were assayed for isoprene production after 4 hours of induction. All colonies showed the production of isoprene. The inducer IPTG had a strong growth inhibitory effect as was evident from the 3 to 4.6-fold reduced cell density in going from 50 to 900 uM concentration of the inducer (data not shown). The graph shows that higher induction, yields a higher specific titer of isoprene. For Figure 70B, the production culture was inoculated from a washed overnight culture at 1 to 10 dilution. The culture was grown for several hours and induced with 50 uM IPTG. The left bar shows isoprene assay results four hours after induction followed by a one hour isoprene accumulation assay. The middle bar shows the one hour normalized value for the same culture with the same induction period but analyzed by a 12 hour isoprene accumulation assay. The right bar shows the value for a one hour isoprene accumulation assay of the culture that was induced for 13 hours.

Figure 71 is a map of the *E. coli-Streptomyces* shuttle vector pUWL201PW (6400 bp) used for cloning isoprene synthase from Kudzu. Tsr, thiostrepton resistance gene. Picture is taken from Doumith et al., Mol. Gen. Genet. 264: 477-485, 2000.

Figure 72 shows isoprene formation by *Streptomyces albus* wild type strain ("wt") and strains harboring plasmid pUWL201PW (negative control) or pUWL201_iso (encoding isoprene synthase from Kudzu).

Figure 73A is a map of the *M. mazei* archaeal Lower Pathway operon.

Figures 73B and 73C are the nucleotide sequence of the *M. mazei* archaeal lower Pathway operon (SEQ ID NO:113).

Figure 74A is a map of MCM376 - MVK from *M. mazei* archaeal Lowerin pET200D.

Figures 74B and 74C are the nucleotide sequence of MCM376 - MVK from *M. mazei* archaeal Lowerin pET200D (SEQ ID NO:114).

Figures 75A-75D show growth and specific productivity of isoprene production for EWL256 compared to RM11608-2. Growth (OD550) is represented by the white diamonds; specific productivity of isoprene is represented by the solid bars. The x-axis is time (hours) post-induction with either 200 (Figures 75A and 75B) or 400 (Figures 75C and 75D) uM IPTG. Y-1 axis is productivity of isoprene (ug/L/OD/hr) and Y-2 is arbitrary units of optical density at a wavelength of 550. These values for the OD550 must be multiplied by 6.66 to obtain the actual OD of the culture.

Figure 76 is a map of plasmid pBBRCMPGI1.5-pgl.

Figures 77A and 77B are the nucleotide sequence of plasmid pBBRCMPGI1.5-pgl (SEQ ID NO:122).

Figures 78A-78F are graphs of isoprene production by *E. coli* strain expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, and *pgl* (RHM111608-2), and grown in fed-batch culture at the 15-L

scale. Figure 78A shows the time course of optical density within the 15-L bioreactor fed with glucose. Figure 78B shows the time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Method for calculating isoprene: cumulative isoprene produced in 59 hrs, g/Fermentor volume at 59 hrs, L [=] g/L broth. Figure 78C also shows the time course of isoprene titer within the 15-L bioreactor fed with glucose. Method for calculating isoprene: $\int$(Instantaneous isoprene production rate, g/L/hr)dt from t = 0 to 59 hours [=] g/L broth. Figure 78D shows the time course of total isoprene produced from the 15-L bioreactor fed with glucose. Figure 78E shows volumetric productivity within the 15-L bioreactor fed with glucose. Figure 78F shows carbon dioxide evolution rate (CER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

Figure 79A is a map of plasmid pJ201:19813.

Figures 79B and 79C are the nucleotide sequence of pJ201:19813 (SEQ ID NO:123).

DETAILED DESCRIPTION OF THE INVENTION

[0026] The disclosure features compositions and methods for the production of increased amounts of isoprene. In particular, these compositions and methods 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 ~ 23.6 molar % yield (10.7 weight % yield) of the carbon that the cells consume from a cell culture medium into isoprene (% carbon yield). As shown in the Examples and Table 2, approximately 60.5 g of isoprene per liter of broth was generated. Isoprene was produced at a peak specific rate of 1.88 x 10^5 nmol/OD/hr (1.88 x 10^5 nmole/g_{wcm}/hr). 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. The cells 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.

[0027] 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) or *Populus alba* (Poplar) isoprene synthase polypeptide was expressed in a variety of host cells, such as *Escherichia coli, Panteoa citrea, Bacillus subtilis, Yarrowia lipolytica,* and *Trichoderma reesei.* As also shown in the Examples, a heterologous *Methanosarcina mazei* (*M. mazei*) mevalonate kinase (MVK) was expressed in host cells such as *Escherichia coli* to increase isoprene production. 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 60.5 g 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 x 10^3)
E. coli BL21/MCM127 with kudzu IS and entire MVA	23.0	874.1 (1.28 x 10^4)
pathway		
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 x 10^3 (4.8 x 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	1094	250	875 (1.28×10^4)
Bacillus subtilis wild-type	1.5	2.5	0.8 (11.7)
Bacillus pBS Kudzu IS	16.6	~30 (over 100 hours)	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)
E. coli BL21/pCLPtrcUpper Pathway and gil.2KKDyl and pTrcAlba-mMVK	2.03×10^4	3.22×10^4	5.9×10^3 (8.66×10^4)
E. coli BL21 /pCLPtrcUpper Pathway and gi1.2KKDyl and pTrcAlba-mMVK plus pBBRCMPGI1.5pgl	3.22×10^4	6.05×10^4	1.28×10^4 (1.88×10^5)
**Normalized to an off-gas flow rate of 1 vvm (1 volume off-gas per 1 L_{broth} per minute).			

[0028] 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.

[0029] 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 of IPP that is converted into DMAPP, which in turn is converted into isoprene.

[0030] 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). As another example, fermentation *of E. coli* with *M. mazei* mevalonate kinase (MVK), *P. alba* isoprene synthase, the upper MVA pathway, and the integrated lower MVA pathway was used to produce isoprene. The levels of isoprene varied from 32 to 35.6 g/L over a time period of 67 hours (Example 10, part III).

[0031] In yet another example, fermentation *of E. coli* with *M. mazei* mevalonate kinase (MVK), *P. alba* isoprene synthase, pgl over-expression (RHM111608-2), the upper MVA pathway, and the integrated lower MVA pathway were used to produce isoprene. The levels of isoprene vary from 33.2 g/L to 40.0 g/L over a time period of 40 hours or 48.6 g/L to 60.5 g/L over a time period of 59 hours (Example 13, part (ii)).

[0032] 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.

[0033] In some embodiments, the production of isoprene by cells that contain a heterologous isoprene synthase nucleic acid is augmented by increasing the amount of a MVA polypeptide expressed by the cells (Figures 19A and 19B). Exemplary MVA pathways polypeptides include any of the following polypeptides: acetyl-CoA acetyltransferase (AA-CoA thiolase) polypeptides, 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) polypeptides, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) polypeptides, mevalonate kinase (MVK) polypeptides, phosphomevalonate kinase (PMK) polypeptides, diphosphomevalonte decarboxylase (MVD) polypeptides, phosphomevalonate decarboxylase (PMDC) polypeptides, isopentenyl phosphate kinase (IPK) polypeptides, IDI polypeptides, and polypeptides (e.g., fusion polypeptides) having an activity of two or more MVA pathway polypeptides. For example, one or more MVA pathway nucleic acids can be introduced into the cells. In some embodiments, the cells contain the upper MVA pathway, which includes AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase nucleic acids. In some embodiments, the cells contain the lower MVA pathway, which includes MVK, PMK, MVD, and IDI nucleic acids. In some

embodiments, the cells contain the entire MVA pathway, which 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.

[0034] 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 nmol/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 µg/L_{broth}/hr/OD) 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).

[0035] As another example, *E. coli* cells containing a nucleic acid encoding a *P. alba* isoprene synthase polypeptide and a nucleic acid encoding *M. mazei* MVK polypeptide generated 320.6 g (at a peak specific rate of 9.54 x 10^4 nmol/L_{broth}/OD_{600}/hr (*i.e.* 9.5 x 10^{-5} mol/L_{broth}/OD_{600}/hr)) of isoprene during a 67 hour fermentation in the absence of yeast extract feeding or 395.5 g (at a peak specific rate of 8.66 x 10^4 nmol/L_{broth}/OD_{600}/hr) during a 68 hour fermentation in the presence of yeast extract feeding *(see* Example 10).

[0036] 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.

[0037] 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 using *E. coli* cells with kudzu isoprene synthase, *S. cerevisia* IDI, and *E. coli* DXS nucleic acids to produce isoprene. In particular, 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). 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.

[0038] 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 and Figures 69A and 69B). *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). *E. coli* cells expressing *P. alba* isoprene synthase and the MVA pathway produced isoprene at a higher initial growth rate from ammonia fiber expansion (AFEX) pretreated corn stover than from the equivalent amount of glucose. (Figures 69A and 69B). 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.

[0039] Additionally, invert sugar was shown to function as a carbon source for the generation of isoprene (Figure 47D).

[0040] Additionally, xylose, acetate, and glycerol were also shown to function as a carbon source for the generation of isoprene (Figures 69A-69D). For example, *E. coli* cells with *P. alba* isoprene synthase and the MVA pathway grown on acetate as the only carbon source had a specific productivity of isoprene about twice as high as during growth on glucose (Example 10, Part IV; Figures 69A and 69B).

[0041] 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). As another example, *E. coli* fadR atoC mutant cells containing the upper and lower MVA pathway plus kudzu isoprene synthase produced isoprene when cultured in a cell medium containing palm oil and a source of glucose (Example 12, part II). 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.

Exemplary Polypeptides and Nucleic Acids

[0042] Various isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids can be used in the compositions and methods of the invention.

[0043] As used herein, "polypeptides" includes polypeptides, proteins, peptides, fragments of polypeptides, and fusion polypeptides that include part or all of a first polypeptide (*e.g.*, an isoprene synthase, DXS, IDI, or MVA pathway polypeptide) and 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.

[0044] 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.

[0045] 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.

[0046] 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.

[0047] 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 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.

[0048] 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.

[0049] 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.

[0050] "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.

[0051] 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 referred to 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 referred to 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 December 11, 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

[0052] 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 mLs of cells are pelleted by centrifugation at 7000 x g for 10 minutes and resuspended in 5 ml of PEB without glycerol. The cells are lysed using a French Pressure cell using standard procedures. Alternatively the cells are treated with lysozyme (Ready-Lyse lysozyme solution; EpiCentre) after a freeze/thaw at -80C.

[0053] 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 referred to, 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.

[0054] 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.

[0055] 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, Populus alba x tremula* (CAC35696), or *Populus alba*) (Sasaki et al., FEBS Letters 579(11): 2514-2518, 2005; 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 referred to 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 referred to 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

[0056] 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

[0057] 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

[0058] 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.

[0059] 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.*

[0060] 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.*

[0061] 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.*

[0062] Mevalonate kinase (MVK) polypeptide 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.*

[0063] 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.*

[0064] 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.*

[0065] 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.*

[0066] 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.*

[0067] Exemplary IDI polypeptides and nucleic acids are described above.

Exemplary Methods for Isolating Nucleic Acids

[0068] 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 referred to 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 referred to particularly with respect to PCR methods) to obtain amounts of DNA suitable for transformation using appropriate vectors.

[0069] 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.

[0070] 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 referred to particularly with respect to transformation methods.

[0071] 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.

[0072] 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 referred to particularly with respect to identification of nucleic acids involved in the synthesis of isoprene).

[0073] 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

[0074] Any of the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid described herein can be included in one or more vectors. Accordingly, the isclosure 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.

[0075] 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.

[0076] 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, *andpyr4.* 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 referred to 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 referred to 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.

[0077] 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 referred to particularly with respect to the use of vectors).

[0078] 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 referred to 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*).

[0079] 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 referred to, 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).

[0080] 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.

[0081] 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 referred to 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 referred to, particularly with respect to vectors. Particularly useful vectors include pFB6, pBR322, PUC18, pUC100, and pENTR/D.

[0082] 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, hjb*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 cbhl,* which is an inducible promoter and has been deposited in GenBank under Accession No. D86235, which is referred to 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 referred to 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 referred to particularly with respect to promoters).

[0083] 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 referred to 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.

[0084] 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.

[0085] 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 referred to 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 referred to particularly with respect to oligonucleotide linkers). Additionally, vectors can be constructed using known recombination techniques *(e.g.,* Invitrogen Life Technologies, Gateway Technology).

[0086] 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 referred to particularly with respect to cloning techniques.

[0087] 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 referred to particularly with respect to molecular biology and cloning techniques.

Exemplary Source Organisms

[0088] 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 (Figure 19). 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.

[0089] 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.

[0090] 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.*

[0091] 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 referred to 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 referred to 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 referred to particularly with respect to strains of T. reesei.

[0092] In some embodiments, the source organism is a yeast, such as *Saccharomyces sp., Schizosaccharomyces sp., Pichia sp.,* or *Candida sp.*

[0093] 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. albus, S. lividans,* or *S. rubiginosus,* or strains of *Escherichia* such as *E. coli.*

[0094] 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.*

[0095] In some embodiments, the source organism is a gram-positive bacterium. Non-limiting examples include strains of *Streptomyces* (*e.g., S. albus, 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.*

[0096] 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 or *Populus alba*) (Sasaki et al., FEBS Letters 579(11): 2514-2518, 2005), aspen (such as *Populus tremuloides*), or *Quercus robur.*

[0097] In some embodiments, the source organism is an algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates.

[0098] 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

[0099] 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

[0100] 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 referred to 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 referred to 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 referred to 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.

[0101] Any method known in the art may be used to select transformants. In one non-limiting example, stable transformants including an *amdS* 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.

[0102] 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 referred to 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.

[0103] 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.

[0104] 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 referred to particularly with respect to transformation methods).

[0105] 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.

[0106] 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 referred to particularly with respect to transformation methods.

Exemplary Cell Culture Media

[0107] 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.

[0108] 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.

[0109] 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), acetate, 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. In some embodiment, the carbohydrate is xylose or glucose.

[0110] 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.

[0111] 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.

[0112] Exemplary lipids are any substance containing one or more fatty acids that are C4 and above fatty acids that are saturated, unsaturated, or branched.

[0113] 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.

[0114] 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.

[0115] 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.

[0116] 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.

[0117] 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 acetate, 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.

[0118] 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 referred to 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.

[0119] 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.

[0120] 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.

[0121] 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).

[0122] 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 referred to particularly with respect to carbon sources) and in bacteria (Hunter et. al., Biochemistry, 24, 4148-4155, 1985, which is hereby referred to 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 referred to 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.

[0123] 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 referred to particularly with respect to carbon sources). Similarly, various species of *Candida* metabolize alanine or oleic acid (Suiter et al., Arch. Microbiol. 153(5), 485-9, 1990, which is hereby referred to particularly with respect to carbon sources).

[0124] 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 referred to 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.

[0125] 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 referred to 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

[0126] 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 referred to by 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.

[0127] 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 referred to 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.

[0128] 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.

[0129] 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 referred to particularly with respect to cell culture and fermentation conditions.

[0130] 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.

[0131] 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 referred to particularly with respect to cell culture and fermentation conditions.

[0132] In some embodiments, cells are immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isoprene production.

[0133] 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.

[0134] 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).

[0135] 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 Production of Isoprene

[0136] 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.

[0137] By "peak specific productivity" is meant the maximum amount of isoprene produced per cell during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak specific productivity time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per cell is at a maximum. The peak specific productivity is determined by dividing the total productivity by the amount of cells, as determined by optical density at 600nm (OD_{600}). In some embodiments, the isoprene amount is measured at the peak specific productivity time point. In some embodiments, the peak specific productivity for the cells is about any of the isoprene amounts per cell disclosed herein.

[0138] By "peak volumetric productivity" is meant the maximum amount of isoprene produced per volume of broth (including the volume of the cells and the cell medium) during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak specific volumetric productivity time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per volume of broth is at a maximum. The peak specific volumetric productivity is determined by dividing the total productivity by the volume of broth and amount of time. In some embodiments, the isoprene amount is measured at the peak specific volumetric productivity time point. In some embodiments, the peak specific volumetric productivity for the cells is about any of the isoprene amounts per volume per time disclosed herein.

[0139] By "peak concentration" is meant the maximum amount of isoprene produced during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak concentration time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per cell is at a maximum. In some embodiments, the isoprene amount is measured at the peak concentration time point. In some embodiments, the peak concentration for the cells is about any of the isoprene amounts disclosed herein.

[0140] By "average volumetric productivity" is meant the average amount of isoprene produced per volume of broth (including the volume of the cells and the cell medium) during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). The average volumetric productivity is determined by dividing the total productivity by the volume of broth and amount of time. In some embodiments, the average specific volumetric productivity for the cells is about any of the isoprene amounts per volume per time disclosed herein.

[0141] 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.

[0142] 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, 12,500, 20,000, 30,000, 40,000, 50,000, 75,000, 100,000, 125,000, 150,000, 188,000, 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 200,000 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, about 2,000 to about 5,000 nmole/g_{wcm}/hr, about 5,000 to about 10,000 nmole/g_{wcm}/hr, about 10,000 to about 50,000 nmole/g_{wcm}/hr, about 50,000 to about 100,000 nmole/g_{wcm}/hr, about 100,000 to about 150,000 nmole/g_{wcm}/hr, or about 150,000 to about 200,000 nmole/g_{wcm}/hr. In some embodiments, the amount of isoprene is between about 20 to about 200,000 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, about 400 to about 1,000 nmole/g_{wcm}/hr, about 1,000 to about 5,000 nmole/g_{wcm}/hr, about 2,000 to about 20,000 nmole/g_{wcm}/hr, about 5,000 to about 50,000 nmole/g_{wcm}/hr, about 10,000 to about 100,000 nmole/g_{wcm}/hr, about 20,000 to about 150,000 nmole/g_{wcm}/hr, or about 20,000 to about 200,000 nmole/g_{wcm}/hr.

[0143] 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 referred to 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, 111.) 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 referred to 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.

[0144] 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).

[0145] 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}.

[0146] 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.

[0147] In some embodiments, the cells in culture have an average volumetric productivity of isoprene at greater than or about 0.1, 1.0, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1100, 1200, 1300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400, 2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, or more mg of isoprene/L of broth/hr (mg/L_{broth}/hr, wherein the volume of broth includes the volume of the cells and the cell medium). In some embodiments, the average volumetric productivity of isoprene is between about 0.1 to about 3,500 mg/L_{broth}/hr, such as between about 0.1 to about 100 mg/L_{broth}/hr, about 100 to about 500 mg/L_{broth}/hr, about 500 to about 1,000 mg/L_{broth}/hr, about 1,000 to about 1,500 mg/L_{broth}/hr, about 1,500 to about 2,000 mg/L_{broth}/hr, about 2,000 to about 2,500 mg/L_{broth}/hr, about 2,500 to about 3,000 mg/L_{broth}/hr, or about 3,000 to about 3,500 mg/L_{broth}/hr. In some embodiments, the average volumetric productivity of isoprene is between about 10 to about 3,500 mg/L_{broth}/hr, about 100 to about 3,500 mg/L_{broth}/hr, about 200 to about 1,000 mg/L_{broth}/hr, about 200 to about 1,500 mg/L_{broth}/hr, about 1,000 to about 3,000 mg/L_{broth}/hr, or about 1,500 to about 3,000 mg/L_{broth}/hr.

[0148] In some embodiments, the cells in culture have a peak volumetric productivity of isoprene at greater than or about 0.5, 1.0, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1100, 1200, 1300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400,

2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, 3,750, 4,000, 4,250, 4,500, 4,750, 5,000, 5,250, 5,500, 5,750, 6,000, 6,250, 6,500, 6,750, 7,000, 7,250, 7,500, 7,750, 8,000, 8,250, 8,500, 8,750, 9,000, 9,250, 9,500, 9,750, 10,000, 12,500, 15,000, or more mg of isoprene/L of broth/hr (mg/L_{broth}/hr, wherein the volume of broth includes the volume of the cells and the cell medium). In some embodiments, the peak volumetric productivity of isoprene is between about 0.5 to about 15,000 mg/L_{broth}/hr, such as between about 0.5 to about 10 mg/L_{broth}/hr, about 1.0 to about 100 mg/L_{broth}/hr, about 100 to about 500 mg/L_{broth}/hr, about 500 to about 1,000 mg/L_{broth}/hr, about 1,000 to about 1,500 mg/L_{broth}/hr, about 1,500 to about 2,000 mg/L_{broth}/hr, about 2,000 to about 2,500 mg/L_{broth}/hr, about 2,500 to about 3,000 mg/L_{broth}/hr, about 3,000 to about 3,500 mg/L_{broth}/hr, about 3,500 to about 5,000 mg/L_{broth}/hr, about 5,000 to about 7,500 mg/L_{broth}/hr, about 7,500 to about 10,000 mg/L_{broth}/hr, about 10,000 to about 12,500 mg/L_{broth}/h, or about 12,500 to about 15,000 mg/L_{broth}/hr. In some embodiments, the peak volumetric productivity of isoprene is between about 10 to about 15,000 mg/L_{broth}/hr, about 100 to about 2,500 mg/L_{broth}/hr, about 1,000 to about 5,000 mg/L_{broth}/hr, about 2,500 to about 7,500 mg/L_{broth}/hr, about 5,000 to about 10,000 mg/L_{broth}/hr, about 7,500 to about 12,500 mg/L_{broth}/hr, or about 10,000 to about 15,000 mg/L_{broth}/hr.

[0149] 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}.

[0150] 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, 2.0, 2.2, 2.4, 2.6, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0, 15.0, 16.0, 17.0, 18.0, 19.0, 20.0, 21.0, 22.0, 23.0, 23.2, 23.4, 23.6, 23.8, 24.0, 25.0, 30.0, 31.0, 32.0, 33.0, 35.0, 37.5, 40.0, 45.0, 47.5, 50.0, 55.0, 60.0, 65.0, 70.0, 75.0, 80.0, 85.0, or 90.0 molar % of the carbon in the cell culture medium into isoprene. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about 90.0 molar %, such as 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%, about 1.0 to about 1.6%, about 1.6 to about 3.0%, about 3.0 to about 5.0%, about 5.0 to about 8.0%, about 8.0 to about 10.0%, about 10.0 to about 15.0%, about 15.0 to about 20.0%, about 20.0 to about 25.0%, about 25.0% to 30.0%, about 30.0% to 35.0%, about 35.0% to 40.0%, about 45.0% to 50.0%, about 50.0% to 55.0%, about 55.0% to 60.0%, about 60.0% to 65.0%, about 65.0% to 70.0%, about 75.0% to 80.0%, about 80.0% to 85.0%, or about 85.0% to 90.0%. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about 0.4 molar %, 0.002 to about 0.16 molar %, 0.04 to about 0.16 molar %, about 0.005 to about 0.3 molar %, about 0.01 to about 0.3 molar %, about 0.05 to about

0.3 molar %, about 0.1 to 0.3 molar %, about 0.3 to about 1.0 molar %, about 1.0 to about 5.0 molar %, about 2 to about 5.0 molar %, about 5.0 to about 10.0 molar %, about 7 to about 10.0 molar %, about 10.0 to about 20.0 molar %, about 12 to about 20.0 molar %, about 16 to about 20.0 molar %, about 18 to about 20.0 molar %, about 18 to 23.2 molar %, about 18 to 23.6 molar %, about 18 to about 23.8 molar %, about 18 to about 24.0 molar %, about 18 to about 25.0 molar %, about 20 to about 30.0 molar %, about 30 to about 40.0 molar %, about 30 to about 50.0 molar %, about 30 to about 60.0 molar %, about 30 to about 70.0 molar %, about 30 to about 80.0 molar %, or about about 30 to about 90.0 molar %

[0151] 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

[0152] 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%

[0153] 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.

[0154] 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)

[0155]

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

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)

[0156]

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)

[0157] 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

[0158] 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.

[0159] 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.

Exemplary Isoprene Purification Methods

[0160] 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, 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 referred to particularly with respect to isoprene recovery and purification methods). 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. 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.

[0161] 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.

[0162] 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.

[0163] 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.

[0164] Additional methods and compositions are described in United States Provisional patent application No. 61/097,186, filed on September 15, 2008, United States Provisional patent application No. 61/097,189, filed on September 15, 2008, and United States Provisional patent application No. 61/097,163, filed on September 15, 2008, all of which are referred to particular with respect to compositions and methods for producing isoprene.

EXAMPLES

[0165] 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. In particular, all publications cited herein are expressly referred to for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention. The foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding.

Example 1: Production of isoprene in *E. coli* expressing recombinant kudzu isoprene synthase

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

[0166] The protein sequence for the kudzu (*Pueraria montana*) isoprene synthase gene (IspS) was obtained from GenBank (AAQ84170). A kudzu isoprene synthase gene, optimized for *E. coli* codon usage, was purchased from DNA2.0 (SEQ ID NO: 1). The isoprene synthase gene was removed from the supplied plasmid by restriction endonuclease digestion with *Bsp*LU11I /*Pst*I, gel-purified, and ligated into pTrcHis2B (Invitrogen) that had been digested with *Nco*I/*Pst*I. The construct was designed such that the stop codon in the isoprene synthase gene 5' to the *Pst*I/ site. As a result, when the construct was expressed the His-Tag is not attached to the isoprene synthase protein. The resulting plasmid, pTrcKudzu, was verified by sequencing (Figures 2 and 3).

[0167] The isoprene synthase gene was also cloned into pET16b (Novagen). In this case, the isoprene synthase gene was inserted into pET16b such that the recombinant isoprene synthase protein contained the N-terminal His tag. The isoprene synthase gene was amplified from pTrcKudzu by PCR using the primer set pET-His-Kudzu-2F: 5'-CGTGAGATCATATGTGTGCGACCTCTTCTCAATTTAC (SEQ ID NO:3) and pET-His-Kudzu-R: 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:4). These primers added an *Nde*I site at the 5'-end and a *Bam*H1 site at the 3' end of the gene respectively. The plasmid pTrcKudzu, described above, was used as template DNA, Herculase polymerase (Stratagene) was used according to manufacture's directions, and primers were added at a concentration of 10 pMols. The PCR was carried out in a total volume of 25 µl. The PCR product was digested with *Nde*I/*Bam*H1 and cloned into pET16b digested with the same enzymes. The ligation mix was transformed into *E. coli* Top10 (Invitrogen) and the correct clone selected by sequencing. The resulting plasmid, in which the kudzu isoprene synthase gene was expressed from the T7 promoter, was designated pETNHisKudzu (Figures 4 and 5).

[0168] The kudzu isoprene synthase gene was also cloned into the low copy number plasmid pCL1920. Primers were used to amplify the kudzu isoprene synthase gene from pTrcKudzu described above. The forward primer added a *Hind*III site and an *E. coli* consensus RBS to the 5' end. The *Pst*I cloning site was already present in pTrcKudzu just 3' of the stop codon so the reverse primer was constructed such that the final PCR product includes the *Pst*I site. The sequences of the primers were: HindIII-rbs-Kudzu F: 5'-CATATGAAAGCTTGTATCGATTAAATAAGGAGGAATAAACC (SEQ ID NO:6) and BamH1-Kudzu R:

[0169] 5'- CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:4). The PCR product was amplified using Herculase polymerase with primers at a concentration of 10 pmol and with 1 ng of template DNA (pTrcKudzu). The amplification protocol included 30 cycles of (95° C for 1 minute, 60° C

for 1 minute, 72° C for 2 minutes). The product was digested with *Hind*III and *Pst*I and ligated into pCL1920 which had also been digested with *Hind*III and *Pst*I. The ligation mix was transformed into *E. coli* Top10. Several transformants were checked by sequencing. The resulting plasmid was designated pCL-lac-Kudzu (Figures 6 and 7).

II. Determination of isoprene production

[0170] For the shake flask cultures, one ml of a culture was transferred from shake flasks to 20 ml CTC headspace vials (Agilent vial cat# 5188 2753; cap cat# 5188 2759). The cap was screwed on tightly and the vials incubated at the equivalent temperature with shaking at 250 rpm. After 30 minutes the vials were removed from the incubator and analyzed as described below *(see* Table 1 for some experimental values from this assay).

[0171] In cases where isoprene production in fermentors was determined, samples were taken from the off-gas of the fermentor and analyzed directly as described below *(see* Table 2 for some experimental values from this assay).

[0172] The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (30 m x 0.25 mm; 0.25 µm film thickness) was used for separation of analytes. The sampler was set up to inject 500 µL of headspace gas. The GC/MS method utilized helium as the carrier gas at a flow of 1 ml/minutes The injection port was held at 250° C with a split ratio of 50:1. The oven temperature was held at 37° C for the 2 minute duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 67. The detector was switched off from 1.4 to 1.7 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at 1.78 minutes. A calibration table was used to quantify the absolute amount of isoprene and was found to be linear from 1 µg/L to 200 µg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method.

III. Production of isoprene in shake flasks containing *E. coli* cells expressing recombinant isoprene synthase

[0173] The vectors described above were introduced to *E. coli* strain BL21 (Novagen) to produce strains BL21/ptrcKudzu, BL21/pCL-lac-Kudzu and BL21/pETHisKudzu. The strains were spread for isolation onto LA (Luria agar) and carbenicillin (50 µg/ml) and incubated overnight at 37° C. Single colonies were inoculated into 250 ml baffled shake flasks containing 20 ml Luria Bertani broth (LB) and carbenicillin (100 µg/ml). Cultures were grown overnight at 20° C with shaking at 200 rpm. The OD_{600} of the overnight cultures were measured and the cultures were diluted into a 250 ml baffled shake flask containing 30 ml MagicMedia (Invitrogen) and carbenicillin (100 µg/ml) to an OD_{600} ~ 0.05. The culture was incubated at 30° C with shaking at 200 rpm. When the OD_{600} ~ 0.5 - 0.8, 400 µM IPTG was added and the cells were incubated for a further 6 hours at 30° C with shaking at 200 rpm. At 0, 2, 4 and 6 hours after induction with IPTG, 1 ml aliquots of the cultures were collected, the OD_{600} was determined and the amount of isoprene produced was measured as described above. Results are shown in Figure 8.

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

[0174] Large scale production of isoprene from *E. coli* containing the recombinant kudzu isoprene synthase gene was determined from a fed-batch culture. The recipe for the fermentation media (TM2) per liter of fermentation medium was as follows: K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, MgSO4 * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with potassium hydroxide (KOH) and q.s. to volume. The final product was filter sterilized with 0.22 µ filter (only, do not autoclave). The recipe for 1000X Modified Trace Metal Solution was as follows: Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 µ filter.

[0175] This experiment was 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 was prepared in soytone-yeast extract-glucose medium. After the inoculum grew to OD_{550} = 0.6, two 600 ml flasks were centrifuged and the contents resuspended in 70 ml supernatant to transfer the cell pellet (70 ml of OD 3.1 material) to the bioreactor. At various times after inoculation, samples were removed and the amount of isoprene produced was determined as described above. Results are shown in Figure 9.

Example 2: Production of isoprene in *E. coli* expressing recombinant poplar isoprene synthase

[0176] The protein sequence for the poplar (*Populus alba* x *Populus tremula*) isoprene synthase (Schnitzler, J-P, et al. (2005) Planta 222:777-786) was obtained from GenBank (CAC35696). A gene, codon optimized for *E. coli,* was purchased from DNA2.0 (p9796-poplar, Figures 30 and 31). The isoprene synthase gene was removed from the supplied plasmid by restriction endonuclease digestion with *Bsp*LU11I/*Pst*I, gel-purified, and ligated into pTrcHis2B that had been digested with *Nco*I/*Pst*I. The construct is cloned such that the stop codon in the insert is before the *Pst*I site, which results in a construct in which the His-Tag is not attached to the isoprene synthase protein. The resulting plasmid pTrcPoplar (Figures 32 and 33), was verified by sequencing.

Example 3: Production of isoprene in *Panteoa citrea* expressing recombinant kudzu isoprene synthase

[0177] The pTrcKudzu and pCL-lac Kudzu plasmids described in Example 1 were electroporated into *P. citrea* (U.S. Pat. No. 7,241,587). Transformants were selected on LA containing carbenicillin (200 µg/ml) or spectinomycin (50 µg/ml) respectively. Production of isoprene from shake flasks and determination of the amount of isoprene produced was performed as described in Example 1 for *E. coli* strains expressing recombinant kudzu isoprene synthase. Results are shown in Figure 10.

Example 4: Production of isoprene in *Bacillus subtilis* expressing recombinant kudzu isoprene

synthase

I. Construction of a *B. subtilis* replicating plasmid for the expression of kudzu isoprene synthase

[0178] The kudzu isoprene synthase gene was expressed in *Bacillus subtilis aprEnprE Pxyl-comK* strain (BG3594comK) using a replicating plasmid (pBS19 with a chloramphenicol resistance cassette) under control of the *aprE* promoter. The isoprene synthase gene, the *aprE* promoter and the transcription terminator were amplified separately and fused using PCR. The construct was then cloned into pBS19 and transformed into *B. subtilis.*

a) Amplification of the *aprE* promoter

[0179] The *aprE* promoter was amplified from chromosomal DNA from *Bacillus subtilis* using the following primers:

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

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

b) Amplification of the isoprene synthase gene

[0180] The kudzu isoprene synthase gene was amplified from plasmid pTrcKudzu (SEQ ID NO:2). The gene had been codon optimized for *E. coli* and synthesized by DNA 2.0. The following primers were used:

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

[0181] The terminator from the alkaline serine protease of *Bacillus amyliquefaciens* was amplified from a previously sequenced plasmid pJHPms382 using the following primers:

CF 07-44 (+) Fuse the 3' end of kudzu isoprene synthase to the terminator
5'- GATTAACCAGCTGATGTATGTCTAAAAAAAACCGGCCTTGG (SEQ ID NO:62)

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

[0182] The kudzu fragment was fused to the terminator fragment using PCR with the following primers:

CF 07-42 (+) Fuse the *aprE* promoter to kudzu isoprene synthase gene (GTG start codon)
5'- TAAAAGGAGAGGGTAAAGAGTGTGTGCGACCTCTTCTCAAT (SEQ ID NO:61)

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

[0183] The kudzu-terminator fragment was fused to the promoter fragment using PCR with the following primers:

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

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

[0184] The fusion PCR fragment was purified using a Qiagen kit and digested with the restriction enzymes *Mfe*I and *Bam*HI. This digested DNA fragment was gel purified using a Qiagen kit and ligated to a vector known as pBS19, which had been digested with *Eco*RI and *Bam*HI and gel purified.

[0185] The ligation mix was transformed into *E. coli* Top 10 cells and colonies were selected on LA and 50 carbenicillin plates. A total of six colonies were chosen and grown overnight in LB and 50 carbenicillin and then plasmids were isolated using a Qiagen kit. The plasmids were digested with *Eco*RI and *Bam*HI to check for inserts and three of the correct plasmids were sent in for sequencing with the following primers:

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

[0186] The plasmid designated pBS Kudzu #2 (Figrues 52 and 12) was correct by sequencing and was transformed into BG 3594 comK, a *Bacillus subtilis* host strain. Selection was done on LA and 5 chloramphenicol plates. A transformant was chosen and struck to single colonies on LA and 5 chloramphenicol, then grown in LB and 5 chloramphenicol until it reached an OD_{600} of 1.5. It was stored frozen in a vial at -80° C in the presence of glycerol. The resulting strain was designated CF 443.

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

[0187] Overnight cultures were inoculated with a single colony of CF 443 from a LA and Chloramphenicol (Cm, 25 µg/ml). Cultures were grown in LB and Cm at 37° C with shaking at 200 rpm. These overnight cultures (1 ml) were used to inoculate 250 ml baffled shake flasks containing 25 ml Grants II media and chloramphenicol at a final concentration of 25 µg/ml. Grants II Media recipe was 10 g soytone, 3 ml 1M K_2HPO_4, 75 g glucose, 3.6 g urea, 100 ml 10X MOPS, q.s. to 1 L with H_2O, pH 7.2; 10X MOPS recipe was 83.72 g MOPS, 7.17 g tricine, 12 g KOH pellets, 10 ml 0.276M K_2SO_4 solution, 10 ml 0.528M $MgCl_2$ solution, 29.22 g NaCl, 100 ml 100X micronutrients, q.s. to 1 L with H_2O; and 100X micronutrients recipe was 1.47 g $CaCl_2$*$2H_2O$, 0.4 g $FeSO_4$*$7H_2O$, 0.1 g $MnSO_4$*H_2O, 0.1 g $ZnSO_4$*H_2O, 0.05 g $CuCl_2$*$2H_2O$, 0.1 g $CoCl_2$*$6H_2O$, 0.1 g Na_2MoO_4*$2H_2O$, q.s. to 1 L with H_2O. Shake flasks were incubated at 37° C and samples were taken at 18, 24, and 44 hours. At 18 hours the headspaces of CF443 and the control strain were sampled. This represented 18 hours of accumulation of isoprene. The amount of isoprene was determined by gas chromatography as described in Example 1. Production of isoprene was enhanced significantly by expressing recombinant isoprene synthase (Figure 11).

III. Production of isoprene by CF443 in 14 L fermentation

[0188] Large scale production of isoprene from *B. subtilis* containing the recombinant kudzu isoprene synthase gene on a replication plasmid was determined from a fed-batch culture. Bacillus strain CF 443, expressing a kudzu isoprene synthase gene, or control stain which does not express a kudzu isoprene synthase gene were cultivated by conventional fed-batch fermentation in a nutrient medium containing soy meal (Cargill), sodium and potassium phosphate, magnesium sulfate and a solution of citric acid, ferric chloride and manganese chloride. Prior to fermentation the media is macerated for 90 minutes using a mixture of enzymes including cellulases, hemicellulases and pectinases (see, WO95/04134). 14-L batch fermentations are fed with 60% wt/wt glucose (Cargill DE99 dextrose, ADM Versadex greens or Danisco invert sugar) and 99% wt/wt oil (Western Family soy oil, where the 99% wt/wt is the concentration of oil before it was added to the cell culture medium). Feed was started when glucose in the batch was non-detectable. The feed rate was ramped over several hours and was adjusted to add oil on an equal carbon basis. The pH was controlled at 6.8 - 7.4 using 28% w/v ammonium hydroxide. In case of foaming, antifoam agent was added to the media. The fermentation

temperature was controlled at 37°C and the fermentation culture was agitated at 750 rpm. Various other parameters such as pH, DO%, airflow, and pressure were monitored throughout the entire process. The DO% is maintained above 20. Samples were taken over the time course of 36 hours and analyzed for cell growth (OD_{550}) and isoprene production. Results of these experiments are presented in Figures 53A and 53B. IV. Integration of the kudzu isoprene synthase (ispS) in *B. subtilis.*

[0189] The kudzu isoprene synthase gene was cloned in an integrating plasmid (pJH101-cmpR) under the control of the *aprE* promoter. Under the conditions tested, no isoprene was detected.

Example 5: Production of isoprene in Trichoderma

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

[0190] The *Yarrowia lipolytica* codon-optimized kudzu IS gene was synthesized by DNA 2.0 (SEQ ID NO:8) (Figure 13). This plasmid served as the template for the following PCR amplification reaction: 1 µl plasmid template (20 ng/ul), 1 µl Primer EL-945 (10 uM) 5'-GCTTATGGATCCTCTAGACTATTACACGTACATCAATTGG (SEQ ID NO: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 contained an additional 4 nucleotides at the 5'-end that did not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but was required for cloning into the pENTR/D-TOPO vector. The reverse primer contained an additional 21 nucleotides at the 5'-end that did not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but were inserted for cloning into other vector backbones. Using the MJ Research PTC-200 Thermocycler, the PCR reaction was performed as follows: 95° C for 2 minutes (first cycle only), 95° C for 30 seconds, 55° C for 30 seconds, 72° C for 30 seconds (repeat for 27 cycles), 72° C for 1 minute after the last cycle. The PCR product was analyzed on a 1.2% E-gel to confirm successful amplification of the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene.

[0191] The PCR product was then cloned using the TOPO pENTR/D-TOPO Cloning Kit following manufacturer's protocol: 1 µl PCR reaction, 1 µl Salt solution, 1 µl TOPO pENTR/D-TOPO vector and 3 µl water in a total reaction volume of 6 µl. The reaction was incubated at room temperature for 5 minutes. One microliter of TOPO reaction was transformed into TOP10 chemically competent *E. coli* cells. The transformants were selected on LA and 50 µg/ml kanamycin plates. Several colonies were picked and each was inoculated into a 5 ml tube containing LB and 50 µg/ml kanamycin and the cultures grown overnight at 37° C with shaking at 200 rpm. Plasmids were isolated from the overnight culture tubes using QIAprep Spin Miniprep Kit, following manufacturer's protocol. Several plasmids were sequenced to verify that the DNA sequence was correct.

[0192] A single pENTR/D-TOPO plasmid, encoding a *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, was used for Gateway Cloning into a custom-made pTrex3g vector. Construction of pTrex3g is described in WO 2005/001036 A2. The reaction was performed following manufacturer's protocol for the Gateway LR Clonase II Enzyme Mix Kit (Invitrogen): 1 µl *Y. lipolytica* codon-optimized kudzu isoprene synthase gene pENTR/D-TOPO donor vector, 1 µl pTrex3g destination vector, 6 µl TE buffer, pH 8.0 in a total reaction volume of 8 µl. The reaction was incubated at room temperature for 1

hour and then 1 µl proteinase K solution was added and the incubation continued at 37° C for 10 minutes. Then 1 µl of reaction was transformed into TOP10 chemically competent *E. coli* cells. The transformants were selected on LA and 50 µg/ml carbenicillin plates. Several colonies were picked and each was inoculated into a 5 ml tube containing LB and 50 µlg/ml carbenicillin and the cultures were grown overnight at 37° C with shaking at 200 rpm. Plasmids were isolated from the overnight culture tubes using QIAprep Spin Miniprep Kit (Qiagen, Inc.), following manufacturer's protocol. Several plasmids were sequenced to verify that the DNA sequence was correct.

[0193] Biolistic transformation of *Y. lipolytica* codon-optimized kudzu isoprene synthase pTrex3g plasmid (Figure 14) into a quad delete *Trichoderma reesei* strain was performed using the Biolistic PDS-1000/HE Particle Delivery System (see WO 2005/001036 A2). Isolation of stable transformants and shake flask evaluation was performed using protocol listed in Example 11 of patent publication WO 2005/001036 A2.

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

[0194] One ml of 15 and 36 hour old cultures of isoprene synthase transformants described above were transferred to head space vials. The vials were sealed and incubated for 5 hours at 30° C. Head space gas was measured and isoprene was identified by the method described in Example 1. Two of the transformants showed traces of isoprene. The amount of isoprene could be increased by a 14 hour incubation. The two positive samples showed isoprene at levels of about 0.5 µg/L for the 14 hour incubation. The untransformed control showed no detectable levels of isoprene. This experiment shows that *T. reesei* is capable of producing isoprene from endogenous precursor when supplied with an exogenous isoprene synthase.

Example 6: Production of isoprene in *Yarrowia*

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

[0195] The starting point for the construction of vectors for the expression of the kudzu isoprene synthase gene in *Yarrowia lipolytica* was the vector pSPZ1(MAP29Spb). The complete sequence of this vector (SEQ ID No:11) is shown in Figure 15.

[0196] The following fragments were amplified by PCR using chromosomal DNA of a *Y. lipolytica* strain GICC 120285 as the template: a promotorless form of the URA3 gene, a fragment of 18S ribosomal RNA gene, a transcription terminator of the *Y. lipolytica* XPR2 gene and two DNA fragments containing the promoters of XPR2 and ICL1 genes. The following PCR primers were used:

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

[0197] For PCR amplification the PfuUltraII polymerase (Stratagene), supplier-provided buffer and dNTPs, 2.5 µM primers and the indicated template DNA were used as per the manufacturer's instructions. The amplification was done using the following cycle: 95° C for 1 min; 34x (95° C for 30 sec; 55° C for 30 sec; 72° C for 3 min) and 10 min at 72° C followed by a 4° C incubation.

[0198] Synthetic DNA molecules encoding the kudzu isoprene synthase gene, codon-optimized for expression in *Yarrowia,* was obtained from DNA 2.0 (Figure 16; SEQ ID NO: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 were also constructed (Figure 18E and 18F).

[0199] 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.*

[0200] Vectors pYLA(KZ1), pYLI(KZ1), pYLA(MAP29) and pYLI(MAP29) were digested with *Sac*II and used to transform the strain *Y. lipolytica* CLIB 122 by a standard lithium acetate/polyethylene glycol

procedure to uridine prototrophy. Briefly, the yeast cells grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) overnight, were collected by centrifugation (4000 rpm, 10 min), washed once with sterile water and suspended in 0.1 M lithium acetate, pH 6.0. Two hundred µl aliquots of the cell suspension were mixed with linearized plasmid DNA solution (10-20 µg), incubated for 10 minutes at room temperature and mixed with 1 ml of 50% PEG 4000 in the same buffer. The suspensions were further incubated for 1 hour at room temperature followed by a 2 minutes heat shock at 42° C. Cells were then plated on SC his leu plates (0.67% yeast nitrogen base, 2% glucose, 100 mg/L each of leucine and histidine). Transformants appeared after 3-4 days of incubation at 30° C.

[0201] Three isolates from the pYLA(KZ1) transformation, three isolates from the pYLI(KZ1) transformation, two isolates from the pYLA(MAP29) transformation and two isolates from the pYLI(MAP29) transformation were grown for 24 hours in YEP7 medium (1% yeast extract, 2% peptone, pH 7.0) at 30° C with shaking. Cells from 10 ml of culture were collected by centrifugation, resuspended in 3 ml of fresh YEP7 and placed into 15 ml screw cap vials. The vials were incubated overnight at room temperature with gentle (60 rpm) shaking. Isoprene content in the headspace of these vials was analyzed by gas chromatography using mass-spectrometric detector as described in Example 1. All transformants obtained with pYLA(KZ1) and pYLI(KZ1) produced readily detectable amounts of isoprene (0.5 µg/L to 1 µg/L, Figure 20). No isoprene was detected in the headspace of the control strains carrying phytase gene instead of an isoprene synthase gene.

Example 7: Production of isoprene in *E. coli* expressing kudzu isoprene synthase and *idi,* or *dxs,* or *idi* and *dxs*

I. Construction of vectors encoding kudzu isoprene synthase and *idi,* or *dxs,* or *idi* and *dxs* for the production of isoprene in *E. coli*

i) Construction of pTrcKudzuKan

[0202] The *bla* gene of pTrcKudzu (described in Example 1) was replaced with the gene conferring kanamycin resistance. To remove the *bla* gene, pTrcKudzu was digested with *Bsp*HI, treated with Shrimp Alkaline Phosphatase (SAP), heat killed at 65° C, then end-filled with Klenow fragment and dNTPs. The 5 kbp large fragment was purified from an agarose gel and ligated to the kan^r gene which had been PCR amplified from pCR-Blunt-II-TOPO using primers MCM22 5'-GATCAAGCTTAACCGGAATTGCCAGCTG (SEQ ID NO:14) and MCM23 5'-GATCCGATCGTCAGAAGAACTCGTCAAGAAGGC (SEQ ID NO:15), digested with *Hind*III and *Pvu*I, and end-filled. A transformant carrying a plasmid conferring kanamycin resistance (pTrcKudzuKan) was selected on LA containing kanamycin 50 µg/ml.

ii) Construction of pTrcKudzu yIDI Kan

[0203] pTrcKudzuKan was digested with *PstI,* treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *idi* from *S. cerevisiae* with a synthetic RBS. The primers for PCR

were NsiI-YIDI 1 F 5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC (SEQ ID NO:16) and PstI-YIDI 1 R 5'- CCTTCTGCAGGACGCGTTGTTATAGC (SEQ ID NO:17); and the template was *S. cerevisiae* genomic DNA. The PCR product was digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The ligation mixture was transformed into chemically competent TOP10 cells and selected on LA containing 50 µg/ml kanamycin. Several transformants were isolated and sequenced and the resulting plasmid was called pTrcKudzu-yIDI(kan) (Figures 34 and 35).

iii) Construction of pTrcKudzu DXS Kan

[0204] Plasmid pTrcKudzuKan was digested with *PstI,* treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *dxs* from *E. coli* with a synthetic RBS. The primers for PCR were MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAATAC CCG (SEQ ID NO:18) and MCM14 5'- CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:19); and the template was *E. coli* genomic DNA. The PCR product was digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The resulting transformation reaction was transformed into TOP10 cells and selected on LA with kanamycin 50 µg/ml. Several transformants were isolated and sequenced and the resulting plasmid was called pTrcKudzu-DXS(kan) (Figures 36 and 37).

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

[0205] pTrcKudzu-yIDI(kan) was digested with *PstI,* treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *E. coli dxs* with a synthetic RBS (primers MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAATAC CCG (SEQ ID NO:18) and MCM14 5'- CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:19); template TOP 10 cells) which had been digested with *Nsi*I and *Pst*I and gel purified. The final plasmid was called pTrcKudzu-yIDI-dxs (kan) (Figures 21 and 22).

v) Construction of pCL PtrcKudzu

[0206] A fragment of DNA containing the promoter, structural gene and terminator from Example 1 above was digested from pTrcKudzu using *Ssp*I and gel purified. It was ligated to pCL1920 which had been digested with *Pvu*II, treated with SAP and heat killed. The resulting ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 µg/ml. Several clones were isolated and sequenced and two were selected. pCL PtrcKudzu and pCL PtrcKudzu (A3) have the insert in opposite orientations (Figures 38-41).

vi) Construction of pCL PtrcKudzu yIDI

[0207] The *Nsi*I-*Pst*I digested, gel purified, IDI PCR amplicon from (ii) above was ligated into pCL PtrcKudzu which had been digested with *Pst*I, treated with SAP, and heat killed. The ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 µg/ml. Several clones were isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu yIDI (Figures 42 and 43).

vii) Construction of pCL PtrcKudzu DXS

[0208] The *NsiI-PstI* digested, gel purified, DXS PCR amplicon from (iii) above was ligated into pCL PtrcKudzu (A3) which had been digested with *PstI,* treated with SAP, and heat killed. The ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 µg/ml. Several clones were isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu DXS (Figures 44 and 45).

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

[0209] Cultures of *E. coli* BL21(λDE3) previously transformed with plasmids pTrcKudzu(kan) (A), pTrcKudzu-yIDI kan (B), pTrcKudzu-DXS kan (C), pTrcKudzu-yIDI-DXS kan (D) were grown in LB kanamycin 50 µg/mL. Cultures of pCL PtrcKudzu (E), pCL PtrcKudzu, pCL PtrcKudzu-yIDI (F) and pCL PtrcKudzu-DXS (G) were grown in LB spectinomycin 50 µg/mL. Cultures were induced with 400 µM IPTG at time 0 (OD_{600} approximately 0.5) and samples taken for isoprene headspace measurement (see Example 1). Results are shown in Figure 23A-23G.

[0210] Plasmid pTrcKudzu-yIDI-dxs (kan) was introduced into *E. coli* strain BL21 by transformation. The resulting strain BL21/pTrc Kudzu IDI DXS was grown overnight in LB containing kanamycin (50 µg/ml) at 20° C and used to inoculate shake flasks of TM3 (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_2O, and filter sterilized) containing 1% glucose. Flasks were incubated at 30° C until an OD_{600} of 0.8 was reached, and then induced with 400 µM IPTG. Samples were taken at various times after induction and the amount of isoprene in the head space was measured as described in Example 1. Results are shown in Figure 23H.

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

[0211] The strain BL21 pTrcKudzuIDIDXS was tested for the ability to generate isoprene from three types of biomass; bagasse, corn stover and soft wood pulp with glucose as a control. Hydrolysates of the biomass were prepared by enzymatic hydrolysis (Brown, L and Torget, R., 1996, NREL standard assay method Lap-009 "Enzymatic Saccharification of Lignocellulosic Biomass") and used at a dilution based upon glucose equivalents. In this example, glucose equivalents were equal to 1% glucose. A single colony from a plate freshly transformed cells of BL21 (DE3) pTrcKudzu yIDI DXS (kan) was used to inoculate 5 ml of LB plus kanamycin (50 µg/ml). The culture was incubated overnight at 25° C with shaking. The following day the overnight culture was diluted to an OD_{600} of 0.05 in 25 ml of TM3 and 0.2% YE and 1% feedstock. The feedstock was corn stover, bagasse, or softwood pulp. Glucose was used as a positive control and no glucose was used as a negative control. Cultures were incubated at 30° C with shaking at 180 rpm. The culture was monitored for OD_{600} and when it reached an OD_{600} of ~0.8, cultures were analyzed at 1 and 3 hours for isoprene production as described in Example 1.

Cultures are not induced. All cultures containing added feedstock produce isoprene equivalent to those of the glucose positive control. Experiments were done in duplicate and are shown in Figure 46.

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

[0212] A single colony from a plate freshly transformed cells of BL21 (λDE3)/pTrcKudzu yIDI DXS (kan) was used to inoculate 5 mL of LB and kanamycin (50 µg/ml). The culture was incubated overnight at 25° C with shaking. The following day the overnight culture was diluted to an OD_{600} of 0.05 in 25 ml of TM3 and 0.2% YE and 1% feedstock. Feedstock was glucose, inverted glucose or corn stover. The invert sugar feedstock (Danisco Invert Sugar) was prepared by enzymatically treating sucrose syrup. AFEX corn stover was prepared as described below (Part V). The cells were grown at 30° C and the first sample was measured when the cultures reached an OD_{600} ~0.8-1.0 (0 hour). The cultures were analyzed for growth as measured by OD_{600} and for isoprene production as in Example 1 at 0, 1 and 3 hours. Results are shown in Figure 47.

V. Preparation of hydrolysate from AFEX pretreated corn stover

[0213] AFEX pretreated corn stover was obtained from Michigan Biotechnology Institute. The pretreatment conditions were 60% moisture, 1:1 ammonia loading, and 90 °C for 30 minutes, then air dried. The moisture content in the AFEX pretreated corn stover was 21.27%. The contents of glucan and xylan in the AFEX pretreated corn stover were 31.7% and 19.1% (dry basis), respectively. The saccharification process was as follows; 20 g of AFEX pretreated corn stover was added into a 500 ml flask with 5 ml of 1 M sodium citrate buffer pH 4.8, 2.25 ml of Accellerase 1000, 0.1 ml of Grindamyl H121 (Danisco xylanase product from Aspergillus niger for bread-making industry), and 72.65 ml of DI water. The flask was put in an orbital shaker and incubated at 50° C for 96 hours. One sample was taken from the shaker and analyzed using HPLC. The hydrolysate contained 38.5 g/l of glucose, 21.8 g/l of xylose, and 10.3 g/l of oligomers of glucose and/or xylose.

VI. The effect of yeast extract on isoprene production in *E. coli* grown in fed-batch culture

[0214] Fermentation was performed at the 14-L scale as previously described with *E. coli* cells containing the pTrcKudzu yIDI DXS plasmid described above. Yeast extract (Bio Springer, Montreal, Quebec, Canada) was fed at an exponential rate. The total amount of yeast extract delivered to the fermentor was varied between 70-830 g during the 40 hour fermentation. Optical density of the fermentation broth was measured at a wavelength of 550 nm. The final optical density within the fermentors was proportional to the amount of yeast extract added (Figure 48A). The isoprene level in the off-gas from the fermentor was determined as previously described. The isoprene titer increased over the course of the fermentation (Figure 48B). The amount of isoprene produced was linearly proportional to the amount of fed yeast extract (Figure 48C).

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

[0215] 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 (λDPE3 pTrc Kudzu dxs yidi) was used to produce isoprene. The levels of isoprene varied from 50 to 300 µg/L over a time period of 15 hours. On the basis of the average isoprene concentrations, the average flow through the device and the extent of isoprene breakthrough, the amount of isoprene collected was calculated to be approximately 17 g.

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

Medium Recipe (per liter fermentation medium):

[0216] K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium gas (NH_3) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotic were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0217] Citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0218] Fermentation was performed in a 500-L bioreactor with E. coli cells containing the pTrcKudzu yIDI DXS plasmid. This experiment was carried out to monitor isoprene formation from glucose and yeast extract at the desired fermentation pH 7.0 and temperature 30° C. An inoculum of *E. coli* strain taken from a frozen vial was prepared in soytone-yeast extract-glucose medium. After the inoculum grew to OD 0.15, measured at 550 nm, 20 ml was used to inoculate a bioreactor containing 2.5-L soytone-yeast extract-glucose medium. The 2.5-L bioreactor was grown at 30° C to OD 1.0 and 2.0-L was transferred to the 500-L bioreactor.

[0219] Yeast extract (Bio Springer, Montreal, Quebec, Canada) and glucose were fed at exponential rates. The total amount of glucose and yeast extract delivered to the bioreactor during the 50 hour fermentation was 181.2 kg and 17.6 kg, respectively. The optical density within the bioreactor over time is shown in Figure 49A. The isoprene level in the off-gas from the bioreactor was determined as previously described. The isoprene titer increased over the course of the fermentation (Figure 49B). The total amount of isoprene produced during the 50 hour fermentation was 55.1 g and the time course of production is shown in Figure 49C.

Example 8: Production of isoprene in *E. coli* expressing kudzu isoprene synthase and recombinant mevalonic acid pathway genes

I. Cloning the lower MVA pathway

[0220] The strategy for cloning the lower mevalonic pathway was as follows. Four genes of the mevalonic acid biosynthesis pathway; mevalonate kinase (MVK), phosphomevalonate kinase (PMK), diphosphomevalonte decarboxylase (MVD) and isopentenyl diphosphate isomerase genes were amplified by PCR from *S. cerevisiae* chromosomal DNA and cloned individually into the pCR BluntII TOPO plasmid (Invitrogen). In some cases, the *idi* gene was amplified from *E. coli* chromosomal DNA. The primers were designed such that an *E. coli* consensus RBS (AGGAGGT (SEQ ID NO:80) or AAGGAGG (SEQ ID NO:81)) was inserted at the 5' end, 8 bp upstream of the start codon and a *Pst*I site was added at the 3' end. The genes were then cloned one by one into the pTrcHis2B vector until the entire pathway was assembled.

[0221] Chromosomal DNA from *S. cerevisiae* S288C was obtained from ATCC (ATCC 204508D). The MVK gene was amplified from the chromosome of *S. cerevisiae* using primers MVKF (5'-AGGAGGTAAAAAAACATGTCATTACCGTTCTTAACTTCTGC, SEQ ID NO: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) was identified by electrophoresis through a 1.2% E-gel (Invitrogen) and cloned into pZeroBLUNT TOPO. The resulting plasmid was designated pMVK1. The plasmid pMVK1 was digested with *Sac*I and *Taq*1 restriction endonucleases and the fragment was gel purified and ligated into pTrcHis2B digested with *Sac*I and *Bst*BI. The resulting plasmid was named pTrcMVK1.

[0222] The second gene in the mevalonic acid biosynthesis pathway, PMK, was 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 was performed using Pfu Turbo polymerase (Stratagene) as per manufacturer's instructions. The correct sized product (1387 bp) was digested with *Pst*I and *Bsi*HKI and ligated into pTrcMVKI digested with *Pst*I. The resulting plasmid was named pTrcKK. The MVD and the *idi* genes were cloned in the same manner. PCR was carried out using the primer pairs PstI-MVD 1 R (5'-GTGCTGGAATTCGCCCTTCTGCAGC, SEQ ID NO: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* was used. To amplify *idi* from *E. coli* chromosomal DNA, the following primer set was used: PstI-CIDI 1 R (5'-GTGTGATGGATATCTGCAGAATTCG, SEQ ID NO:29) and NsiI-CIDI 1 F (5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAACATG, SEQ ID NO:30). Template DNA was chromosomal DNA isolated by standard methods from *E. coli* FM5 (WO 96/35796 and WO 2004/033646, which are each hereby referred to particularly with respect to isolation of nucleic acids). The final plasmids were named pKKDIy for the construct encoding the yeast *idi* gene or pKKDIc for the construct encoding the *E. coli idi* gene. The plasmids were transformed into *E. coli* hosts BL21 for subsequent analysis. In some cases the isoprene synthase from kudzu was cloned into pKKDIy yielding plasmid pKKDIyIS.

[0223] The lower MVA pathway was also cloned into pTrc containing a kanamycin antibiotic resistance marker. The plasmid pTrcKKDIy was digested with restriction endonucleases *Apa*I and *Pst*I, the 5930 bp fragment was separated on a 1.2% agarose E-gel and purified using the Qiagen Gel Purification kit

according to the manufacturer's instructions. The plasmid pTrcKudzuKan, described in Example 7, was digested with restriction endonucleases *Apa*I and *Pst*I, and the 3338 bp fragment containing the vector was purified from a 1.2% E-gel using the Qiagen Gel Purification kit. The 3338 bp vector fragment and the 5930 bp lower MVA pathway fragment were ligated using the Roche Quick Ligation kit. The ligation mix was transformed into *E. coli* TOP10 cells and tranformants were grown at 37° C overnight with selection on LA containing kanamycin (50 µg/ml). The transformants were verified by restriction enzyme digestion and one was frozen as a stock. The plasmid was designated pTrcKanKKDIy.

II. Cloning a kudzu isoprene synthase gene into pTrcKanKKDIy

[0224] The kudzu isoprene synthase gene was amplified by PCR from pTrcKudzu, described in Example 1, using primers MCM50 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGTGTGCGACCTCTTCTCAATTT ACT (SEQ ID NO:31) and MCM53 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:32). The resulting PCR fragment was cloned into pCR2.1 and transformed into *E. coli* TOP10. This fragment contains the coding sequence for kudzu isoprene synthase and an upstream region containing a RBS from *E. coli.* Transformants were incubated overnight at 37° C with selection on LA containing carbenicillin (50 µg/ml). The correct insertion of the fragment was verified by sequencing and this strain was designated MCM93.

[0225] The plasmid from strain MCM93 was digested with restriction endonucleases *Nsi*I and *Pst*I to liberate a 1724 bp insert containing the RBS and kudzu isoprene synthase. The 1724 bp fragment was separated on a 1.2% agarose E-gel and purified using the Qiagen Gel Purification kit according to the manufacturer's instructions. Plasmid pTrcKanKKDIy was digested with the restriction endonuclease *Pst*I, treated with SAP for 30 minutes at 37° C and purified using the Qiagen PCR cleanup kit. The plasmid and kudzu isoprene synthase encoding DNA fragment were ligated using the Roche Quick Ligation kit. The ligation mix was transformed into *E. coli* TOP10 cells and transformants were grown overnight at 37° C with selection on LA containing Kanamycin at 50 µg/ml. The correct transformant was verified by restriction digestion and the plasmid was designated pTrcKKDyIkISKan (Figures 24 and 25). This plasmid was transformed into BL21(λDE3) cells (Invitrogen).

III. Isoprene production from mevalonate in *E. coli* expressing the recombinant lower mevalonate pathway and isoprene synthase from kudzu.

[0226] Strain BL21/pTrcKKDyIkISKan was cultured in MOPS medium (Neidhardt et al., (1974) J. Bacteriology 119:736-747) adjusted to pH 7.1 and supplemented with 0.5% glucose and 0.5% mevalonic acid. A control culture was also set up using identical conditions but without the addition of 0.5% mevalonic acid. The culture was started from an overnight seed culture with a 1% inoculum and induced with 500 µM IPTG when the culture had reached an OD_{600} of 0.3 to 0.5. The cultures were grown at 30° C with shaking at 250 rpm. The production of isoprene was analyzed 3 hours after induction by using the head space assay described in Example 1. Maximum production of isoprene was 6.67 x 10^4 nmol/L_{broth}/OD_{600}/hr where L_{broth} is the volume of broth and includes both the volume of the cell medium and the volume of the cells. The control culture not supplemented with mevalonic acid did not produce measurable isoprene.

IV. Cloning the upper MVA pathway

[0227] The upper mevalonate biosynthetic pathway, comprising two genes encoding three enzymatic activities, was cloned from *Enterococcus faecalis.* The *mvaE* gene encodes a protein with the enzymatic activities of both acetyl-CoA acetyltransferase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the first and third proteins in the pathway, and the *mvaS* gene encodes second enzyme in the pathway, HMG-CoA synthase. The *mvaE* gene was amplified from *E. faecalis* genomic DNA (ATCC 700802D-5) with an *E. coli* ribosome binding site and a spacer in front using the following primers:

CF 07-60 (+) Start of *mvaE* w/ RBS + ATG start codon *Sac*I
5' - GAGACATGAGCTCAGGAGGTAAAAAAACATGAAAACAGTAGTTATTATTG (SEQ ID NO:34)

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

[0228] The *mvaS* gene was amplified from *E. faecalis* genomic DNA (ATCC 700802D-5) with a RBS and spacer from *E. coli* in front using the following primers:

CF 07-61 (+) Fuse *mvaE* to *mvaS* with RBS in between
5' - GATTTAAGAAAACAATAAAGGAGGTAAAAAAACATGACAATTGGGATTGATAAA (SEQ ID NO:36)

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

[0229] The PCR fragments were fused together with PCR using the following primers:

CF 07-60 (+) Start *of mvaE* w/ RBS + ATG start codon *Sac*I
5' -GAGACATGAGCTCAGGAGGTAAAAAAACATGAAAACAGTAGTTATTATTG (SEQ ID NO:34)

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

[0230] The fusion PCR fragment was purified using a Qiagen kit and digested with the restriction enzymes *Sac*I and *Bgl*II. This digested DNA fragment was gel purified using a Qiagen kit and ligated into the commercially available vector pTrcHis2A, which had been digested with *Sac*I and *Bgl*II and gel purified.

[0231] The ligation mix was transformed into *E. coli* Top 10 cells and colonies were selected on LA and 50 µg/ml carbenicillin plates. A total of six colonies were chosen and grown overnight in LB and 50 µg/ml carbenicillin and plasmids were isolated using a Qiagen kit. The plasmids were digested with *Sac*I and *Bgl*II to check for inserts and one correct plasmid was sequenced with the following primers:

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

5' -ATGAAAACAGTAGTTATTATTGATGC (SEQ ID NO: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)

[0232] The plasmid called pTrcHis2AUpperPathway#1 was correct by sequencing and was transformed into the commercially available *E. coli* strain BL21. Selection was done on LA and 50 µg/ml carbenicillin. Two transformants were chosen and grown in LB and 50 µg/ml carbenicillin until they reached an OD_{600} of 1.5. Both strains were frozen in a vial at -80° C in the presence of glycerol. Strains were designated CF 449 for pTrcHis2AUpperPathway#1 in BL21, isolate #1 and CF 450 for pTrcHis2AUpperPathway#1 in BL21, isolate #2. Both clones were found to behave identically when analyzed.

V. Cloning of UpperMVA Pathway into pCL1920

[0233] The plasmid pTrcHis2AUpperPathway was digested with the restriction endonuclease *Ssp*I to release a fragment containing pTrc-*mvaE*-*mvaS*-(His tag)-terminator. In this fragment, the his-tag was not translated. This blunt ended 4.5 kbp fragment was purified from a 1.2% E-gel using the Qiagen Gel Purification kit. A dephosphorylated, blunt ended 4.2 kbp fragment from pCL1920 was prepared by digesting the vector with the restriction endonuclease *Pvu*II, treating with SAP and gel purifying from a 1.2% E-gel using the Qiagen Gel Purification kit. The two fragments were ligated using the Roche Quick Ligation Kit and transformed into TOP10 chemically competent cells. Transformants were selected on LA containing spectinomycin (50 µg/ml). A correct colony was identified by screening for the presence of the insert by PCR. The plasmid was designated pCL PtrcUpperPathway (Figures 26 and 27).

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

[0234] To obtain a strain with a complete mevalonic acid pathway plus kudzu isoprene synthase,

plasmids pTrcKKDyIkISkan and pCLpTrcUpperPathway were both transformed into BL21(λDE3) competent cells (Invitrogen) and transformants were selected on LA containing kanamycin (50 µg/ml) and Spectinomycin (50 µg/ml). The transformants were checked by plasmid prep to ensure that both plasmids were retained in the host. The strain was designated MCM127.

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

[0235] Single colonies of the BL21/pTrcHis2A-*mvaE*/*mvaS* or FM5/p pTrcHis2A-*mvaE*/*mvaS* are inoculated into LB and carbenicillin (100 µg/ml) and are grown overnight at 37° C with shaking at 200 rpm. These cultures were diluted into 50 ml medium in 250 ml baffled flasks to an OD_{600} of 0.1. The medium was TM3, 1 or 2% glucose, carbenicillin (100 ug/ml) or TM3, 1% glucose. hydrolyzed soy oil, and carbenicillin (100 ug/ml) or TM3 and biomass (prepared bagasse, corn stover or switchgrass). Cultures were grown at 30° C with shaking at 200 rpm for approximately 2-3 hours until an OD_{600} of 0.4 was reached. At this point the expression from the *mvaE mvaS* construct was induced by the addition of IPTG (400 µM). Cultures were incubated for a further 20 or 40 hours with samples taken at 2 hour intervals to 6 hour post induction and then at 24, 36 and 48 hours as needed. Sampling was done by removing 1 ml of culture, measuring the OD_{600}, pelleting the cells in a microfuge, removing the supernatant and analyzing it for mevalonic acid.

[0236] A 14 liter fermentation of *E. coli* cells with nucleic acids encoding *Enterococcus faecalis* AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase polypeptides produced 22 grams of mevalonic acid with TM3 medium and 2% glucose as the cell medium. A shake flask of these cells produced 2-4 grams of mevalonic acid per liter with LB medium and 1% glucose as the cell culture medium. The production of mevalonic acid in these strains indicated that the MVA pathway was functional in *E. coli.*

VIII. Production of isoprene from *E. coli* BL21 containing the upper and lower MVA pathway plus kudzu isoprene synthase.

[0237] The following strains were created by transforming in various combinations of plasmids containing the upper and lower MVA pathway and the kudzu isoprene synthase gene as described above and the plasmids containing the idi, dxs, and dxr and isoprene synthase genes described in Example 7. The host cells used were chemically competent BE21(λBE3) and the transformations were done by standard methods. Transformants were selected on L agar containing kanamycin (50 µg/ml) or kanamycin plus spectinomycin (both at a concentration of 50 µg/ml). Plates were grown at 37° C. The resulting strains were designated as follows:

Grown on Kanamycin plus Spectinomycin (50 µg/ml each)

MCM127 - pCL Upper MVA and pTrcKKDyIkIS (kan) in BL21(λDE3)

MCM131 - pCL1920 and pTrcKKDyIkIS (kan) in BL21(λDE3)

MCM125 - pCL Upper MVA and pTrcHis2B (kan) in BE21(λDPE3

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)

[0238] The above strains were streaked from freezer stocks to LA and appropriate antibiotic and grown overnight at 37° C. A single colony from each plate was used to inoculate shake flasks (25 ml LB and the appropriate antibiotic). The flasks were incubated at 22° C overnight with shaking at 200 rpm. The next morning the flasks were transferred to a 37° C incubator and grown for a further 4.5 hours with shaking at 200 rpm. The 25 ml cultures were centrifuged to pellet the cells and the cells were resuspended in 5 ml LB and the appropriate antibiotic. The cultures were then diluted into 25 ml LB, % glucose, and the appropriate antibiotic to an OD_{600} of 0.1. Two flasks for each strain were set up, one set for induction with IPTG (800 µM) the second set was not induced. The cultures were incubated at 37° C with shaking at 250 rpm. One set of the cultures were induced after 1.50 hours (immediately following sampling time point 1). At each sampling time point, the OD_{600} was measured and the amount of isoprene determined as described in Example 1. Results are presented in Table 3. The amount of isoprene made is presented as the amount at the peak production for the particular strain.

Table 3. Production of isoprene in *E. coli* strains

Strain	Isoprene ($\mu g/L_{broth}/hr/OD$
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

[0239] Mevalonolactone (1.0 g, 7.7 mmol) (CAS# 503-48-0) was supplied from Sigma-Aldrich (WI, USA) as a syrup that was dissolved in water (7.7 mL) and was treated with potassium hydroxide (7.7 mmol) in order to generate the potassium salt of mevalonic acid. The conversion to mevalonic acid was confirmed by ^1H NMR analysis. Samples for HPLC analysis were prepared by centrifugation at 14,000 rpm for 5 minutes to remove cells, followed by the addition of a 300 µl aliquot of supernatant to 900 µl of H_2O. Perchloric acid (36 µl of a 70% solution) was then added followed by mixing and cooling on ice for 5 minutes. The samples were then centrifuged again (14,000 rpm for 5 min) and the supernatant transferred to HPLC. Mevalonic acid standards (20, 10, 5, 1 and 0.5 g/L) were prepared in the same fashion. Analysis of mevalonic acid (20 uL injection volume) was performed by HPLC using a BioRad Aminex 87-H+ column (300 mm by 7.0 mm) eluted with 5 mM sulfuric acid at 0.6 mL/min with refractive index (RI) detection. Under these conditions mevalonic acid eluted as the lactone form at 18.5 minutes.

Example 9. Construction of the upper and lower MVA pathway for integration into *Bacillus*

subtilis

I. Construction of the Upper MVA pathway in *Bacillus subtilis*

[0240] 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.

[0241] 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

[0242] The product is digested with restriction endonucleases *Pst*I/*Bam*HI and ligated to pJM102 (Perego, M. 1993. Integrational vectors for genetic manipulation in Bacillus subtilis, p. 615-624. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), Bacillus subtilis and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.) which is digested with *Pst*I/*Bam*HI. The ligation is transformed into *E.coli* TOP 10 chemically competent cells and transformants are selected on LA containing carbenicillin (50 µg/ml). The correct plasmid is identified by sequencing and is designated pJMUpperpathway2 (Figures 50 and 51). 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.

[0243] 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.

[0244] This strain is used subsequently as a host for the integration of the lower mevalonic acid pathway.

[0245] The following primers are used to sequence the various constructs above.

Sequencing primers:

[0246]

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)

[0247] 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 µng/ml).

II. Construction of the Lower MVA pathway in *Bacillus subtilis*

[0248] 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: Production of isoprene in *E. coli* expressing *M. mazei* mevalonate kinase and *P. alba* isoprene synthase

I. Construction of vectors and strains encoding *M. mazei* mevalonate kinase (MVK) and *P. alba* isoprene synthase

(i) Construction of strain EWL201 (BL21, Cm-GI1.2-KKDyI)

[0249] *E. coli* BL21 (Novagen brand, EMD Biosciences, Inc.) was a recipient strain, transduced with MCM331 P1 lysate (lysate prepared according to the method described in Ausubel, et al., Current Protocols in Molecular Biology. John Wiley and Sons, Inc.). Transductants were selected for by spreading cells onto L Agar and 20 µg/(µl chloramphenicol. The plates were incubated overnight at 30°C. Analysis of transductants showed no colonies on control plates (water + cells control plate for reversion and water and P1 lysate control plate for lysate contamination.

[0250] Four transductants were picked and used to inoculate 5 mL L Broth and 20 µg/µl chloramphenicol. The cultures were grown overnight at 30°C with shaking at 200 rpm. To make genomic DNA preps of each transductant for PCR analysis, 1.5mL of overnight cell culture were centrifuged. The cell pellet was resuspended with 400µl Resuspension Buffer (20mM Tris, 1mM EDTA, 50mM NaCl, pH 7.5) and 4µl RNase, DNase-free (Roche) was added. The tubes were incubated at 37°C for 30 minutes followed by the addition of 4µl 10% SDS and 4µl of 10mg/ml Proteinase K stock solution (Sigma-Aldrich). The tubes were incubated at 37°C for 1 hour. The cell lysate was transferred into 2ml Phase Lock Light Gel tubes (Eppendorf) and 200µl each of saturated phenol pH7.9 (Ambion Inc.) and chloroform were added. The tubes were mixed well and microcentrifuged for 5 minutes. A second extraction was done with 400µl chloroform and the aqueous layer was transferred to a new eppendorf tube. The genomic DNA was precipitated by the addition of 1ml of 100% ethanol and centrifugation for 5 minutes. The genomic DNA pellet was washed with 1ml 70% ethanol. The ethanol was removed and the genomic DNA pellet was allowed to air dry briefly. The genomic DNA pellet was resuspended with 200µl TE.

[0251] Using Pfu Ultra II DNA polymerase (Stratagene) and 200ng/µl of genomic DNA as template, 2 different sets of PCR reaction tubes were prepared according to manufacturer's protocol. For set 1, primers MCM130 and GB Cm-Rev (Table 4) were used to ensure transductants were successfully integrated into the attTn7 locus. PCR parameters for set 1 were 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 25 seconds, 72°C for 25 seconds (repeat steps 2-4 for 28 cycles), 72°C for 1 minute. For set 2, primers MVD For and MVD Rev (Table 4) were used to ensure that the gi1.2-KKDyI operon integrated properly. PCR parameters for set 2 were 95°C for 2 minutes (first cycle only),

95°C for 25 seconds, 55°C for 25 seconds, 72°C for 10 seconds (repeat steps 2-4 for 28 cycles), 72°C for 1 minute. Analysis of PCR amplicons on a 1.2% E-gel (Invitrogen Corp.) showed that all 4 transductant clones were correct (picked one and designated as strain EWL201).

ii) Construction of Strain EWL204 (BL21, 1oopout-GI1.2-KKDyI)

[0252] The chloramphenicol marker was looped out of strain EWL201 using plasmid pCP20 as described by Datsenko and Wanner (2000) (Datsenko et al., Proc Natl. Acad. Sci USA 97:6640-6645, 2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. (Datsenko et al., PNAS, 97: 6640-6645, 2000). EWL201 cells were grown in L Broth to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50µl of cell suspension was mixed with 1 µl of pCP20 and the cell suspension mixture was electroporated in a 2mm cuvette (Invitrogen Corp.) at 2.5 Volts and 25uFd using a Gene Pulser Electroporator (Bio-Rad Inc.). 1ml of LB was immediately added to the cells, then transferred to a 14ml polypropylene tube (Sarstedt) with a metal cap. Cells were allowed to recover by growing for 1 hour at 30°C. Transformants were selected on L Agar and 20µg/µl chloramphenicol and 50µg/µl carbenicillin and incubated at 30°C overnight. The next day, a single clone was grown in 10ml L Broth and 50µg/µl carbenicillin at 30°C until early log phase. The temperature of the growing culture was then shifted to 42°C for 2 hours. Serial dilutions were made, the cells were then spread onto LA plates (no antibiotic selection), and incubated overnight at 30°C. The next day, 20 colonies were picked and patched onto L Agar (no antibiotics) and LA and 20µg/µl chloramphenicol plates. Plates were then incubated overnight at 30°C. Cells able to grow on LA plates, but not LA and 20µg/µl chloramphenicol plates, were deemed to have the chloramphenicol marker looped out (picked one and designated as strain EWL204).

iii) Construction of plasmid pEWL230 (pTrc P. alba)

[0253] Generation of a synthetic gene encoding *Populus alba* isoprene synthase (P. alba HGS) was outsourced to DNA2.0 Inc. (Menlo Park, CA) based on their codon optimization method for *E. coli* expression. The synthetic gene was custom cloned into plasmid pET24a (Novagen brand, EMD Biosciences, Inc.) and delivered lyophilized (Figures 54, 55A and 55B).

[0254] A PCR reaction was performed to amplify the *P. alba* isoprene synthase (*P. alba* HGS) gene using pET24 P. alba HGS as the template, primers MCM182 and MCM192, and Herculase II Fusion DNA polymerase (Stratagene) according to manufacturer's protocol. PCR conditions were as follows: 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 20 seconds, 72°C for 1 minute, repeat for 25 cycles, with final extension at 72°C for 3 minutes.
The *P. alba* isoprene synthase PCR product was purified using QIAquick PCR Purification Kit (Qiagen Inc.).

[0255] *P. alba* isoprene synthase PCR product was then digested in a 20µl reaction containing 1µl *Bsp*HI endonuclease (New England Biolabs) with 2µl 10X NEB Buffer 4. The reaction was incubated for 2 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 20µl reaction containing 1 µl *Pst*I endonuclease (Roche) with 2µl 10X Buffer H. The reaction was incubated for 2 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. Plasmid pTrcHis2B (Invitrogen

Corp.) was digested in a 20µl reaction containing 1 µl *Nco*I endonuclease (Roche), 1µl *Pst*I endonuclease, and 2µl 10X Buffer H. The reaction was incubated for 2 hours at 37°C. The digested pTrcHis2B vector was gel purified using a 1.2% E-gel (Invitrogen Corp.) and extracted using the QIAquick Gel Extraction Kit (Qiagen) (Figure 56). Using the compatible cohesive ends of *Bsp*HI and *Nco*I sites, a 20µl ligation reaction was prepared containing 5µl *P. alba* isoprene synthase insert, 2µl pTrc vector, 1µl T4 DNA ligase (New England Biolabs), 2µl 10X ligase buffer, and 10µl ddH_2O. The ligation mixture was incubated at room temperature for 40 minutes. The ligation mixture was desalted by floating a 0.025µm nitrocellulose membrane filter (Millipore) in a petri dish of ddH_2O and applying the ligation mixture gently on top of the nitrocellulose membrane filter for 30 minutes at room temperature. MCM446 cells (See section II) were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50µl of cell suspension was mixed with 5µl of desalted pTrc P.alba HGS ligation mix. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB is immediately added to the cells, then transferred to a 14ml polypropylene tube (Sarstedt) with a metal cap. Cells were allowed to recover by growing for 2 hour at 30°C. Transformants were selected on L Agar and 50µg/µl carbenicillin and 10mM mevalonic acid and incubated at 30°C. The next day, 6 transformants were picked and grown in 5ml L Broth and 50µg/µl carbenicillin tubes overnight at 30°C. Plasmid preps were performed on the overnight cultures using QIAquick Spin Miniprep Kit (Qiagen). Due to the use of BL21 cells for propagating plasmids, a modification of washing the spin columns with PB Buffer 5X and PE Buffer 3X was incorporated to the standard manufacturer's protocol for achieving high quality plasmid DNA. Plasmids were digested with *Pst*I in a 20µl reaction to ensure the correct sized linear fragment. All 6 plasmids were the correct size and shipped to Quintara Biosciences (Berkeley, CA) for sequencing with primers MCM65, MCM66, EL1000 (Table 4). DNA sequencing results showed all 6 plasmids were correct. Picked one and designated plasmid as EWL230 (Figures 57, 58A and 58B).

iv) Construction of plasmid pEWL244 (pTrc P. alba-mMVK)

[0256] A PCR reaction was performed to amplify the *Methanosarcina mazei* (*M. mazei)* MVK gene using MCM376 as the template (see section v), primers MCM165 and MCM177 (see Table 4), and Pfu Ultra II Fusion DNA polymerase (Stratagene) according to manufacturer's protocol. PCR conditions were as follows: 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 25 seconds, 72°C for 18 seconds, repeat for 28 cycles, with final extension at 72°C for 1 minute. The *M. mazei* MVK PCR product was purified using QIAquick PCR Purification Kit (Qiagen Inc.,)

[0257] The *M. mazei* MVK PCR product was then digested in a 40µl reaction containing 8µl PCR product, 2µl *Pme*I endonuclease (New England Biolabs), 4µl 10X NEB Buffer 4, 4µl 10X NEB BSA, and 22µl of ddH_2O. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 47µl reaction containing 2µl *Nsi*I endonuclease (Roche), 4.7µl 10X Buffer H, and 40µl of PmeI digested *M. mazei* MVK fragment. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then gel purified using a 1.2% E-gel and extracted using the QIAquick Gel Extraction Kit. Plasmid EWL230 was digested in a 40µl reaction containing 10µl plasmid, 2µl PmeI endonuclease, 4µl 10X NEB Buffer 4, 4µl 10X NEB BSA, and 20µl of ddH_2O. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 47µl reaction containing 2µl *Pst*I endonuclease, 4.7µl 10X Buffer H, and 40µl of *Pme*I digested EWL230 linear fragment. The reaction was incubated for 3 hours at 37°C.

The digested PCR fragment was then gel purified using a 1.2% E-gel and extracted using the QIAquick Gel Extraction Kit (Figure 59). Using the compatible cohesive ends of *Nsi*I and *Pst*I sites, a 20µl ligation reaction was prepared containing 8µl *M. mazei* MVK insert, 3µl EWL230 plasmid, 1µl T4 DNA ligase, 2µl 10X ligase buffer, and 6µl ddH_2O. The ligation mixture was incubated at overnight at 16°C. The next day, the ligation mixture was desalted by floating a 0.025µm nitrocellulose membrane filter in a petri dish of ddH_2O and applying the ligation mixture gently on top of the nitrocellulose membrane filter for 30 minutes at room temperature. MCM446 cells were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50µl of cell suspension was mixed with 5µl of desalted pTrc P.alba-mMVK ligation mix. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB is immediately added to the cells, then the cells are transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hour at 30°C. Transformants were selected on LA and 50µg/µl carbenicillin and 5mM mevalonic acid plates and incubated at 30°C. The next day, 6 transformants were picked and grown in 5ml LB and 50µg/µl carbenicillin tubes overnight at 30°C. Plasmid preps were performed on the overnight cultures using QIAquick Spin Miniprep Kit. Due to the use of BL21 cells for propagating plasmids, a modification of washing the spin columns with PB Buffer 5X and PE Buffer 3X was incorporated to the standard manufacturer's protocol for achieving high quality plasmid DNA. Plasmids were digested with PstI in a 20µl reaction to ensure the correct sized linear fragment. Three of the 6 plasmids were the correct size and shipped to Quintara Biosciences for sequencing with primers MCM65, MCM66, EL1000, EL1003, and EL1006 (Table 4). DNA sequencing results showed all 3 plasmids were correct. Picked one and designated plasmid as EWL244 (Figures 60 and 61A-B).

v) Construction of plasmid MCM376 - MVK from *M. mazei* archaeal Lower in pET200D.

[0258] The MVK ORF from the *M. mazei* archaeal Lower Pathway operon (Figures 73A-C) was PCR amplified using primers MCM161 and MCM162 (Table 4) using the Invitrogen Platinum HiFi PCR mix. 45 uL of PCR mix was combined with 1 uL template, 1 uL of each primer at 10 uM, and 2 uL water. The reaction was cycled as follows: 94 °C for 2:00 minutes; 30 cycles of 94 °C for 0:30 minutes, 55 °C for 0:30 minutes and 68 °C for 1:15 minutes; and then 72 °C for 7:00 minutes, and 4 °C until cool. 3 uL of this PCR reaction was ligated to Invitrogen pET200D plasmid according to the manufacturer's protocol. 3 uL of this ligation was introduced into Invitrogen TOP10 cells, and transformants were selected on LA/kan50. A plasmid from a transformant was isolated and the insert sequenced, resulting in MCM376 (Figures 74A-C).

vi) Construction of strain EWL251 (BL21(DE3), Cm-GI1.2-KKDyI, pTrc P.alba-mMVK)

[0259] MCM331 cells (which contain chromosomal construct gil.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase) were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. Mixed 50µl of cell suspension with 1 µl of plasmid EWL244. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB is immediately added to the cells, then the cells were transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hours at 30°C. Transformants were selected on LA and 50µg/µl carbenicillin and 5mM mevalonic acid plates and incubated at 37°C. One colony was selected and designated as strain EWL251.

vii) Construction of strain EWL256 (BL21(DE3), Cm-GI1.2-KKDyI, pTrc P.alba-mMVK, pCL Upper MVA)

[0260] EWL251 cells were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. Mixed 50µl of cell suspension with 1µl of plasmid MCM82 (which is pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS). The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB was immediately added to the cells, then transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hour at 30°C. Transformants were selected on LA and 50µg/µl carbenicillin and 50µg/µl spectinomycin plates and incubated at 37°C. Picked one colony and designated as strain EWL256.

Table 4: Primer Sequences

Primer name	Primer sequence
MCM130	ACCAATTGCACCCGGCAGA (SEQ ID NO:94)
GB Cm Rev	GCTAAAGCGCATGCTCCAGAC (SEQ ID NO:95)
MVD	GACTGGCCTCAGATGAAAGC (SEQ ID NO:96)
For	
MVD Rev	CAAACATGTGGCATGGAAAG (SEQ ID NO:97)
MCM182	GGGCCCGTTTAAACTTTAACTAGACTCTGCAGTTAGCGTTCAAACGGCAGAA (SEQ ID NO:98)
MCM192	CGCATGCATGTCATGAGATGTAGCGTGTCCACCGAAAA (SEQ ID NO:99
MCM65	ACAATTTCACACAGGAAACAGC (SEQ ID NO:100)
MCM66	CCAGGCAAATTCTGTTTTATCAG (SEQ ID NO:101)
EL1000	GCACTGTCTTTCCGTCTGCTGC (SEQ ID NO:102)
MCM165	GCGAACGATGCATAAAGGAGGTAAAAAAACATGGTATCCTGTTCTGCGCCGGG TAAGATTTACCTG (SEQ ID NO:103)
MCM177	GGGCCCGTTTAAACTTTAACTAGACTTTAATCTACTTTCAGACCTTGC (SEQ ID NO:104)
EL1003	GATAGTAACGGCTGCGCTGCTACC (SEQ ID NO:105)
EL1006	GACAGCTTATCATCGACTGCACG (SEQ ID NO:106)
MCM161	CACCATGGTATCCTGTTCTGCG (SEQ ID NO:107)
MCM162	TTAATCTACTTTCAGACCTTGC (SEQ ID NO:108)

II. Construction of MCM442-449: BL21 and BL21(DE3) with FRT-cmR-FRT-gi1.x-mKKDyI

i) Construction of Template for Recombination

[0261] FRT-based recombination cassettes, and plasmids for Red/ET-mediated integration and antibiotic marker loopout were obtained from Gene Bridges GmbH (Germany). Procedures using these materials were carried out according to Gene Bridges protocols. Primers MCM193 and MCM195 were used to amplify the resistance cassette from the FRT-gb2-Cm-FRT template using Stratagene Herculase II Fusion kit according to the manufacturer's protocol. The 50uL reaction was cycled as follows: 95°C, 2 minutes; (95°C, 20 seconds, 55°C, 20 seconds, 72°C, 1 minute) x 5, (95°C, 20 seconds, 60°C, 20 seconds, 72°C, 1 minute) x 25; 72°C, 3 minutes; 4°C until cool. The amplicon was purified by a Qiagen PCR column according to the manufacturer's protocol and eluted in 30uL EB (Elution Buffer). DNA was digested with *Nde*I and *Pci*I in a 20uL reaction with 1x Roche H buffer and 0.5uL BSA. Plasmid MCM376 was digested in a 10uL reaction containing 1uL each of *Nde*I, *Nco*I, and Roche H buffer. Reactions proceeded overnight at 37°C, and then cut DNA was purified on Qiagen PCR columns and eluted in 30uL EB. The PCR product was ligated into MCM376 in a reaction containing 1uL vector, 3uL PCR product, 1uL Roche Quick Ligase Buffer 2, 5uL Buffer1, and 1uL Ligase. The reaction proceeded at room temperature for 3 hours and then 5uL was transformed into Invitrogen TOP10 cells according to the manufacturer's protocol. Transformants were selected on L agar (LA) and chloramphenicol (10 ug/mLO) at 37°C overnight.

[0262] Transformant colonies were patched onto LA containing chloramphenicol (30 ug/mL) and kanamycin (50 ug/ml) for storage and sent to Quintara (Berkeley, CA) for sequencing. Four clones, one each with the four different nucleotides at the "N" in primer MCM195, were found to have the correct sequence for the inserted promoter. Clones were grown in 5mL LB containing chloramphenicol (30 ug/mL) and kanamycin (50 ug/mL) and used for the preparation of plasmid DNA. This plasmid was retransformed into TOP10 cells and strains were frozen as:

Table 5: MCM484-487

MCM484	cmR-gi1.6-MVK(mazei) in pET (clone AI-3, variable nt A)
MCM485	cmR-gi1.0-MVK(mazei) in pET (clone B4-6, variable nt C)
MCM486	cmR-gi1.2-MVK(mazei) in pET (clone C1-5, variable nt G)
MCM487	cmR-gi1.5-MVK(mazei) in pET (clone C3-3, variable nt T)

ii) Creation of Recombination Target Strains MCM349 and MCM441

[0263] The chloramphenicol resistance (cmR) marker was looped out of strain MCM331 using plasmid pGB706 (GeneBridges) according to Manufacturer's instructions. MCM331 cells were grown to mid-log in LB and washed three times in iced, sterile water. A 1uL aliquot of pGB706 DNA was added to 50uL of cell suspension and this mixture was electroporated in a 2mm cuvette at 2.5 volts, 25uFd followed immediately by recovery in 500uL LB for one hour at 30C. Transformants were selected on LB containing tetracycline (5 ug/ml) at 30°C. The following day, a clone was grown up at 30°C in LB containing tetracycline (5 ug/ml) until visibly turbid (OD600~0.5-0.8). This culture was streaked onto LB and grown overnight at 37°C. A clone that was unable to grow on LB containing chloramphenicol (10 ug/mL) or LB containing tetracycline (5 ug/mL) was frozen as MCM348. Plasmid MCM356 (pRedET carbencillin; GeneBridges) was electroporated in as described above and transformants were selected on LB containing carbenicillin (50 ug/mL) at 30°C. A clone was grown in LB carbenicillin (50 ug/mL) at

30°C and frozen as MCM349.

[0264] Strain MCM441 was created by electrotransforming plasmid MCM356 into EWL204 as above.

iii) Recombination of FRT-cmR-FRT-gi1.x-mMVK into MCM349 and MCM441

[0265] Plasmids MCM484-487 were used as template for PCR amplification with primers MCM120 and MCM196 and Herculase II Fusion kit, according to the manufacturer's protocol. Three reactions per template were carried out, with 0, 1, or 3uL DMSO. The 50uL reactions were cycled as follows: 95°C, 2 minutes; (95°C, 20 seconds; 55°C 20 seconds; 72°C, 1.5 minutes) for five cycles; (95°C, 20 seconds; 60°C 20 seconds; 72°C, 1.5 minutes) for 25 cycles; 72°C for 3 minutes; 4°C, overnight.] The three reactions from a given template were pooled and purified on Qiagen PCR columns and eluted with 30 uL EB at 60°C. 5uL DNA was digested with 1uL *Dpn*I in 1x Roche Buffer A for 3 hours at 37°C. This DNA was then microdialyzed against excess water for 30 minutes.

[0266] Strains were grown in 5mL LB containing carbenicillin (50 ug/mL) from fresh streaks at 30C to an OD600 of ~0.5. 40mM L-arabinose was added and cultures were incubated at 37C for 1.5 hours. Cells were harvested and electroporated with 3uL dialyzed amplicons above, and then recovered in 500uL SOC at 37C for 1.5-3 hours. Transformants were selected on LA plates containing chloramphenicol (5 ug/mL) at 37°C.

[0267] Kanamycin sensitive clones were screened by PCR for insertion of the amplicon. PCR products from positive clones were sequenced to verify the sequence of inserted DNA. Amplicons were consistent with the FRT-gi1.2-yKKDyI at attTn7 in MCM441 and 348 being replaced by FRT-cmR-FRT-gi1.x-mKKDyI (The yK and mK designations refer to the mevalonate kinase from *Saccharomyces cerevisiae* and *Methanosarcina mazei* respectively).

Table 6A: The following strains were grown in LB containing chloramphenicol (5 ug/mL) and frozen.

Strain ID	Name	Parent	Recombination Amplicon Template
MCM442	BL21(DE3) cmR-gi1.6mKKDyI A1, clone37 (A)	MCM349	MCM484
MCM443	BL21(DE3) cmR-gi1.0mKKDyI B4, clone27 (C)	MCM349	MCM485
MCM444	BL21(DE3) cmR-gi1.2mKKDyI C1, clone 16 (G)	MCM349	MCM486
MCM445	BL21(DE3) cmR-gi1.5mKKDyI C3, clone7 (T)	MCM349	MCM487
MCM446	BL21 cmR-gi1.6mKKDvI A1-3 (A)	MCM441	MCM484
MCM447	BL21 cmR-gi1.0mKKDyI B4-6 (C)	MCM441	MCM485
MCM448	BL21 cmR-gi1.2mKKDyI C1-5 (G)	MCM441	MCM486
MCM449	BL21 cmR-gi1.5mKKDyI C3-3 (T)	MCM441	MCM487

Table 6B: Primers

MCM120	AAAGTAGCCGAAGATGACGGTTTGTCACATGGAGTTGGCAGGAT GTTTGATTAAAAGCAATTAACCCTCACTAAAGGGCGG (SEQ ID NO:109)
MCM193	GATATACATATGAATTAACCCTCACTAAAGG (SEQ ID NO:110)
MCM195	GCATGCATGACATGTTTTTTTACCTCCTTTGTTATCCGCTCACAAT TAGTGGTTGAATTATTTGCTCAGGATGTGGCATNGTCAAGGGCG CGGCCGCGATCTAATACGACTCACTATAGGGCTCG (SEQ ID NO:111)
MCM196	AGGCTCTCAACTCTGACATGTTTTTTTCCTCCTTAAGGGTGCAGG CCTATCGCAAATTAGCTTAATCTACTTTCAGACCTTGCTCGG (SEQ ID NO:112)

III. The effect of yeast extract on isoprene production in *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium):

[0268] K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0269] Citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0270] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL Upper), the integrated lower MVA pathway (gi1.2KKDyI), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from *P. alba* (pTrcAlba-mMVK). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. A frozen vial of the *E. coli* strain was thawed and inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 15-L bioreactor bringing the initial volume to 5-L.

i) Production of isoprene in *E.coli* cells (EL256) grown in fed-batch culture without yeast

extract feeding

[0271] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 67 hour fermentation was 3.9 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 102 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 192 uM when OD_{550} reached 140. The OD_{550} profile within the bioreactor over time is shown in Figure 67A. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a final value of 35.6 g/L (Figure 67B). The total amount of isoprene produced during the 67 hour fermentation was 320.6 g and the time course of production is shown in Figure 67C. The metabolic activity profile, as measured by TCER, is shown in Figure 67D. The molar yield of utilized carbon that went into producing isoprene during fermentation was 17.9%. The weight percent yield of isoprene from glucose was 8.1%.

Production of isoprene in *E.coli* cells (EL256) grown in fed-batch culture with yeast extract feeding

[0272] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 68 hour fermentation was 7.1 kg. A total of 1.06 kg of yeast extract was also fed during the fermentation. Induction was achieved by adding IPTG. The IPTG concentration was brought to 208 uM when the optical density at 550 nm (OD_{550}) reached a value of 7. The IPTG concentration was raised to 193 uM when OD_{550} reached 180. The OD_{550} profile within the bioreactor over time is shown in Figure 68A. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 32.2 g/L (Figure 68B). The total amount of isoprene produced during the 68 hour fermentation was 395.5 g and the time course of production is shown in Figure 68C. The time course of volumetric productivity is shown in Figure 68D and shows that an average rate of 1.1 g/L/hr was maintained for between 23 and 63 hours. The metabolic activity profile, as measured by CER, is shown in Figure 68E The molar yield of utilized carbon that went into producing isoprene during fermentation was 10.3%. The weight percent yield of isoprene from glucose was 5.2%.

IV. Production of isoprene from different carbon sources in *E. coli* harboring the mevalonic acid (MVA) pathway and isoprene synthase (EWL256)

Media Recipe (per liter fermentation media):

[0273] K_2HPO_4 13.6 g, KH_2PO_4 13.6g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 0.2 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were dissolved sequentially in diH_2O. The pH was adjusted to 6.8 with

ammonium hydroxide (30%) and brought to volume. Media was filter sterilized with a 0.22 micron filter. Carbon source was added to a final concentration of 1%. Required antibiotics were added after sterilization and pH adjustment.

1000X Trace Metal Solution (per liter fermentation media):

[0274] Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, $ZnSO_4$ * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, and then brought to volume and filter sterilized with a 0.22 micron filter.

i) Preparation of AFEX biomass hydrolysate

[0275] AFEX pretreated corn stover was hydrolyzed to prepare biomass hydrolysate containing both xylose, glucose and acetate.

[0276] AFEX pretreated corn stover, received from Michigan Biotechnology Institute, was used. The pretreatment conditions were, 60% moisture, 1:1 ammonia loading, and 90 °C for 30 minutes, then air dried. The moisture content in the AFEX pretreated corn stover was 21.27%. Content of glucan and xylan in the AFEX pretreated corn stover were 31.7% and 19.1% (dry basis) respectively. The enzyme used was accellerase 1000, Grindamyl H121 (Danisco xylanase product from Aspergillus niger for bread-making industry).

[0277] For saccharification, 20 g of AFEX pretreated corn stover was added into a 500 ml flask, together with 5 ml of 1 M pH 4.8 sodium citrate buffer, 2.25 ml of Accellerase 1000, 0.1 ml of Grindamyl H121, and 72.65 ml of DI water. The flask was put in an orbital shaker, and incubated at 50°C for 96 hours.

[0278] For analysis, one sample was taken from the shaker, and analyzed using HPLC. The hydrolysate contained 37.2 g/l of glucose and 24.3 g/L of xylose, and 7.6 g/L of oligomers of glucose and/or xylose. Additionally, the hydrolysate also contains 1.17 g/L acetate.

ii) Experimental procedure

[0279] An inoculum of the *E. coli* strain EWL256 containing the MVA pathway and isoprene synthase was taken from a frozen vial and streaked onto an LB broth agar plate containing spectinomycin (50 ug/mL) and carbinicllin (50 ug/mL) and incubated at 30°C overnight. A single colony was inoculated into TM3 media containing glucose, xylose, glycerol, acetate or biomass as only carbon source and grown overnight at 30°C. Cells grow on acetate reached a significantly lower optical density. Cells grown on glucose, glycerol, biomass hydrolysate or acetate were diluted into 20 mL of TM3 media containing the respective carbon sources to reach an optical density of between 0.1 measured at 600nM. A negative control not containing any carbon source was prepared from the glucose overnight culture. A separate experiment was performed with glucose and xylose, where the cultures were diluted to an optical density of 0.05. All culture conditions (except for acetate and glycerol) were tested in duplicates and the

presented results are averaged between these cultures. Production of isoprene was induced with 200µM IPTG from the beginning of the experiment. The flasks were incubated at 30°C in an orbital shaker (200 rpm) and growth was followed by measuring optical density. After the glucose fed cultures had reached an optical density of approximately 0.4, samples were analyzed for isoprene production from all the tested carbon sources every hour for three hours. Samples of 100 µL were transferred in duplicates to 2 mL glass vials, sealed and incubated for 30 min at 30°C. The bacteria were then heat killed by incubation at 80°C for 8 minutes. The amount of produced isoprene was measured using GC-MS and specific productivity (µg/L*hr) was calculated.

iii) Results

[0280] Significant production of isoprene could be demonstrated during growth on all the tested carbon sources. These carbon sources are examples of common alcohols, organic acids, sugars containing 5 or 6 carbon units (C5 or C6), and biomass hydrolysate.

[0281] The initial growth rate on biomass hydrolysate was comparable to the growth rate on glucose (Figure 69A). The initial specific productivity during growth on biomass hydrolysate was significantly higher than during growth on glucose. This demonstrates that biomass hydrolysate can be used as an efficient source of carbon for the production of isoprene. The specific productivity declined after 255 minutes of growth on biomass hydrolysate (Figure 69B). The bacteria had a slower growth rate with xylose as only carbon source when compared to glucose (Figure 69C), but a significant specific isoprene productivity was measured (Figure 69D). This shows that both C5 and C6 sugars can be utilized for the production of isoprene via the mevalonate acid pathway.

[0282] Suprisingly, bacteria grown on acetate as the only carbon source had a specific productivity of isoprene approximately twice as high as during growth on glucose (Figure 69A). The bacteria grew slower on acetate when compared to glucose (Figure 69B), but the performed experiment demonstrates that acetate can also be used as a carbon source for the production of isoprene. Acetate was also present in the biomass hydrolysate as measured by HPLC.

[0283] The bacteria grew well with glycerol as only carbon source (Figure 69A) and significant production of isoprene was demonstrated (Figure 69B). This shows that common alcohols may also be used as carbon sources for production of isoprene via the mevalonate acid pathway.

Example 11: Expression of isoprene-synthase from plant in *Streptomyces sp.*

[0284] The gene for isoprene synthase Kudzu was obtained from plasmid pJ201:19813. Plasmid pJ201:19813 encodes isoprene synthase from *Pueraia lobata* (Kudzu plant) and was codon-optimized for *Pseudomonas fluorescens*, *Pseudomonas putida, Rhodopseudomonas palustris* and *Corynebacterium* (Figures 79A-79C (SEQ ID NO:123)). Digestion of plasmid pJ201:19813 with restriction enzymes NdeI and BamHI liberated gene iso19813 that was ligated into the *Streptomyces-E.coli* shuttle vector pUWL201PW (Doumith et al., Mol. Gen. Genet. 264: 477-485, 2000; Figure 71) to generate pUWL201_iso. Successful cloning was verified by restriction analysis of pUWL201_iso. Expression of isoprene synthase iso19813 was under control of the *erm*-promoter which allows for constitutive expression in *Streptomycetes* species, but not for expression in *E. coli.*

[0285] PUWL201PW (no insert) and pUWL201_iso were introduced in *Streptomyces albus* J1074 (Sanchez et al., Chem. Biol. 9:519-531, 2002) by transformation of protoplasts as described by Hopwood et al., The John innesfoundation, Norwich, 1985.

[0286] A 200 µl aliquot of protoplast suspensions was transformed with 1.9 µg pUWL201PW or 2.9 µg pUWL201_iso. After incubation overnight at 28°C on non-selective R5-agarplates, positive transformants were selected by further incubation for 4 days in R3-overlay agar containing thiostrepton (250 µg/ml). Thiostrepton resistant transformants were examined for presence of the pUWL-plasmids by plasmid preparation using Plasmid Mini Kit (Qiagen). Prepared plasmid DNA was reintroduced in *E. coli* DH5α to generate sufficient amounts of plasmid DNA to be analyzed by restriction analysis. Positive transformants were selected on ampicillin-containing L-agar plates and insert analysis was done by digestion of plasmid DNA with *Nde*I and *Bam*HI endonucleases. Isoprene synthase was identified as a 1.7 kb fragment in positive pUWL201 iso clones while in the control strains (pUWL201PW) no such fragment was observed.

[0287] Wild type strain and transformants of *S. albus* containing control plasmid pUWL201PW or isoprene synthase encoding pUWL201_iso were analyzed for isoprene formation. Strains were cultivated in duplicate on solid media (tryptic soy broth agar, TSB; 2.5 ml) in presence or absence of thiostrepton (200 µg/ml) and incubated for 4 days at 28°C in sealed head-space vials (total volume 20 ml). 500 µl head-space samples (end point measurements) were analyzed by GC-MS in SIM-mode and isoprene was identified according to reference retention times and molecular masses (67 m/z). Isoprene present in head-space samples was quantified by previously generated calibration curves. While wild-type *S. albus* and control strains harboring pUWL201PW produced isoprene in concentrations slightly higher than the detection limit (0.04 - 0.07 ppm), *S. albus* harboring pUWL201_iso produced isoprene in at least tenfold excess compared to controls (0.75 ppm; Figure 72). The results demonstrate successful expression of plant-derived isoprene synthase in a prokaryotic organism of the *Actinomycetes* group.

Example 12: Production of isoprene or mevalonate from fatty acid or palm oil in *E. coli fadR atoC* LS5218 containing the upper or upper and lower Mevalonic Acid pathway plus kudzu isoprene synthase.

[0288] *Escherichia coli fadR atoC* strain LS5218 (#6966) was obtained from the Coli Genetic Stock Center. FadR encodes a transcription repressor that negatively regulates expression of the genes encoding fatty acid degradation enzymes (Campbell et al., J. Bacteriol. 183: 5982-5990, 2001). AtoC is a response regulator in a two-component regulatory system with AtoS, regulates acetolactate metabolism. The fadR atoC strain allows constitutive expression of the fatty acid degradation genes and incorporates long chain fatty acids into long-chain-length polyhydroxyalkanoates. When palm oil is used as a carbon source for either mevalonate or isoprene production, the palm oil was converted to glycerol plus fatty acid. Methods for this are well known in the art, and it can be done either enzymatically by incubation with a lipase (for example Porcine pancreatic lipase, *Candida rugosa* lipase, or other similar lipases) or chemically by saponification with a base such as sodium hydroxide.

i) *E. coli fadR atoC* strain expressing the upper Mevalonic Acid pathway

[0289] Strain WW4 was created by electroporating pCLPtrcUpperPathway into LS5218 using standard methods (Sambrooke et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989). Incorporation of the plasmid was demonstrated by the production of mevalonic acid (MVA) when cells were cultured in TM3 medium supplemented with either C12 fatty acid (FA) or palm oil as the carbon source. To demonstrate production of MVA by WW4 from fatty acid, cells from an overnight culture were diluted 1 to 100 into 5 mL of modified TM3 medium (TM3 without yeast extract) supplemented with 0.25% C12 FA (Sigma cat # L9755). The first sign of MVA production (24 mg/L) was apparent after overnight incubation at 30°C of the IPTG induced culture. Production increased over three days with the final level of 194 mg/L of MVA produced. To demonstrate production of MVA by WW4 from oil, cells from an overnight culture were diluted 1 to 100 into modified TM3 medium supplemented with 200 mg of digested palm oil per 5 mL of TM3 medium. The first sign of MVA production (50 mg/L) was apparent after overnight incubation of the IPTG induced culture at 30°C. Production increased over three days with a final level of 500 mg/L of MVA produced.

ii) *E. coli fadR atoC* strain expressing the upper and lower MVA pathway plus kudzu isoprene synthase

[0290] *Escherichia coli* strain WW4 (LS5218 fadR atoC pCLPtrcUpperPathway) was transformed with pMCM118 [pTrcKKDyIkIS] to yield WW10. The incorporation of the plasmid was demonstrated by evidence of production of isoprene when the strain was cultured in TM3 and glucose and induced with IPTG (100, 300, or 900 uM). The strain was relatively sensitive to IPTG and showed a significant growth defect even at 100 uM IPTG. These results are shown in Figure 70A.

[0291] To test isoprene production from dodecanoic acid, WW10 was cultured overnight in L broth containing spectinomycin (50 ug/ml), and kanamycin (50 ug/ml) at 37C with shaking at 200 rpm. The cells were washed with modified TM3 medium by centrifugation and resuspension in their original culture volume with this medium. The washed and resuspended cells from this starter culture were diluted 1 to 100 and 1 to 10 into 5 mL of modified TM3 medium containing 0.125% C12 Fatty Acid (Sigma cat # L9755).

[0292] To demonstrate production of mevalonate from palm oil, the oil was predigested with lipase at 37°C and 250 rpm for several days to release the fatty acids (evidence of hydrolysis was judged by the foam formed when tubes were shaken).

[0293] In addition, a culture was set up by diluting the washed cells at 1 to 10 into modified TM3 medium contained in test tubes with palm oil. A further tube was set up by the addition of 0.125% C12FA to the remainder (2.5 mL) of the washed cells without further dilution (bioconversion). After 3.75 hours of growth at 30°C with shaking at 250 rpm all of the cultures were induced by the addition of 50 uM IPTG. Incubation was continued for 4 hours after which time 200 uL of each of the cultures was assayed for isoprene accumulation with a modified head space assay (1 hour accumulation at 30°C with shaking at 500 rpm). An additional isoprene assay was conducted by a 12 hour incubation of the assay glass block prior to GCMS analysis. Incubation of the induced cultures was continued overnight and 200 uL aliquots were again assayed for isoprene production (1 hour, 30 deg, 500 rpm Shel-Lab shaker) the following morning. Analysis of these cultures showed the production of significant levels of isoprene. The highest levels of isoprene were observed in the culture which was seeded at 1/10 dilution from the overnight starter culture after it had been incubated and induced overnight. This result

suggests that this culture continued to grow and increase in cell density. These results are shown in Figure 70B. Cell density could not be measured directly because the fatty acid suspension had a turbid appearance. Cell density of this culture was therefore determined by plating an aliquot of the culture and showed $8x10^7$ colony forming units. This corresponds approximately to an OD_{600} of 0.1. Nevertheless, this culture provided significant isoprene production; no isoprene is observed for similar strains without the pathway described in this example.

Example 13: Improvement of isoprene production by constitutive expression of ybhE in *E. coli.*

[0294] This example shows production of isoprene in a strain constitutively expressing *ybhE* (pg1) compared to a control strain with wild type *ybhE.* The gene *ybhE* (pg1) encodes a 6-phosphogluconolactonase that suppresses posttranslational gluconylation of heterologously expressed proteins and improves product solubility and yield while also improving biomass yield and flux through the pentose phosphate pathway (Aon et al. Applied and Environmental Microbiology, 74(4): 950-958, 2008).

[0295] The BL21 strain of *E. coli* producing isoprene (EWL256) was constructed with constitutive expression of the *ybhE* gene on a replicating plasmid pBBR1MCS5(Gentamycin) (obtained from Dr. K. Peterson, Louisiana State University).

[0296] FRT-based recombination cassettes, and plasmids for Red/ET-mediated integration and antibiotic marker loopout were obtained from Gene Bridges GmbH (Germany). Procedures using these materials were carried out according to Gene Bridges protocols. Primers Pgl-F and PglGI1.5-R were used to amplify the resistance cassette from the FRT-gb2-Cm-FRT template using Stratagene Herculase II Fusion kit according to the manufacturer's protocol. The PCR reaction (50 uL final volume) contained: 5 uL buffer, 1 uL template DNA (FRT-gb2-Cm-F from Gene Bridges), 10 pmols of each primer, and 1.5 uL 25mM dNTP mix, made to 50 uL with dH_2O. The reaction was cycled as follows: 1 x 2 minutes, 95°C then 30 cycles of (30 seconds at 95°C; 30 seconds at 63°C; 3 minutes at 72°C).

[0297] The resulting PCR product was purified using the QiaQick PCR purification kit (Qiagen) and electroporated into electrocompetent MG1655 cells harboring the pRed-ET recombinase-containing plasmid as follows. Cells were prepared by growing in 5 mLs of L broth to and OD600~0.6 at 30°C. The cells were induced for recombinase expression by the addition of 4% arabinose and allowed to grow for 30 minutes at 30°C followed by 30 minutes of growth at 37°C. An aliquot of 1.5 mLs of the cells was washed 3-4 times in ice cold dH_2O. The final cell pellet was resuspended in 40 uL of ice cold dH_2O and 2-5 uL of the PCR product was added. The electroporation was carried out in 1-mm gap cuvettes, at 1.3 kV in a Gene Pulser Electroporator (Bio-Rad Inc.). Cells were recovered for 1-2 hours at 30°C and plated on L agar containing chloramphenicol (5 ug/mL). Five transformants were analyzed by PCR and sequencing using primers flanking the integration site (2 primer sets: pgl and 49 rev and 3' EcoRV-pglstop; Bottom Pgb2 and Top GB's CMP (946)). A correct transformant was selected and this strain was designated MG1655 GI1.5-pgl::CMP.

[0298] The chromosomal DNA of MG1655 GI1.5-pgl::CMP was used as template to generate a PCR fragment containing the FRT-CMP-FRT-GI1.5 - ybhE construct. This construct was cloned into pBBR1MCS5(Gentamycin) as follows. The fragment, here on referred to as CMP-GI1.5-pgl, was amplified using the 5' primer Pglconfirm-F and 3' primer 3' EcoRV-pglstop. The resulting fragment was

cloned using the Invitrogen TOPO-Blunt cloning kit into the plasmid vector pCR-Blunt II-TOPO as suggested from the manufacturer. The *Nsi*I fragment harboring the CMP-GI1.5-pgl fragment was cloned into the *Pst*I site of pBBR1MCS5(Gentamycin). A 20µl ligation reaction was prepared containing 5µl CMP-GI1.5-pgl insert, 2µl pBBR1MCS5(Gentamycin) vector, 1µl T4 DNA ligase (New England Biolabs), 2µl 10X ligase buffer, and 10µl ddH_2O. The ligation mixture was incubated at room temperature for 40 minutes then 2-4 uL were electroporated into electrocompetent Top10 cells (Invitrogen) using the parameters disclosed above. Transformants were selected on L agar containing 10 ug/ml chloramphenicol and 5 ug/ml Gentamycin. The sequence of the selected clone was determined using a number of the primers described above as well as with the in-house T3 and Reverse primers provided by Sequetech, CA. This plasmid was designated pBBRCMPGI1.5-pg1 (Figures 77A-B and SEQ ID NO:122).

[0299] Plasmid pBBRCMPGI1.5-pgl was electroporated into EWL256, as described above in Example 10 and transformants were plated on L agar containing Chloramphenicol (10 ug/mL), Gentamycin (5 ug/mL), spectinomycin (50 ug/mL), and carbenicillin (50 ug/mL). One transformant was selected and designated RM11608-2.

Primers:

[0300]

Pgl-F
5'-
ACCGCCAAAAGCGACTAATTTTAGCTGTTACAGTCAGTTGAATTAACCCTCACTAAA
GGGCGGCCGC-3' (SEQ ID NO:115)

PglGI1.5-R
5'-
GCTGGCGATATAAACTGTTTGCTTCATGAATGCTCCTTTGGGTTACCTCCGGGAAAC
GCGGTTGATTTGTTTAGTGGTTGAATTATTTGCTCAGGATGTGGCATAGTCAAGGGC
GTGACGGCTCGCTAATACGACTCACTATAGGGCTCGAG-3' (SEQ ID NO:116)

3' EcoRV-pglstop:
5'-CTT GAT ATC TTA GTG TGC GTT AAC CAC CAC (SEQ ID NO:117)

pgl +49 rev: CGTGAATTTGCTGGCTCTCAG (SEQ ID NO:118)

Bottom Pgb2: GGTTTAGTTCCTCACCTTGTC (SEQ ID NO:119)

Top GB's CMP (946): ACTGAAACGTTTTCATCGCTC (SEQ ID NO:120)

Pglconfirm-F
5'-ACCGCCAAAAGCGACTAATTTTAGCT-3' (SEQ ID NO:121)

i) Small scale analysis

Media Recipe (per liter fermentation media):

[0301] K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 1 g, 1000X Trace Metals Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media was filter-sterilized with a 0.22 micron filter. Glucose 5.0 g and antibiotics were added after sterilization and pH adjustment.

1000X Trace Metal Solution (per liter fermentation media):

[0302] Citric Acid * H_2O 40g, $MnSO_4$ * H_2O 30g, NaCl 10g, $FeSO_4$ * $7H_2O$ 1g, $CoCl_2$ * $6H_2O$ 1g, $ZnSO_4$*$7H_2O$ 1g, $CuSO_4$ * $5H_2O$ 100mg, H_3BO_3 100mg, $NaMoO_4$ * $2H_2O$ 100mg. Each component is dissolved one at a time in diH_2O. The pH is adjusted to 3.0 with HCl/NaOH, and then the solution is brought to volume and filter-sterilized with a 0.22 micron filter.

a) Experimental procedure

[0303] Isoprene production was analyzed by growing the strains in a Cellerator™ from MicroReactor Technologies, Inc. The working volume in each of the 24 wells was 4.5 mL. The temperature was maintained at 30°C, the pH setpoint was 7.0, the oxygen flow setpoint was 20 sccm and the agitation rate was 800 rpm. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 30°C. A single colony was inoculated into media with antibiotics and grown overnight. The bacteria were diluted into 4.5 mL of media with antibiotics to reach an optical density of 0.05 measured at 550 nm.

[0304] Off-gas analysis of isoprene was performed using a gas chromatograph-mass spectrometer (GC-MS) (Agilent) headspace assay. Sample preparation was as follows: 100 µL of whole broth was placed in a sealed GC vial and incubated at 30°C for a fixed time of 30 minutes. Following a heat kill step, consisting of incubation at 70°C for 5 minutes, the sample was loaded on the GC.

[0305] Optical density (OD) at a wavelength of 550 nm was obtained using a microplate reader (Spectramax) during the course of the run. Specific productivity was obtained by dividing the isoprene concentration (µg/L) by the OD reading and the time (hour).

[0306] The two strains EWL256 and RM11608-2 were assessed at 200 and 400 uM IPTG induction levels. Samples were analyzed for isoprene production and cell growth (OD550) at 1, 2.5, 4.75, and 8 hours post-induction. Samples were done in duplicate.

b) Results

[0307] The experiment demonstrated that at 2 different concentrations of IPTG the strain expressing the ybhE (pg1) had a dramatic 2-3 fold increase in specific productivity of isoprene compared to the control strain.

ii) Isoprene fermentation from *E. coli* expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, and *pgl* over-expression (RHM111608-2) and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium)

[0308] K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified trace Metal Solution:

[0309] Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, $ZnSO_4$* $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter

[0310] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL Upper), the integrated lower MVA pathway (gil.2KKDyI), high expression of mevalonate kinase from *M. mazei* and isoprene synthase from *P. alba* (pTrcAlba-mMVK), and high expression of *pgl* (pBBR-pgl). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 34°C. A frozen vial of the *E. coli* strain was thawed and inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 15-L bioreactor bringing the initial volume to 5-L.

[0311] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 40 hour (59 hour) fermentation was 3.1 kg (4.2 kg at 59 hour). Induction was achieved by adding IPTG. The IPTG concentration was brought to 110 uM when the optical density at 550 nm (OD_{550}) reached a value of 4. The IPTG concentration was raised to 192 uM when OD_{550} reached 150. The OD_{550} profile within the bioreactor over time is shown in Figure 78A. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 33.2 g/L at 40 hours (48.6 g/L at 59 hours) (Figure 78B). The isoprene titer increased over the course of the fermentation to a maximum value of 40.0 g/L at 40 hours (60.5 g/L at 59 hours) (Figure 78C). The total amount of isoprene produced during the 40-hour (59-hour) fermentation was 281.3 g (451.0 g at 59 hours) and the time course of production is shown in Figure 78D. The time course of volumetric productivity is shown in Figure 78E and shows that an average rate of 1.0 g/L/hr was maintained between 0 and 40 hours (1.4 g/L/hour between 19 and 59 hour). The metabolic activity profile, as measured by CER, is shown in Figure 78F. The molar yield of utilized carbon that went into producing isoprene during

fermentation was 19.6% at 40 hours (23.6% at 59 hours). The weight percent yield of isoprene from glucose was 8.9% at 40 hours (10.7% at 59 hours).

[0312] 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.

[0313] 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.

[0314] For use herein, unless clearly indicated otherwise, use of the terms "a", "an," and the like refers to one or more.

[0315] 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.

[0316] 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

[0317]

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: XO02017(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: Csa1_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: BRAD02161(dxs)

BBT: BBta_2479(dxs)

RPA: RPA0952(dxs)

RPB: RPB_4460

RPC: RPC_149

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: SP00247(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: ZM01234(dxs) ZM01598(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: BPUM2159

GKA: GK2392

GTN: GTNG_2322

LMO: 1mo1365(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: Swo1_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: CPA_1060(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

[0318]

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

WY: WA1043 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: Sba1195_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: Pat1_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 Mmwyll_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 Beep1808_5644

BUR:

Bcep18194A3629 Bcep18194_A5080 Bcep18194_A5091

Beep18194_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(th1A)

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: Sium_2280 Sium_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: b110226(atoB) b113949 b117400 b117819 blr3724(phbA)

BRA: BRAD00562(phbA) BRAD00983(pimB) BRAD03110 BRAD03134(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(thl) 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: 1we1431

LLA: L11745(thiL) L25946(fadA)

LLC: LACR_1665 LACR_1956

LLM: 11mg_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: CB03200(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: Swo1_0308 Swo1_0675 Swo1_0789 Swo1_1486 Swo1_1934 Swo1_2051

TTE: TTE0549(paaJ)

MTA: Moth_1260

MTU: Rv_1135A 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: Ace1_0626 Ace1_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 Rxy1_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: Tme1_1134

FNO: Fnod_0314

PMO: Pmob 0515

HMA:

rrnAC0896(acaB3) rrnAC2815(aca2) rrnAC3497(yqeF)

rmB0240(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: Pis1_0029 Pis1_1301

PCL: Pcal_0781

PAS: Pars_0309 Pars_1071

CMA: Cmaq_1941

Exemplary HMG-CoA synthase nucleic acids and polypeptides

[0319]

HSA: 3157(HMGCS1) 3158(HMGCS2)

PTR: 457169(HMGCS2) 461892(HMGCS1)

MCC: 702553(HMGCS1) 713541(HMGCS2)

MMU: 15360(Hmgcs2) 208715(Hmgcs1)

RNO: 24450(Hmgcs2) 29637(Hmgcsl)

CFA: 479344(HMGCS1) 607923(HMGCS2)

BTA: 407767(HMGCS1)

SSC: 397673(CH242-38B5.1)

GGA: 396379(HMGCS1)

XLA: 380091(hmgcsl) 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: YP01457

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: BURPS 1710b_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

[0320]

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: Pat1_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

[0321]

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: lp12017

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: lhng_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

[0322]

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

[0323]

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: lp12018

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(mvdl)

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

[0324]

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

[0325]

HSA: 3422(IDI1) 91734(IDI2)

PTR: 450262(IDI2) 450263(IDI1)

MCC: 710052(LOC710052) 721730(LOC721730)

MMU: 319554(Idil)

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(idil)

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: lp12029

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: NCg12223(cg12305)

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: SS00063

STO: ST2059

SAI: Saci_0091

MSE: Msed_2136

PAI: PAE0801

PIS: PisI_1093

PCL: Pcal_0017

PAS: Pars_0051

TPE: Tpen_0272

Exemplary isoprene synthase nucleic acids and polypeptides

[0326] Genbank Accession Nos.

AY341431

AY316691

AY279379

AJ457070

AY182241

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Bakterie-, svampe-, gær- eller planteceller omfattende en eller flere nukleinsyrer, som koder for:

(a) et isopren-syntase-polypeptid, hvor isopren-syntase-polypeptidet er kodet af en heterolog nukleinsyre, og

(b) et isopentenyl-diphosphat-delta-isomerase-(IDI)-polypeptid, hvor IDI-polypeptidet er kodet af en heterolog nukleinsyre; og/eller hvor IDI-polypeptidet er kodet af en indsættelse af en kopi af en endogen nukleinsyre, som koder for et IDI-polypeptid og

(c)

(i) et 1-Deoxyxylulose-5-phosphat-syntase-(DXS)-polypeptid, hvor DXS-polypeptidet er kodet af en heterolog nukleinsyre; og/eller hvor DXS-polypeptidet er kodet af en indsættelse af en kopi af en endogen nukleinsyre, som koder for et DXS-polypeptid
og/eller

(ii) et eller flere mevalonat-(MVA)-signalvej-polypeptider, hvor nævnte ene eller flere MVA-signalvej-polypeptider er kodet af en heterolog nukleinsyre; og/eller hvor nævnte ene eller flere MVA-signalvej-polypeptider er kodet af en indsættelse af en kopi af en endogen nukleinsyre, som koder for et eller flere MVA-signalvej-polypeptider,

hvor cellerne producerer mere end omkring 400 nmol/g_{wcm}/time af isopren.

2. Bakterie-, svampe-, gær- eller planteceller omfattende en eller flere nukleinsyrer, som koder for:

(a) et isopren-syntase-polypeptid, hvor isopren-syntase-polypeptidet er kodet af en heterolog nukleinsyre, og

(b) et isopentenyl-diphosphat-delta-isomerase-(IDI)-polypeptid, hvor IDI-polypeptidet er kodet af en heterolog nukleinsyre; og/eller hvor IDI-polypeptidet er kodet af en indsættelse af en kopi af en endogen nukleinsyre, som koder for et IDI-polypeptid og

(c)

(i) et 1-Deoxyxylulose-S-phosphat-syntase-(DXS)-polypeptid hvor DXS-polypeptidet er kodet af en heterolog nukleinsyre; og/eller hvor DXS-polypeptidet er kodet af en indsættelse af en kopi af en endogen nukleinsyre, som koder for et DXS-polypeptid og/eller

(ii) et eller flere mevalonat-(MVA)-signalvej-polypeptider, hvor nævnte ene eller flere MVA-signalvej-polypeptider er kodet af en heterolog nukleinsyre; og/eller hvor nævnte ene eller flere MVA-signalvej-polypeptider er kodet af en indsættelse af en kopi af en endogen nukleinsyre, som koder for et eller flere MVA-signalvej-polypeptider,

hvor cellerne omdanner mere end omkring 0,2 molprocent af kulstoffet, som cellerne forbruger fra et celledyrkningsmedium, til isopren.

3. Cellerne ifølge krav 1 eller krav 2, hvor den heterologe nukleinsyre er funktionelt forbundet til en promoter.

4. Cellerne ifølge et hvilket som helst af kravene 1-3, hvor isopren-syntase-polypeptidet er et plante-isopren-syntase-polypeptid.

5. Cellerne ifølge et hvilket som helst af kravene 1-4, hvor MVA-signalvej-polypeptidet er en mevalonat-kinase (MVK), hvor MVK eventuelt er et polypeptid fra *Methanosarcina.*

6. Cellerne ifølge et hvilket som helst af kravene 1-5, hvor isopren-syntase-polypeptidet er et naturligt forekommende polypeptid fra *Pueraria* eller *Populus.*

7. Cellerne ifølge et hvilket som helst af kravene 1-6, hvor cellerne er bakterieceller, eventuelt Bacillus-, Escherichia- eller Pantoea-celler, eller hvor cellerne er svampeceller, eventuelt *Trichoderma*-celler, eller hvor cellerne er gærceller, eventuelt *Yarrowia- eller Saccharomyces*-celler.

8. Fremgangsmåde til fremstilling af isopren, hvilken fremgangsmåde omfatter at dyrke celler ifølge et hvilket som helst af kravene 1-7 under betingelser tilstrækkelige til fremstilling af isopren.

9. Fremgangsmåden ifølge krav 8, hvilken fremgangsmåde omfatter:

(a) at dyrke celler omfattende en heterolog nukleinsyre som koder for et isopren-syntase-polypeptid under egnede dyrkningsbetingelser til fremstillingen af isopren, hvor cellerne producerer mere end omkring 400 nmol/g_{wcm}/time af isopren, og

(b) at fremstille isopren.

10. Fremgangsmåden ifølge krav 8, hvilken fremgangsmåde omfatter:

(a) at dyrke celler omfattende en heterolog nukleinsyre som koder for et isopren-syntase-polypeptid under egnede dyrkningsbetingelser til fremstillingen af isopren, hvor cellerne omdanner mere end omkring 0,2 molprocent af kulstoffet, som cellerne forbruger fra et celledyrkningsmedium, til isopren, og

(b) at fremstille isopren.

11. Fremgangsmåden ifølge et hvilket som helst af kravene 8-10, yderligere omfattende at genvinde isoprenen fremstillet af cellerne.

12. Anvendelse af celler ifølge et hvilket som helst af kravene 1-7 i fremstillingen af isopren.

13. Fremgangsmåden ifølge et hvilket som helst af kravene 8-10, yderligere omfattende trinnet at oprense isoprenen fremstillet af cellerne, og/eller omfattende trinnet at polymerisere isoprenen fremstillet af cellerne.

DRAWINGS

Figure 1

1-
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Figure 2

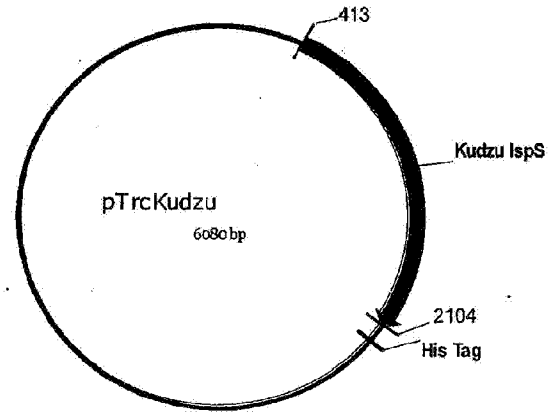

Figure 3A

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Figure 3B

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Figure 3C

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Figure 4

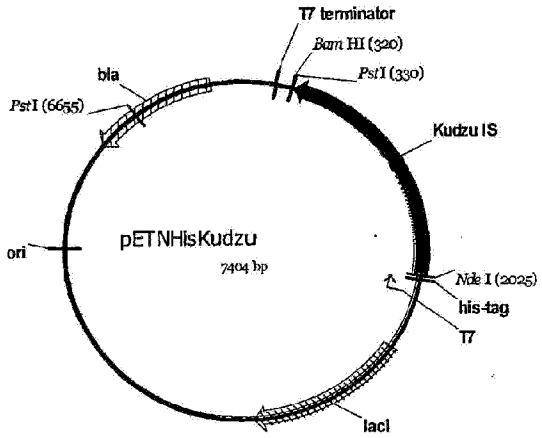
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Figure 5A

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Figure 5B

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Figure 5C

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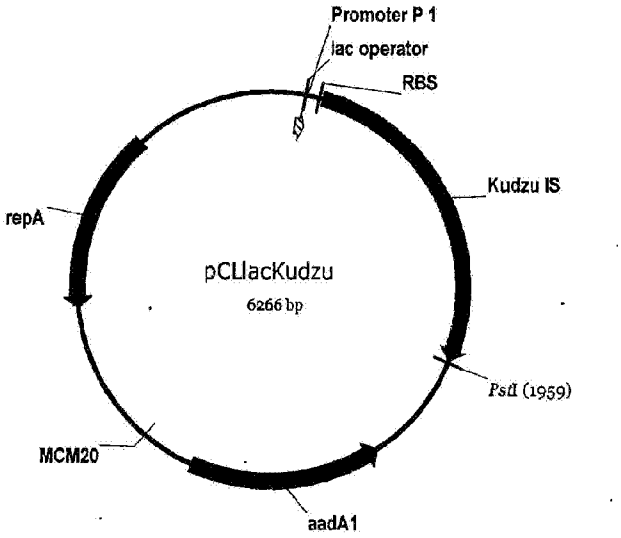
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Figure 7A

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Figure 7B

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Figure 7C

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(SEQ ID NO:7)

Figure 8A

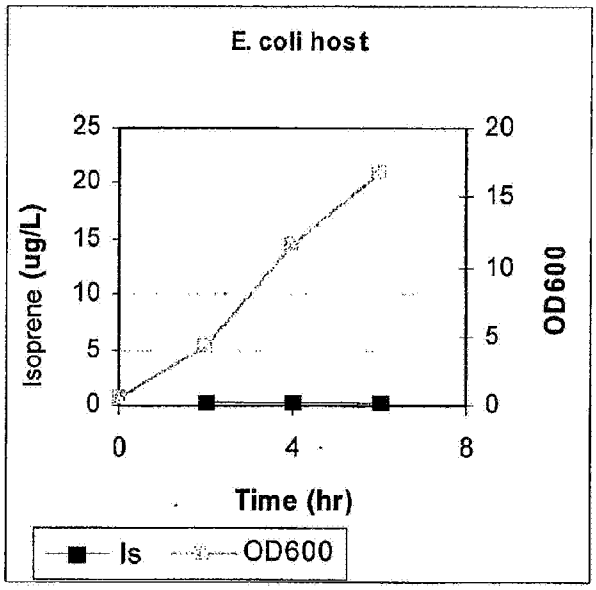

E. coli host
Isoprene (ug/L)
OD600
Time (hr)
Is
OD600
0
4
8
25
20
15
10
5
0
20
15
10
5
0

Figure 8B

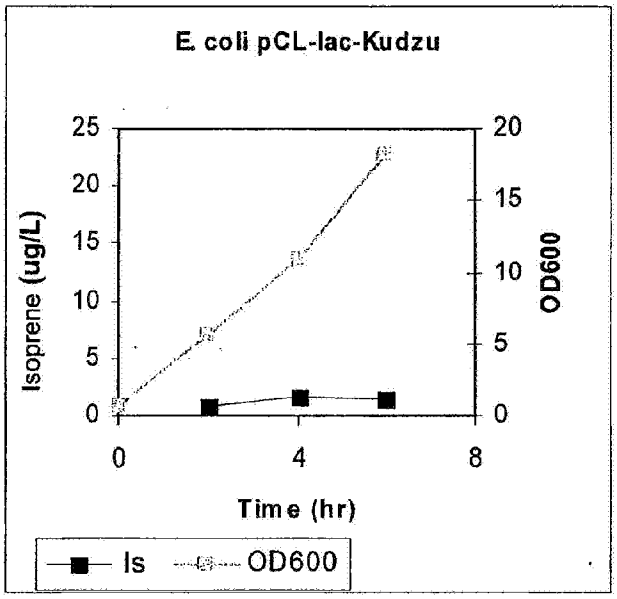

E. coli pCL-lac-Kudzu
Isoprene (ug/L)
OD600
Time (hr)
Is
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8

Figure 8C

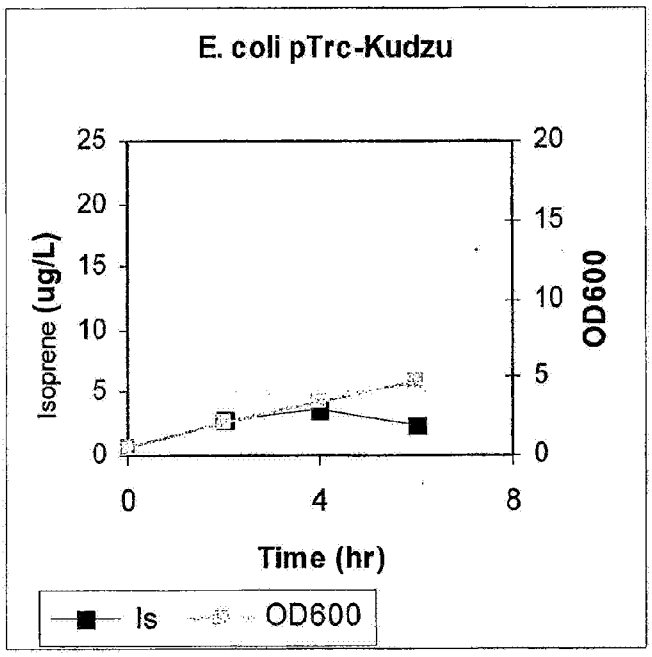

E. coli pTrc-Kudzu
Isoprene (ug/L)
OD600
Time (hr)
0
4
8
0
5
10
15
20
25
Is
OD600

Figure 8D

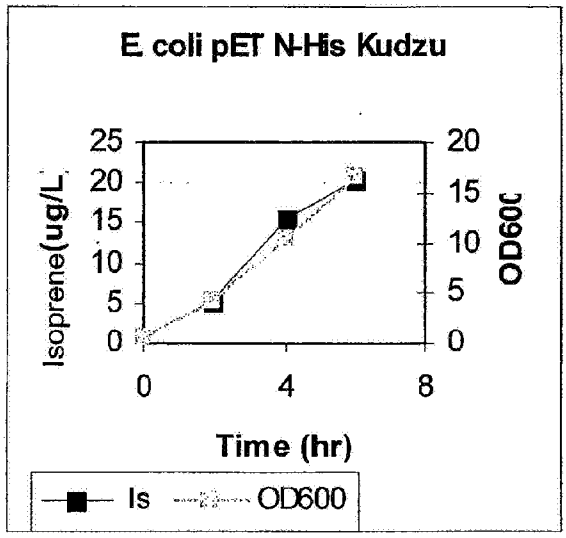
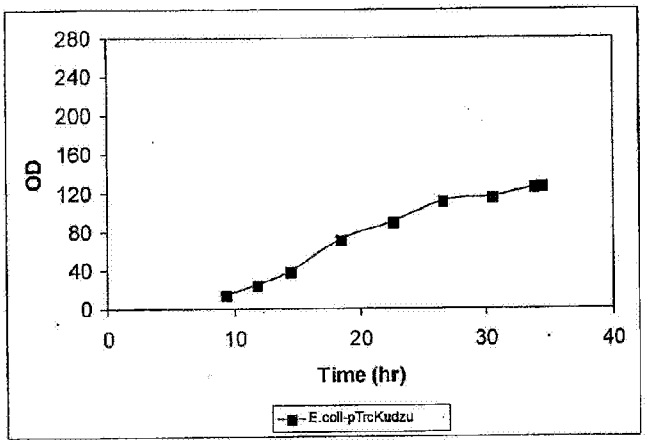

E coli pET N-His Kudzu
Isoprene(ug/L
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 9

A.


OD
280
240
200
160
120
80
40
0
0
10
20
30
40
Time (hr)
E.coli-pTrcKudzu

B.

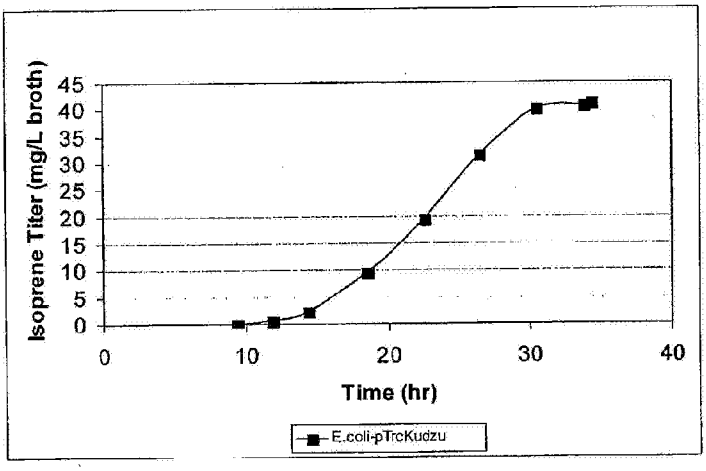

Isoprene Titer (mg/L broth)
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
Time (hr)
E.coli-pTrcKudzu

Figure 10A

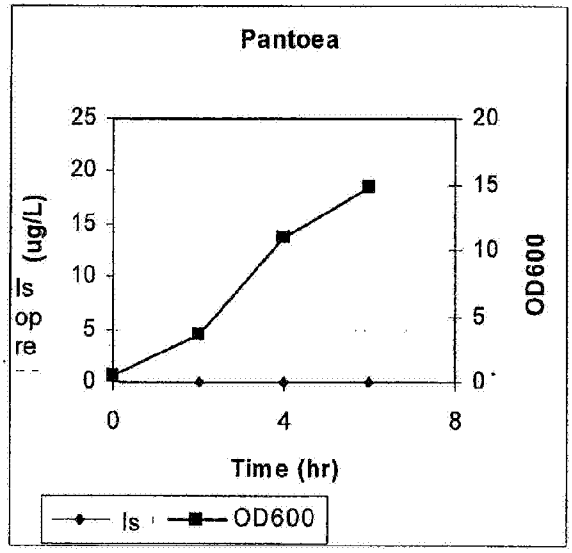

Pantoea
25
20
15
10
5
0
Is (ug/L)
op
re
20
15
10
5
0
OD600
0
4
8
Time (hr)
Is
OD600

Figure 10B

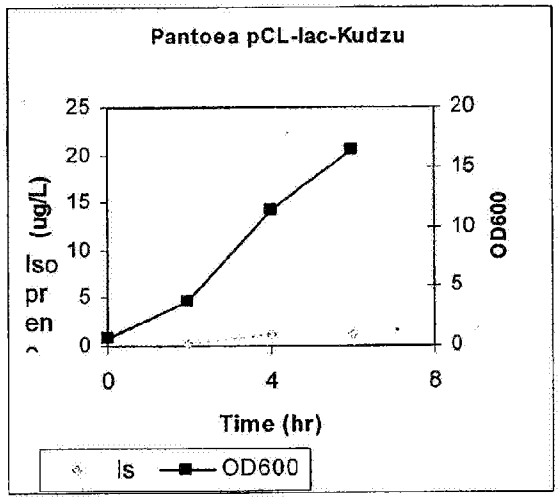

Pantoea pCL-lac-Kudzu
25
20
15
10
5
0
(ug/L)
Iso
pr
en
20
15
10
5
0
OD600
0
4
8
Time (hr)
Is
OD600

Figure 10C

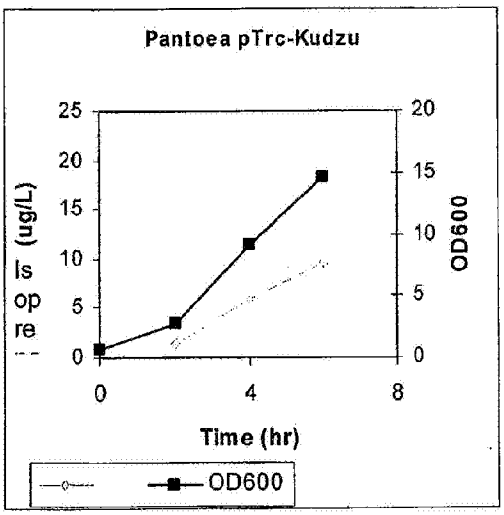

Pantoea pTrc-Kudzu
25
20
15
10
5
0
Is
op
re
(ug/L)
20
15
10
5
0
OD600
0
4
8
Time (hr)
OD600

Figure 11

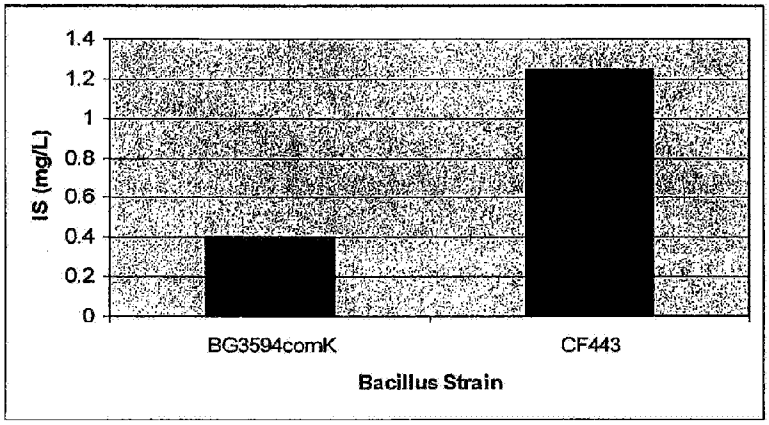


Figure 12A

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Figure 12B

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Figure 12C

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(SEQ ID NO:57)

Figure 13

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GACGTCTTCGAGAGATTTAAGGACAAGGAGGGAGGATTTAGCGGCGAGCTGAAGGGAGACGTTC
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Figure 14

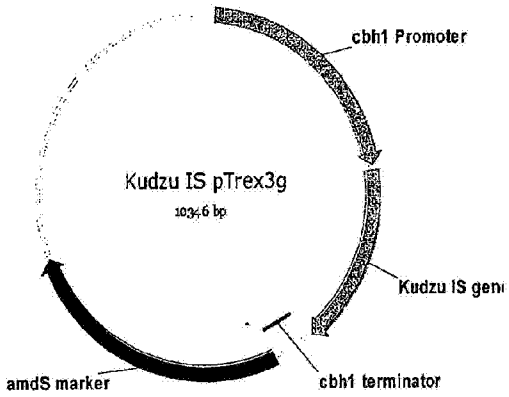
cbh1 Promoter
Kudzu IS pTrex3g
10346 bp
Kudzu IS gen
amdS marker
cbh1 terminator

Figure 15A

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 121 CTTTTCTTTT CTCTCATTCA ACGCACTCCA TCGTATCCGT ATTCCTCTTA TTTTTTCTCT
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 961 GTCACGTCAC GGTGTTAGGG CCCCTACAAA AATGACTCAA ACCATGCGTG ATGTCACTCC
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1801 TGATAACAAA ATATTGTTCA TTGCAGGTCA TGACACAAAT ATTGCTAATA TAGCCGGCAT
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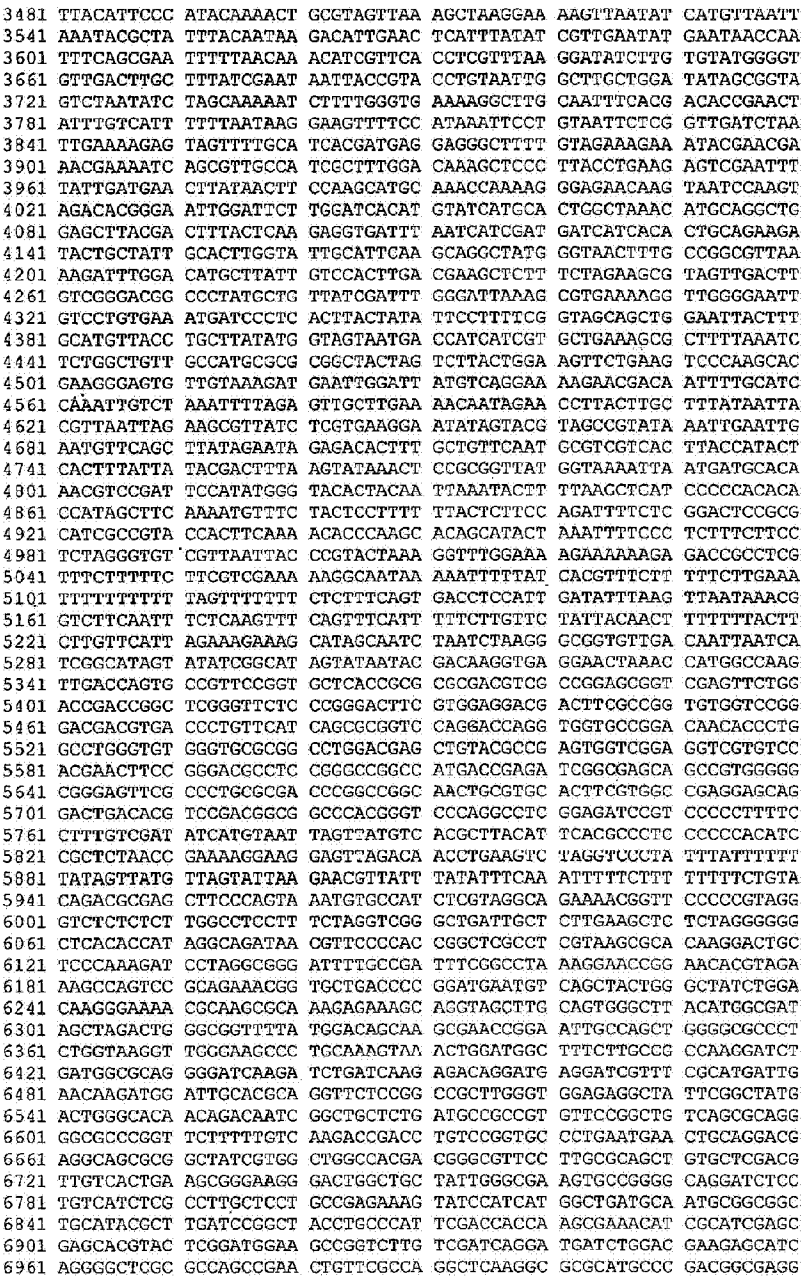
Figure 15B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4261 GTCGGGACGG CCCTATGCTG TTATCGATTT GGGATTAAAG CGTGAAAAGG TTGGGGAATT
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6901 GAGCACGTAC TCGGATGGAA GCCGGTCTTG TCGATCAGGA TGATCTGGAC GAAGAGCATC
6961 AGGGGCTCGC GCCAGCCGAA CTGTTCGCCA GGCTCAAGGC GCGCATGCCC GACGGCGAGG

Figure 15C

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7201 TTTACGGTAT CGCCGCTCCC GATTCGCAGC GCATCGCCTT CTATCGCCTT CTTGACGAGT
7261 TCTTCTGAAT TGAAAAAGGT ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAAACT
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7621 CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT TACCAGTGGC
7681 TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACGAT AGTTACCGGA
7741 TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA CAGCCCAGCT TGGAGCGAAC
7801 GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCATTGA GAAAGCGCCA CGCTTCCCGA
7861 AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG AGCGCACGAG
7921 GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC GCCACCTCTG
7981 ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA AAAACGCCAG
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(SEQ ID NO:11)

Figure 16

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  61 CTCGACGATC TGCTAACTAC CAGCCGAACC TTTGGAACTT TGAGTTTCTC CAGTCTCTCG
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 181 GATGTATGAT CAACAGAGTT GACACCCAAC CCCTGTCTTT GCTGGAGCTG ATCGACGATG
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 421 ACAAGGAGGG AGGATTTAGC GGCGAGCTGA AGGGAGACGT TCAGGGTCTT CTCTCCTTGT
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 541 CCATCACTCA CCTTAAGAAT AACCTTAAGG AGGGAATTAA CACCAAGGTG GCCGAGCAGG
 601 TTTCTCACGC CCTGGAGCTC CCCTACCACC AACGGCTCCA TAGACTGGAG GCTCGTTGGT
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 841 ATTTTTGGGC CCTTGGAATG GCGCCTGACC CCCAGTTCGG AGAGTGCCGG AAGGCGGTGA
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(SEQ ID NO:12)

Figure 17

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 121 TTCTGACACC GACGAGTCGA TCGAGGTTTA TAAGGATAAG GCCAAGAAAC TTGAGGCCGA
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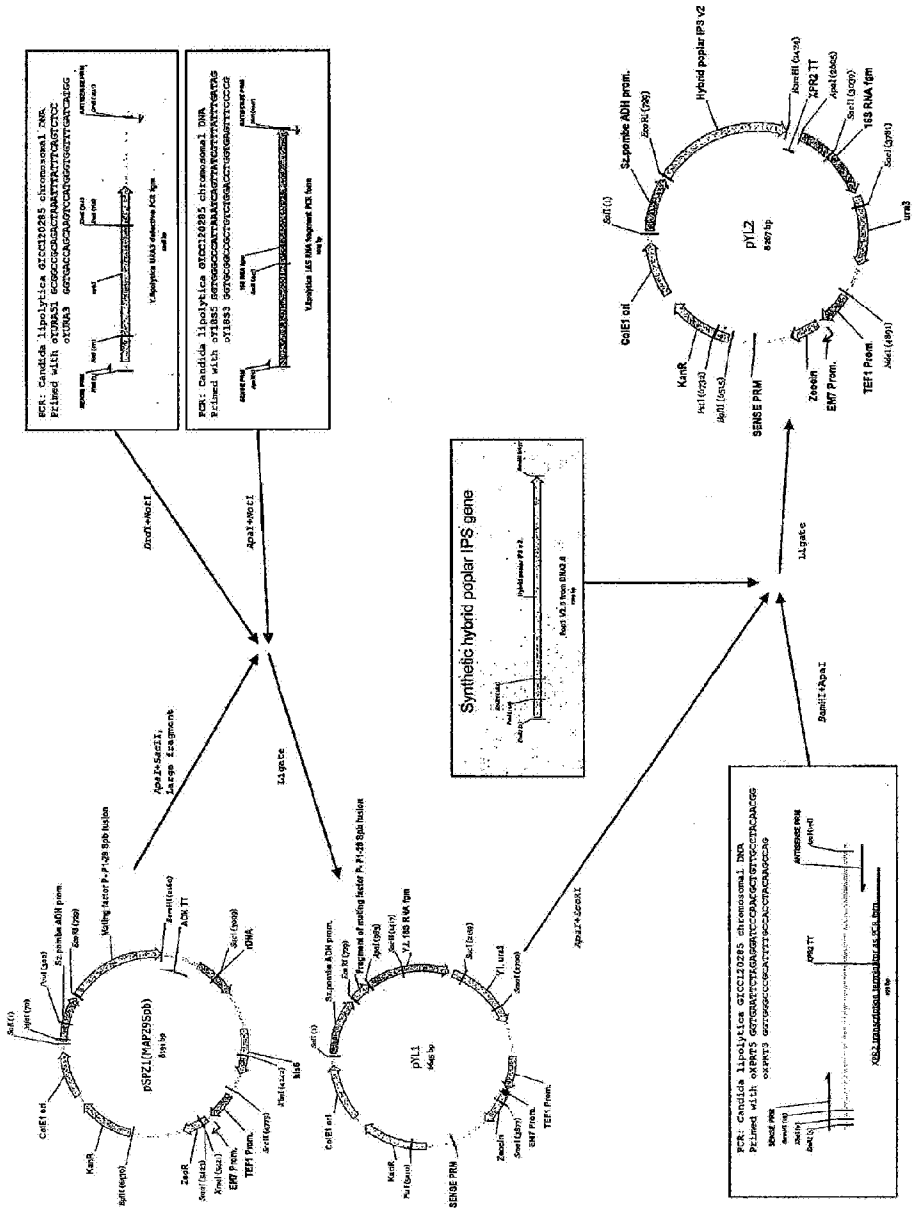
(SEQ ID NO:13)

Figure 18A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Ligate
SENSE PRM
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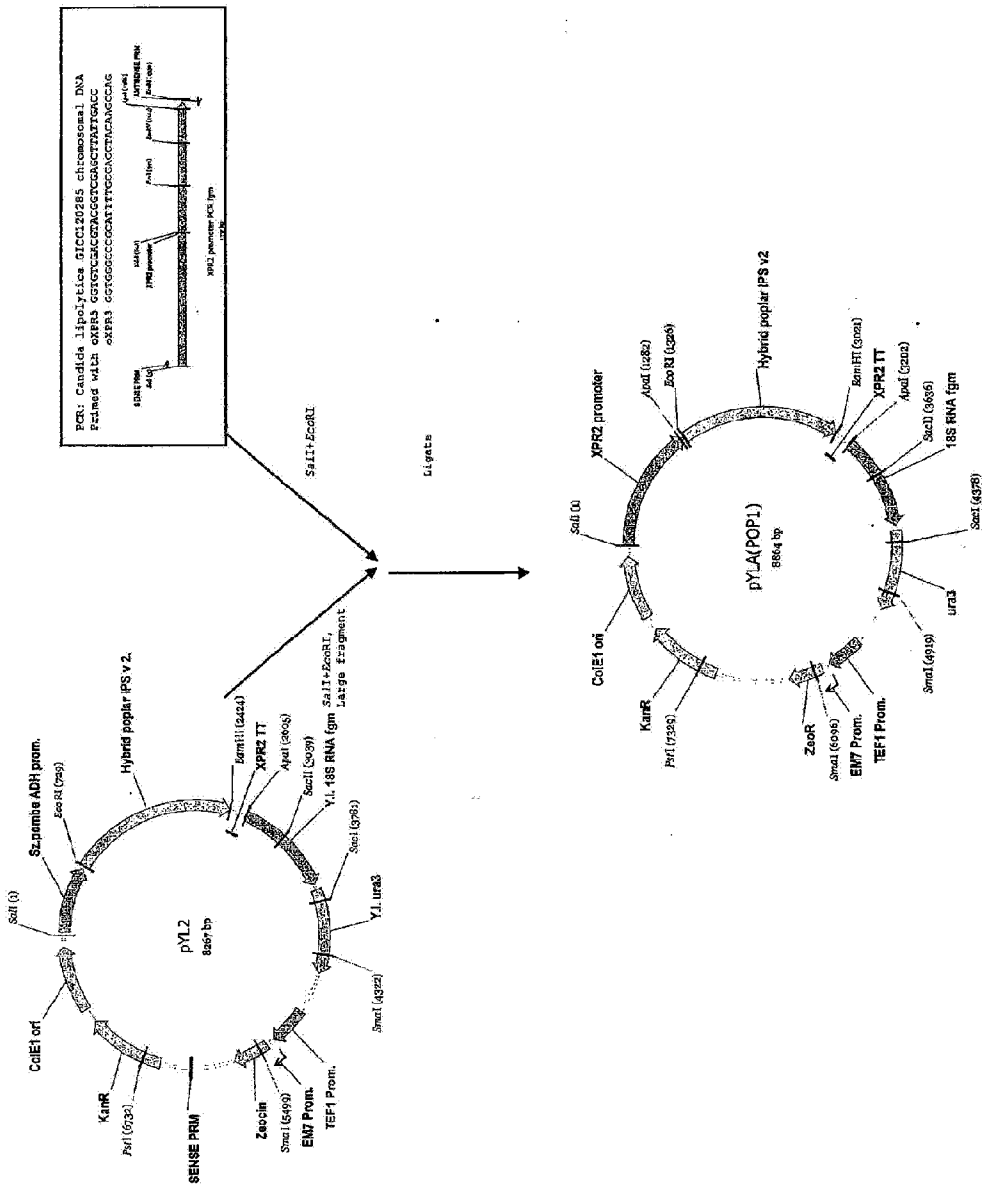
EMF Prom.
Zeocin
KanR
ColE1 ori
pYL2
pYL1
pSPZL(MAPZ95ob)

Figure 18B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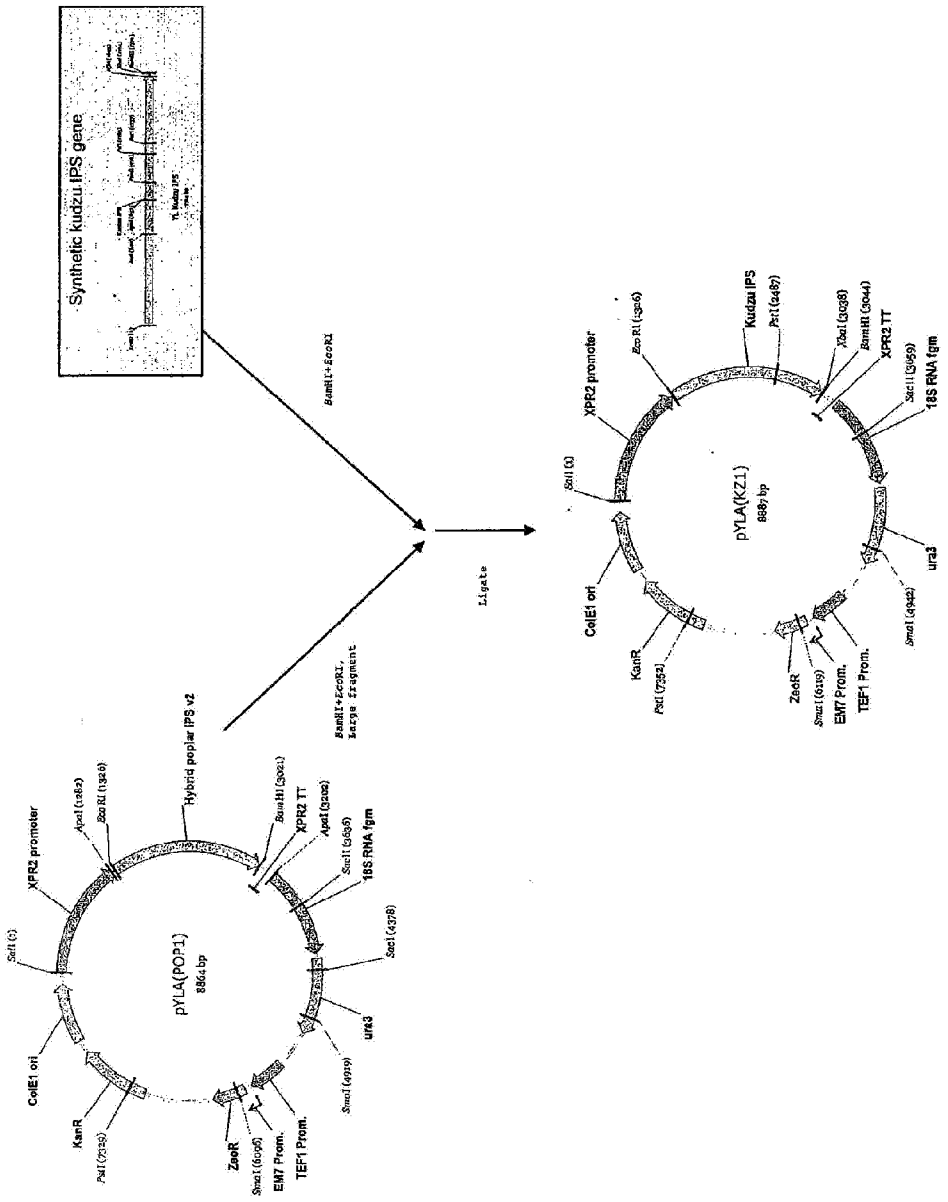
XPR2 promoter PCR fgm
SalI+EcoRI
Ligate
pYL2
8269 bp
Sz.pombe ADH prom.
Hybrid poplar IPS v2
BamHI (6424)
XPR2 TT
ApaI (6605)
SacII (6699)
Y.l. 18S RNA fgm
SacI (6782)
Y.l. ura3
SmaI (6922)
TEF1 Prom.
EM7 Prom.
Zeocin
SmaI (6499)
SENSE PRM
KanR
PstI (6032)
ColE1 ori
SalI (1)
SalI+EcoRI,
Large fragment
pYLA(POP1)
8864 bp
XPR2 promoter
AvaI (1282)
EcoRI (1286)
Hybrid poplar IPS v2
BamHI (5004)
XPR2 TT
ApaI (5202)
SacII (5656)
18S RNA fgm
SacI (6378)
ura3
SmaI (6919)
TEF1 Prom.
EM7 Prom.
ZeoR
SmaI (6598)
KanR
PstI (7386)
ColE1 ori
SalI (1)

Figure 18C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BamHI+EcoRI, Large fragment
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8864 bp
XPR2 promoter
ApaI (1484)
EcoRI (1928)
Hybrid poplar IPS v2
BamHI (3001)
XPR2 TT
ApaI (3206)
SacII (3439)
18S RNA fgm
SacII (4378)
ura3
SmaI (4959)
TEF1 Prom.
EM7 Prom.
SmaI (5090)
ZeoR
KanR
PstI (7038)
ColE1 ori
SalI (1)
pYLA(KZ1)
8887 bp
XPR2 promoter
EcoRI (3016)
Kudzu IPS
PstI (5497)
XbaI (5038)
BamHI (5044)
XPR2 TT
SacII (5459)
18S RNA fgm
ura3
SmaI (4942)
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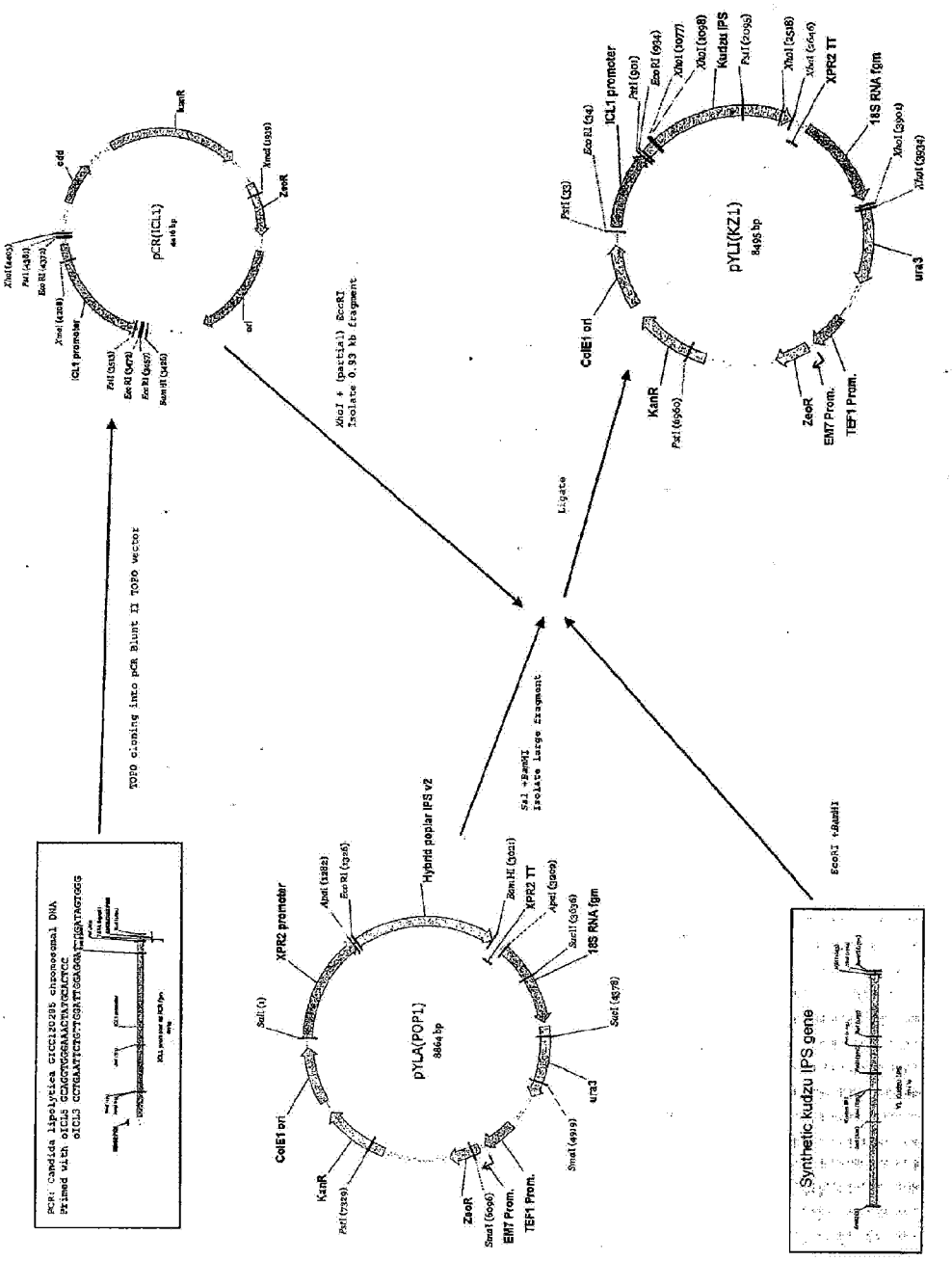
ZeoR
KanR
PstI (7253)
ColE1 ori
SalI (1)

Figure 18D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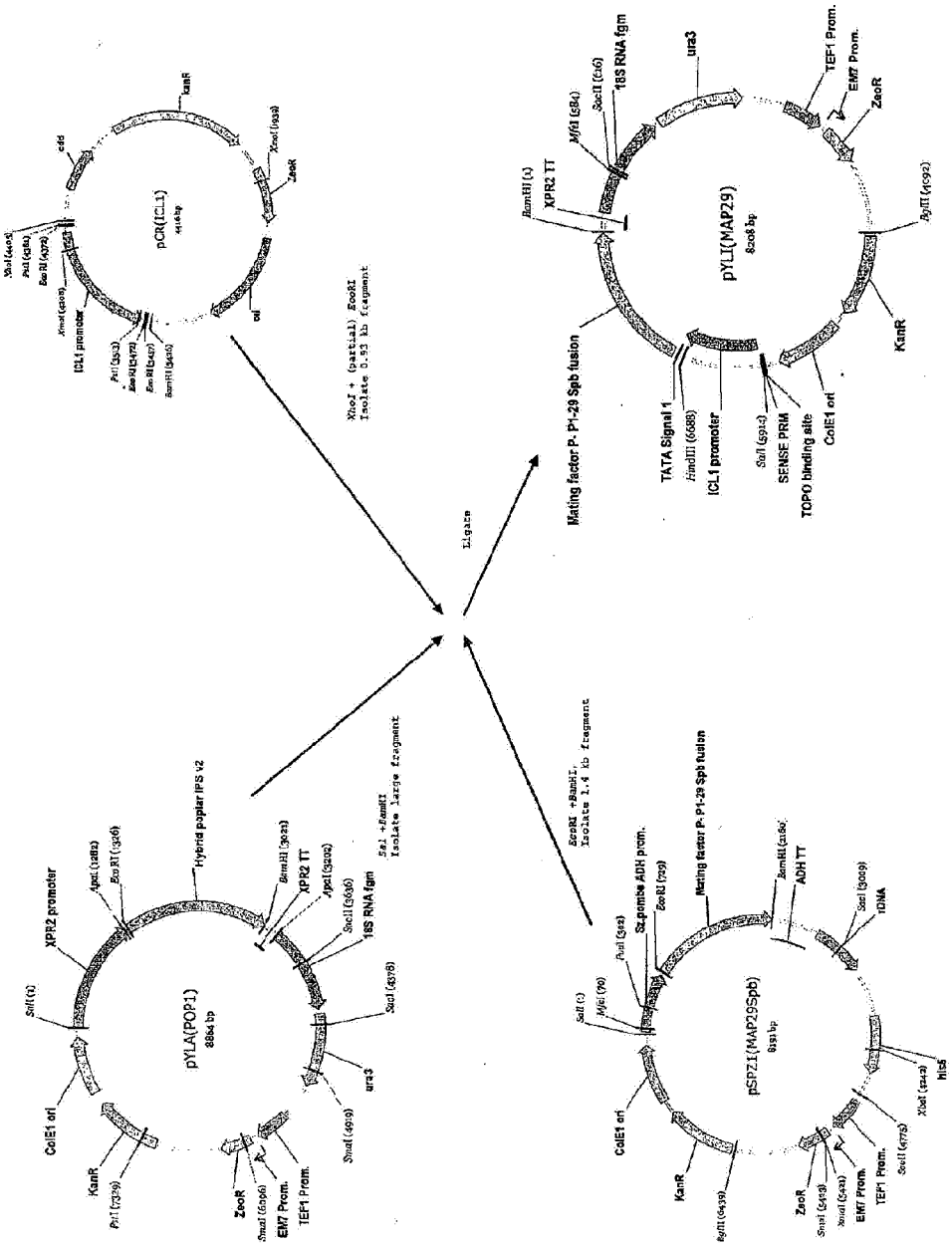
TOPO cloning into pCR Blunt II TOPO vector
Ligate
ICL1 promoter
Hybrid poplar IPS v2
Kudzu IPS
XPR2 promoter
XPR2 TT
18S RNA fgm
ColE1 ori
KanR
ZeoR
EM7 Prom.
TEF1 Prom.
ura3

Figure 18E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4164bp
KanR
ZeoR
ori
ICL1 promoter
XhoI + (partial) EcoRI
Isolate 0.93 kb fragment
pYLI(MAP29)
8208 bp
ura3
TEF1 Prom.
EM7 Prom.
ZeoR
AgeI (4092)
KanR
ColE1 ori
TOPO binding site
SENSE PRM
ICL1 promoter
TATA Signal 1
Mating factor P. P1-29 Spb fusion
XPR2 TT
18S RNA fgm
Ligase
pYLA(POP1)
8864 bp
XPR2 promoter
Hybrid poplar IFS v2
XPR2 TT
18S RNA fgm
ura3
ColE1 ori
KanR
ZeoR
EM7 Prom.
TEF1 Prom.
Sal +BamHI
Isolate large fragment
pSPZL(MAP29Spb)
8191bp
Mating factor P. P1-29 Spb fusion
ADH TT
rDNA
his5
ColE1 ori
KanR
ZeoR
EM7 Prom.
TEF1 Prom.
BamHI +BamHI
Isolate 1.4 kb fragment

Figure 18F

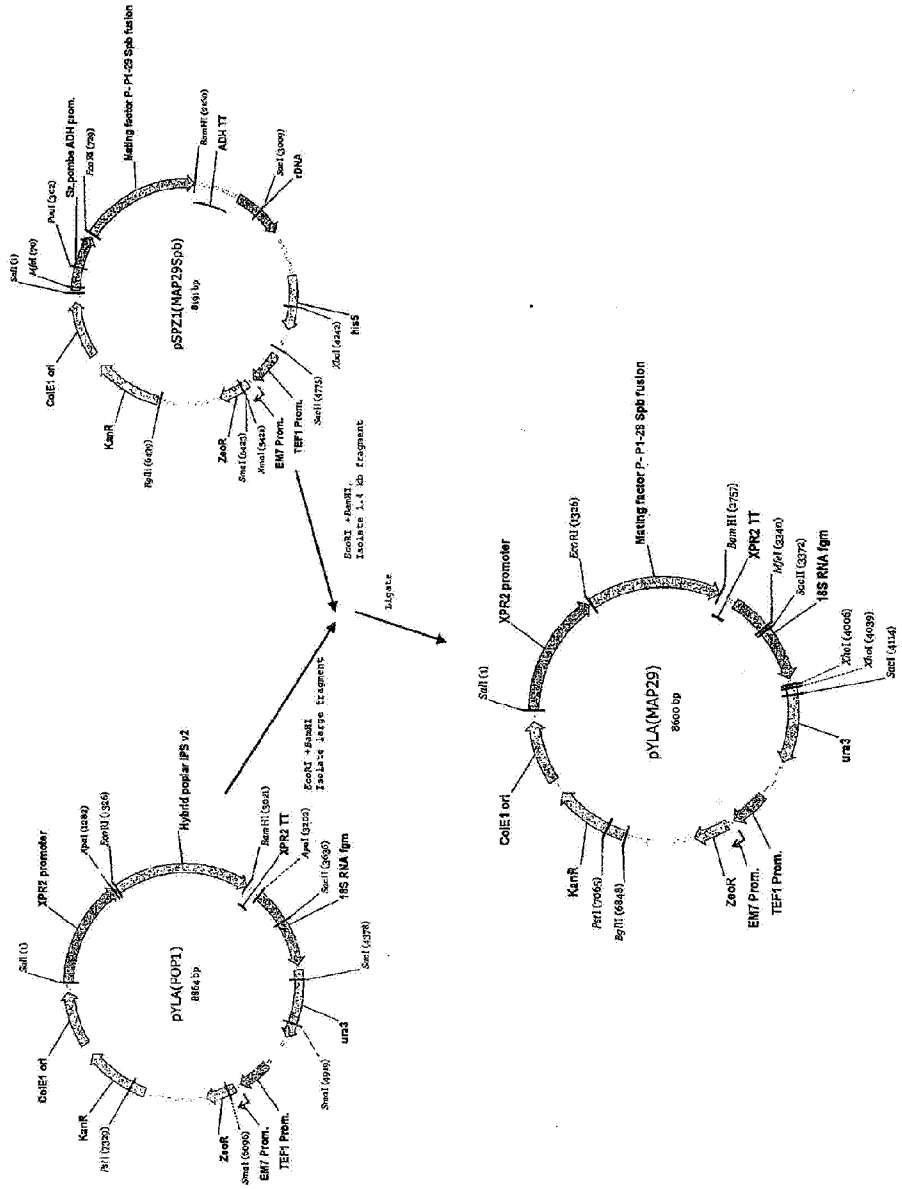
pSPZ1(MAP29Spb)
8915 bp
Mating factor P-P1-29 Spb fusion
St. pombe ADH prom.
ADH TT
rDNA
his5
ColE1 ori
KanR
ZeoR
EM7 Prom.
TEF1 Prom.
BamHI + BamHI
Isolate 1.4 kb fragment
Ligate
pYLA(HOP1)
8864 bp
Hybrid poplar IPS v2
XPR2 promoter
XPR2 TT
18S RNA fgm
ura3
BamHI + BamHI
Isolate large fragment
pYLA(MAP29)
9600 bp
XPR2 promoter
Mating factor P-P1-29 Spb fusion
XPR2 TT
18S RNA fgm
ura3
ColE1 ori
KanR
ZeoR
EM7 Prom.
TEF1 Prom.

Figure 19A

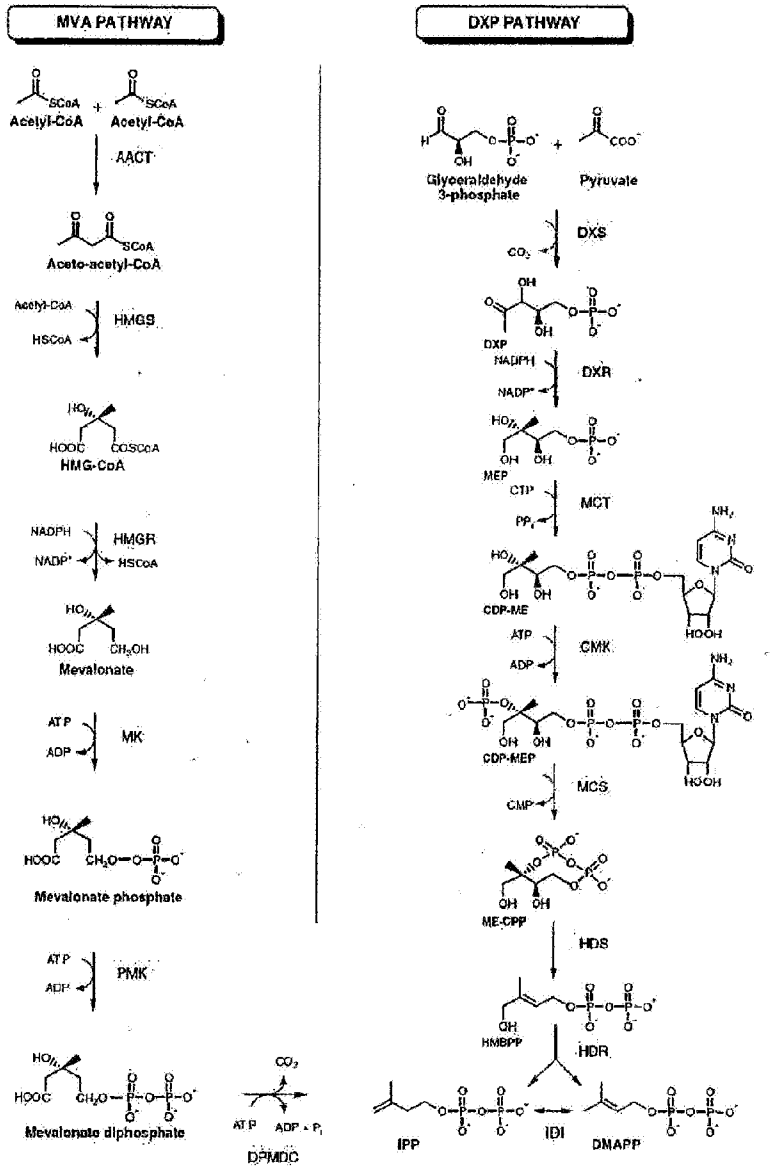
MVA PATHWAY
DXP PATHWAY
Acetyl-CoA
Acetyl-CoA
AACT
Aceto-acetyl-CoA
HMGS
HMG-CoA
HMGR
Mevalonate
MK
Mevalonate phosphate
PMK
Mevalonate diphosphate
DPMDC
Glyceraldehyde 3-phosphate
Pyruvate
DXS
DXP
DXR
MEP
MCT
CDP-ME
CMK
CDP-MEP
MCS
ME-CPP
HDS
HMBPP
HDR
IPP
IDI
DMAPP

Figure 19B

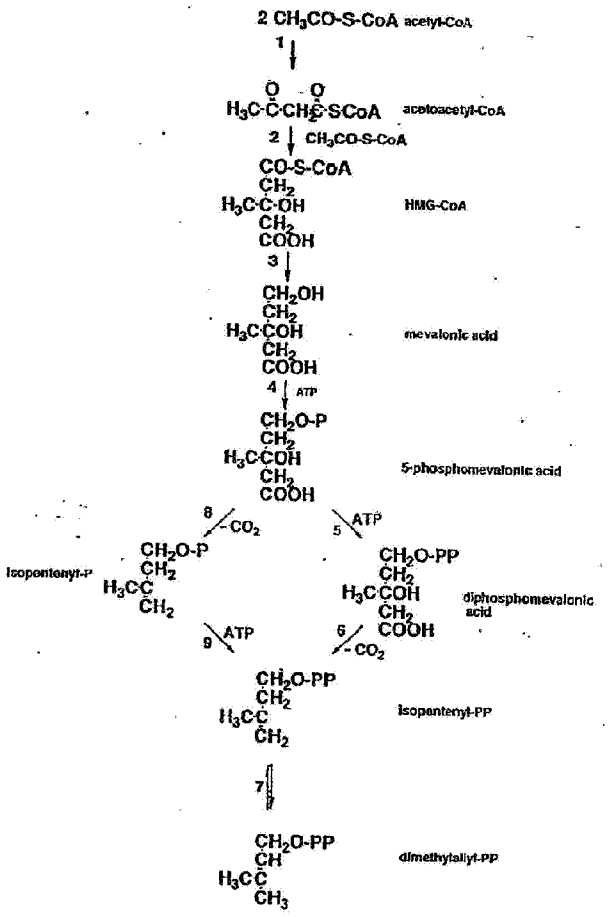
2 CH3CO-S-CoA
acetyl-CoA
1
acetoacetyl-CoA
2
CH3CO-S-CoA
HMG-CoA
3
mevalonic acid
4
ATP
5-phosphomevalonic acid
6
-CO2
5
ATP
Isopentenyl-P
diphosphomevalonic acid
9
ATP
6
-CO2
Isopentenyl-PP
7
dimethylallyl-PP

Figure 20

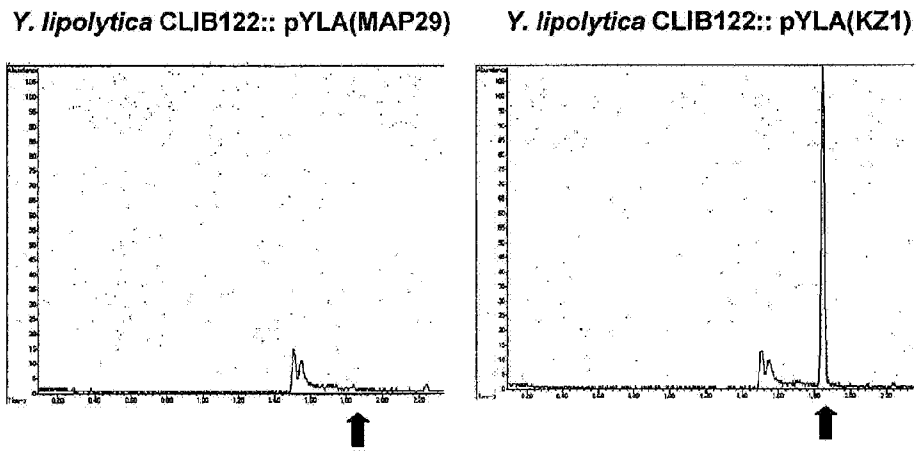
Y. lipolytica CLIB122:: pYLA(MAP29)
Y. lipolytica CLIB122:: pYLA(KZ1)

Figure 21

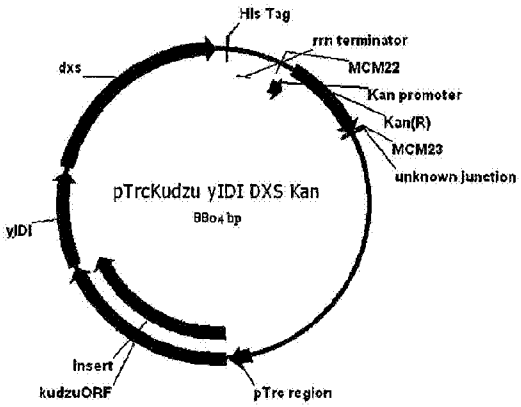
His Tag
rrn terminator
MCM22
Kan promoter
Kan(R)
MCM23
unknown junction
dxs
pTrcKudzu yIDI DXS Kan
8804 bp
yIDI
Insert
kudzuORF
pTre region

Figure 22A

1-

gctggtaccatatgggaattcgaagctttctagaacaaaaactcatctcagaagaggatctgaa
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acagaatttgcctggcggcagtagcgcggtggtcccacctgaccccatgccgaactcagaagtg
aaacgccgtagcgccgatggtagtgtggggtctccccatgcgagagtagggaactgccaggcat
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Figure 22B

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Figure 22C

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Figure 22D

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acgccacgctggtcgatatgcgttttgtgaaaccgcttgatgaagcgttaattctggaaatggc
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(SEQ ID NO:20)

Figure 23A

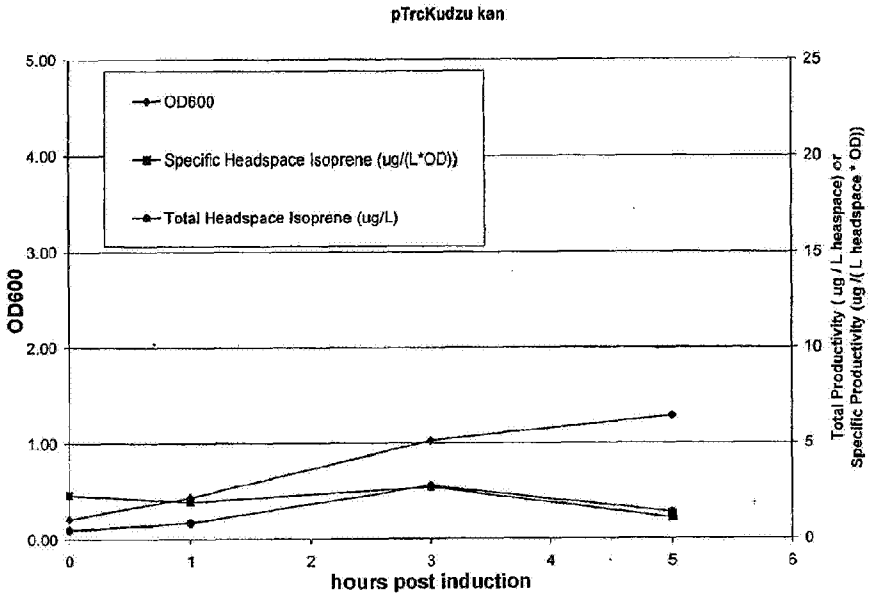

pTrcKudzu kan
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
OD600
Total Productivity (ug / L headspace) or Specific Productivity (ug /(L headspace * OD))
hours post induction
5.00
4.00
3.00
2.00
1.00
0.00
25
20
15
10
5
0
0
1
2
3
4
5
6

Figure 23B

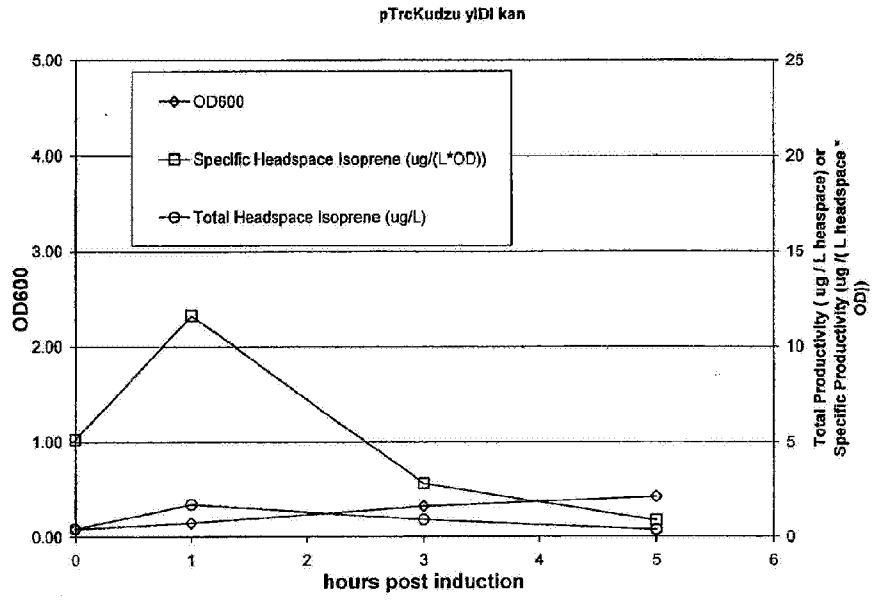

pTrcKudzu yIDI kan
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
OD600
Total Productivity (ug / L headspace) or Specific Productivity (ug /(L headspace * OD))
hours post induction
5.00
4.00
3.00
2.00
1.00
0.00
25
20
15
10
5
0
0
1
2
3
4
5
6

Figure 23C

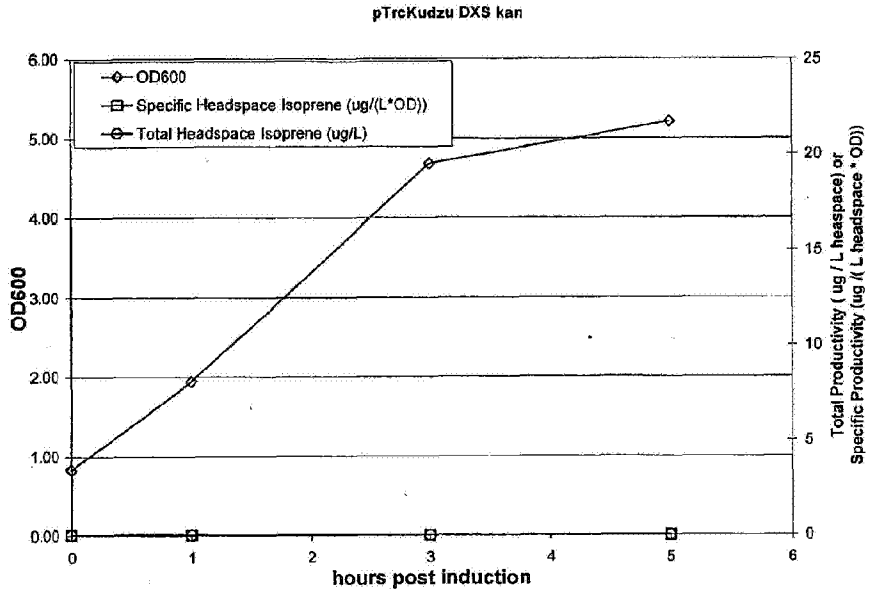

pTrcKudzu DXS kan
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
OD600
Total Productivity (ug / L headspace) or Specific Productivity (ug /(L headspace * OD))
hours post induction
6.00
5.00
4.00
3.00
2.00
1.00
0.00
25
20
15
10
5
0
0
1
2
3
4
5
6

Figure 23D

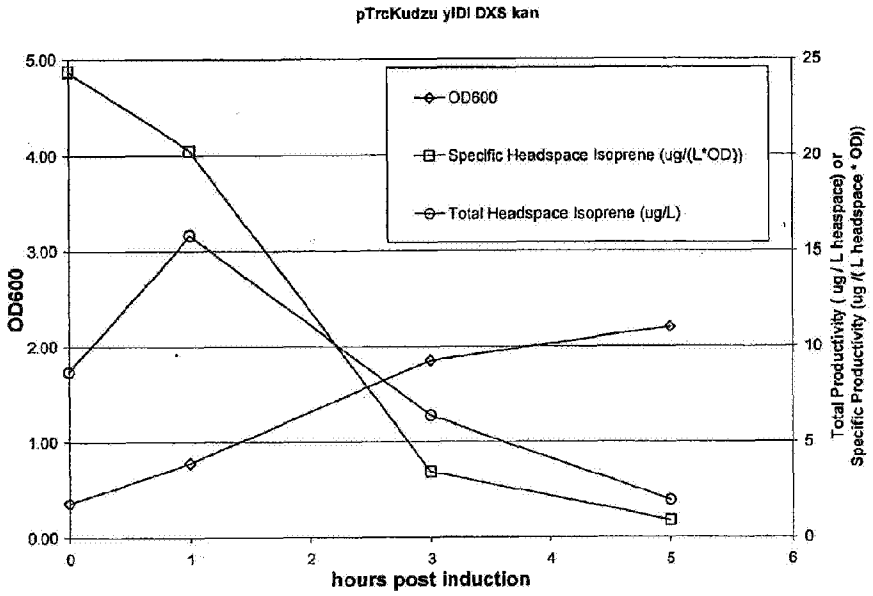

pTrcKudzu yIDI DXS kan
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
5.00
4.00
3.00
2.00
1.00
0.00
0
1
2
3
4
5
6
25
20
15
10
5
0
hours post induction
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))

Figure 23E

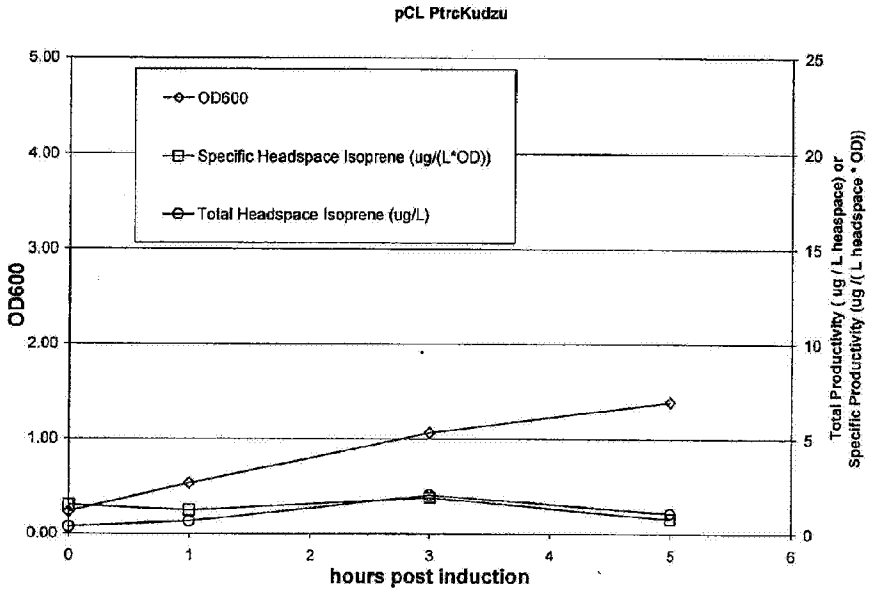

pCL PtrcKudzu
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
5.00
4.00
3.00
2.00
1.00
0.00
25
20
15
10
5
0
0
1
2
3
4
5
6

Figure 23F

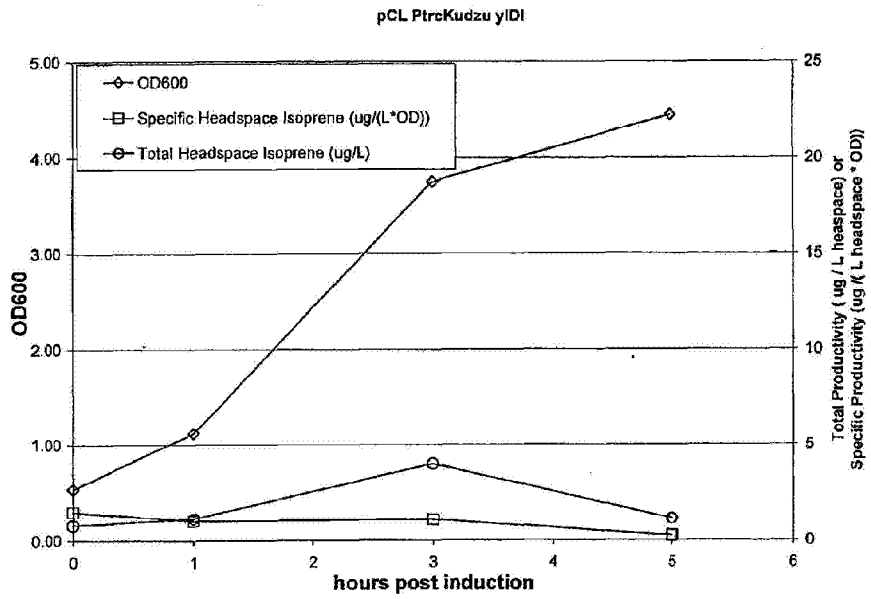

pCL PtrcKudzu yIDI
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
5.00
4.00
3.00
2.00
1.00
0.00
0
1
2
3
4
5
6
hours post induction
25
20
15
10
5
0
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))

Figure 23G

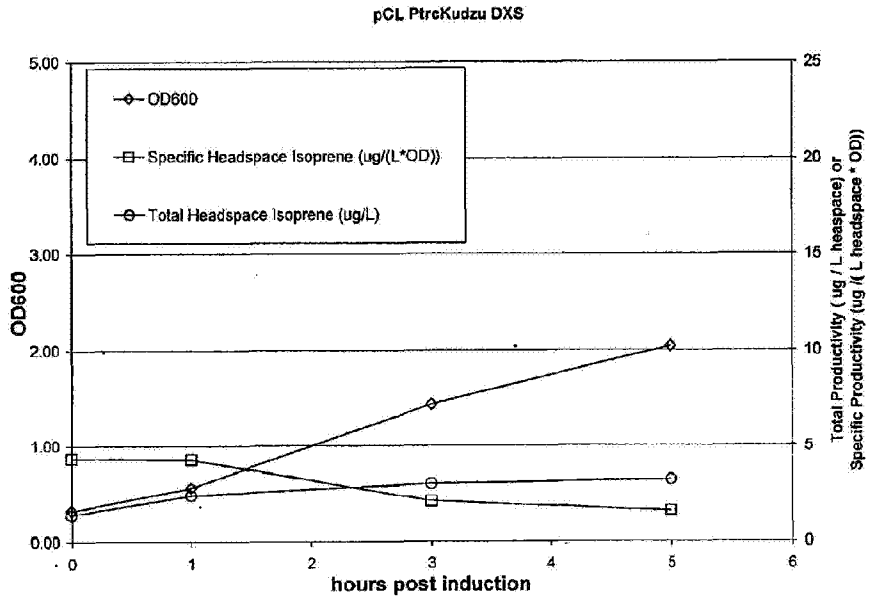

pCL PtrcKudzu DXS
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
5.00
4.00
3.00
2.00
1.00
0.00
25
20
15
10
5
0
0
1
2
3
4
5
6
hours post induction
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))

Figure 23H

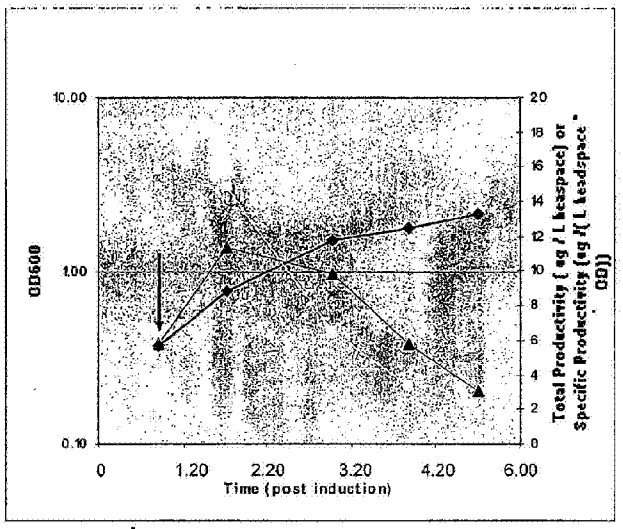
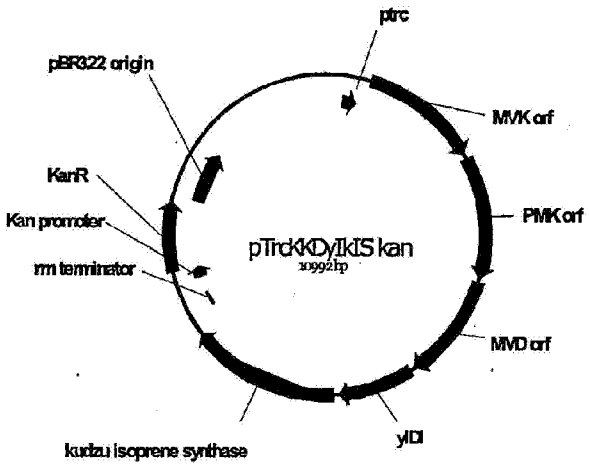

10.00
1.00
0.10
OD600
20
18
16
14
12
10
8
6
4
2
0
Total Productivity (ug / L keaspace) or
Specific Productivity (ug /(L keadspace *
OD))
0
1.20
2.20
3.20
4.20
6.00
Time (post induction)

Figure 24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MVK orf
KanR
PMK orf
Kan promoter
pTrcKKDyIkIS kan
10992 bp
rrn terminator
MVD orf
yIDI
kudzu isoprene synthase

Figure 25A

5'-
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Figure 25B

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Figure 25C

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Figure 25D

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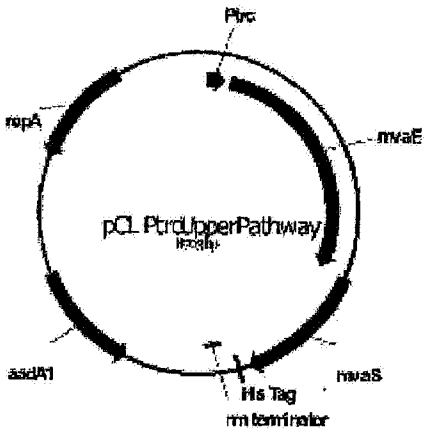
Figure 26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mvaS
His Tag
rrnB terminator

Figure 27A

5'-

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Figure 27B

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Figure 27C

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Figure 27D

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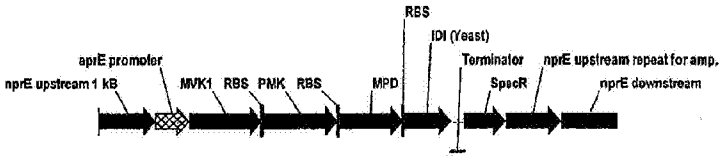
Figure 28



Lower Pathway Bacillus Cassette

9371 bp

Figure 29A

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Figure 29B

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Figure 29C

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Figure 29D

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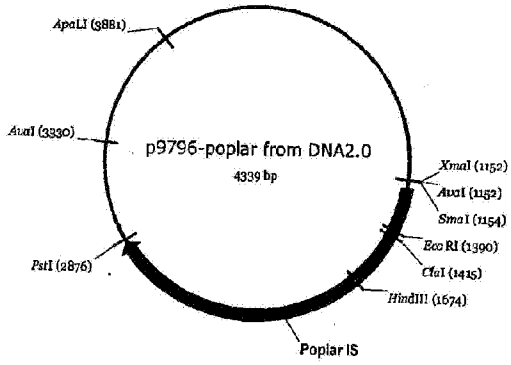
Figure 30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Poplar IS

Figure 31A

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Figure 31B

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Figure 32

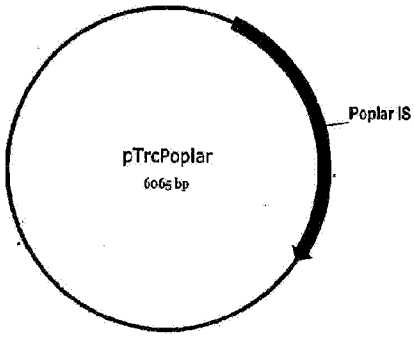

Figure 33A

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Figure 33B

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Figure 33C

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Figure 34

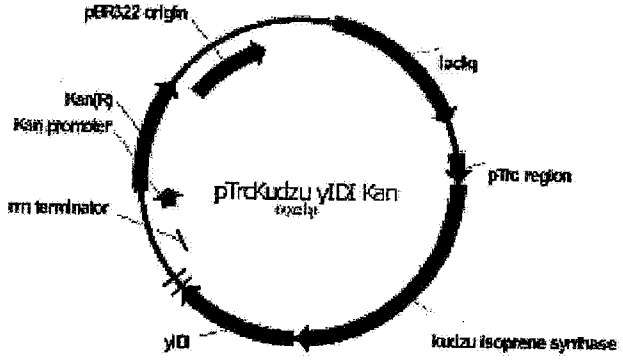
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Figure 35A

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Figure 35B

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Figure 35C

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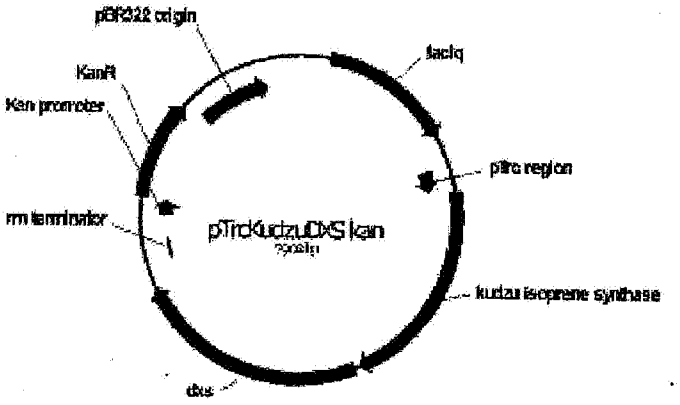
Figure 36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dxs

Figure 37A

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Figure 37B

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Figure 37C

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Figure 38

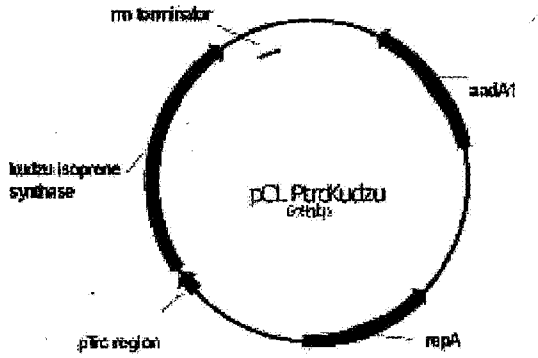
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Figure 39A

5′-

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Figure 39B

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Figure 39C

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Figure 40

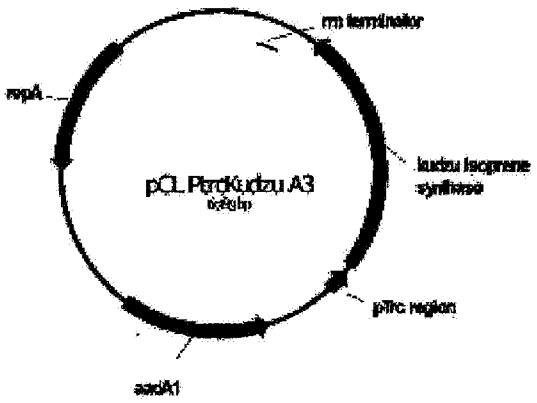

Figure 41A

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Figure 41B

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Figure 41C

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Figure 42

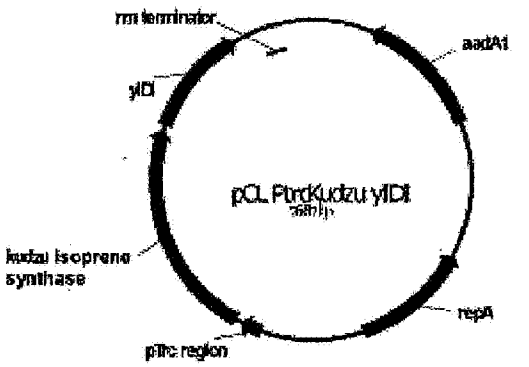
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Figure 43A

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Figure 43B

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Figure 43C

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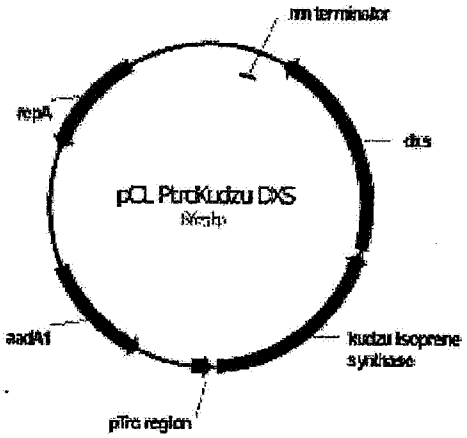
Figure 44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repA
dxs
pCL PtrcKudzu DXS
aadA1
kudzu isoprene synthase
ptrc region

Figure 45A

5'-

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Figure 45B

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Figure 45C

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Figure 45D

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Figure 46A

A.

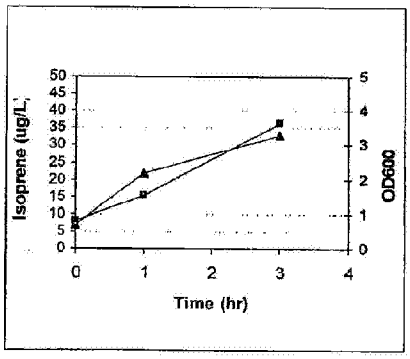


Figure 46B

B.

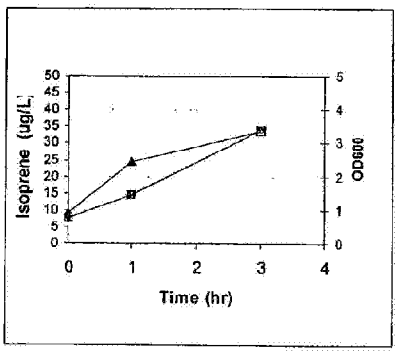


Figure 46C

C.

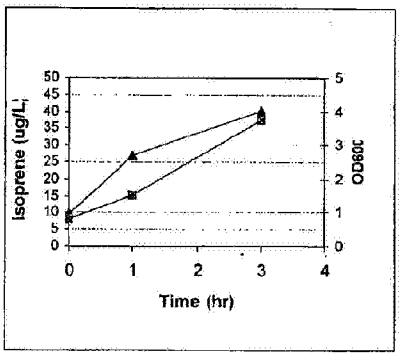


Figure 46D

D.

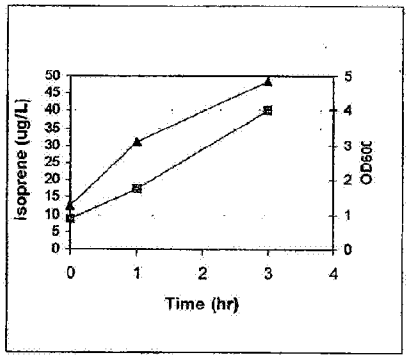


Figure 46E

E.

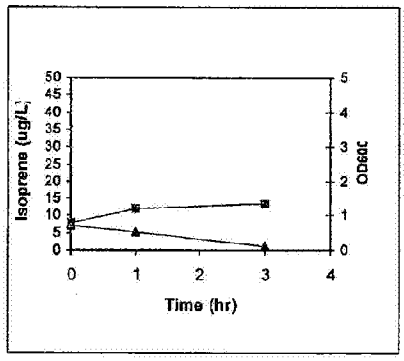

Isoprene (ug/L)
OD600
Time (hr)
0
5
10
15
20
25
30
35
40
45
50
0
1
2
3
4
5
0
1
2
3
4

Figure 47A

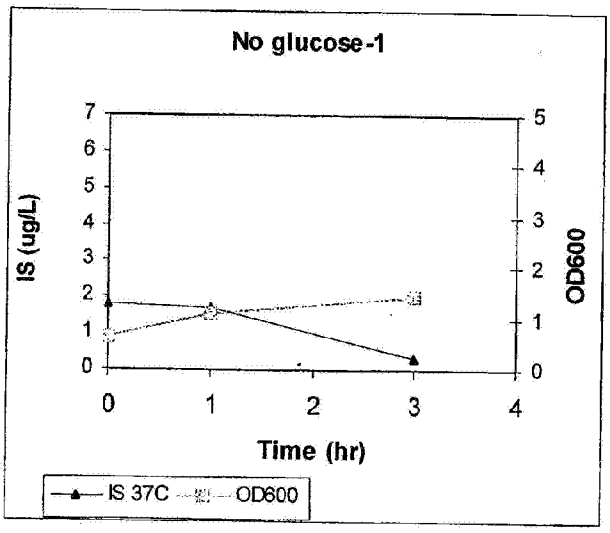


Figure 47B

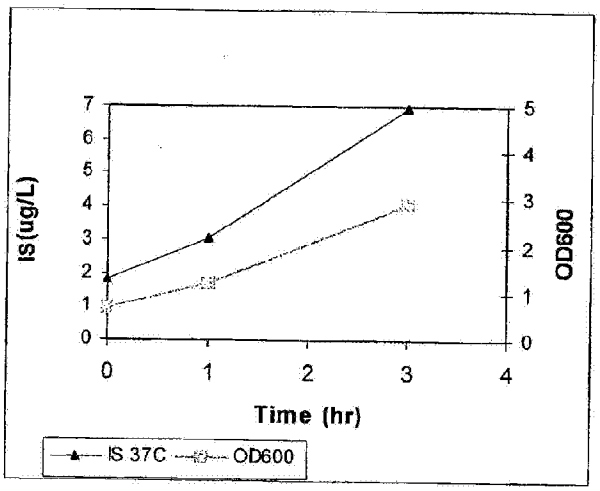


Figure 47C

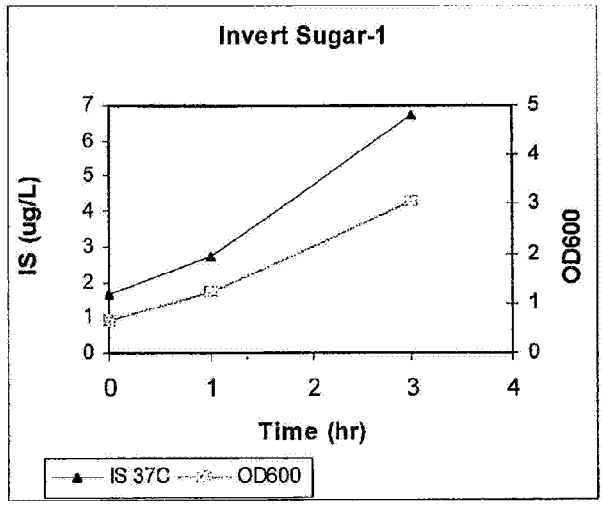


Figure 47D

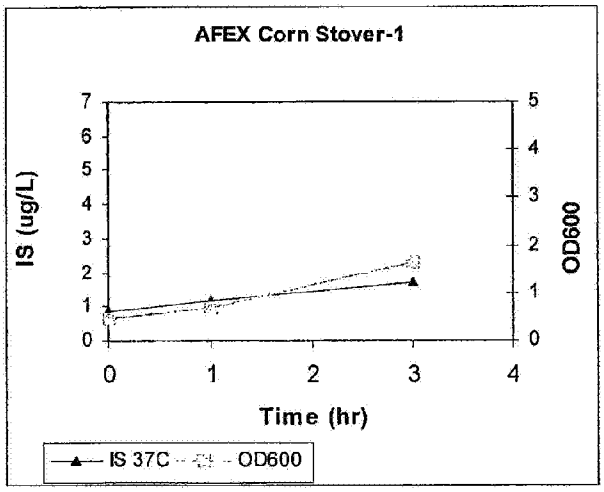


Figure 48A

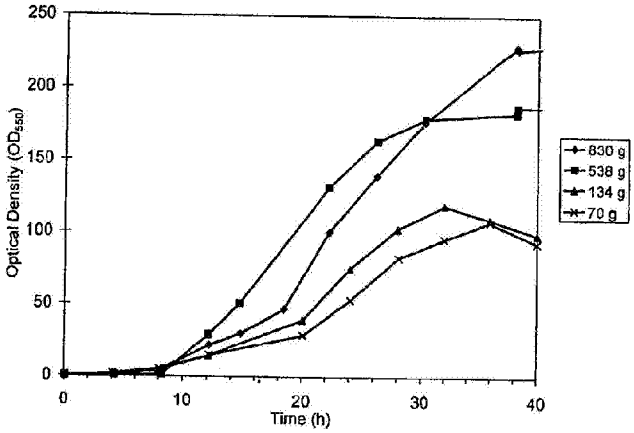


Figure 48B

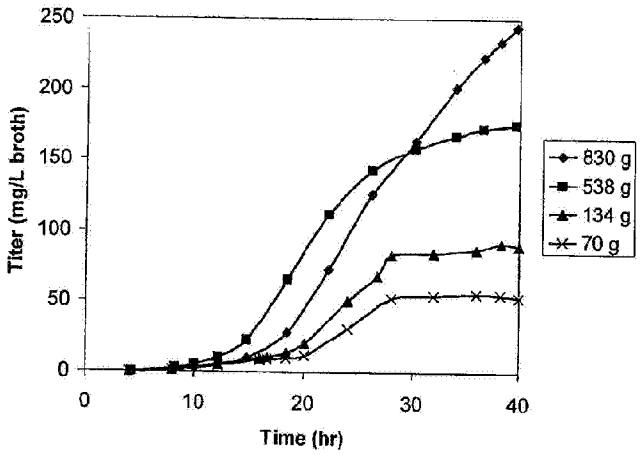
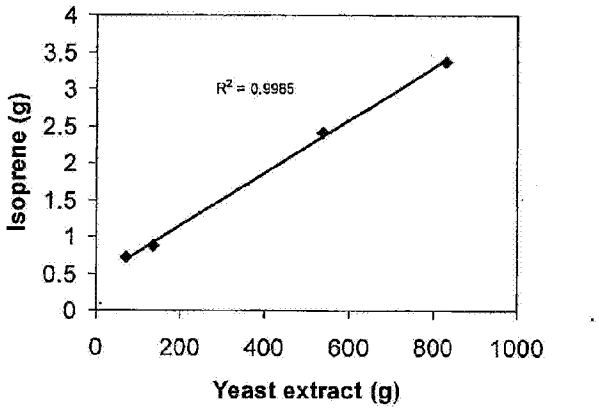


Figure 48C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3.5
3
2.5
2
1.5
1
0.5
0
Isoprene (g)
R^2 = 0.9985
0
200
400
600
800
1000
Yeast extract (g)

Figure 49A

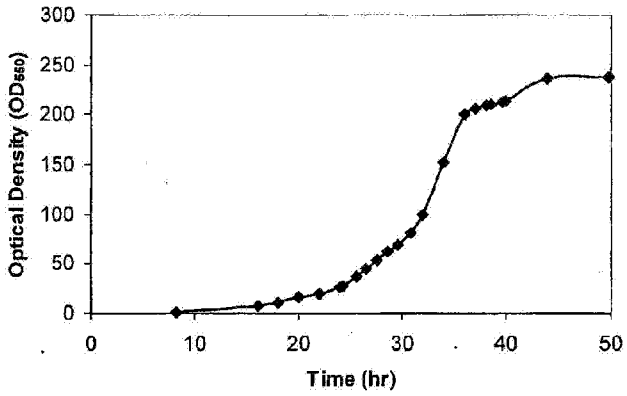

Figure 49B

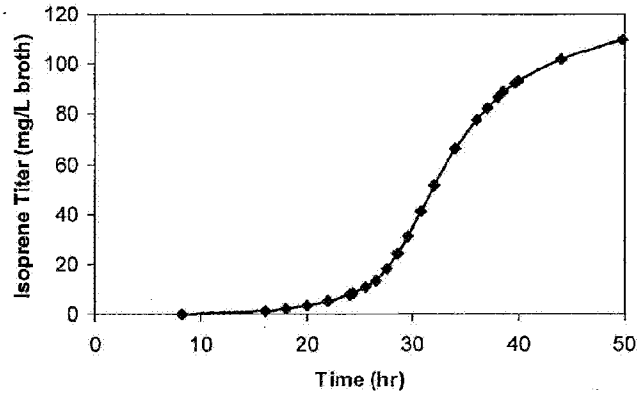

Figure 49C

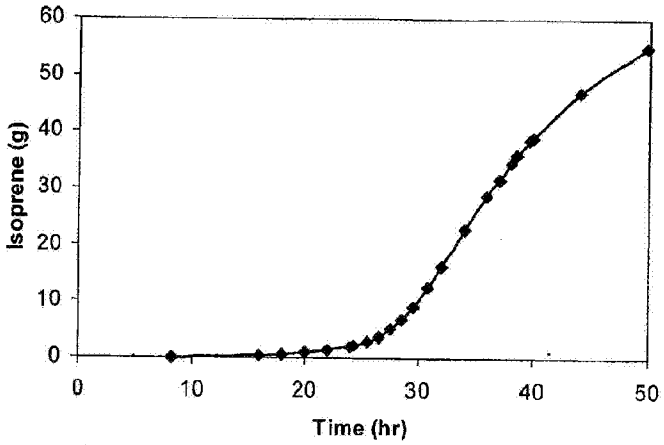

Figure 50

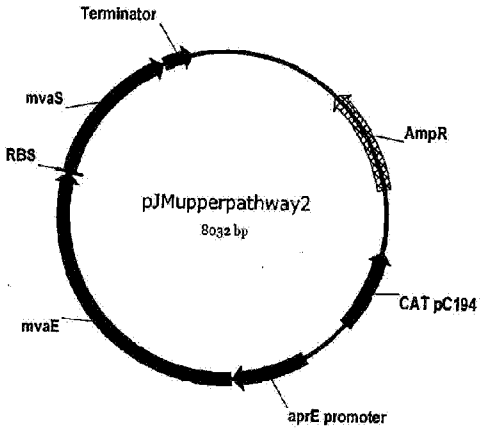
Terminator
mvaS
RBS
AmpR
pJMupperpathway2
8032 bp
CAT pC194
mvaE
aprE promoter

Figure 51A

5′-
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Figure 51B

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Figure 51C

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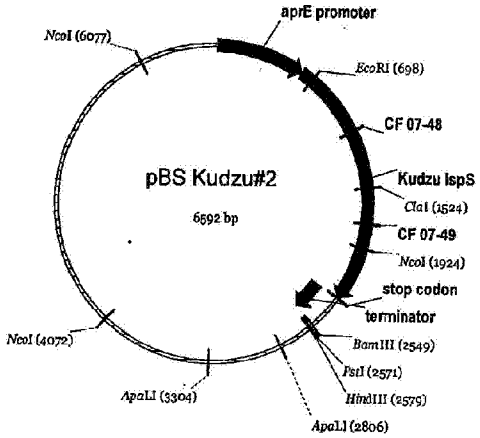
(SEQ ID NO:56)

Figure 52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NcoI (6077)
EcoRI (698)
CF 07-48
pBS Kudzu#2
6592 bp
Kudzu IspS
ClaI (1524)
CF 07-49
NcoI (1924)
stop codon
terminator
BamHI (2549)
PstI (2571)
HindIII (2579)
NcoI (4079)
ApaLI (3304)
ApaLI (2806)

Figure 53A

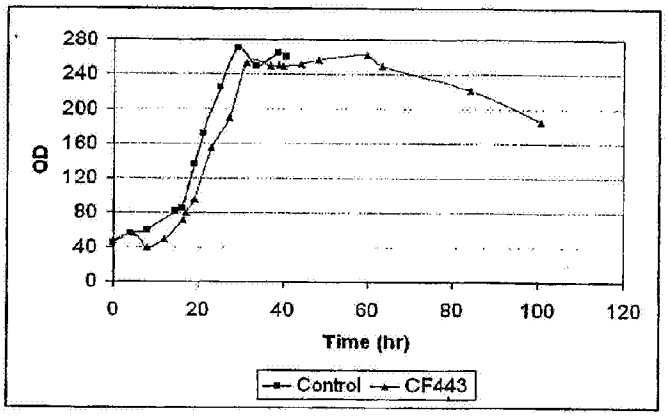


Figure 53B

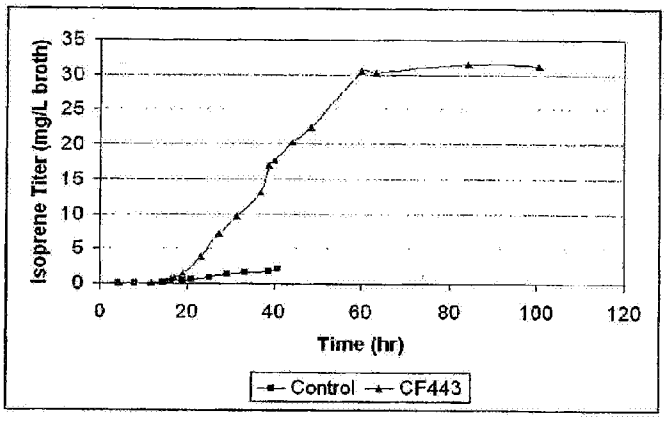
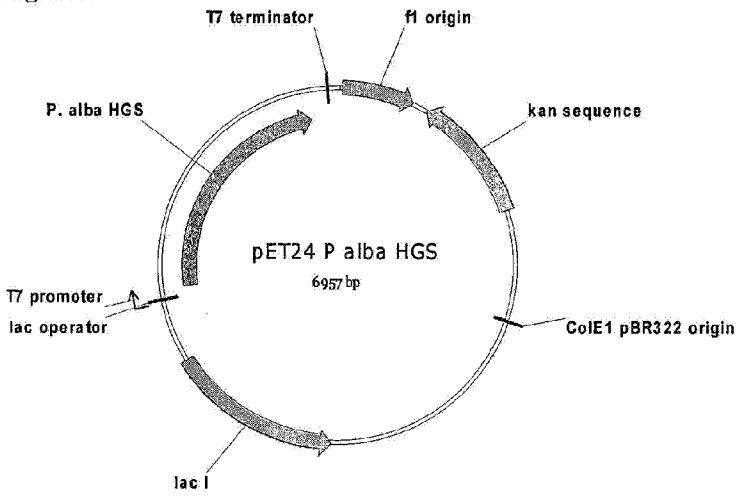


Figure 54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f1 origin
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lac operator
ColE1 pBR322 origin
lac I

Figure 55A

1-

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Figure 55B

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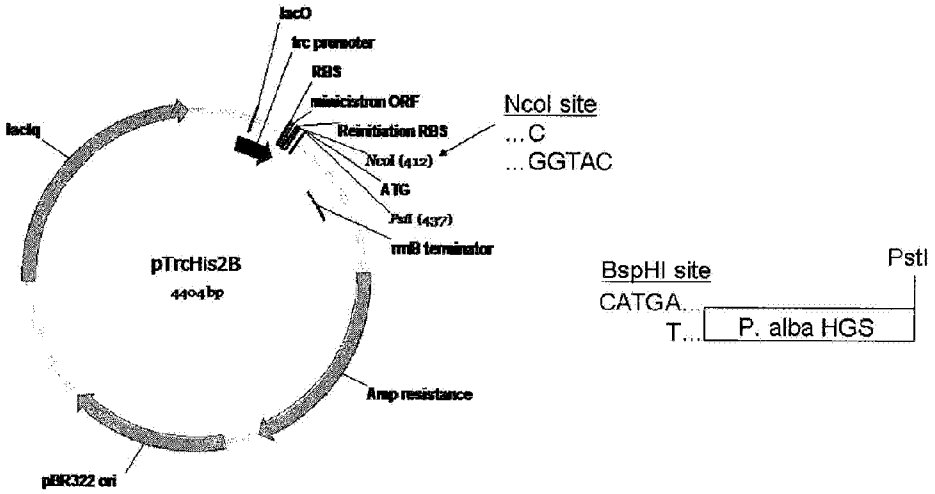
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Figure 57

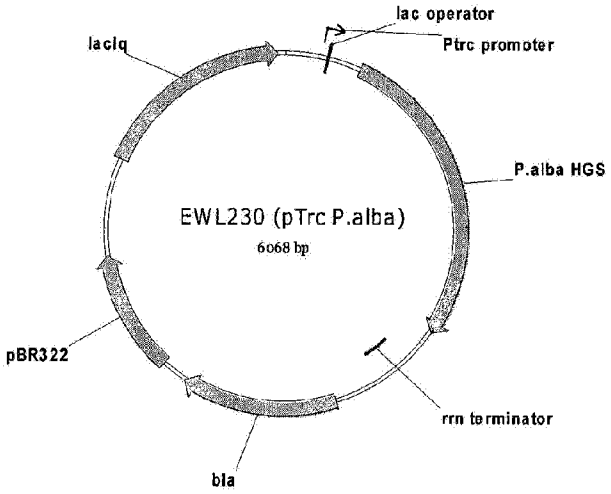
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Figure 58A

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Figure 58B

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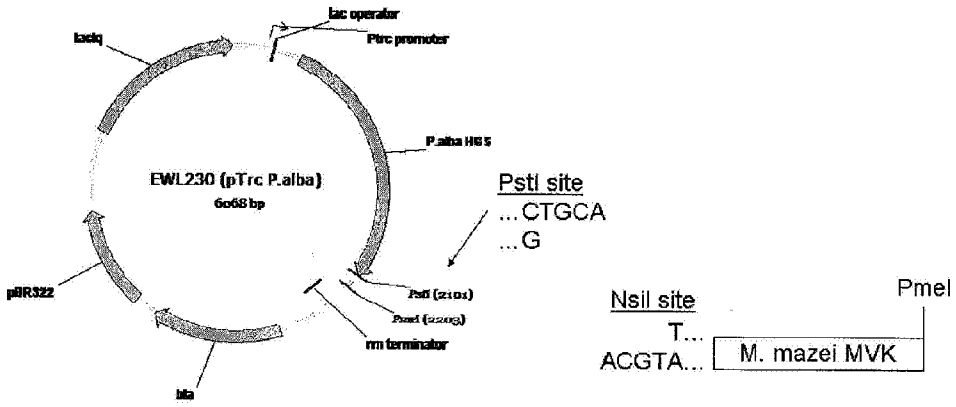
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Figure 60

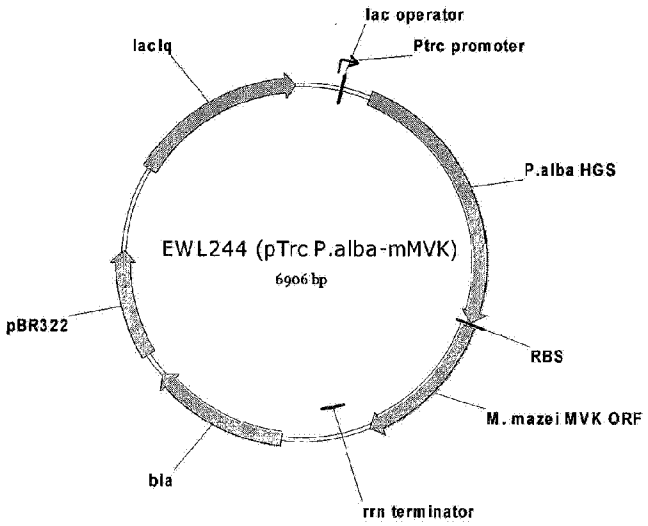
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Figure 61A

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Figure 61B

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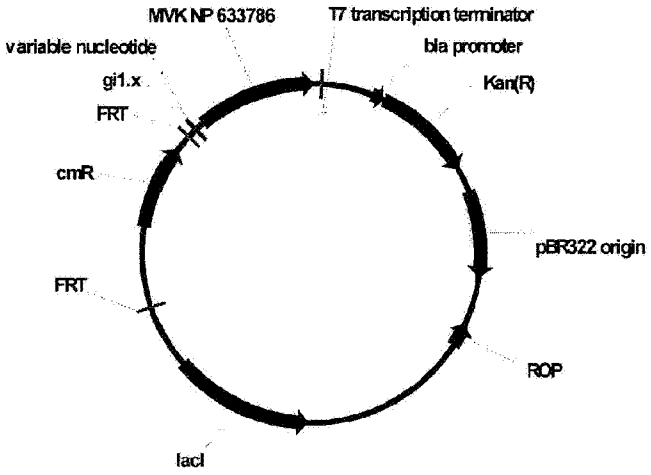
Figure 62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8123 bp

Figure 63A

1-

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Figure 63B

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Figure 64A

1-

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Figure 64B

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Figure 64C

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Figure 65A

1-

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Figure 65B

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Figure 65C

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Figure 66A

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Figure 66B

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Figure 66C

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Figure 67A

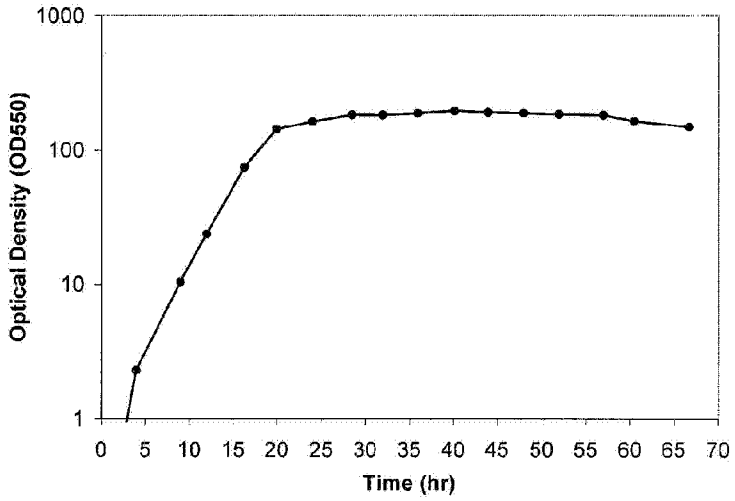


Figure 67B

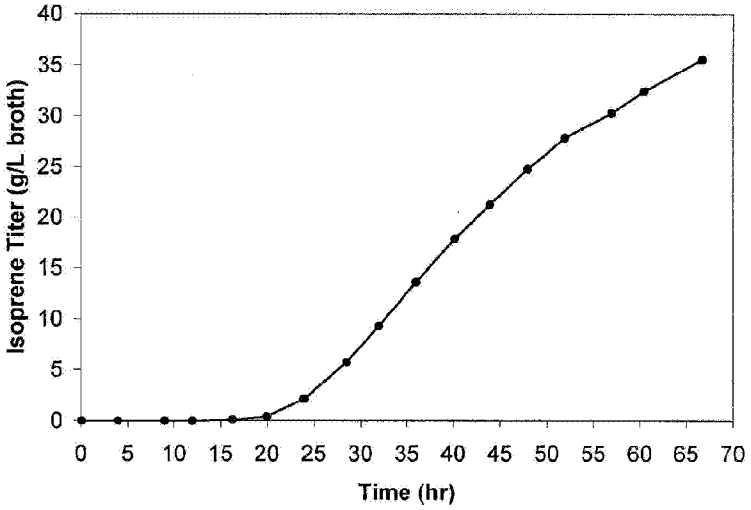


Figure 67C

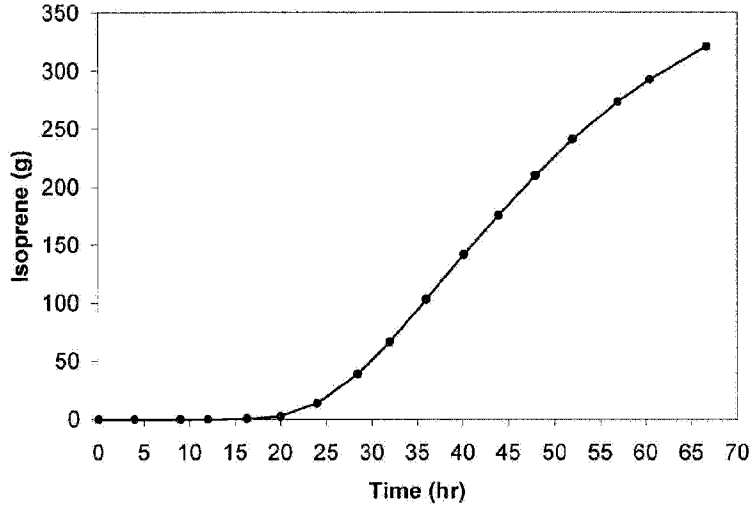


Figure 67D

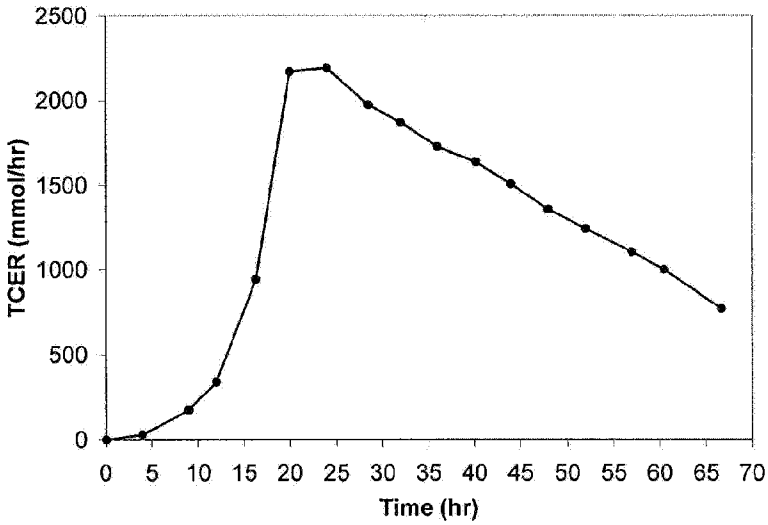


Figure 68A

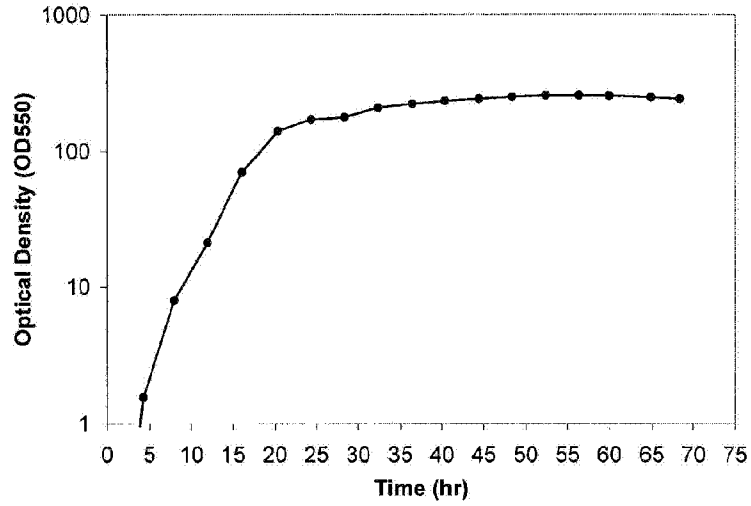


Figure 68B

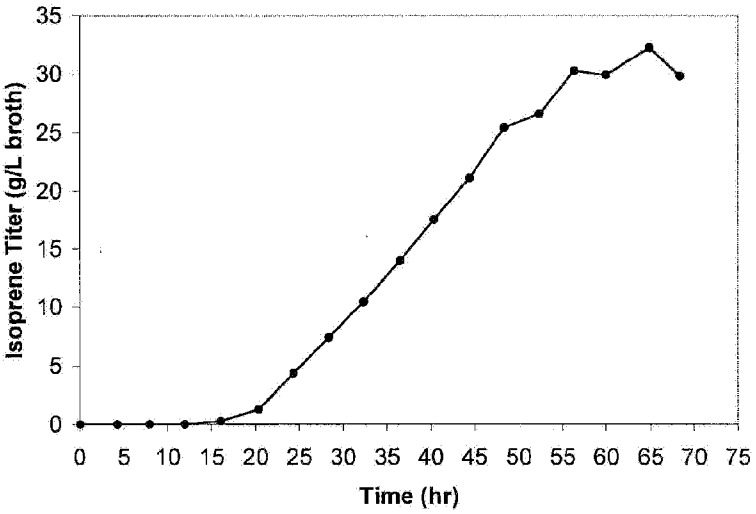


Figure 68C

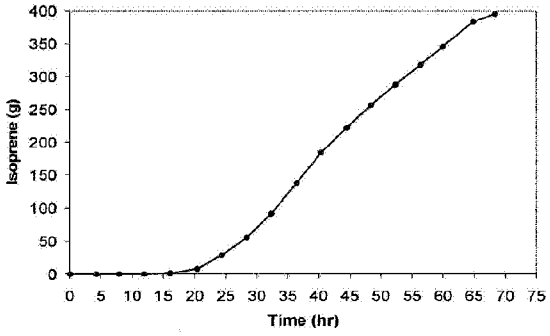
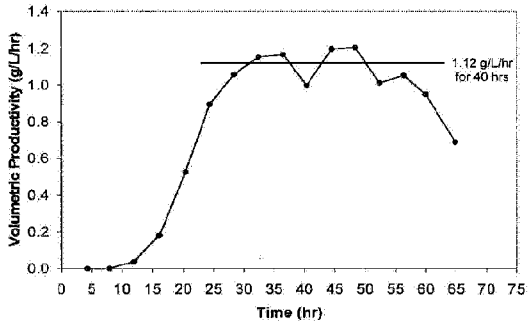


Figure 68D



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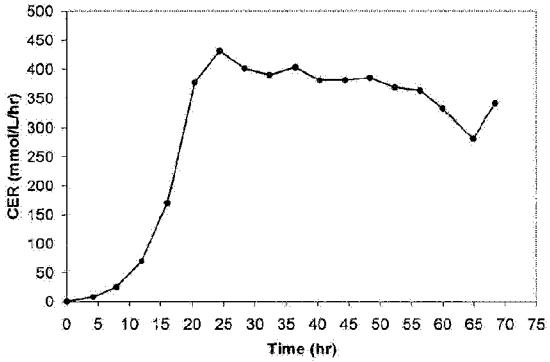

Figure 69A

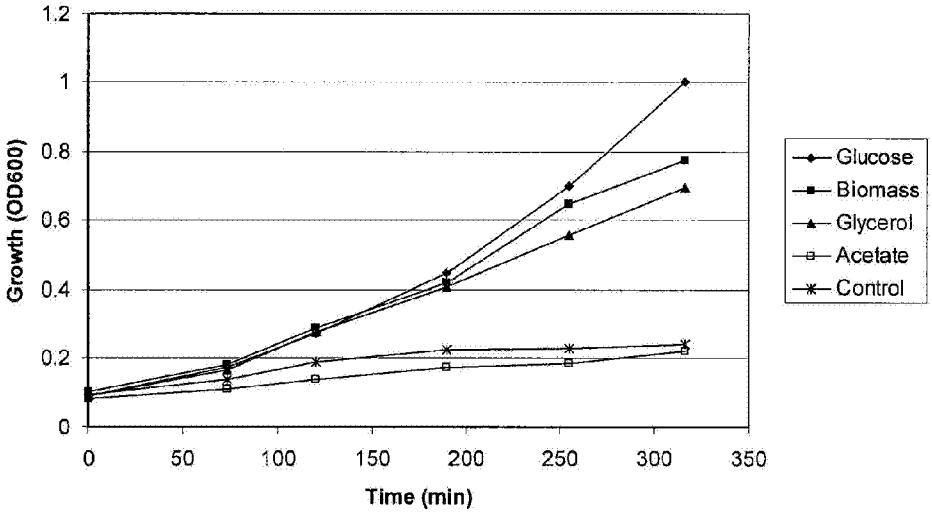

Figure 69B

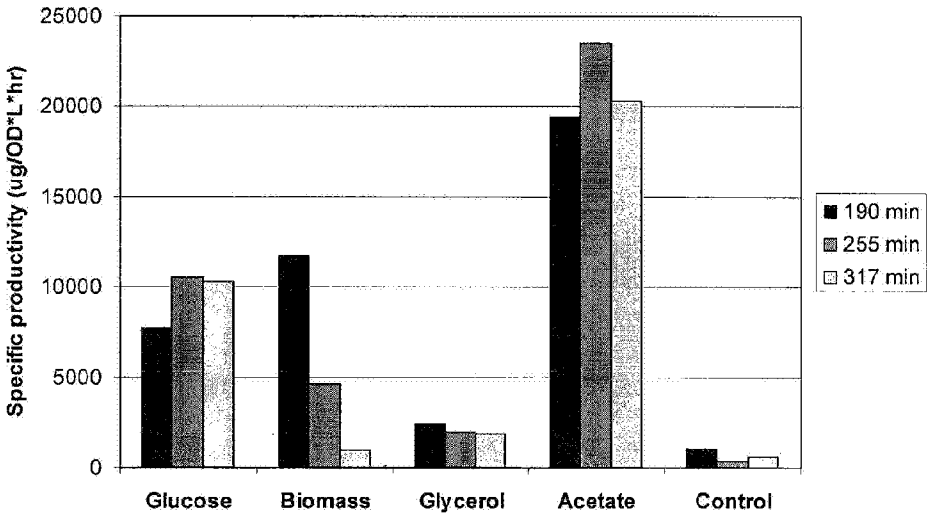

Figure 69C

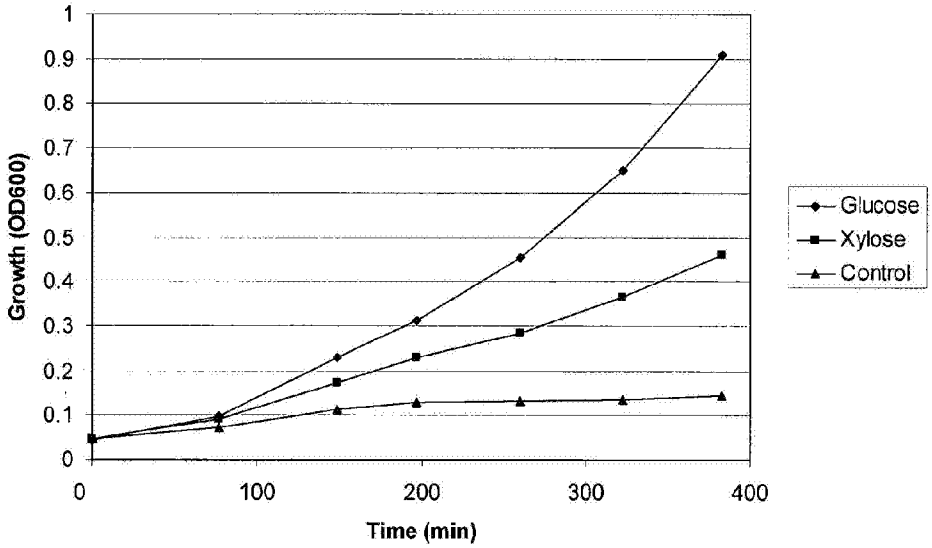

Figure 69D

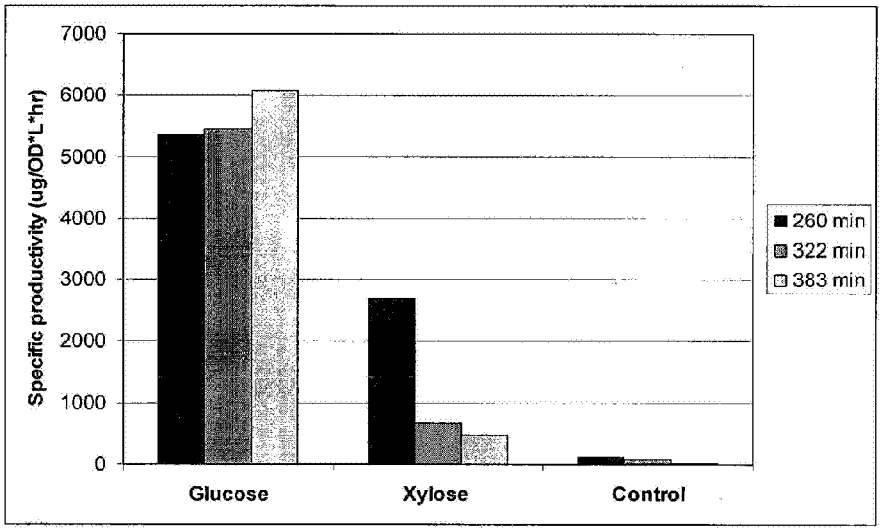

Figure 70A

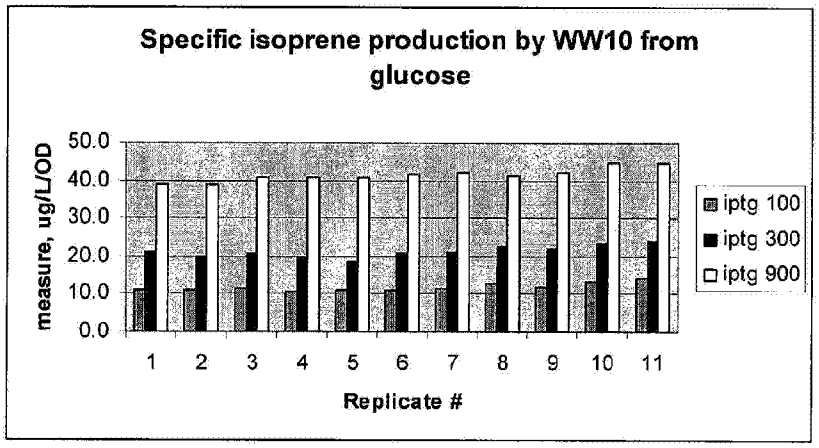

Figure 70B

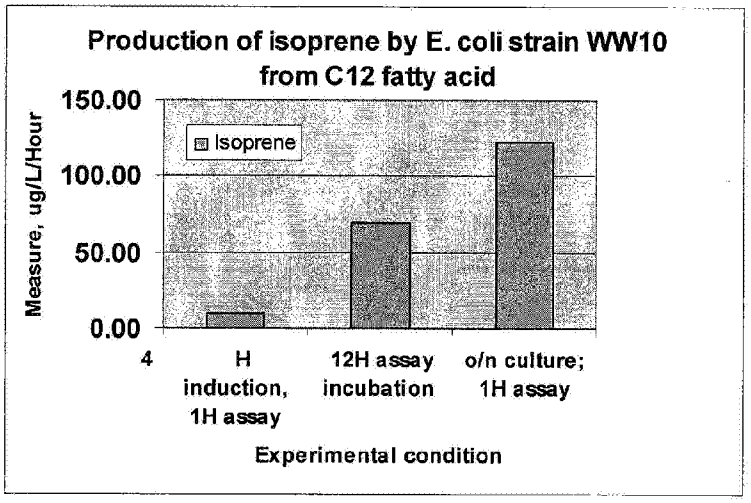

Figure 71

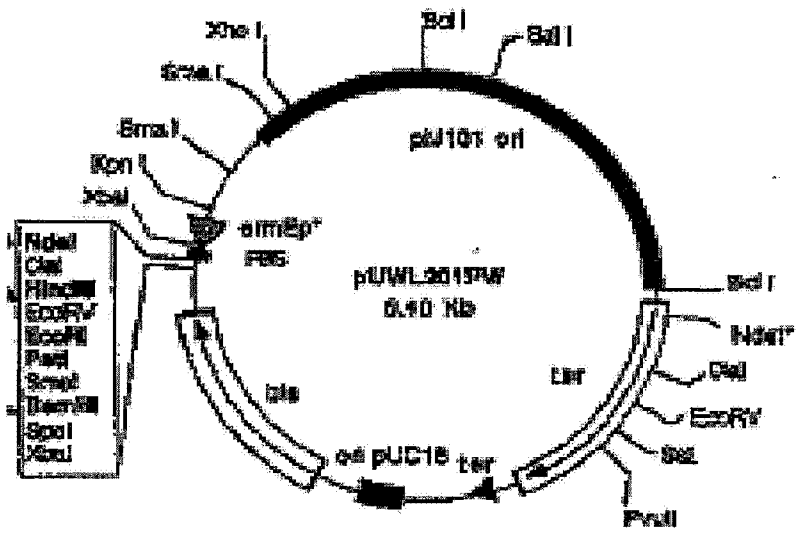
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Figure 72

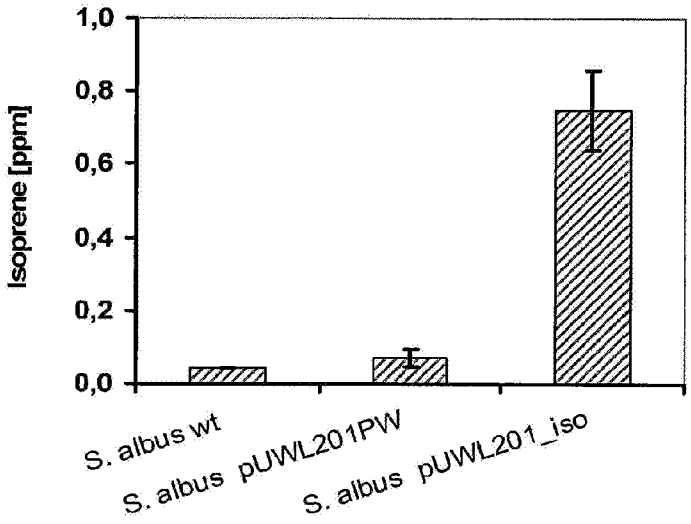
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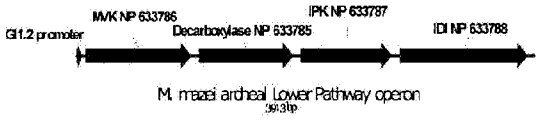
Figure 73A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IPK NP 633787
GI1.2 promoter
Decarboxylase NP 633785
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M. mazei archeal Lower Pathway operon

Figure 73B

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Figure 73C

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Figure 74A

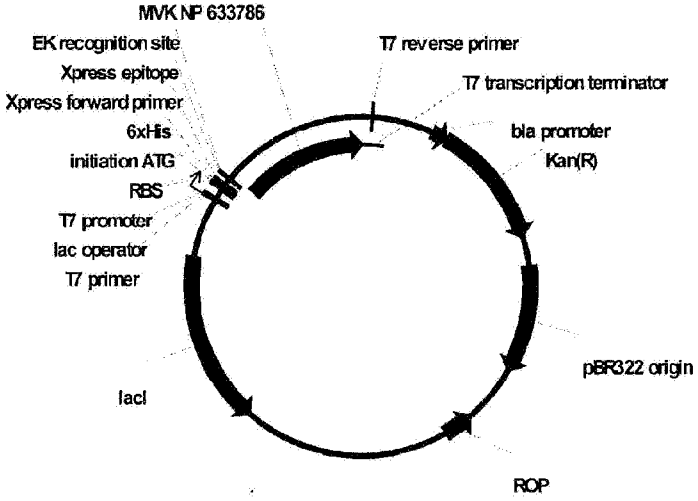
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Figure 74B

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Figure 74C

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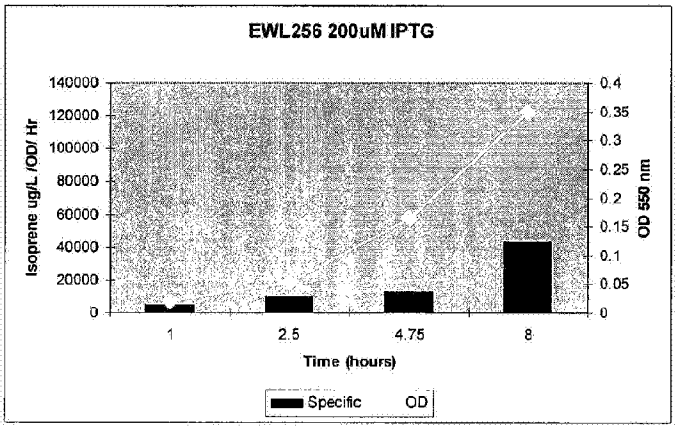

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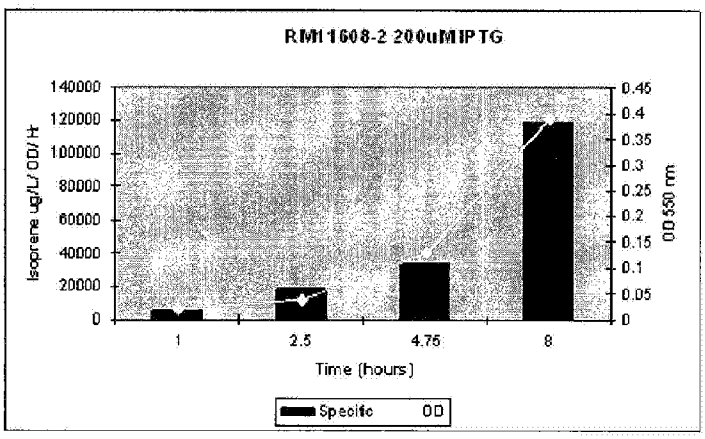

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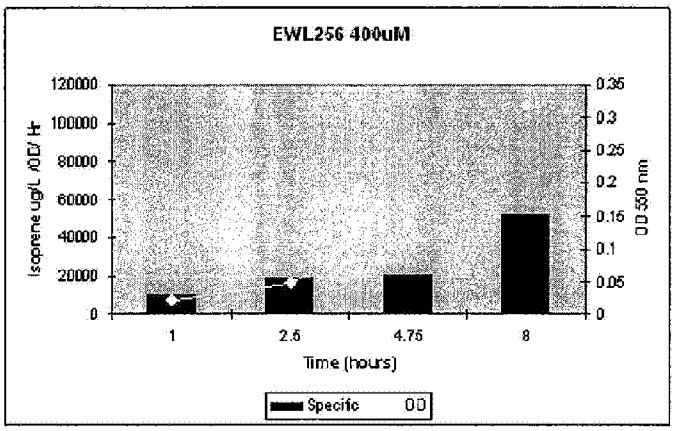

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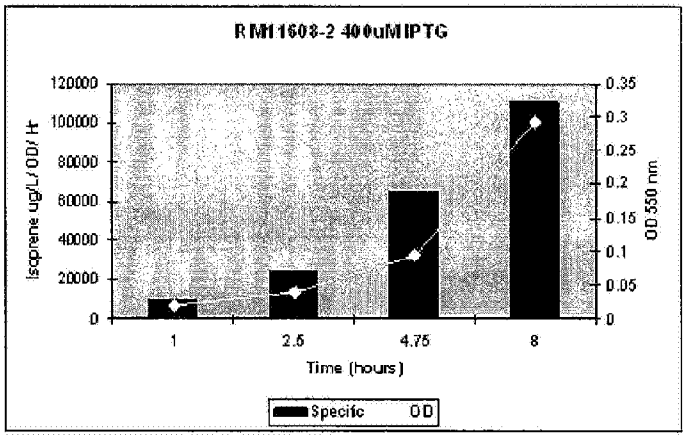
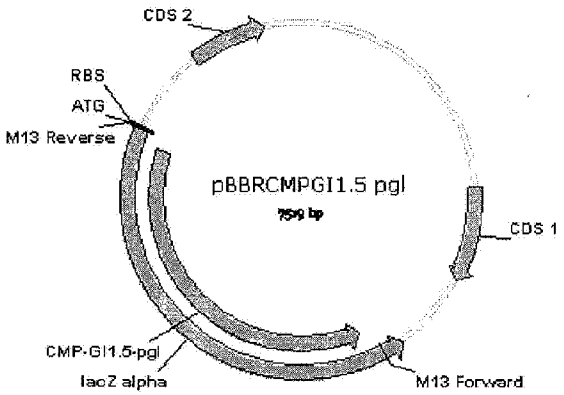


Figure 76



CDS 2: Gentamycin resistance gene; CDS1: E. coli replication protein.

Figure 77A

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Figure 77B

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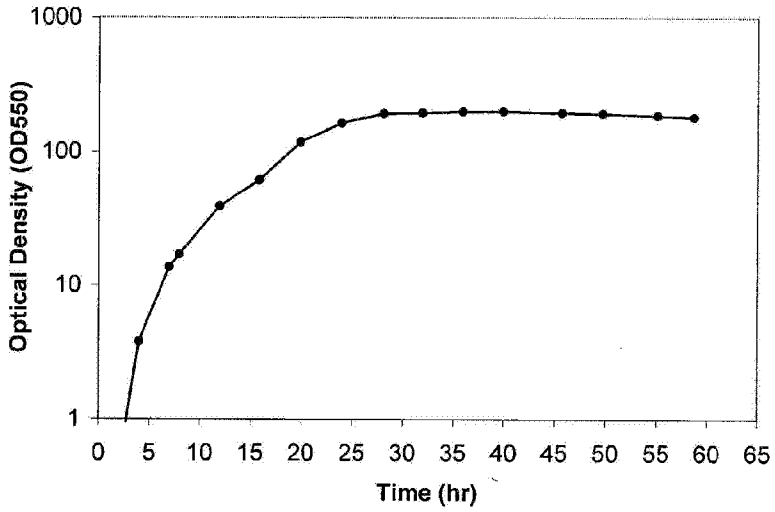
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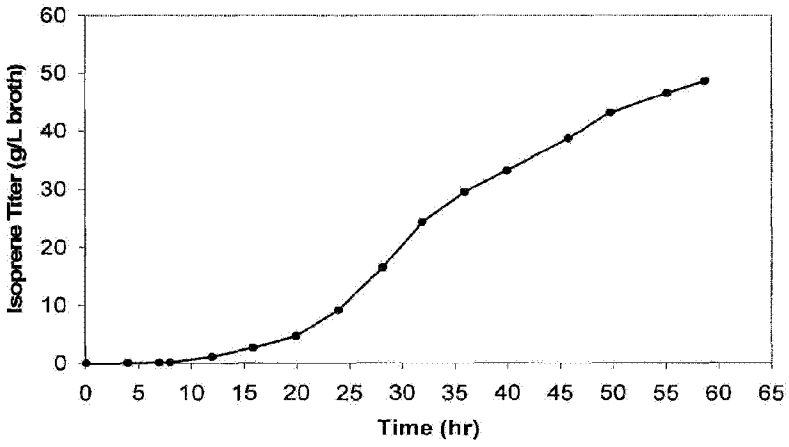
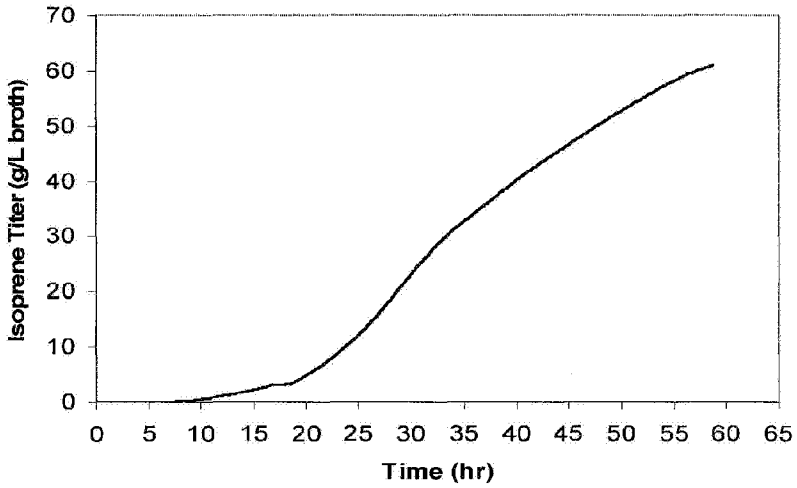
55
60
65
Time (hr)

Figure 78B



Titer 78C



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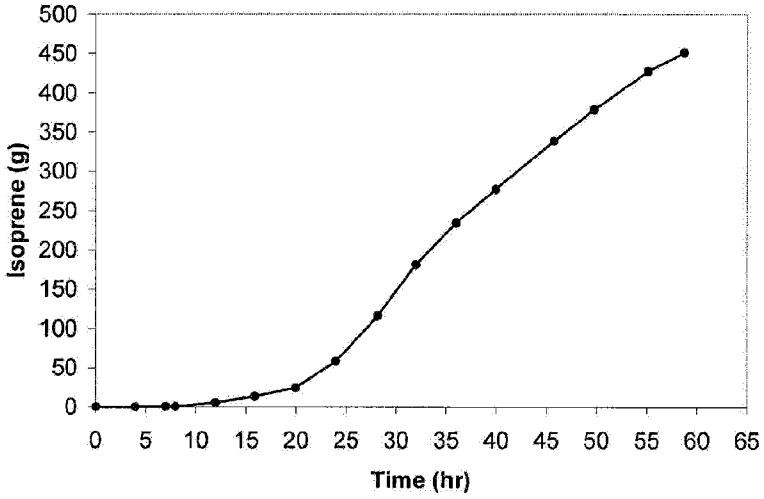

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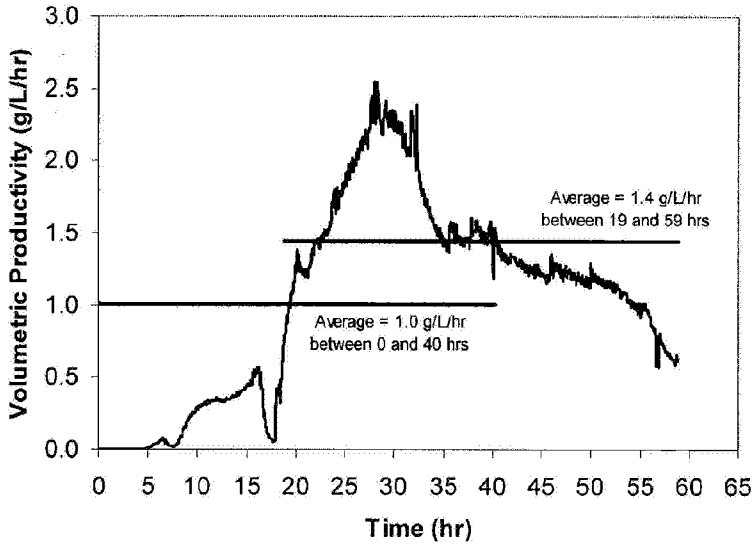

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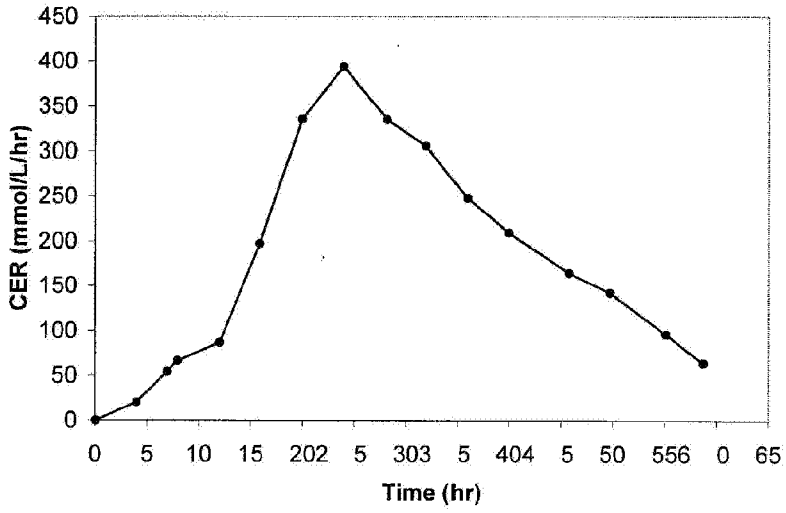
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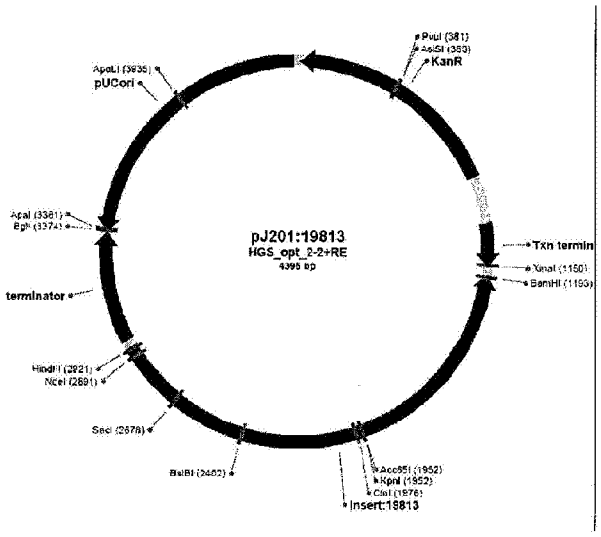
250
200
150
100
50
0
0 5 10 15 202 5 303 5 404 5 50 556 0 65
Time (hr)

Figure 79A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KanR
ApoLI (3935)
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pJ201:19813
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4395 bp
Txn termin
XmaI (1150)
BamHI (1159)
terminator
HindIII (2921)
NcoI (2891)
SacI (2878)
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Acc65I (1952)
KpnI (1952)
ClaI (1876)
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Figure 79B

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Figure 79C

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