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(54) **Title**: IMPROVED ISOPRENE PRODUCTION USING THE DXP AND MVA PATHWAY

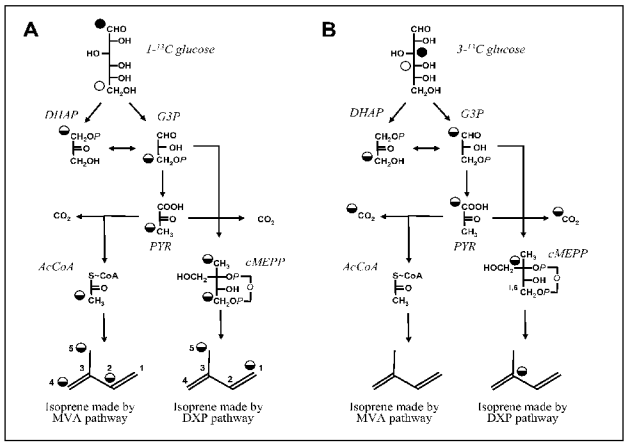

Figure 100

(57) **Abstract**: The invention provides for methods for producing isoprene from cultured cells using various components of the DXP pathway and MVA pathway, or components associated with the DXP pathway and MVA pathway, iron-sulfur cluster-interacting redox polypeptides, and isoprene synthase. The invention also provides compositions that include these cultured cells.

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IMPROVED ISOPRENE PRODUCTION USING THE DXP AND MVA PATHWAY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/187,941, filed June 17, 2009; U.S. Provisional Patent Application No. 61/187,930, filed June 17, 2009; U.S. Provisional Patent Application No. 61/314,985, filed March 17, 2010; U.S. Provisional Patent Application No. 61/314,979, filed March 17, 2010; the disclosure of all of these applications are hereby incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to compositions and methods for improving the production of isoprene from cultured cells using the DXP pathway and MVA pathway.

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 (Figure 19A). 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 invention provides, *inter alia*, compositions and methods for the production of isoprene in increased amounts using various DXP pathway genes and polypeptides and various MVA pathway genes and polypeptides, iron-sulfur cluster-interacting redox genes and polypeptides, isoprene synthase, and optionally, various genes and polypeptides associated with the DXP pathway, various genes and polypeptides associated with the MVA pathway, and IDI genes and polypeptides. In one aspect, the invention features cells or cells in culture which have been engineered for producing isoprene in increased amounts by using a combination of various DXP pathway genes and polypeptides, various MVA pathway genes and polypeptides, iron-sulfur cluster-interacting redox genes and polypeptides, isoprene synthase genes and polypeptides, and optionally, DXP pathway associated genes and polypeptides, MVA pathway associated genes and polypeptides, and IDI genes and polypeptides.

[0007] In some embodiments, the cells or cells in culture comprise (i) a heterologous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide and/or (ii) a duplicate copy of an endogenous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide. In some embodiments, the cells or cells in culture comprise (i) one or more copies of heterologous or endogenous nucleic

acid encoding an iron-sulfur cluster-interacting redox polypeptide, (ii) one or more copies of heterologous or endogenous nucleic acid encoding a DXP pathway polypeptide and/or a MVA pathway polypeptide, and (iii) one or more copies of heterologous or endogenous nucleic acid encoding an isoprene synthase polypeptide. In some embodiments, the iron-sulfur cluster-interacting redox polypeptide, the DXP pathway polypeptide, a MVA pathway polypeptide, and isoprene synthase polypeptide are operably linked to a promoter.

[0008] In some embodiments, the DXP pathway polypeptide is selected from the group consisting of DXS (1-deoxy-D-xylulose-5-phosphate synthase), DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase), MCT (4-diphosphocytidyl-2C-methyl-D-erythritol synthase), CMK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), MCS (2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), HDS (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase), and HDR (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase). In some embodiments, the DXP pathway polypeptide is DXS, HDS, or HDR. In some embodiments, the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide.

[0009] In some embodiments, the MVA pathway polypeptide is selected from the group consisting acetyl-CoA acetyltransferase (AA-CoA thiolase), 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), diphosphomevalonte decarboxylase (MVD), phosphomevalonate decarboxylase (PMDC) and isopentenyl phosphate kinase (IPK). In some embodiments, the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide.

[0010] In one embodiment, both the DXP and MVA pathways can be present in any ratio to produce isoprene from each pathway in any proportion in cells or cells in culture. In another embodiment, about 10% to 50% of the isoprene is produced utilizing the DXP pathway and the remainder is produced utilizing the MVA pathway. In another

embodiment, at least about 50% of the isoprene is produced utilizing the DXP pathway and the remainder is produced utilizing the MVA pathway.

[0011] In some embodiments, the invention provides cells or 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 or cells in culture convert more than about 0.002% of the carbon in a cell culture medium into isoprene.

[0012] In some embodiments, the invention provides cells or cells in culture where the level of HMBPP and DMAPP are maintained below 1 mM for the duration of the fermentation run. In other embodiments, the invention provides cells in culture where the level of HMBPP and DMAPP are maintained below 1 mM during the exponential phase of the fermentation. In other embodiments, the invention provides cells or cells in culture in which late DXP pathway enzymes, particularly IspG and IspH are maintained at levels consistent with minimizing phosphorylation level of Dxr.

[0013] In some embodiments of any of the aspects of the invention, the iron-sulfur cluster-interacting redox polypeptide comprises flavodoxin (*e.g.*, flavodoxin I), flavodoxin reductase, ferredoxin (*e.g.*, ferredoxin I), ferredoxin-NADP+ oxidoreductase, and genes or polypeptides encoding thereof *(e.g., fpr* and *fldA*).

[0014] In some embodiments, the cells or cells in culture comprise (i) a heterologous nucleic acid encoding a ferredoxin polypeptide, a ferredoxin-NADP+ oxidoreductase polypeptide, a DXP pathway polypeptide, and an isoprene synthase polypeptide and/or (ii) a duplicate copy of an endogenous nucleic acid encoding a ferredoxin polypeptide, a ferredoxin-NADP+ oxidoreductase polypeptide, a DXP pathway polypeptide, and an isoprene synthase polypeptide. In some embodiments, the cells or cells in culture comprise IspG and fldA. In another embodiment, the cells or cells in culture comprise IspG, fldA, and IspH. In some embodiments, the ferredoxin polypeptide, the ferredoxin-NADP+ oxidoreductase, the DXP pathway polypeptide, and isoprene synthase polypeptide are operably linked to a promoter. In some embodiments, the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding an IDI polypeptide.

[0015] In some embodiments, the cells in culture comprise (i) a heterologous nucleic acid encoding a flavodoxin polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide and/or (ii) a duplicate copy of an endogenous nucleic acid encoding a flavodoxin polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide. In some embodiments, the flavodoxin polypeptide, the DXP pathway polypeptide, MVA pathway polypeptide, and isoprene synthase polypeptide are operably linked to a promoter. In some embodiments, the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding an IDI polypeptide.

[0016] In some embodiments, the cells are cultured in a culture medium that includes a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing. In some embodiments, the cells are cultured under limited glucose conditions.

[0017] In other aspects, the invention provides for methods of producing isoprene, the method comprising (a) culturing cells comprising (i) a heterologous nucleic acid encoding a heterologous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide or (ii) a duplicate copy of an endogenous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide under suitable culture conditions for the production of isoprene, and (b) producing isoprene. In one embodiment, the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide. In other embodiments, the cells in culture produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In other embodiments, more than about 0.02 molar percent of the carbon that the cells consume from a cell culture medium is converted into isoprene.

[0018] In one aspect, the invention features methods of producing isoprene, such as methods of using any of the cells described herein to produce isoprene. In some embodiments, the method involves culturing cells comprising (i) a heterologous nucleic acid encoding a heterologous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, and an isoprene synthase polypeptide, and/or (ii) a duplicate copy of an endogenous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, and an isoprene synthase polypeptide. In some embodiments, the cells are cultured under suitable culture conditions for the production of isoprene, and isoprene is produced. In some embodiments, the iron-sulfur cluster-interacting redox polypeptide, isoprene synthase polypeptide, and DXP pathway polypeptide are operably linked to a promoter. In some embodiments, the DXP pathway polypeptide is selected from the group consisting of DXS (1-deoxy-D-xylulose-5-phosphate synthase), DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase), MCT (4-diphosphocytidyl-2C-methyl-D-erythritol synthase), CMK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), MCS (2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), HDS (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase), and HDR (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase). In some embodiments, the DXP pathway polypeptide is DXS, HDS, or HDR. In some embodiments, the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide. 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 involves culturing cells under conditions sufficient to convert more than about 0.002% (mol/mol) of the carbon in a cell culture medium into isoprene.

[0019] In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2 or more times the amount of isoprene produced during the growth phase for the same length of time. In some embodiments, the gas phase comprises greater than or about 9.5 % (volume) oxygen, and the concentration of isoprene in the gas phase is less than the lower

flammability limit or greater than the upper flammability limit. In particular embodiments, (i) the concentration of isoprene in the gas phase is less than the lower flammability limit or greater than the upper flammability limit, and (ii) the cells produce greater than about 400 nmole/g_{wcm}/hr of isoprene.

[0020] In some embodiments, isoprene is only produced in stationary phase. In some embodiments, isoprene is produced in both the growth phase and stationary phase. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of isoprene produced during the growth phase for the same length of time.

[0021] In one aspect, the invention features compositions and systems that comprise isoprene. In some embodiments, the composition comprises greater than or about 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 mg of isoprene. In some embodiments, the composition comprises greater than or about 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 g of isoprene(w/w) of the volatile organic fraction of the composition is isoprene.

[0022] In some embodiments, the composition comprises greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such as 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne by

weight compared to the total weight of all C5 hydrocarbons in the composition. In particular embodiments, the composition has greater than about 2 mg of isoprene and has greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition.

[0023] In some embodiments, the composition has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In particular embodiments, the composition also has greater than about 2 mg of isoprene.

[0024] In some embodiments, the composition comprises (i) a gas phase that comprises isoprene and (ii) cells in culture that produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the composition comprises a closed system, and the gas phase comprises greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ug/L of isoprene when normalized to 1 mL of 1 OD_{600} cultured for 1 hour. In some embodiments, the composition comprises an open system, and the gas phase comprises greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ug/L of isoprene when sparged at a rate of 1 vvm. In some embodiments, the volatile organic fraction of the gas phase comprises greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the volatile organic fraction. In some embodiments, the volatile organic fraction of the gas phase comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such as 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the volatile organic fraction. In some embodiments, the volatile organic fraction of the gas phase has less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne by weight compared to the total weight of all C5

hydrocarbons in the volatile organic fraction. In particular embodiments, the volatile organic fraction of the gas phase has greater than about 2 mg of isoprene and has greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the volatile organic fraction.

[0025] In some embodiments, the volatile organic fraction of the gas phase has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the volatile organic fraction of the gas phase that inhibits the polymerization of isoprene. In particular embodiments, the volatile organic fraction of the gas phase also has greater than about 2 mg of isoprene.

[0026] In some embodiments of any of the compositions of the invention, at least a portion of the isoprene is in a gas phase. In some embodiments, at least a portion of the isoprene is in a liquid phase (such as a condensate). In some embodiments, at least a portion of the isoprene is in a solid phase. In some embodiments, at least a portion of the isoprene is adsorbed to a solid support, such as a support that includes silica and/or activated carbon. In some embodiments, the composition includes ethanol. In some embodiments, the composition includes between about 75 to about 90% by weight of ethanol, such as between about 75 to about 80%, about 80 to about 85%, or about 85 to about 90% by weight of ethanol. In some embodiments, the composition includes between about 4 to about 15% by weight of isoprene, such as between about 4 to about 8%, about 8 to about 12%, or about 12 to about 15% by weight of isoprene.

[0027] In some embodiments, the invention also features systems that include any of the cells and/or compositions described herein. In some embodiments, the system includes a reactor that chamber comprises cells in culture that produce greater than about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments, the system is not a closed system. In some embodiments, at least a portion of the isoprene is removed from the system. In some embodiments, the system includes a gas phase comprising isoprene. In various embodiments, the gas phase comprises any of the compositions described herein.

[0028] In one aspect, the invention provides a tire comprising polyisoprene. In some embodiments, the polyisoprene is produced by (i) polymerizing isoprene in any of the compositions described herein or (ii) polymerizing isoprene recovered from any of the compositions described herein. In some embodiments, the polyisoprene comprises *cis*-1,4-polyisoprene.

[0029] In some embodiments of any of the compositions, systems, and methods of the invention, a nonflammable concentration of isoprene in the gas phase is produced. In some embodiments, the gas phase comprises less than about 9.5 % (volume) oxygen. In some embodiments, the gas phase comprises greater than or about 9.5 % (volume) oxygen, and the concentration of isoprene in the gas phase is less than the lower flammability limit or greater than the upper flammability limit. In some embodiments, the portion of the gas phase other than isoprene comprises between about 0% to about 100% (volume) oxygen, such as between about 10% to about 100% (volume) oxygen. In some embodiments, the portion of the gas phase other than isoprene comprises between about 0% to about 99% (volume) nitrogen. In some embodiments, the portion of the gas phase other than isoprene comprises between about 1% to about 50% (volume) CO_2.

[0030] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding a DXP pathway associated polypeptide.

[0031] In some embodiments of any of the aspects of the invention, the cells in culture produce isoprene at greater than or about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments of any of the aspects of the invention, the cells in culture convert greater than or about 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6%, or more of the carbon in the cell culture medium into isoprene. In some embodiments of any of the aspects of the invention, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 100,000, or more ng of isoprene/gram of cells for the wet weight of the cells /hr

($ng/g_{wcm}/h$). In some embodiments of any of the aspects of the invention, the cells in culture produce a cumulative titer (total amount) of isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 50,000, 100,000, or more mg of isoprene/L of broth (mg/L_{broth}, wherein the volume of broth includes the volume of the cells and the cell medium). Other exemplary rates of isoprene production and total amounts of isoprene production are disclosed herein.

[0032] In some embodiments of any of the aspects of the invention, isoprene production can be further increased by using a mutant DXP pathway polypeptide and nucleic acid derived from thereof. In some embodiments, the mutant DXP pathway polypeptide is a HDR polypeptide with the iron-sulfur cluster regulator (iscR) removed. In some embodiments, the mutant DXP pathway polypeptide is a mutant HDR polypeptide that produces solely DMAPP or a majority of DMAPP relative to IPP.

[0033] In some embodiments of any of the aspects of the invention, isoprene production can be further increased by increasing the carbon flux through the DXP pathway and/or MVA pathway. In some embodiments, the carbon flux can be increased by avoiding any feedback inhibition of DXS activity by metabolites downstream the DXP pathway or/and intermediates of other pathways that use a DXP pathway polypeptide as a substrate. In some embodiments, the other pathway that uses DXP pathway polypeptide as a substrate (*e.g.*, DXP) is the thiamine (Vitamin B1) or pyridoxal (Vitamin B6) pathway. In some embodiments, the carbon flux can be increased by expressing a DXP pathway polypeptide from a different organism that is not subject to inhibition by downstream products of the DXP pathway. In some embodiments, the carbon flux can be increased by deregulating glucose uptake. In other embodiments, the carbon flux can be increased by maximizing the balance between the precursors required for the DXP pathway and/or MVA pathway. In some embodiments, the balance of the DXP pathway precursors, pyruvate and glyceraldehydes-3-phosphate (G-3-P), can be achieved by redirecting the carbon flux with the effect of elevating or lowering pyruvate or G-3-P separately. In some embodiments, the carbon flux can be increased by using a CRP (cAMP Receptor Protein)-deleted mutant.

[0034] In some embodiments, the carbon flux can be increased by using a strain (containing one or more DXP pathway genes or one or more both DXP pathway and MVA pathway genes) containing a pyruvate dehydrogenase E1 subunit variant. In some embodiments, the pyruvate dehydrogenase (PDH) E1 subunit variant has an E636Q point mutation.

[0035] In some embodiments of any of the aspects of the invention, isoprene production can be further increased by utilizing the downstream genes or polypeptides of the DXP pathway by introducing a heterologous terpene synthase nucleic acid or a duplicate copy of an endogenous terpene synthase nucleic acid into the cells, which includes, but is not limited to ocimene synthase, farnesene synthase, and artemesinin synthase.

[0036] In some embodiments of any of the aspects of the invention, in some embodiments, the vector comprises a selective marker, such as an antibiotic resistance nucleic acid.

[0037] 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.

[0038] In some embodiments, iron-sulfur cluster-interacting redox nucleic acid, any one or more of the nucleic acids in the DXP pathway, MVA pathway, and isoprene synthase nucleic acid are placed under the control of a promoter or factor that is more

active in stationary phase than in the growth phase. In one embodiment, IDI nucleic acid is also included for IDI expression to produce a higher amount of isoprene than when IDI is not used. For example, one or more iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, IDI nucleic acid, or isoprene synthase nucleic acid may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, MVA pathway nucleic acid, IDI nucleic acid, or isoprene synthase nucleic acid are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

[0039] In some embodiments of any of the aspects of the invention, cells expressing iron-sulfur cluster-interacting redox polypeptide, isoprene synthase polypeptide, and DXP pathway polypeptide are grown under non-inducing conditions. In some embodiments of any of the aspects of the invention, cells expressing iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, IDI polypeptide, and isoprene synthase polypeptide are grown under non-inducing conditions. For example, the non-inducing condition is that IPTG-induced expression from the Trc promoter regulated gene constructs is not performed.

[0040] In some embodiments of any of the aspects of the invention, the cells express a second DXP pathway polypeptide, in addition to the first DXP pathway polypeptide, including DXS (1-deoxy-D-xylulose-5-phosphate synthase), DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase), MCT (4-diphosphocytidyl-2C-methyl-D-erythritol synthase), CMK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), MCS (2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), HDS (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase), and HDR (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase). In some embodiments of any of the aspects of the invention, the cells express two or more DXP pathway polypeptides, in addition to the first DXP pathway polypeptide as described above. In some embodiments of any of the aspects of the invention, the cells express 2, 3, 4, 5, 6, or 7 DXP pathway polypeptides, in addition to the first DXP pathway polypeptide as described above.

[0041] In some embodiments of any of the aspects of the invention, the cells express a second MVA pathway polypeptide, in addition to the first MVA pathway polypeptide, including acetyl-CoA acetyltransferase (AA-CoA thiolase), 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), diphosphomevalonte decarboxylase (MVD), phosphomevalonate decarboxylase (PMDC) and isopentenyl phosphate kinase (IPK). In some embodiments of any of the aspects of the invention, the cells express two or more MVA pathway polypeptides, in addition to the first MVA pathway polypeptide as described above. In some embodiments of any of the aspects of the invention, the cells express 2, 3, 4, 5, 6, or 7 MVA pathway polypeptides, in addition to the first MVA pathway polypeptide as described above.

[0042] In some embodiments of any of the aspects of the invention, at least a portion of the cells maintain the heterologous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, and 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, at least a portion of the cells maintain the heterologous iron-sulfur cluster-interacting redox nucleic acid, IDI nucleic acid, DXP pathway nucleic acid, and 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, at least a portion of the cells maintain the heterologous isoprene synthase nucleic acid, DXS nucleic acid, IDI nucleic acid, and iron-sulfur cluster-interacting redox 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 iron-sulfur cluster-interacting redox nucleic acid, isoprene synthase nucleic acid, DXP pathway nucleic acid, and/or IDI nucleic acid also comprises a selective marker, such as an antibiotic resistance nucleic acid.

[0043] In some embodiments of any of the aspects of the invention, the isoprene synthase polypeptide is a polypeptide from a plant such as *Pueraria* (*e.g., Pueraria*

montana or Pueraria lobata) or *Populus* (*e.g.*, *Populus tremuloides*, *Populus alba*, *Populus nigra*, *Populus trichocarpa*, or the hybrid, *Populus alba x Populus tremula*).

[0044] In some embodiments of any of the aspects of the invention, the cells are bacterial cells, such as gram-positive bacterial cells (*e.g.*, *Bacillus* cells such as *Bacillus subtilis* cells or *Streptomyces* cells such as *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus* cells) or cyanobacterial cells (*e.g.*, *Thermosynechococcus* cells such as *Thermosynechococcus elongates* 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) or cyanobacterial cells (*e.g.*, *Thermosynechococcus* cells such as *Thermosynechococcus elongates* cells). In some embodiments of any of the aspects of the invention, the cells are fungal, cells such as filamentous fungal cells (*e.g.*, *Trichoderma* cells such as *Trichoderma reesei* cells or *Aspergillus* cells such as *Aspergillus oryzae* and *Aspergillus niger*), or yeast cells (*e.g.*, *Yarrowia* cells such as *Yarrowia lipolytica* cells).

[0045] 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.

[0046] In one aspect, the invention features a product produced by any of the compositions or methods of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0047] 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.

[0048] Figure 2 is a map of pTrcKudzu.

[0049] Figure 3A, B, and C 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.

[0050] Figure 4 is a map of pETNHisKudzu.

[0051] Figure 5A, B, and C is the nucleotide sequence of pETNHisKudzu (SEQ ID NO:5).

[0052] Figure 6 is a map of pCL-lac-Kudzu.

[0053] Figure 7A, B, and C is the nucleotide sequence of pCL-lac-Kudzu (SEQ ID NO:7).

[0054] Figure 8A is a graph showing the production of isoprene in *E. coli* BL21 cells with no vector.

[0055] Figure 8B is a graph showing the production of isoprene in *E. coli* BL21 cells with pCL-lac-Kudzu

[0056] Figure 8C is a graph showing the production of isoprene in *E. coli* BL21 cells with pTrcKudzu.

[0057] Figure 8D is a graph showing the production of isoprene in *E. coli* BL21 cells with pETN-HisKudzu.

[0058] Figure 9A is a graph showing OD over time of fermentation of *E. coli* BL21/pTrcKudzu in a 14 liter fed batch fermentation.

[0059] Figure 9B is a graph showing isoprene production over time of fermentation of *E. coli* BL21/pTrcKudzu in a 14 liter fed batch fermentation.

[0060] 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}.

[0061] 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}.

[0062] 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}.

[0063] 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.

[0064] Figure 12 is the nucleotide sequence of pBS Kudzu #2 (SEQ ID NO:56).

[0065] Figure 13 is the nucleotide sequence of kudzu isoprene synthase codon-optimized for expression in *Yarrowia* (SEQ ID NO:8).

[0066] Figure 14 is a map of pTrex3g comprising a kudzu isoprene synthase gene codon-optimized for expression in *Yarrowia*.

[0067] Figure 15 is the nucleotide sequence of vector pSPZ1(MAP29Spb) (SEQ ID NO:11).

[0068] Figure 16 is the nucleotide sequence of the synthetic kudzu (*Pueraria montana*) isoprene gene codon-optimized for expression in *Yarrowia* (SEQ ID NO:12).

[0069] 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.

[0070] Figure 18A shows a schematic outlining construction of vectors pYLA 1, pYL1 and pYL2.

[0071] Figure 18B shows a schematic outlining construction of the vector pYLA(POP1).

[0072] Figure 18C shows a schematic outlining construction of the vector pYLA(KZ1)

[0073] Figure 18D shows a schematic outlining construction of the vector pYLI(KZ1)

[0074] Figure 18E shows a schematic outlining construction of the vector pYLI(MAP29)

[0075] Figure 18F shows a schematic outlining construction of the vector pYLA(MAP29)

[0076] Figure 19 shows the MVA and DXP metabolic pathways for isoprene (based on F. Bouvier *et al.*, Progress in Lipid Res. 44: 357-429, 2005). The following description includes alternative names for each polypeptide in the pathways and a reference that discloses an assay for measuring the activity of the indicated polypeptide (each of these references are each hereby incorporated by reference in their entireties, particularly with respect to assays for polypeptide activity for polypeptides in the MVA and DXP pathways). **Mevalonate Pathway: AACT**; Acetyl-CoA acetyltransferase, MvaE, EC 2.3.1.9. Assay: J. Bacteriol., 184: 2116–2122, 2002; **HMGS**; Hydroxymethylglutaryl-CoA synthase, MvaS, EC 2.3.3.10. Assay: J. Bacteriol., 184: 4065–4070, 2002; **HMGR**; 3-Hydroxy-3-methylglutaryl-CoA reductase, MvaE, EC 1.1.1.34. Assay: J. Bacteriol., 184: 2116–2122, 2002; **MVK**; Mevalonate kinase, ERG12, EC 2.7.1.36. Assay: Curr Genet 19:9-14, 1991. **PMK**; Phosphomevalonate kinase, ERG8, EC 2.7.4.2, Assay: Mol Cell Biol., 11:620–631, 1991; **DPMDC**; Diphosphomevalonate decarboxylase, MVD1, EC 4.1.1.33. Assay: Biochemistry, 33:13355-13362, 1994; **IDI**; Isopentenyl-diphosphate delta-isomerase, IDI1, EC 5.3.3.2. Assay: J. Biol. Chem. 264:19169-19175, 1989. **DXP Pathway: DXS**; 1-deoxy-D-xylulose-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, GcpE, 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, LytB, EC 1.17.1.2. Assay: JACS, 126:12847-12855, 2004.

[0077] 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.

[0078] Figure 21 is a map of pTrcKudzu yIDI DXS Kan.

[0079] Figure 22A-D is the nucleotide sequence of pTrcKudzu yIDI DXS Kan (SEQ ID NO:20).

[0080] 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).

[0081] 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).

[0082] 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).

[0083] 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).

[0084] 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).

[0085] 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).

[0086] 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).

[0087] 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).

[0088] Figure 24 is a map of p9796-poplar.

[0089] Figures 25A-25B are a nucleotide sequence of p9796-poplar (SEQ ID NO:21).

[0090] Figure 26 is a map of pTrcPoplar.

[0091] Figures 27A-27C are a nucleotide sequence of pTrcPoplar (SEQ ID NO:22).

[0092] Figure 28 is a map of pTrcKudzu yIDI Kan.

[0093] Figure 29 is a nucleotide sequence of pTrcKudzu yIDI Kan (SEQ ID NO:23).

[0094] Figure 30 is a map of pTrcKudzuDXS Kan.

[0095] Figures 31A-33C are a nucleotide sequence of pTrcKudzuDXS Kan (SEQ ID NO:24).

[0096] Figure 32 is a map of pCL PtrcKudzu.

[0097] Figures 33A-33C are a nucleotide sequence of pCL PtrcKudzu (SEQ ID NO:25).

[0098] Figure 34 is a map of pCL PtrcKudzu A3.

[0099] Figures 35A-35C are a nucleotide sequence of pCL PtrcKudzu A3 (SEQ ID NO:26).

[0100] Figure 36 is a map of pCL PtrcKudzu yIDI.

[0101] Figures 37A-37C are a nucleotide sequence of pCL PtrcKudzu yIDI (SEQ ID NO:27).

[0102] Figure 38 is a map of pCL PtrcKudzu DXS.

[0103] Figures 39A-39D are a nucleotide sequence of pCL PtrcKudzu DXS (SEQ ID NO:28).

[0104] Figure 40 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.

[0105] Figure 41A 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).

[0106] Figure 41B 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).

[0107] Figure 41C 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).

[0108] Figure 41D 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).

[0109] Figure 42 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.

[0110] Figure 43 shows graphs demonstrating isoprene production from a 500 L bioreactor with *E. coli* cells containing the pTrcKudzu + yIDI + DXS plasmid. Panel A shows the time course of optical density within the 500-L bioreactor fed with glucose and yeast extract. Panel B shows the time course of isoprene titer within the 500-L bioreactor fed with glucose and yeast extract. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Panel C shows the time course of total isoprene produced from the 500-L bioreactor fed with glucose and yeast extract.

[0111] Figure 44 is a map of pBS Kudzu #2.

[0112] Figure 45A 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).

[0113] Figure 45B 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).

[0114] Figures 46A-46D depict the growth rate and specific productivity of isoprene generation for the empty vector (control), HgS, and HgS-FldA strains.

[0115] Figure 46E is a map of pBAD33.

[0116] Figures 46F and 46G are the nucleotide sequence of pBAD33 (SEQ ID NO:51).

[0117] Figure 46H is a map of pTrcHgS-pBAD33.

[0118] Figures 46I and 46J are the nucleotide sequence of pTrcHgS-pBAD33 (SEQ ID NO:52).

[0119] Figure 46K is a map of pTrcHgSfldA-pBAD33.

[0120] Figures 46L and 46M are the nucleotide sequence of pTrcHgSfldA-pBAD33 (SEQ ID NO:53).

[0121] Figure 47 shows the growth and isoprene production of strains REM19-22 compared to REM23-26. The expression of isoprene synthase in both sets of strains and the expression of the *T. elongatus* genes in the test set of strains was induced with 200uM IPTG at time 0 when the cultures were at an $OD_{\lambda 600nm}$ of approximately 0.2-0.25. The data shown in the figure is that obtained 4 hours after the addition of IPTG to the cultures. Cells were grown shaking in the TM3 at 30ºC. Comparison of the parental to test set strains indicates that isoprene production increases 10%, 20%, 30%, and 80% over the parental strains for the GI1.0-*dxs*, GI1.2-*dxs*, GI1.5-*dxs*, and GI-1.6-*dxs* test strains, respectively.

[0122] Figure 48 shows the increased levels of the GcpE product, HDMAPP, accumulate in strains REM23-2. The concentrations of DXP metabolites and larger isoprenoid molecules were determined for REM19-26 (strain indicated on the x-axis) at a 5 hour IPTG-induction period. The DXP metabolites and isoprenoids measured are indicated in the figure legend; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; cMEPP, 2-*C*-methyl-D-erythritol-2, 4-cyclodiphosphate; HDMAPP, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; FPP, farnesyl pyrophosphate.

[0123] Figure 49 shows specific productivity of isoprene production in strain REM29 compared to REMH86.

[0124] Figure 50 depicts a cartoon representation of the strategy used to insert the GI 1.X-promoter series in front of *dxs* using the RED/ET system. REM29 (blue) and REMH86 (yellow) were assayed for growth rate (strains grew comparably) and isoprene production every 30 minutes across a 3 hour shake flask fermentation. At time 0 both

cultures were induced with 400uM IPTG. Over the course of the fermentation beginning at the first time point after induction, the test strain produced approximately 16% higher isoprene levels than the parental strain.

[0125] Figure 51 is a map of T7-MEARR alba/pBBR1MCS-5.

[0126] Figure 52 is a map of the Ptac-gcpE-petF-petH/pK184 construct that was used to generate strains REM23-26.

[0127] Figures 53A-53B show a cartoon representation of the T7-(-3) alba/pBBR1MCS-5 (top) and T7-MTE alba/pBBR1MCS-5 (bottom) constructs that were used to generate strains REMH76 and REMH86.

[0128] Figure 54 is a map of the Ptac-gcpE-lytB-petF-petH/pK184 construct that was used to generate strains REM31 and REM29.

[0129] Figures 55A-55C are the nucleotide sequence of T7-MEARR alba/pBBR1MCS-5 (SEQ ID NO:73).

[0130] Figures 56A-56B are the nucleotide sequence of Ptac-gcpE-petF-petH/pK184 (SEQ ID NO:74).

[0131] Figures 57A-57C are the nucleotide sequence of T7-(-3_alba/pBBR1MCS-5 (SEQ ID NO:75).

[0132] Figures 58A-58C are the nucleotide sequence of T7-MTE alba/pBBR1MCS-5 (SEQ ID NO:76).

[0133] Figures 59A-59B are the nucleotide sequence of Ptab-gcpE-LytB-petF-petH/pK184 (SEQ ID NO:77).

[0134] Figures 60A-60B show that *ΔiscR* BL21(DE3) supports increased isoprene production. Panel 60A shows the specific productivity of REM12 compared to the otherwise isogenic *ΔiscR* strain REM13. Isoprene levels were determined 4.5 hours and 8 hours after induction of the IPTG-inducible isoprene synthase and DXP enzymes

harbored by the strains. Data from three groups (A-C) of three biological replicates for each strain are shown. Error bars depict the standard deviation occurring between the biological replicates of each group. From this data it was determined that isoprene levels generated from the *ΔiscR* strain were an average of 40% and 73% higher than that produced by the wild-type strain at the 4.5 hour and 8 hour time point, respectively. Panel 60B shows the growth rate of REM12 and REM13 isoprene-producing strains. The growth rate of the same strains depicted in panel A was monitored over the course of the eight hour experiment by periodically measuring the optical density of the cultures at 600nm. Time 0 corresponds to the time that 50uM IPTG was added to the cultures. Cells were grown shaking in TM3 at 30ºC. The higher isoprene-producing strain *ΔiscR* (REM13) grows at a reduced rate relative to the lower isoprene-producing wild-type (REM12) strain.

[0135] Figure 61 is a cartoon representation of the strategy used to delete the *iscR* locus using the RED/ET system.

[0136] Figure 62 is a cartoon representation of the T7-MEARR alba/pBBR1MCS-5.

[0137] Figure 63 is a cartoon representation of the DXP operon pET24a.

[0138] Figures 64A-64C are the nucleotide sequence of T7-MEARR alba/pBBR1MCS-5 (SEQ ID NO:78).

[0139] Figures 65A-65D are the nucleotide sequence of DXP operon pETt24a (SEQ ID NO:79).

[0140] Figure 66 is a cartoon representation of the strategy used to delete *ispG* and *ispH* using the RED/ET system.

[0141] Figure 67 is a cartoon representation of the GI 1.6-gcpE-lytB-yidi/pCR-Blunt II-TOPO construct that was used to generate strain MD09-219/ GI1.6-gcpE-lytB-yidi/pCRII-TOPO (Kan).

[0142] Figure 68 depicts the Pentose Phosphate (PPP) and Entner-Doudoroff (ED) pathways (Fraenkel, *J.* Bact. 95:1267-1271 (1965), which is hereby incorporated by reference in its entirety).

[0143] Figure 69 is a map of pDu-39.

[0144] Figure 70 is a map of pMCM596 pET24(MEA)alba-dxs-yIDI.

[0145] Figures 71A-71B are the nucleotide sequence of pDu-39(SEQ ID NO:108).

[0146] Figures 72A-72C are the nucleotide sequence of MCM596 (SEQ ID NO:109).

[0147] Figures 73A-C are the nucleotide sequence of pMCM596 (SEQ ID NO:110).

[0148] Figure 74 shows comparison of DXS sequences in microorganisms synthesizing isoprenoids via the DXP pathway (*E.coli*, *Chlorobium tepidum* TLS, *Synechocystis* sp. PCC6803, *Gloeobacter violaceus* PCC 7421, *Clostridium botulinum* B1 str. Okra, *Mycobacterium tuberculosis* CDC1551) and via the MVA pathway (*Myxococcus xanthus* DK 1622, *Gramella forsetii* KT0803, *Flavobacterium johnsoniae* UW101, *Lactobacillus johnsonii* NCC 533, *Lactobacillus gasseri* ATCC 33323, and *Lactococcus lactis* subsp. *lactis* Il1403). Note the difference in amino acid sequence at positions 200-260 in the two groups of microorganisms.

[0149] Figures 75A and 75B are the nucleotide sequence of pDU-9.

[0150] Figure 76 is a map of pDu9-pET-16b rev-yIDI.

[0151] Figure 77 depicts GB-CMP-GI1.X-yidi construct design. The final construct consists of Fragment A (Frag A) fused to Fragment B (Frag B) to create a GI1.X promoter library transcribing yIDI with the chloramphenicol antibiotic resistance marker upstream, and flanking 50bp regions of homology to the desired integration site on the chromosome.

[0152] Figure 78 depicts a plasmid map of pDW33. pBR322- plasmid origin of replication; lacIq- lac repressor; Ptrc – the trc promoter; lac operator – lac repressor

binding site; P. alba IspS (MEA)- gene encoding the isoprene synthase; rrn terminator-transcription terminator; bla- beta lactamase gene.

[0153] Figure 79 (includes five panels: Figures 79A, 79B, 79C, 79D, and 79E) shows the results of 15-L scale fermentation comparison of strains CMP272, REMG39, and REM H8_12 for growth, isoprene production, and product yield on carbon. Panel (A) isoprene titer (g/L broth); Panel (B) specific productivity of isoprene generating cultures; Panel (C) cell growth depicted by optical density (550nm); Panel (D) cell growth shown by respiration (carbon evolution rate, CER); Panel (E) overall percent yield of product from carbon (weight in grams of isoprene/weight in grams of carbon fed*100). The fermentation conditions are described in Example 24 Section F (CMP272), G (REMG39), and Example 29 Section E (REM H8_12).

[0154] Figure 80 (includes three panels, 80A, 80B, and 80C) shows the results of large scale fermentation comparison of strains CMP272, REMG39, and REM H8_12 for DXP metabolites. Panels (A-C) The same cells described in Figure 79 are presented here. A legend describing the metabolite profiles is shown at the bottom of each panel. DXP, 1-Deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-Methyl-D-erythritol 4-phosphate; CDP-ME, 4-(Cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol; CDP-MEP, 2-Phospho-4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol; cMEPP, 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate; HDMAPP, 1-Hydroxy-2methyl-2-buten-4-yl 4-diphosphate; DMAPP, Dimethylallyl diphosphate; IPP, Isopentenyl diphosphate; FPP, faresyl pyrophosphate.

[0155] Figure 81 (includes two panels: Figure 81A and 81B) depicts one strategy for inserting GI1.X fldA into the BL21(DE3) chromosome. Panel (A) The endogenous 150 bp BL21(DE3) *fldA* locus is shown. The regions of homology within the GI1.X fldA PCR fragment to the desired 5' and 3' integration sites on the chromosome are depicted as gray block arrows. The half-arrowhead lines show where the PCR primers used to verify the construct anneal to the chromosome. The ribosome binding site (RBS), start codon of the encoded *fldA* mRNA, and the endogenous DNA upstream of the *fldA* to be replaced by the GI1.X proter series is shown. Panel (B) The 313 bp BL21(DE3) GI1.X *fldA* region generated via Gene Bridges methods (GI1.6 *fldA* of strain REM I6_4) is shown. The

inserted GI1.X promoter sequence(s) is illustrated as a black block arrow; the placement of the FTR scar sequences generated from use of the Gene Bridges insertion method is indicated.

[0156] Figure 82 depicts a plasmid map of GI1.6fldA/pCL. repA – plasmid replication protein; aad – aminoglycoside adenyltransferase; M13 for and M13 rev – binding sites for the respective primers; RBS – ribosome binding site; fldA – *E. coli fldA* gene.

[0157] Figure 83 depicts a plasmid map of GI1.6fldA-IspG/pCL. Same plasmid base as in Figure 82: FldA- *E. coli fldA* gene; IspG – E. coli *ispG* gene.

[0158] Figure 84 depicts a plasmid map of GI1.6IspG/pCL. Same plasmid base as Figures 82 and 83: IspG – *E. coli ispG* gene.

[0159] Figure 85 (includes two panels, Figure 85A and 85B) depicts small scale comparison of strains, REMC9_12, REME7_12, and REMD6_12. Panel (A) Specific productivity (SP) of isoprene production relative to growth. The y1 axis, specific productivity of isoprene production (ug/L/OD/hr); y2 axis, cell density (OD_{600}). Specific productivity (solid bars) and OD_{600} (diamonds). Measurements were taken at 3 and 4.5 h post-induction (600 uM IPTG) from at least 2 biological replicates. Panel (B) Intracellular metabolite concentrations. cMEPP: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HDMAPP- hydroxydimethylallyl diphosphate; DMAPP – dimethylallyl diphosphate; IPP- isopentenyl diphosphate. Y-axis: metabolite concentration in mM. Measurments shown were taken at 3.75 h post-induction (600 uM IPTG); separate experimental samples from (A); replicates produced similar results.

[0160] Figure 86 (includes two panels: Figure 86A and 86B) shows the results of small scale comparisons of strains REMG2_11, REMG4_11 and REMG39. Panel (A) Specific productivity of isoprene production relative to growth of. The y1 axis, specific productivity of isoprene production (ug/L/OD/hr); y2 axis, cell density (OD_{600}). Specific productivity (solid bars) and OD600 (diamonds). Measurements are shown at 1 and 3.5 h post-induction (400 uM IPTG) from at least 2 biological replicates. Panel (B) Intracellular metabolite concentrations of strains. The y-axis is metabolite concentration

in mM. cMEPP: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HDMAPP-hydroxydimethylallyl diphosphate. Measurements are shown for the 3.5 h post-induction (400 uM IPTG) samples from (A); replicates produced similar results (rows 1-3: REM G2_11; rows 4-6: REM G4_11; rows 7-9: REMG39).

[0161] Figure 87 depicts a plasmid map of pEWL454. The plasmid base is pK184. p15A ori – plasmid origin of replication; RBS – ribosome binding site; kan – kanamycin antibiotic resistance marker.

[0162] Figure 88 depicts a plasmid map of PtacAnabaenaAspA terminator/pEWL454. This is the same plasmid base as in Figure 87. Anabaena IspH – gene encoding the IspH enzyme from Anabaena.

[0163] Figure 89 depicts the specific productivity of isoprene production and intracellular metabolites of strains REMI7_11, and REMH8_12. The two strains were compared at 3 and 3.75 h following induction (500 uM IPTG). Isoprene measurements are shown from at least 2 biological replicates; replicates are not shown for the metabolite data, but produced similar results.

[0164] Figure 90 (includes three panels: Figure 90A, 90B and 90C) depicts the results from a 15-L scale fermentation of strain REM H8_12 and REM G4_11 (A). Panel (A) isoprene titer (g/L broth) for REMH 8_12 (open squares) and REM G4_11 (open circles); Panel (B) cell growth depicted by optical density (550nm); Panel (C) DXP metabolites. A legend describing the metabolite profiles is shown at the bottom of (C); see Figure 80 for metabolite descriptions.

[0165] Figure 91 depicts results from a preparative scale inactivation of Dxr by DMAPP.

[0166] Figure 92 (includes two panels: 92A and 92B) depicts isoprene production by strains REM H8_12 and REM I7_11 harboring an engineered DXP pathway and a lower MVA pathways. The top panel shows isoprene production specifically due to MVA fed at indicated concentrations to cultures grown on [U-^{13}C]-glucose. The lower panel shows isoprene production specifically arising from [U-^{13}C]-glucose]. Isoprene measurements

were taken at indicated times after induction of the cultures with IPTG. Isoprene evolved was monitored by GC-MS with detection at m/z = 67 as well as m/z = 73. While m/z = 67 reports on isoprene from MVA (all ^{12}C), m/z = 73 reports on isoprene derived from [U-^{13}C]-glucose.

[0167] Figure 93 depicts a plasmid map of pDW15. mob – plasmid mobilization region; AacC1 (Gent Resistance) – aminoglycoside acetyltransferase, gentamicin resistance gene; M13 Reverse and M13 Forward – binding sites for the respective primers; Ptrc, Trc promoter; mvaE and mvaS –*E. faecalis* genes encoding the Acetoacetyl-Coenzyme A Thiolase/3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase and 3-Hydroxy-3-Methylglutaryl-Coenzyme A Synthase, respectively; RepA – plasmid replication protein.

[0168] Figure 94 depicts a plasmid map of PTrp mMVK/pDW15. Same plasmid base as in 1). Trp promoter; encoded M. mazei MVK - *M. mazei* gene encoding Mevalonate Kinase; aspA terminator.

[0169] Figure 95 depicts a plasmid map of pMCM900. FRT - Flip recombinase target site; core Trc promoter - RNA polymerase binding site; lac operator - LacI binding site; PMK orf - yeast phosphomevalonate kinase coding sequence; MVD orf - yeast diphosphomevalonate decarboxylase coding sequence; yIDI - yeast isopentenyl diphosphate isomerase coding sequence; aspA terminator - aspA transcriptional terminator; attTn7 downstream – glmS - downstream recombination targetting sequence; KanR - kanamycin resistance gene; R6K ori - plasmid origin of replication; attTn7 upstream (pstS) - upstream recombination targetting sequence.

[0170] Figure 96 depicts the results for experiments for determining the specific productivity relative to culture density in the presence and absence of fosmidomycin for strain REM A2_17 grown on unlabeled glucose. The y1 axis, specific productivity of isoprene production (ug/L OD hr); y2 axis, cell density (OD_{600nm}). Specific productivity (solid bars) and Cell density (diamonds). Measurements were taken approx. 45 minutes post-introduction of either 0mM or 2mM fosmidomycin; both occurring approx. 3 hours after induction with 400uM IPTG. The data presented is the average of 3 biological; error

bars are shown for specific productivity and cell density values. The data suggests a contribution of roughly 59% and 41% for isoprene generated via the MVA pathway and DXP pathway, respectively; MVA flux was determined by the fraction of isoprene produced during exposure to fosmidomycin relative to the amount of isoprene produced in the absence of the inhibitor.

[0171] Figure 97 depicts the results for experiments for determining the effect of fosmidomycin on accumulation of the DXP and MVA pathway metabolites and isoprene emission rate in REM A2_17 strain. The metabolite concentrations in pelleted cells (same cells depicted in Figure 96) and isoprene emission rates were measured in the cultures at the end of a 45 min. incubation in the presence and in the absence of 2 mM fosmidomycin ("+FM" and "-FM", respectively). The results are expressed as an average ratio of the obtained concentrations and rates measured in three different cultures.

[0172] Figure 98 depicts the results for experiments for determining the specific productivity relative to culture density in the presence and absence of fosmidomycin for strain REM A2_17 grown on unlabeled and 1-^{13}C labeled glucose. The y1 axis, specific productivity of isoprene production (ug/L OD hr); y2 axis, cell density (OD_{600nm}). Specific productivity (solid bars) and Cell density (diamonds). In lane 1: unlabeled culture without tryptophan and without fosmidomycin; lane 2: 1-^{13}C glucose culture without tryptophan and without fosmidomycin; lane 3: 1-^{13}C glucose culture with 50uM tryptophan and without fosmidomycin; lane 4: unlabeled culture without tryptophan and with 2mM fosmidomycin; lane5: 1-^{13}C glucose culture with 50uM tryptophan and with 2mM fosmidomycin; lane 6: 1-^{13}C glucose culture with 50uM tryptophan and with 2mM fosmidomycin. Measurements were taken approx. 45 minutes post-introduction of either 0mM or 2mM fosmidomycin; both occurring approx. 3 hours after induction with 400uM IPTG. The data presented is the average of 2 technical replicates; error bars are shown for specific productivity values. The data suggests a contribution of roughly 52% and 48% for isoprene generated via the MVA pathway and DXP pathway, respectively for the unlabeled culture. Similarly, the data shows a 57% MVA-flux to 43% DXP-flux and 49% MVA-flux to 51% DXP-flux contribution to the isoprene generated by the 1-^{13}Cglucose culture without and with 50uM tryptophan, respectively. The repressed expression of the

MVK enzyme mediated by the presence of tryptophan in the growth media for cultures represented by lanes 3 and 6 was reflected in the data as a 24% to 34% decrease in overall-flux compared to the cultures grown without the addition of tryptophan to the growth media. MVA flux was determined by the fraction of isoprene produced during exposure to fosmidomycin relative to the amount of isoprene produced in the absence of the inhibitor for each particular culture type.

[0173] Figure 99 depicts the results for experiments for determining the specific productivity relative to culture density in the presence and absence of fosmidomycin for strain REM A2_17 grown on 3-^{13}C glucose. The y1 axis, specific productivity of isoprene production (ug/L OD hr); y2 axis, cell density (OD_{600nm}). Specific productivity (solid bars) and Cell density (diamonds). Measurements were taken approx. 1 hour post-introduction of either 0mM or 2mM fosmidomycin; both occurring approx. 3 hours after induction with 400uM IPTG. The data presented is the average of 2 technical replicates; error bars are shown for specific productivity values. The data suggests a contribution of roughly 58% and 42% for isoprene generated via the MVA pathway and DXP pathway, respectively; MVA flux was determined by the fraction of isoprene produced during exposure to fosmidomycin relative to the amount of isoprene produced in the absence of the inhibitor.

[0174] Figure 100 (panels A and B) depicts the DXP and MVA pathway-specific labeling pattern of isoprene resulting from: A) 1-^{13}C glucose and B) 3-^{13}C glucose catabolism via glycolysis. Black circles indicate 100% abundance of ^{13}C atoms at specified positions. Half-black circles indicate ^{13}C abundance of 50% with the rest 50% being ^{12}C atoms coming from the positions in glucose shown by open circles.

[0175] Figure 101 (panels A and B) depicts the calculated distributions of isoprene and cMEPP cumomers in REM A2_17 strain grown on: A) 1-^{13}C glucose or B) 3-^{13}C glucose in the presence or in the absence of fosmidomycin (+FM and –FM, respectively).

[0176] Figure 102 (panels A and B) depicts the GC-MS spectra of: A) unlabeled (synthetic) isoprene standard having natural abundance of ^{13}C and B) isoprene produced by the REM A2_17 strain grown on 3-^{13}C glucose. Note that intensities of m/z 68, 69 and

70 peaks relative to the m/z 67 peak are higher in the REM A2_17 strain compared to the isoprene standard because of ^{13}C enrichment.

[0177] Figure 103 depicts results of isoprene ^{13}C isotope enrichment as a function of MVA/MEP pathway ratio.

[0178] Figure 104 depicts an exemplary apparatus for generation, collection and analysis of Bioisoprene™ product.

[0179] Figure 105 depicts results showing the ^{13}C NMR spectrum of natural ^{13}C-abundance isoprene.

[0180] Figure 106 depicts results showing the ^{13}C NMR spectrum of isoprene derived from a MVA/MEP dual pathway strain. Both C-1 and C-2/C-4 are ^{13}C-enriched relative to C-3, with a signal intensity equal or less than the noise level demonstrates the contribution of the both the MVA and MEP pathways to isoprene synthesis in this strain.

[0181] Figure 107 depicts a diagram for a portion of the PL.6 *fkpB* locus. The nucleotide sequence of the region depicted in the figure is indicated by the 323 bases listed below the diagram. The 5' and 3' regions of homology used to integrate the PL.6 promoter upstream of *fkpB* are shown in gray. The sequence highlighted in black bold text represents the exogenous sequence left in the region after loopout of the Gene Bridges chloramphenicol resistance cassette, referred to in the figure as the Gene Bridges scar, with the remaining FRT (Flipase recognition target) site underlined. The PL.6 promoter sequence is shown in regular black text. The -35, -10, and RBS (ribosome binding site) positions are indicated in the figure.

[0182] Figure 108 (panels A,B,C,D) depicts a comparison of the isoprene productivity of 4 strains. Panel A, typical isoprene productivity of strain WW119 (parent to strains in panel B and C) at two time points at 200 uM IPTG. This experiment was performed as is described in the text for strains in panels B and C, except that isoprene monitoring was limited to 2 and 4 hours. OD_{600} was monitored throughout culture period for all strains at hourly intervals. Panel B shows isoprene specific productivity for strains REM 6_15 (PL.6 fkpB-ispH Δ*iscR*) at several IPTG concentrations. Panel C shows isoprene specific

productivity for strain REM D8_15 (PL.6 fkpB-ispH) at several IPTG concentrations. The data is consistent with that *ΔiscR* rescues isoprene productivity lost upon introduction of PL.6*fkpB-ispH*. Panel D shows isoprene specific productivity for strain REM D7_15 at severl IPTG concentrations.

[0183] Figure 109 shows an image of *E. coli* ispH western blot. Lane description is as follows: Lane 1, SeeBlue® Plus2 Pre-Stained Standard, Invitrogen, Lane 2, *E. coli* ispH purified standard (0.4 µg), Lane 3, REM A7_15 soluble fraction, Lane 4, REM A7_15 insoluble fraction, Lane 5, REM A8_15 soluble fraction, Lane 6, REM A8_15 insoluble fraction, Lane 6, REM D1_14 soluble fraction, Lane 7, REM D1_14 insoluble fraction, Lane 8, WW103 soluble fraction and, Lane 10, WW103 insoluble fraction. Development method: 1°Ab Anti-Rabbit *E. coli* ispH at 1:10,000 dilution, 2°Ab Alexa Fluor® 488 goat anti-rabbit IgG (H+L), Invitrogen, 1:1,000 dilution; see text for additional details. Gel was a Novagen 4 to 12% BT gel. Loading was normalized to equal OD_{600}. Pel, pellet; sup, supernatant.

[0184] Figure 110 shows *E. coli* ispH western blot quantitation. Quantitation of the western data was by ImageQuant 5.2 (Molecular Dynamics). Light shaded bars represent amount of ispH found in the soluble fraction. Dark shaded bars represent amount of ispH found in the insoluble fraction.

DETAILED DESCRIPTION OF THE INVENTION

[0185] The invention provides, *inter alia*, compositions and methods for the production of isoprene in increased amounts using various DXP pathway genes and polypeptides, various MVA pathway genes and polypeptides, iron-sulfur cluster-interacting redox genes and polypeptides, isoprene synthase genes and polypeptides, and optionally, IDI genes and polypeptides and various genes and polypeptides associated with the DXP pathway and/or MVA pathway.

[0186] 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. 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.

[0187] As used herein, the term "isoprene" or "2-methyl-1,3-butadiene" (CAS# 78-79-5) refers to the direct and final volatile C5 hydrocarbon product from the elimination of pyrophosphate from 3,3-dimethylallyl pyrophosphate (DMAPP), and does not involve the linking or polymerization of one or more isopentenyl diphosphate (IPP) molecules to one or more DMAPP molecules. The term "isoprene" is not generally intended to be limited to its method of production.

[0188] As used herein, the phrase, "various genes and polypeptides associated with the DXP pathway," or "DXP pathway associated nucleic acid(s) or polypeptide(s)" refers to any nucleic acid or polypeptide that interacts with DXP pathway polypeptides or nucleic acids, including, but not limited to, a terpene synthase (*e.g.*, ocimene synthase, farnesene synthase, and artemesinin synthase), either directly or indirectly.

[0189] For use herein, unless clearly indicated otherwise, use of the terms "a", "an," and the like refers to one or more.

[0190] 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.

[0191] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments.

[0192] The present invention is based in part on the surprising discovery that an increased amount of an iron-sulfur cluster-interacting redox polypeptide increases the activity demonstrated by the DXP pathway polypeptides (such as HDS (GcpE or IspG) or HDR polypeptide(IspH or LytB). While not intending to be bound to a particular theory, it is believed that the increased expression of one or more endogenous or heterologous iron-sulfur interacting redox nucleic acids or polypeptides improve the rate of formation and the amount of DXP pathway polypeptides containing an iron sulfur cluster (such as HDS or HDR), and/or stabilize DXP pathway polypeptides containing an iron sulfur cluster (such as HDS or HDR). This in turn increases the carbon flux to isoprene synthesis in cells by increasing the synthesis of HMBPP and/or DMAPP and decreasing the cMEPP and HMBPP pools in the DXP pathway. For example, overexpression of an iron-sulfur cluster- interacting redox polypeptide (flavodoxin I) in cells overexpressing a DXP pathway polypeptide (DXS), isoprene synthase polypeptide, and IDI polypeptide resulted in increased production of isoprene by about 1- to 2-fold in comparison to cells overexpressing DXP pathway polypeptide, isoprene synthase polypeptide, and IDI polypeptide only. *See* Example 8. Overexpression of one or more iron-sulfur cluster-interacting redox polypeptide (ferredoxin and ferredoxin-NADP+ oxidoreductase), one or more DXP pathway polypeptide, isoprene synthase polypeptide, and IDI polypeptide resulted in increased production of isoprene. *See* Example 9.

[0193] Accordingly, in one aspect of the invention, cells in culture comprise (i) a heterologous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a heterologous nucleic acid encoding DXP pathway polypeptide, and an heterologous nucleic acid encoding isoprene synthase and/or (ii) a duplicate copies of endogenous nucleic acids encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, and an isoprene synthase polypeptide. In some embodiments, the cells in culture comprise (i) one or more copies of heterologous or endogenous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, (ii) one or more copies

of heterologous or endogenous nucleic acid encoding a DXP pathway polypeptide, and (iii) one or more copies of heterologous or endogenous nucleic acid encoding an isoprene synthase polypeptide.

[0194] In another aspect of the invention, provided are methods of producing isoprene. In one embodiments, the method comprises (a) culturing cells comprising (i) a heterologous nucleic acid encoding a heterologous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, and an isoprene synthase polypeptide and/or (ii) a duplicate copy of an endogenous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, and an isoprene synthase polypeptide under suitable culture conditions for the production of isoprene, and (b) producing isoprene.

[0195] As used herein, iron-sulfur cluster-interacting redox polypeptide is a polypeptide that is capable of transferring electrons to a polypeptide containing an iron-sulfur cluster. An iron-sulfur cluster-interacting redox polypeptide includes, but is not limited to, flavodoxin (*e.g.*, flavodoxin I), flavodoxin reductase, ferredoxin (*e.g.*, ferredoxin I), ferredoxin-NADP+ oxidoreductase, and genes or polypeptides encoding thereof (*e.g.*, *fpr* or *fldA*). For example, DXP pathway polypeptide HDS (GcpE) is a metallo-enzyme possessing a $[4Fe\text{-}4S]^{2+}$ center and catalyzes the reduction of cMEPP into HMBPP via two successive one-electron transfers mediated by the reduction of $[4Fe\text{-}4S]^{2+}$ center in the presence of flavodoxin/flavodoxin reductase (*see*, Wolff *et al.*, *FEBS Letters*, 541:115-120 (2003)), which is hereby incorporated by reference in its entirety). Similarly, DXP pathway polypeptide HDR (LytB) is also a Fe/S protein catalyzing the reduction of HMBPP into IPP or DMAPP via two successive one-electron transfers in the presence of flavodoxin/flavodoxin reductase/NADPH system. *See*, for example, Seemann, M. *et al. Agnew. Chem. Int. Ed.*, 41: 4337-4339 (2002); Wolff, M. *et al.*, *FEBS Letters*, 541: 115-120 (2003), which are each hereby incorporated by reference in their entirety, particularly with respect to the description of GcpE, LytB, and flavodoxin/flavodoxin reductase/NADPH system).

[0196] As used herein, flavodoxin is a protein that is capable of transferring electrons and contains the prosthetic group flavin mononucleotide. In *Escherichia coli* (*E. coli*), flavodoxin is encoded by the *fldA* gene and reduced by the FAD-containing protein NADPH:ferredoxin oxidoreductase, and plays an essential role in the DXP pathway for isoprenoid biosynthesis (*see*, example, Kia-Joo, P. *et al. FEBS Letters*, 579: 3802-3806, 2005, which is hereby incorporated by reference in its entirety).

[0197] As used herein, ferredoxin is a protein that is capable of transferring electron and contains iron and labile sulfur in equal amounts and plays an essential role in the DXP pathway for isoprenoid biosynthesis. For example, HDS from plants and cyanobacteria have been shown to be ferredoxin, rather than flavodoxin-dependent, enzymes (Seemann *et al.*, *FEBS Lett.*, 580(6):1547-52 (2006), which is hereby incorporated by reference in its entirety).

[0198] As used herein, *Fpr* encodes flavodoxin/ferredoxin NADPH-oxidoreductase and provides the necessary electron derived from NADPH via FldA for HDS and HDR to perform their catalytic functions (reviewed in report by L. A. Furgerson, *The Mevalonate-Independent Pathway to Isoprenoid Compounds: Discovery, Elucidation, and Reaction Mechanisms*, published February 13, 2006, which is hereby incorporated by reference in its entirety).

[0199] As used herein, the encoded DXS, DXR, MCT, CMK, MCS, HDS, and HDR polypeptides are part of the DXP pathway for the biosynthesis of isoprene (Figure 19A).

[0200] DXS polypeptides convert pyruvate and D-glyceraldehyde-3-phosphate into 1-deoxy-D-xylulose-5-phosphate (DXP). 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.

[0201] DXR polypeptides convert 1-deoxy-D-xylulose 5-phosphate (DXP) into 2-C-methyl-D-erythritol 4-phosphate (MEP). 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.

[0202] MCT polypeptides convert 2-C-methyl-D-erythritol 4-phosphate (MEP) into 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-Me). While not intending to be bound by any particular theory, it is believed that increasing the amount of MCT polypeptide increases the flow of carbon through the DXP pathway, leading to greater isoprene production.

[0203] CMK polypeptides convert 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME) into 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-MEP). While not intending to be bound by any particular theory, it is believed that increasing the amount of CMK polypeptide increases the flow of carbon through the DXP pathway, leading to greater isoprene production.

[0204] MCS polypeptides convert 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-MEP) into 2-C-methyl-D-erythritol 2, 4-cyclodiphoshphate (ME-CPP or cMEPP). While not intending to be bound by any particular theory, it is believed that increasing the amount of MCS polypeptide increases the flow of carbon through the DXP pathway, leading to greater isoprene production.

[0205] HDS polypeptides convert 2-C-methyl-D-erythritol 2, 4-cyclodiphoshphate (ME-CPP or cMEPP) into (E)-4-hydroxy-3-methylbut-2-en-1-yl-diphosphate (HMBPP or HDMAPP). While not intending to be bound by any particular theory, it is believed that increasing the amount of HDS polypeptide increases the flow of carbon through the DXP pathway, leading to greater isoprene production.

[0206] HDR polypeptides convert (E)-4-hydroxy-3-methylbut-2-en-1-yl-diphosphate (HMBPP) into 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 HDR polypeptide increases the flow of carbon through the DXP pathway, leading to greater isoprene production.

[0207] Isoprene synthase polypeptides convert dimethylallyl diphosphate (DMAPP) into isoprene.

[0208] Heterologous iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, and isoprene synthase polypeptide can be expressed in a variety of host cells, such as *Escherichia coli (E. coli), Panteoa citrea, Bacillus subtilis, Yarrowia lipolytica,* and *Trichoderma reesei*. All of these cells produced more isoprene than the naturally occurring DXP pathway alone.

[0209] As discussed further below, isoprene production by cells can be enhanced by increasing the amount of expression of an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, and an isoprene synthase polypeptide. The DXP pathway polypeptides include DXS, DXR, MCT, CMK, MCS, HDS, and HDR. For example, one or more DXP pathway nucleic acids can be introduced into the cells, which includes DXS, DXR, MCT, CMK, MCS, HDS, and HDR. The DXS, DXR, MCT, CMK, MCS, HDS, or HDR nucleic acid may be a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid. Similarly, the iron-sulfur cluster-interacting redox nucleic acid may be a heterologous nucleic acid or duplicate copy of an endogenous nucleic acid. Similarly, the isoprene synthase nucleic acid may be a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid. In some embodiments, the amount of one or more iron-sulfur cluster-interacting redox polypeptide, one or more of DXS, DXR, MCT, CMK, MCS, HDS, or HDR polypeptide, and isoprene synthase polypeptide are increased by replacing one or more endogenous iron-sulfur cluster-interacting redox promoters or regulatory regions, one or more of the endogenous DXS, DXR, MCT, CMK, MCS, HDS, or HDR promoters or regulatory regions, and isoprene synthase promoter or regulatory region with other promoters and/or regulatory regions that result in greater transcription of iron-sulfur cluster-interacting redox nucleic acids, one or more of DXS, DXR, MCT, CMK, MCS, HDS, or HDR nucleic acids, and isoprene synthase nucleic acid.

[0210] In some embodiments, the presence of heterologous or extra endogenous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, and isoprene synthase nucleic acid cause cells to grow more reproducibly and/or remain viable for longer compared to the corresponding cell with only one or two of these heterologous or extra endogenous nucleic acids. While not intending to be bound to a particular theory, it

is believed that the overexpressing an iron sulfur cluster-interacting redox polypeptide can increase the rate of formation or the amount of one or more DXP pathway polypeptides (*e.g.*, GcpE and/or LytB) or stabilizes one or more DXP pathway polypeptides (*e.g.*, GcpE and/or LytB), so that one or more DXP pathway polypeptides are active for a longer period of time, which in turn cause cells containing heterologous or extra endogenous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, and isoprene synthase nucleic acid to grow more reproducibly and/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 iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, and isoprene synthase nucleic acid grow better than cells with only a DXP pathway nucleic acid, with only a heterologous iron-sulfur cluster-interacting redox nucleic acid, with a heterologous iron-sulfur cluster-interacting redox nucleic acid and DXP pathway nucleic acid, iron-sulfur cluster-interacting redox nucleic acid and isoprene synthase nucleic acid, or DXP pathway nucleic acid and isoprene synthase nucleic acid. Also, large amounts of iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, and isoprene synthase polypeptide can be expressed in the cells without causing an excessive amount of toxicity to the cells.

[0211] In some embodiments of any of the aspects of the invention, the cells express a second DXP pathway polypeptide, in addition to the first DXP pathway polypeptide, including DXS (1-deoxy-D-xylulose-5-phosphate synthase), DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase), MCT (4-diphosphocytidyl-2C-methyl-D-erythritol synthase), CMK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), MCS (2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), HDS (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase), and HDR (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase). In some embodiments of any of the aspects of the invention, the cells express two or more DXP pathway polypeptides, in addition to the first DXP pathway polypeptide as described above. In some embodiments of any of the aspects of the invention, the cells express 2, 3, 4, 5, 6, or 7 DXP pathway polypeptides, in addition to the first DXP pathway polypeptide as described above.

[0212] Additionally, isoprene production by cells that contain a heterologous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid (*e.g.*, DXS, DXR, MCT, CMK, MCS, HDS, or HDR), and isoprene synthase nucleic acid can be enhanced by increasing the amount of an IDI polypeptide expressed by the cells.

[0213] In some embodiments, isoprene production by cells that contain a heterologous iron-sulfur cluster-interacting redox nucleic acid, DXS nucleic acid, and isoprene synthase nucleic acid can be enhanced by increasing the amount of an IDI polypeptide expressed by the cells. In other embodiments, isoprene production by cells that contain a heterologous iron-sulfur cluster-interacting redox nucleic acid, HDS (IspG or GcpE), and isoprene synthase nucleic acids can be enhanced by increasing the amount of an IDI polypeptide expressed by the cells. In some embodiments, the cells comprise IspG and fldA. In another embodiment, the cells comprise IspG, fldA, and IspH.

[0214] In some embodiments, isoprene production by cells that contain a heterologous flavodoxin nucleic acid, DXS nucleic acid, and isoprene synthase nucleic acid can be enhanced by increasing the amount of an IDI polypeptide expressed by the cells. In other embodiments, isoprene production by cells that contain a heterologous flavodoxin nucleic acid, HDS (IspG or GcpE) nucleic acid, and isoprene synthase nucleic acid can be enhanced by increasing the amount of an IDI polypeptide expressed by the cells.

[0215] In some embodiments, isoprene production by cells that contain a heterologous ferredoxin nucleic acid, ferredoxin-NADP+ oxidoreductase nucleic acid, DXS nucleic acid, and isoprene synthase nucleic acid can be enhanced by increasing the amount of an IDI polypeptide expressed by the cells. In other embodiments, isoprene production by cells that contain a heterologous ferredoxin nucleic acid, ferredoxin-NADP+ oxidoreductase nucleic acid, HDS (IspG or GcpE) nucleic acid, and isoprene synthase nucleic acid can be enhanced by increasing the amount of an IDI polypeptide expressed by the cells.

[0216] In some embodiments, isoprene production by cells that contain a heterologous iron-sulfur cluster-interacting redox nucleic acid, HDR (IspH or LytB) nucleic acid, and isoprene synthase nucleic acid can be enhanced by increasing the amount of an IDI

(isopentenyl-diphosphate delta-isomerase) polypeptide expressed by the cells. In some embodiments, the cells comprise IspG and fldA. In another embodiment, the cells comprise IspG, fldA, and IspH.

[0217] In some embodiments, isoprene production by cells that contain a heterologous flavodoxin, HDR (IspH or LytB), and isoprene synthase nucleic acids can be enhanced by increasing the amount of an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide expressed by the cells.

[0218] In some embodiments, isoprene production by cells that contain a heterologous ferredoxin nucleic acid, ferredoxin-NADP+ oxidoreductase nucleic acid, HDR (IspH or LytB) nucleic acid, and isoprene synthase nucleic acids can be enhanced by increasing the amount of an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide expressed by the cells.

[0219] In some embodiments, isoprene production by cells that contain a heterologous ferredoxin nucleic acid, ferredoxin-NADP+ oxidoreductase nucleic acid, HDS and HDR nucleic acids, and isoprene synthase nucleic acid can be enhanced by increasing the amount of an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide expressed by the cells.

[0220] IDI polypeptides catalyze the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). While not intending to be bound by any particular theory, it is believed that increasing the amount of IDI polypeptide in cells increases the amount (and conversion rate) of IPP that is converted into DMAPP, which in turn is converted into isoprene.

[0221] 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 iron-sulfur cluster-interacting redox polypeptide, one or more of DXP pathway polypeptide (*e.g.*, DXS, DXR, MCT, CMK, MCS, HDS, or HDR), isoprene synthase polypeptide, and IDI polypeptide are increased by replacing endogenous iron-sulfur cluster-interacting redox promoter or regulatory region, one or more of the endogenous DXP pathway promoter or

regulatory region, and IDI promoters or regulatory region with other promoters and/or regulatory regions that result in greater transcription of iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, isoprene synthase nucleic acid, and IDI nucleic acid.

[0222] Heterologous IDI polypeptides can also be expressed in a variety of host cells in the presence of isoprene synthase, such as *Escherichia coli (E. coli), Panteoa citrea, Bacillus subtilis, Yarrowia lipolytica,* and *Trichoderma reesei*. All of these cells produced more isoprene than when IDI is not used.

[0223] Additionally, isoprene production by cells that contain a heterologous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid (*e.g.*, DXS, DXR, MCT, CMK, MCS, HDS, or HDR), isoprene synthase nucleic acid, and optionally IDI nucleic acid, can be enhanced by increasing the amount of a DXP pathway associated polypeptide expressed by the cells

[0224] In some embodiments of any of the aspects of the invention, isoprene production can be further increased by using a mutant DXP pathway polypeptide or nucleic acid derived from thereof. In some embodiments, the mutant DXP pathway polypeptide is a HDR polypeptide with the iron-sulfur cluster regulator (iscR) removed. In some embodiments, the mutant DXP pathway polypeptide is a mutant HDR polypeptide that produces solely DMAPP or a majority of DMAPP relative to IPP. For example, the use of the LytBG120D in a DXP pathway-mediated isoprene production strain allows the unique generation of an isoprenoid product that is derived almost entirely from DMAPP. *See* Example 18.

[0225] As used herein, iscR is encoded by an ORF located immediately upstream of genes coding for the *E. coli* Fe-S cluster assembly proteins. In the DXP pathway, the implementation of a gene cassette directing the overexpression of the isc operon involved in the assembly of iron-sulfur clusters into an *E. coli* strain engineered for HDR protein anaerobically purified from this strain by a factor of at least 200. (Gräwert *et al.*, *J Am Chem Soc.* 126(40):12847-55 (2004); Schwartz *et al.*, *PNAS,* 98(26):14751-3 (2001);

Akhtar and Jones, *Appl. Microbiol. Biotechnol.* 78(5):853-62 (2008), which are each hereby incorporated by reference in their entireties).

[0226] In some embodiments of any of the aspects of the invention, isoprene production can be further increased by increasing the carbon flux through the DXP pathway. In some embodiments, the carbon flux can be increased by avoiding any feedback inhibition of DXS activity by metabolites downstream the DXP pathway or/and intermediates of other pathways that use a DXP pathway polypeptide as a substrate (e.g., DXR). In some embodiments, the feedback inhibition by some DXP pathway polypeptides (e.g., DXR) can be alleviated by rebalancing pathyway enzymes and maintaining levels of HMBPP and DMAPP at concentrations below 1 to 2 mM DMAPP and 1 to 2 mM HMBPP. In some embodiments, the level of HMBPP and DMAPP are maintained below 1 mM for the duration of the fermentation run. In other embodiments, the level of HMBPP and DMAPP are maintained below 1 mM during the exponential phase of the fermentation. In other embodiments, late DXP pathway enzymes, particularly IspG and IspH, are maintained at levels consistent with minimizing phosphorylation level of Dxr.

[0227] In some embodiments, the other pathway that uses DXP pathway polypeptide as a substrate (*e.g.*, DXP) is the thiamine (Vitamin B1) or pyridoxal (Vitamin B6) pathway. In some embodiments, the carbon flux can be increased by expressing a DXP pathway polypeptide from a different organism that is not subject to inhibition by downstream products of the DXP pathway. In some embodiments, the carbon flux can be increased by deregulating glucose uptake. In other embodiments, the carbon flux can be increased by maximizing the balance between the precursors required for the DXP pathway. In some embodiments, the balance of the DXP pathway precursors, pyruvate and glyceraldehydes-3-phosphate (G-3-P) can be achieved by redirecting the carbon flux with the effect of elevating or lowering pyruvate or G-3-P separately. In some embodiments, the carbon flux can be increased by using a strain (containing one or more DXP pathway genes or one or more both DXP pathway and MVA pathway genes) containing a pyruvate dehydrogenase E1 subunit variant. In some embodiments, the pyruvate dehydrogenase (PDH) E1 subunit variant has an E636Q point mutation. In some

embodiments, the carbon flux can be increased by using a CRP-deleted mutant. As used herein, CRP (cAMP Receptor Protein) is a positive regulator protein activated by cyclic AMP. It is required for RNA polymerase to initiate transcription of certain (catabolite-sensitive) operons of *E. coli*.

[0228] In some embodiments of any of the aspects of the invention, isoprene production can be further increased by utilizing the downstream genes or polypeptides of the DXP pathway by introducing a heterologous terpene synthase nucleic acid or a duplicate copy of an endogenous terpene synthase nucleic acid into the cells, which includes, but is not limited to ocimene synthase, farnesene synthase, and artemesinin synthase.

[0229] In some embodiments, a renewable carbon source is used for the production of isoprene. In some embodiments, the concentrations of isoprene and any oxidants are within the nonflammable ranges to reduce or eliminate the risk that a fire may occur during production or recovery of isoprene. See for example, U.S. Appl. No. 61/133,947, which is hereby incorporated by reference in its entirety, particularly with respect to flammability modeling and testing of isoprene in Example 13 and WO2010/003007. The compositions and methods of the present invention are desirable because they allow high isoprene yield per cell, high carbon yield, high isoprene purity, high productivity, low energy usage, low production cost and investment, and minimal side reactions. This efficient, large scale, biosynthetic process for isoprene production provides an isoprene source for synthetic isoprene-based rubber and provides a desirable, low-cost alternative to using natural rubber.

[0230] In some embodiments, at least a portion of the cells maintain the heterologous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, and isoprene synthase 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, at least a portion of the cells maintain the heterologous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic

acid, and IDI 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 iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, isoprene synthase nucleic acid, and/or IDI nucleic acid and DXP pathway associated nucleic acid also comprises a selective marker, such as a kanamycin, ampicillin, carbenicillin, gentamicin, hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol antibiotic resistance nucleic acid.

[0231] The amount of isoprene produced can be further increased by adding yeast extract to the cell culture medium. For example, the amount of isoprene produced that are linearly proportional to the amount of yeast extract in the cell medium for the concentrations are tested. Increasing the amount of yeast extract in the presence of glucose can result in more isoprene being produced than increasing the amount of glucose in the presence of yeast extract. Also, increasing the amount of yeast extract can allow the cells to produce a high level of isoprene for a longer length of time and improved the health of the cells.

[0232] Isoprene production can also be demonstrated using three types of hydrolyzed biomass (bagasse, corn stover, and soft wood pulp) as the carbon source. 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.

[0233] In some embodiments, an oil is included in the cell medium. *See*, for example, U.S. 61/134,094, which is hereby incorporated by reference in its entirety, particularly with respect to oils included in the cell medium 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 glyceraldehyde 3-phosphate and/or pyruvate in the cells, thereby increasing the carbon flow through the DXP 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 DXP pathway to produce isoprene or are genetically modified to contain nucleic acids for the entire DXP 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

[0234] Various iron-sulfur cluster-interacting redox polypeptides and nucleic acids, DXP pathway polypeptides and nucleic acids, DXP pathway associated polypeptides and nucleic acids, isoprene synthase polypeptides and nucleic acids, and IDI polypeptides and nucleic acids can be used in the compositions and methods of the invention.

[0235] As used herein, "polypeptides" includes polypeptides, proteins, peptides, fragments of polypeptides, and fusion polypeptides. In some embodiments, the fusion polypeptide includes part or all of a first polypeptide (*e.g.*, an iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, and IDI polypeptide, or catalytically active fragment thereof) and may optionally include part or all of a second polypeptide (*e.g.*, a peptide that facilitates purification or detection of the fusion polypeptide, such as a His-tag). In some embodiments, the fusion polypeptide has an activity of two or more DXP pathway polypeptides.

[0236] 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 iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, or IDI 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) iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, or IDI polypeptide.

[0237] 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.

[0238] In some embodiments, the polypeptide is a heterologous polypeptide. By "heterologous polypeptide" is meant a polypeptide whose amino acid sequence is not identical to that of another polypeptide naturally expressed in the same host cell. In particular, a heterologous polypeptide is not identical to a wild-type nucleic acid that is found in the same host cell in nature.

[0239] 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 hereby incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA, a genomic DNA fragment, or a cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In various embodiments, a nucleic acid is a recombinant nucleic acid. In some embodiments, an iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI 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 iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, and/or IDI and all or part of another polypeptide (*e.g.*, a peptide that facilitates purification or detection of the fusion polypeptide, such as a His-tag). In some embodiments, part or all of a recombinant nucleic acid is chemically synthesized. It is to be understood that mutations, including single nucleotide mutations, can occur within a nucleic acid as defined herein.

[0240] 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.

[0241] In particular embodiments, the nucleic acid includes a segment of or the entire nucleic acid sequence of any naturally-occurring iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic, or IDI 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 iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic, or IDI 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) iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI 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 iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid. In some embodiments, the nucleic acid is a degenerate variant of any nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, or IDI polypeptide.

[0242] "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.

[0243] The accession numbers of exemplary isoprene synthase and DXP pathway polypeptides and nucleic acids are listed in Appendix 1 (the accession numbers of Appendix 1 and their corresponding sequences are herein incorporated by reference in their entireties, particularly with respect to the amino acid and nucleic acid sequences of isoprene synthase and/or DXP pathway polypeptides and nucleic acids). The Kegg database also contains the amino acid and nucleic acid sequences of numerous exemplary isoprene synthase and/or DXP pathway polypeptides and nucleic acids (*see*, for example, the world-wide web at "genome.jp/kegg/pathway/map/map00100.html" and the sequences therein, which are each hereby incorporated by reference in their entireties, particularly with respect to the amino acid and nucleic acid sequences of isoprene synthase and/or DXP pathway polypeptides and nucleic acids). In some embodiments, one or more of the isoprene synthase and/or DXP pathway polypeptides and/or nucleic acids have a sequence identical to a sequence publicly available on December 12, 2007 or September 14, 2008 such as any of the sequences that correspond to any of the accession numbers in Appendix 1 or any of the sequences present in the Kegg database. Additional exemplary isoprene synthase and/or DXP pathway polypeptides and nucleic acids are described further below.

Exemplary Isoprene Synthase Polypeptides and Nucleic Acids

[0244] As noted above, isoprene synthase polypeptides convert dimethylallyl diphosphate (DMAPP) into isoprene. Exemplary isoprene synthase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of an isoprene synthase polypeptide. Standard methods can be used to determine whether a polypeptide has isoprene synthase polypeptide activity by measuring the ability of the polypeptide to convert DMAPP into isoprene *in vitro*, in a cell extract,

or *in vivo*. In an exemplary assay, cell extracts are prepared by growing a strain (*e.g.*, the *E. coli*/pTrcKudzu strain described herein) in the shake flask method as described in Example 1. After induction is complete, approximately 10 mL of cells are pelleted by centrifugation at 7000 x *g* for 10 minutes and resuspended in 5 ml of PEB without glycerol. The cells are lysed using a French Pressure cell using standard procedures. Alternatively the cells are treated with lysozyme (Ready-Lyse lysozyme solution; EpiCentre) after a freeze/thaw at -80C.

[0245] Isoprene synthase polypeptide activity in the cell extract can be measured, for example, as described in Silver *et al.*, J. Biol. Chem. 270:13010-13016, 1995 and references therein, which are each hereby incorporated by reference in their entireties, particularly with respect to assays for isoprene synthase polypeptide activity. DMAPP (Sigma) is evaporated to dryness under a stream of nitrogen and rehydrated to a concentration of 100 mM in 100 mM potassium phosphate buffer pH 8.2 and stored at -20 °C. To perform the assay, a solution of 5 μL of 1M $MgCl_2$, 1 mM (250 μg/ml) DMAPP, 65 μL of Plant Extract Buffer (PEB) (50 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 5% glycerol, and 2 mM DTT) is added to 25 μL of cell extract in a 20 ml Headspace vial with a metal screw cap and teflon coated silicon septum (Agilent Technologies) and cultured at 37 °C for 15 minutes with shaking. The reaction is quenched by adding 200 μL of 250 mM EDTA and quantified by GC/MS as described in Example 1, part II.

[0246] 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.

[0247] In some embodiments, the isoprene synthase polypeptide or nucleic acid is from the family Fabaceae, such as the Faboideae subfamily. In some embodiments, the isoprene synthase polypeptide or nucleic acid is a polypeptide or nucleic acid from *Pueraria montana* (kudzu) (Sharkey *et al.*, Plant Physiology 137: 700-712, 2005),

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

Exemplary DXP Pathway Polypeptides and Nucleic Acids

[0248] Exemplary DXP pathways polypeptides include, but are not limited to any of the following polypeptides: DXS polypeptides, DXR polypeptides, MCT polypeptides, CMK polypeptides, MCS polypeptides, HDS polypeptides, HDR polypeptides, IDI polypeptides, and polypeptides (*e.g.*, fusion polypeptides) having an activity of one, two, or more of the DXP pathway polypeptides. In particular, DXP pathway polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of a DXP pathway polypeptide. Exemplary DXP 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 a DXP pathway polypeptide. Exemplary DXP 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.

[0249] In particular, DXS polypeptides convert pyruvate and D-glyceraldehyde 3-phosphate into 1-deoxy-d-xylulose 5-phosphate (DXP). Standard methods 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 *in vitro*, in a cell extract, or *in vivo*.

[0250] DXR polypeptides convert 1-deoxy-d-xylulose 5-phosphate (DXP) into 2-C-methyl-D-erythritol 4-phosphate (MEP). Standard methods can be used to determine whether a polypeptide has DXR polypeptides activity by measuring the ability of the polypeptide to convert DXP *in vitro*, in a cell extract, or *in vivo*.

[0251] MCT polypeptides convert 2-C-methyl-D-erythritol 4-phosphate (MEP) into 4-(cytidine 5'-diphospho)-2-methyl-D-erythritol (CDP-ME). Standard methods can be used to determine whether a polypeptide has MCT polypeptides activity by measuring the ability of the polypeptide to convert MEP *in vitro*, in a cell extract, or *in vivo*.

[0252] CMK polypeptides convert 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME) into 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-MEP). Standard methods can be used to determine whether a polypeptide has CMK polypeptides activity by measuring the ability of the polypeptide to convert CDP-ME *in vitro*, in a cell extract, or *in vivo*.

[0253] MCS polypeptides convert 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-MEP) into 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate (ME-CPP or cMEPP). Standard methods can be used to determine whether a polypeptide has MCS polypeptides activity by measuring the ability of the polypeptide to convert CDP-MEP *in vitro*, in a cell extract, or *in vivo*.

[0254] HDS polypeptides convert 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate into (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP or HDMAPP). Standard methods can be used to determine whether a polypeptide has HDS polypeptides activity

by measuring the ability of the polypeptide to convert ME-CPP *in vitro*, in a cell extract, or *in vivo*.

[0255] HDR polypeptides convert (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Standard methods can be used to determine whether a polypeptide has HDR polypeptides activity by measuring the ability of the polypeptide to convert HMBPP *in vitro*, in a cell extract, or *in vivo*.

[0256] IDI polypeptides convert isopentenyl diphosphate into dimethylallyl diphosphate . Standard methods can be used to determine whether a polypeptide has IDI polypeptides activity by measuring the ability of the polypeptide to convert isopentenyl diphosphate *in vitro*, in a cell extract, or *in vivo*.

Exemplary MVA Pathway Polypeptides and Nucleic Acids

[0257] In some aspects of the invention, the cells described in any of the compositions or methods described herein comprise a nucleic acid encoding an MVA pathway polypeptide. In some embodiments, the MVA pathway polypeptide is an endogenous polypeptide. In some embodiments, the cells comprise one or more additional copies of an endogenous nucleic acid encoding an MVA pathway polypeptide. In some embodiments, the endogenous nucleic acid encoding an MVA pathway polypeptide operably linked to a constitutive promoter. In some embodiments, the endogenous nucleic acid encoding an MVA pathway polypeptide operably linked to a constitutive promoter. In some embodiments, the endogenous nucleic acid encoding an MVA pathway polypeptide is operably linked to a strong promoter. In a particular embodiment, the cells are engineered to over-express the endogenous MVA pathway polypeptide relative to wild-type cells.

[0258] In some embodiments, the MVA pathway polypeptide is a heterologous polypeptide. In some embodiments, the cells comprise more than one copy of a heterologous nucleic acid encoding an MVA pathway polypeptide. In some embodiments, the heterologous nucleic acid encoding an MVA pathway polypeptide is

operably linked to a constitutive promoter. In some embodiments, the heterologous nucleic acid encoding an MVA pathway polypeptide is operably linked to a strong promoter.

[0259] 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. In addition, variants of MVA pathway polypeptide that confer the result of better isoprene production can also be used as well.

[0260] Types of MVA pathway polypeptides and/or DXP pathway polypeptides which can be used and methods of making microorganisms (e.g., facultative anaerobes such as E. coli) encoding MVA pathway polypeptides and/or DXP pathway polypeptides are also described in International Patent Application Publication No. WO2009/076676; U.S. Patent Application Nos. 12/496,573, 12/560,390, 12/560,317, 12/560,370, 12/560,305, and 12/560,366; and U.S. Provisional Patent Application Nos. 61/187,930, 61/187,934, and 61/187,959.

[0261] One of skill in the art can readily select and/or use suitable promoters to optimize the expression of isoprene synthase or and one or more MVA pathway polypeptides and/or one or more DXP pathway polypeptides. Similarly, one of skill in

the art can readily select and/or use suitable vectors (or transfer vehicle) to optimize the expression of isoprene synthase or and one or more MVA pathway polypeptides and/or one or more DXP pathway polypeptides. In some embodiments, the vector contains a selective marker. 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. In some embodiments, an isoprene synthase or MVA pathway nucleic acid integrates into a chromosome of the cells without a selective marker.

Exemplary Iron-sulfur Cluster-Interacting Redox Polypeptides and Nucleic Acids

[0262] As noted above, the iron-sulfur cluster-interacting redox polypeptide plays an essential role in the DXP pathway for isoprenoid biosynthesis. Exemplary iron-sulfur cluster-interacting redox polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of a iron-sulfur cluster-interacting redox polypeptide. Standard methods can be used to determine whether a polypeptide has iron-sulfur cluster-interacting redox polypeptide activity by using a hydrogenase-linked assay measuring the rate of metronidazole[1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] reduction (Chen and Blanchard, Analytical Biochem, 93:216-222 (1979)), which is hereby incorporated by reference in its entirety, especially with respect to the hydrogenase-linked assay for ferredoxin and flavodoxin).

[0263] Exemplary iron-sulfur cluster-interacting redox polypeptide 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 iron-sulfur cluster-interacting redox polypeptide. Exemplary iron-sulfur cluster-interacting redox 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 Methods for Isolating Nucleic Acids

[0264] Iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic, or IDI nucleic acid can be isolated using standard methods. Methods of obtaining desired nucleic acids from a source organism of interest (such as a bacterial genome) are common and well known in the art of molecular biology (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to the isolation of nucleic acids of interest). For example, if the sequence of the nucleic acid is known (such as any of the known nucleic acids described herein), suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes complementary to the desired nucleic acid sequence. Once the sequence is isolated, the DNA may be amplified using standard primer directed amplification methods such as polymerase chain reaction (PCR) (U.S. Patent No. 4,683,202, which is hereby incorporated by reference in its entirety, particularly with respect to PCR methods) to obtain amounts of DNA suitable for transformation using appropriate vectors.

[0265] Alternatively, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic, and/or IDI nucleic acid (such as any isoprene synthase nucleic acid, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, and/or IDI nucleic acid with a known nucleic acid sequence) can be chemically synthesized using standard methods.

[0266] Additional iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic, and/or IDI nucleic acid which may be suitable for use in the compositions and methods described herein can be identified using standard methods. For example, cosmid libraries of the chromosomal DNA of organisms known to produce isoprene naturally can be constructed in organisms such as *E. coli*, and then screened for isoprene production. In particular, cosmid libraries may be created where large segments of genomic DNA (35-45 kb) are packaged into vectors and used to transform appropriate hosts. Cosmid vectors are

unique in being able to accommodate large quantities of DNA. Generally cosmid vectors have at least one copy of the cos DNA sequence which is needed for packaging and subsequent circularization of the heterologous DNA. In addition to the cos sequence, these vectors also contain an origin of replication such as ColEI and drug resistance markers such as a nucleic acid resistant to ampicillin or neomycin. Methods of using cosmid vectors for the transformation of suitable bacterial hosts are well described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to transformation methods.

[0267] 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.

[0268] Additional methods for obtaining iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, and/or IDI nucleic acid include screening a metagenomic library by assay (such as the headspace assay (*see* for example, in US Appl. No.: 12/335,071 and PCT/US2008/086809, which are hereby incorporated by reference in their entireties, particularly with respect to headspace assay for isoprene production in Example 1 and 7) 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 iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, and/or IDI polypeptide. Conserved amino acids for isoprene synthase polypeptides can be identified based on aligned sequences of known

isoprene synthase polypeptides. An organism found to produce isoprene naturally can be subjected to standard protein purification methods (which are well known in the art) and the resulting purified polypeptide can be sequenced using standard methods. Other methods are found in the literature (*see*, for example, Julsing *et al.*, *Applied. Microbiol. Biotechnol.* 75: 1377-84, 2007; Withers *et al.*, *Appl Environ Microbiol.* 73(19):6277-83, 2007, which are each hereby incorporated by reference in their entireties, particularly with respect to identification of nucleic acids involved in the synthesis of isoprene).

[0269] Additionally, standard sequence alignment and/or structure prediction programs can be used to identify additional DXP pathway polypeptides and nucleic acids based on the similarity of their primary and/or predicted polypeptide secondary structure with that of known DXP 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, flavodoxin I, DXP pathway, and/or IDI polypeptides and nucleic acids. The secondary and/or tertiary structure of an iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, and/or IDI 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 iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, and/or IDI polypeptide can be determined using standard methods. Additional iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, and/or IDI nucleic acid can also be identified by hybridization to probes generated from known iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, and/or IDI nucleic acid.

Exemplary Promoters and Vectors

[0270] Any of the iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid described herein can be included in one or more vectors. Accordingly, the invention also features vectors with one more nucleic acids encoding any of the iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, and/or IDI polypeptide 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.

[0271] 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.

[0272] In some embodiments, the vector contains a selective marker. The term "selective marker" refers to a nucleic acid capable of expression in a host cell that allows for ease of selection of those host cells containing an introduced nucleic acid or vector. Examples of selectable markers include, but are not limited to, antibiotic resistance nucleic acids (*e.g.*, kanamycin, ampicillin, carbenicillin, gentamicin, hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol) and/or nucleic acids that confer a metabolic advantage, such as a nutritional advantage on the host cell. Exemplary nutritional selective markers include those markers known in the art as *amd*S, *arg*B, and *pyr4*. Markers useful in vector systems for transformation of *Trichoderma* are known in the art (*see*, *e.g.*, Finkelstein, Chapter 6 in Biotechnology of Filamentous Fungi, Finkelstein *et al., Eds.* Butterworth-Heinemann, Boston, MA, Chap. 6., 1992; and

Kinghorn *et al.*, Applied Molecular Genetics of Filamentous Fungi, Blackie Academic and Professional, Chapman and Hall, London, 1992, which are each hereby incorporated by reference in their entireties, particularly with respect to selective markers). In some embodiments, the selective marker is the *amd*S nucleic acid, which encodes the enzyme acetamidase, allowing transformed cells to grow on acetamide as a nitrogen source. The use of an *A. nidulans amd*S nucleic acid as a selective marker is described in Kelley *et al.*, *EMBO J.* 4:475 – 479, 1985 and Penttila *et al.*, *Gene* 61:155-164, 1987 (which are each hereby incorporated by reference in their entireties, particularly with respect to selective markers). In some embodiments, an isoprene synthase, flavodoxin I, DXP pathway, or IDI nucleic acid integrates into a chromosome of the cells without a selective marker.

[0273] Suitable vectors are those which are compatible with the host cell employed. Suitable vectors can be derived, for example, from a bacterium, a virus (such as bacteriophage T7 or a M-13 derived phage), a cosmid, a yeast, or a plant. Protocols for obtaining and using such vectors are known to those in the art (*see*, for example, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to the use of vectors).

[0274] Promoters are well known in the art. Any promoter that functions in the host cell can be used for expression of an iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid in the host cell. Initiation control regions or promoters, which are useful to drive expression of iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, and/or IDI nucleic acid in various host cells are numerous and familiar to those skilled in the art (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to vectors for the expression of nucleic acids of interest). Virtually any promoter capable of driving these nucleic acids is suitable for the present invention including, but not limited to, CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADCI,

TRP1, URA3, LEU2, ENO, and TPI (useful for expression in *Saccharomyces*); AOX1 (useful for expression in *Pichia*); and lac, trp, λP_L, λP_R, T7, tac, and trc (useful for expression in *E. coli*).

[0275] In some embodiments, a glucose isomerase promoter is used (*see*, for example, U.S. Patent No. 7,132,527 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect promoters and plasmid systems for expressing polypeptides of interest). Reported glucose isomerase promoter mutants can be used to vary the level of expression of the polypeptide encoded by a nucleic acid operably linked to the glucose isomerase promoter (U.S. Patent No. 7,132,527). In various embodiments, the glucose isomerase promoter is contained in a low, medium, or high copy plasmid (U.S. Patent No. 7,132,527).

[0276] In various embodiments, an iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI 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 iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid is operably linked to a T7 promoter. In some embodiments, the heterologous or extra endogenous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI 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 iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid is operably linked to a Trc promoter. In some embodiments, the heterologous or extra endogenous is iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI 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 iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid is operably linked to a Lac promoter. In some embodiments, the heterologous or extra endogenous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid operably linked to a Lac promoter is contained in a low copy plasmid. In some embodiments, the heterologous or extra endogenous iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid is operably linked to an endogenous promoter, such as an endogenous *Escherichia, Panteoa, Bacillus, Yarrowia, Streptomyces, Trichoderma* or *Thermosynechococcus* promoter or an endogenous alkaline serine protease iron-sulfur cluster-interacting redox promoter, DXP pathway promoter, DXP pathway associated promoter, isoprene synthase promoter, or IDI promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase nucleic acid, iron-sulfur cluster-interacting redox nucleic acid , DXP pathway nucleic acid, DXP pathway associated nucleic acid, and/or IDI 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.

[0277] In some embodiments, the vector is any vector which when introduced into a fungal host cell is integrated into the host cell genome and is replicated. Reference is made to the Fungal Genetics Stock Center Catalogue of Strains (FGSC, the world-wide web at "fgsc.net" and the references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to vectors) for a list of vectors. Additional examples of suitable expression and/or integration vectors are provided in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, Current Protocols in Molecular Biology (F. M. Ausubel *et al.* (eds) 1987, Supplement 30, section 7.7.18); van den Hondel *et al.* in Bennett and Lasure (Eds.) More Gene Manipulations in Fungi, Academic Press pp. 396-428, 1991; and U.S. Patent No. 5,874,276, which are each hereby incorporated by reference in their entireties,

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

[0278] In some embodiments, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid is operably linked to a suitable promoter that shows transcriptional activity in a fungal host cell. The promoter may be derived from one or more nucleic acids encoding a polypeptide that is either endogenous or heterologous to the host cell. In some embodiments, the promoter is useful in a *Trichoderma* host. Suitable non-limiting examples of promoters include *cbh*1, *cbh*2, *egl*1, *egl*2, *pepA*, *hfb*1, *hfb*2, *xyn*1, and *amy*. In some embodiments, the promoter is one that is native to the host cell. For example, in some embodiments when *T. reesei* is the host, the promoter is a native *T. reesei* promoter. In some embodiments, the promoter is *T. reesei cbh1*, which is an inducible promoter and has been deposited in GenBank under Accession No. D86235, which is hereby incorporated by reference in its entirety, particularly with respect to promoters. In some embodiments, the promoter is one that is heterologous to the fungal host cell. Other examples of useful promoters include promoters from the genes of *A. awamori* and *A. niger* glucoamylase (glaA) (Nunberg *et al.*, *Mol. Cell Biol.* 4:2306-2315, 1984 and Boel *et al.*, *EMBO J.* 3:1581-1585, 1984, which are each hereby incorporated by reference in their entireties, particularly with respect to promoters); *Aspergillus niger* alpha amylases, *Aspergillus oryzae* TAKA amylase, *T. reesei xln*1, and the *T. reesei cellobiohydrolase* 1 (EP 137280, which is hereby incorporated by reference in its entirety, particularly with respect to promoters).

[0279] In some embodiments, the expression vector also includes a termination sequence. Termination control regions may also be derived from various genes native to the host cell. In some embodiments, the termination sequence and the promoter sequence are derived from the same source. In another embodiment, the termination sequence is endogenous to the host cell. A particularly suitable terminator sequence is *cbh*1 derived from a *Trichoderma* strain (such as *T. reesei*). Other useful fungal terminators include the terminator from an *A. niger* or *A. awamori* glucoamylase nucleic acid (Nunberg *et al.*, *Mol. Cell Biol.* 4:2306-2315, 1984 and Boel *et al.*, *EMBO J.* 3:1581-1585, 1984; which

are each hereby incorporated by reference in their entireties, particularly with respect to fungal terminators). Optionally, a termination site may be included. For effective expression of the polypeptides, DNA encoding the polypeptide are linked operably through initiation codons to selected expression control regions such that expression results in the formation of the appropriate messenger RNA.

[0280] In some embodiments, the promoter, coding, region, and terminator all originate from the iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid to be expressed. In some embodiments, the coding region for iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI 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.

[0281] An iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid can be incorporated into a vector, such as an expression vector, using standard techniques (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982, which is hereby incorporated by reference in its entirety, particularly with respect to the screening of appropriate DNA sequences and the construction of vectors). Methods used to ligate the DNA construct comprising a nucleic acid of interest (such as an iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI 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 iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid and the vector. Then, the compatible ends of the cleaved iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid and the cleaved vector can be ligated. Linking is

generally accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide linkers are used in accordance with conventional practice (*see*, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, and Bennett and Lasure, More Gene Manipulations in Fungi, Academic Press, San Diego, pp 70–76, 1991, which are each hereby incorporated by reference in their entireties, particularly with respect to oligonucleotide linkers). Additionally, vectors can be constructed using known recombination techniques (*e.g.*, Invitrogen Life Technologies, Gateway Technology).

[0282] In some embodiments, it may be desirable to over-express iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid at levels far higher than currently found in naturally-occurring cells. This result may be accomplished by the selective cloning of the nucleic acids encoding those polypeptides into multicopy plasmids or placing those nucleic acids under a strong inducible or constitutive promoter. Methods for over-expressing desired polypeptides are common and well known in the art of molecular biology and examples may be found in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to cloning techniques.

[0283] The following resources include descriptions of additional general methodology useful in accordance with the invention: Kreigler, Gene Transfer and Expression; A Laboratory Manual,1990 and Ausubel *et al.*, Eds. Current Protocols in Molecular Biology, 1994, which are each hereby incorporated by reference in their entireties, particularly with respect to molecular biology and cloning techniques.

Exemplary Source Organisms

[0284] Iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid (and their encoded polypeptides) can be obtained from any organism that naturally contains iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, and/or IDI nucleic

acid. 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 19A). Thus, DXS, DXR, MCT, CMK, MCS, HDS, or HDR 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.

[0285] In some embodiments, the nucleic acid sequence of the iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid 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 iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, or IDI polypeptide is identical to the sequence of a polypeptide that is produced by any of the following organisms in nature. In some embodiments, the iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid or its encoded 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.

[0286] 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.*

[0287] In some embodiments, the fungus is *A. nidulans, A. awamori, A. oryzae, A. aculeatus, A. niger, A. japonicus, T. reesei, T. viride, F. oxysporum,* or *F. solani.* Aspergillus strains are disclosed in Ward et al., Appl. Microbiol. Biotechnol. 39:738-743, 1993 and Goedegebuur et al., Curr Gene 41:89-98, 2002, which are each hereby incorporated by reference in their entireties, particularly with respect to fungi. In particular embodiments, the fungus is a strain of Trichoderma, such as a strain of T. reesei. Strains of T. reesei are known and non-limiting examples include ATCC No. 13631, ATCC No. 26921, ATCC No. 56764, ATCC No. 56765, ATCC No. 56767, and NRRL 15709, which are each hereby incorporated by reference in their entireties, particularly with respect to strains of T. reesei. In some embodiments, the host strain is a derivative of RL-P37. RL-P37 is disclosed in Sheir-Neiss *et al.*, Appl. Microbiol. Biotechnology 20:46–53, 1984, which is hereby incorporated by reference in its entirety, particularly with respect to strains of T. reesei.

[0288] In some embodiments, the source organism is a yeast, such as *Saccharomyces sp., Schizosaccharomyces sp.*, *Pichia sp.,* or *Candida sp.*

[0289] In some embodiments, the source organism is a bacterium, such as strains of *Bacillus* such as *B. lichenformis* or *B. subtilis,* strains of *Pantoea* such as *P. citrea*, strains of *Pseudomonas* such as *P. alcaligenes,* strains of *Streptomyces* such as *S. lividans* or *S. rubiginosus,* strains of *Thermosynechococcus* such as *T. elongatus*, strains of *Sinorhizobium* such as *S. meliloti*, strains of *Helicobacter* such as *H. pylori*, strains of *Agrobacterium* such as A. *tumefaciens*, strains of *Deinococcus* such as *D. radiodurans*, strains of *Listeria* such as *L. monocytogenes*, strains of *Lactobacillus* such as *L. spp,* or strains of *Escherichia* such as *E. coli.*

[0290] 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*.

[0291] In some embodiments, the source organism is a gram-positive bacterium. Non-limiting examples include strains of *Streptomyces* (*e.g.*, *S. lividans*, *S. coelicolor*, or *S. griseus*), *Bacillus*, *Listeria* (*e.g.*, *L. monocytogenes*) or *Lactobacillus* (*e.g.*, *L. spp*). In some embodiments, the source organism is a gram–negative bacterium, such as *E. coli*, *Pseudomonas sp*, or *H. pylori.*

[0292] In some embodiments, the source organism is a plant, such as a plant from the family *Fabaceae*, such as the *Faboideae* subfamily. In some embodiments, the source organism is *kudzu, poplar* (such as *Populus alba x tremula* CAC35696), *aspen* (such as *Populus tremuloides*), *Quercus robur, Arabidopsis (*such as *A. thaliana*), or *Zea* (such as *Z. mays*) .

[0293] In some embodiments, the source organism is an algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates.

[0294] In some embodiments, the source organism is a cyanobacterium, such as cyanobacteria classified into any of the following groups based on morphology: *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales*, or *Stigonematales*. In some embodiments, the cyanobacterium is *Thermosynechococcus elongates*.

Exemplary Host Cells

[0295] A variety of host cells can be used to express iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, MVA pathway polypeptide, MVA pathway associated polypeptide, isoprene synthase polypeptide, or IDI polypeptide 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, and one or more DXP pathway polypeptide and iron-sulfur cluster-interacting redox polypeptides are added to enhance production of isoprene using this pathway. In some embodiments, the host cell naturally produces isoprene using the DXP pathway and isoprene synthase, and one or more DXP pathway nucleic acids, one or more iron-sulfur cluster-interacting redox nucleic acids, and IDI are added to enhance production of isoprene using this pathway.

Exemplary Transformation Methods

[0296] iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, isoprene synthase nucleic acid, or IDI nucleic acid or its 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 iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, isoprene synthase polypeptide, and/or IDI polypeptide. Introduction of a DNA construct or vector into a host cell can be performed using techniques such as transformation, electroporation, nuclear microinjection, transduction, transfection (*e.g.*, lipofection mediated or DEAE-Dextrin mediated transfection or transfection using a recombinant phage virus), incubation with calcium phosphate DNA precipitate, high velocity bombardment with DNA-coated microprojectiles, and protoplast fusion. General transformation techniques are known in the art (*see, e.g.*, Current Protocols in Molecular Biology (F. M. Ausubel *et al.* (eds) Chapter 9, 1987; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989; and Campbell *et al.*, *Curr. Genet.* 16:53-56, 1989, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods). The expression of heterologous polypeptide in *Trichoderma* is described in U.S. Patent No. 6,022,725; U.S. Patent No. 6,268,328; U.S. Patent No. 7,262,041;WO 2005/001036; Harkki *et al.*; *Enzyme Microb. Technol.* 13:227-233, 1991; Harkki *et al.*, *Bio Technol.* 7:596-603, 1989; EP 244,234; EP 215,594; and Nevalainen *et al.*, "*The Molecular Biology of Trichoderma and its Application to the Expression of Both Homologous and Heterologous Genes*," in Molecular Industrial Mycology, *Eds.* Leong and Berka, Marcel Dekker Inc., NY pp. 129 – 148, 1992, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation and expression methods). Reference is also made to Cao *et al.*, (*Sci.* 9:991–1001, 2000; EP 238023; and Yelton *et al.*, *Proceedings. Natl. Acad. Sci.* USA 81:1470-1474, 1984 (which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods) for transformation of *Aspergillus* strains. The introduced nucleic acids may be integrated into chromosomal DNA or maintained as extrachromosomal replicating sequences.

[0297] Any method known in the art may be used to select transformants. In one non-limiting example, stable transformants including an *amd*S marker are distinguished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth, rather than ragged outline on solid culture medium containing acetamide. Additionally, in some cases a further test of stability is conducted by growing the transformants on a solid non-selective medium (*e.g.,* a medium that lacks acetamide), harvesting spores from this culture medium, and determining the percentage of these spores which subsequently germinate and grow on selective medium containing acetamide.

[0298] 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 hereby incorporated by reference in its entirety, particularly with respect to transformation methods). In some embodiments, the mycelia are obtained from germinated vegetative spores. The mycelia are treated with an enzyme that digests the cell wall resulting in protoplasts. The protoplasts are then protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate, and the like. Usually the concentration of these stabilizers varies between 0.8 M and 1.2 M. It is desirable to use about a 1.2 M solution of sorbitol in the suspension medium.

[0299] 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.

[0300] Usually a suspension containing the *Trichoderma sp.* protoplasts or cells that have been subjected to a permeability treatment at a density of 10^5 to 10^7/mL (such as 2 x 10^6/mL) are used in the transformation. A volume of 100 µL of these protoplasts or cells in an appropriate solution (*e.g.*, 1.2 M sorbitol and 50 mM $CaCl_2$) are mixed with the desired DNA. Generally, a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension. In some embodiments, about 0.25 volumes are added to the protoplast suspension. Additives such as dimethyl sulfoxide, heparin, spermidine, potassium chloride, and the like may also be added to the uptake solution and aid in transformation. Similar procedures are available for other fungal host cells (*see*, *e.g.*, U.S. Patent Nos. 6,022,725 and 6,268,328, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods).

[0301] 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.

[0302] The transformation of bacterial cells may be performed according to conventional methods, *e.g.*, as described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982, which is hereby incorporated by reference in its entirety, particularly with respect to transformation methods.

Exemplary Cell Culture Media

[0303] 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.

[0304] 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.

[0305] In some embodiments, the carbon source is a carbohydrate (such as monosaccharide, disaccharide, oligosaccharide, or polysaccharids), invert sugar (*e.g.*, enzymatically treated sucrose syrup), glycerol, glycerine (*e.g.*, a glycerine byproduct of a biodiesel or soap-making process), dihydroxyacetone, one-carbon source, oil (*e.g.*, a plant or vegetable oil such as corn, palm, or soybean oil), animal fat, animal oil, fatty acid (*e.g.*, a saturated fatty acid, unsaturated fatty acid, or polyunsaturated fatty acid), lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, polypeptide (*e.g.,* a microbial or plant protein or peptide), renewable carbon source (*e.g.*, a biomass carbon source such as a hydrolyzed biomass carbon source), yeast extract, component from a yeast extract, polymer, acid, alcohol, aldehyde, ketone, amino acid, succinate, lactate, acetate, ethanol, or any combination of two or more of the foregoing. In some embodiments, the carbon source is a product of photosynthesis, including, but not limited to, glucose.

[0306] 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.

[0307] 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.

[0308] In some embodiments, the cells are cultured under limited glucose conditions. By "limited glucose conditions" is meant that the amount of glucose that is added is less than or about 105% (such as about 100%) of the amount of glucose that is consumed by the cells. In particular embodiments, the amount of glucose that is added to the culture medium is approximately the same as the amount of glucose that is consumed by the cells during a specific period of time. In some embodiments, the rate of cell growth is controlled by limiting the amount of added glucose such that the cells grow at the rate that can be supported by the amount of glucose in the cell medium. In some embodiments, glucose does not accumulate during the time the cells are cultured. In various embodiments, the cells are cultured under limited glucose conditions for greater than or about 1, 2, 3, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, or 70 hours. In various embodiments, the cells are cultured under limited glucose conditions for greater than or

about 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 95, or 100% of the total length of time the cells are cultured. While not intending to be bound by any particular theory, it is believed that limited glucose conditions may allow more favorable regulation of the cells.

[0309] In some embodiments, the cells are cultured in the presence of an excess of glucose. In particular embodiments, the amount of glucose that is added is greater than about 105% (such as about or greater than 110, 120, 150, 175, 200, 250, 300, 400, or 500%) or more of the amount of glucose that is consumed by the cells during a specific period of time. In some embodiments, glucose accumulates during the time the cells are cultured.

[0310] Exemplary lipids are any substance containing one or more fatty acids that are C4 and above fatty acids that are saturated, unsaturated, or branched.

[0311] 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.

[0312] 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.

[0313] 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.

[0314] 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.

[0315] Exemplary renewable carbon sources include cheese whey permeate, cornsteep liquor, sugar beet molasses, barley malt, and components from any of the foregoing. Exemplary renewable carbon sources also include glucose, hexose, pentose and xylose

present in biomass, such as corn, switchgrass, sugar cane, cell waste of fermentation processes, and protein by-product from the milling of soy, corn, or wheat. In some embodiments, the biomass carbon source is a lignocellulosic, hemicellulosic, or cellulosic material such as, but are not limited to, a grass, wheat, wheat straw, bagasse, sugar cane bagasse, soft wood pulp, corn, corn cob or husk, corn kernel, fiber from corn kernels, corn stover, switch grass, rice hull product, or a by-product from wet or dry milling of grains (*e.g.*, corn, sorghum, rye, triticate, barley, wheat, and/or distillers grains). Exemplary cellulosic materials include wood, paper and pulp waste, herbaceous plants, and fruit pulp. In some embodiments, the carbon source includes any plant part, such as stems, grains, roots, or tubers. In some embodiments, all or part of any of the following plants are used as a carbon source: corn, wheat, rye, sorghum, triticate, rice, millet, barley, cassava, legumes, such as beans and peas, potatoes, sweet potatoes, bananas, sugarcane, and/or tapioca. In some embodiments, the carbon source is a biomass hydrolysate, such as a biomass hydrolysate that includes both xylose and glucose or that includes both sucrose and glucose.

[0316] In some embodiments, the renewable carbon source (such as biomass) is pretreated before it is added to the cell culture medium. In some embodiments, the pretreatment includes enzymatic pretreatment, chemical pretreatment, or a combination of both enzymatic and chemical pretreatment (*see*, for example, Farzaneh *et al.*, *Bioresource Technology* 96 (18): 2014-2018, 2005; U.S. Patent No. 6,176,176; U.S. Patent No. 6,106,888; which are each hereby incorporated by reference in their entireties, particularly with respect to the pretreatment of renewable carbon sources). In some embodiments, the renewable carbon source is partially or completely hydrolyzed before it is added to the cell culture medium.

[0317] 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.

[0318] 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.

[0319] 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).

[0320] Additionally the carbon source may also be one-carbon substrates such as carbon dioxide, or methanol. Glycerol production from single carbon sources (*e.g.*, methanol, formaldehyde, or formate) has been reported in methylotrophic yeasts (Yamada *et al.*, *Agric. Biol. Chem.,* 53(2) 541-543, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources) and in bacteria (Hunter *et. al.*, *Biochemistry,* 24, 4148-4155, 1985, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources). These organisms can assimilate single carbon compounds, ranging in oxidation state from methane to formate, and produce glycerol. The pathway of carbon assimilation can be through ribulose monophosphate, through serine, or through xylulose-momophosphate (Gottschalk, Bacterial Metabolism, Second Edition, Springer-Verlag: New York, 1986, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources). The ribulose monophosphate pathway involves the condensation of formate with ribulose-5-phosphate to form a six carbon sugar that becomes fructose and eventually the three carbon product glyceraldehyde-3-phosphate. Likewise, the serine pathway assimilates the one-carbon compound into the glycolytic pathway via methylenetetrahydrofolate.

[0321] In addition to one and two carbon substrates, methylotrophic organisms are also known to utilize a number of other carbon containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion *et al.*, *Microb. Growth Cl Compd.,* [Int. Symp.], 7th ed., 415-32. Editors: Murrell *et al.*, Publisher: Intercept, Andover, UK, 1993, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources).

Similarly, various species of *Candida* metabolize alanine or oleic acid (Sulter *et al.*, *Arch. Microbiol.* 153(5), 485-9, 1990, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources).

[0322] In some embodiments, cells are cultured in a standard medium containing physiological salts and nutrients (*see*, *e.g.*, Pourquie, J. *et al.*, Biochemistry and Genetics of Cellulose Degradation, *eds.* Aubert *et al.*, Academic Press, pp. 71-86, 1988 and Ilmen *et al.*, *Appl. Environ. Microbiol.* 63:1298-1306, 1997, which are each hereby incorporated by reference in their entireties, particularly with respect to cell medias). Exemplary growth media are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, or Yeast medium (YM) broth. Other defined or synthetic growth media may also be used, and the appropriate medium for growth of particular host cells are known by someone skilled in the art of microbiology or fermentation science.

[0323] In addition to an appropriate carbon source, the cell medium desirably contains suitable minerals, salts, cofactors, buffers, and other components known to those skilled in the art suitable for the growth of the cultures or the enhancement of isoprene production (*see*, for example, WO 2004/033646 and references cited therein and WO 96/35796 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect cell medias and cell culture conditions). In some embodiments where an isoprene synthase nucleic acid, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, or IDI 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 polypeptide, iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, or IDI 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 isoprene synthase nucleic acid, iron-sulfur cluster-interacting redox nucleic

acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, or IDI nucleic acid.

Exemplary Cell Culture Conditions

[0324] Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Exemplary techniques may be found in Manual of Methods for General Bacteriology Gerhardt *et al.*, *eds*), American Society for Microbiology, Washington, D.C. (1994) or Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA, which are each hereby incorporated by reference in their entireties, particularly with respect to cell culture techniques. In some embodiments, the cells are cultured in a culture medium under conditions permitting the expression of one or more isoprene synthase polypeptide, iron-sulfur cluster-interacting redox polypeptide, DXP pathway polypeptide, DXP pathway associated polypeptide, or IDI polypeptide encoded by a nucleic acid inserted into the host cells.

[0325] Standard cell culture conditions can be used to culture the cells (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to cell culture and fermentation conditions). Cells are grown and maintained at an appropriate temperature, gas mixture, and pH (such as at about 20 to about 37°C, at about 6% to about 84% CO_2, and at a pH between about 5 to about 9). In some embodiments, cells are grown at 35 °C in an appropriate cell medium. In some embodiments, *e.g.*, cultures are cultured at approximately 28 ºC 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.

[0326] 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.

[0327] In some embodiments, a variation on the standard batch system is used, such as the Fed-Batch system. Fed-Batch fermentation processes comprise a typical batch system with the exception that the carbon source is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of carbon source in the cell medium. Fed-batch fermentations may be performed with the carbon source (*e.g.*, glucose) in a limited or excess amount. Measurement of the actual carbon source concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen, and the partial pressure of waste gases such as CO_2. Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in Brock, Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., which is hereby incorporated by reference in its entirety, particularly with respect to cell culture and fermentation conditions.

[0328] 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.

[0329] Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or isoprene production. For example, one method maintains a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allows all other parameters to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration (*e.g.*, the concentration measured by media turbidity) is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, the cell loss due to media being drawn off is balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., which is hereby incorporated by reference in its entirety, particularly with respect to cell culture and fermentation conditions.

[0330] In some embodiments, cells are immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isoprene production.

[0331] 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.

[0332] 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).

[0333] 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 nucleic acid, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, or IDI nucleic acid, operably linked to the promoter. In some embodiments, a compound (such as IPTG) is added to induce expression of the isoprene synthase nucleic acid, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, or IDI nucleic acid operably linked to the promoter.

Exemplary Methods for Decoupling Isoprene Production from Cell Growth.

[0334] The invention provides, *inter alia*, compositions and methods for increasing the production of isoprene from cultured cells. When feedstock is used, it is desirable for the carbon from the feedstock to be converted to isoprene rather than to the growth and maintenance of the cells. In some embodiments, the cells are grown to a low to medium OD_{600}, then production of isoprene is started or increased. This strategy permits a large portion of the carbon to be converted to isoprene.

[0335] In some embodiments, cells reach an optical density such that they no longer divide or divide extremely slowly, but continue to make isoprene for several hours (such as about 2, 4, 6, 8, 10, 15, 20, 25, 30, or more hours). In some cases, the optical density

at 550 nm decreases over time (such as a decrease in the optical density after the cells are no longer in an exponential growth phase due to cell lysis), and the cells continue to produce a substantial amount of isoprene. In some embodiments, the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%) over a certain time period (such as greater than or about 5, 10, 15, 20, 25, 30, 40, 50 or 60 hours), and the cells produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr) during this time period. In some embodiments, the amount of isoprene is between about 2 to about 5,000 nmole/g_{wcm}/hr, such as between about 2 to about 100 nmole/g_{wcm}/hr, about 100 to about 500 nmole/g_{wcm}/hr, about 150 to about 500 nmole/g_{wcm} /hr, about 500 to about 1,000 nmole/g_{wcm}/hr, about 1,000 to about 2,000 nmole/g_{wcm}/hr, or about 2,000 to about 5,000 nmole/g_{wcm}/hr. In some embodiments, the amount of isoprene is between about 20 to about 5,000 nmole/g_{wcm}/hr, about 100 to about 5,000 nmole/g_{wcm}/hr, about 200 to about 2,000 nmole/g_{wcm}/hr, about 200 to about 1,000 nmole/g_{wcm}/hr, about 300 to about 1,000 nmole/g_{wcm}/hr, or about 400 to about 1,000 nmole/g_{wcm}/hr.

[0336] In some embodiments, the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%) over a certain time period (such as greater than or about 5, 10, 15, 20, 25, 30, 40, 50 or 60 hours), and the cells produce a cumulative titer (total amount) of isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 50,000, 100,000, or more mg of isoprene/L of broth (mg/L_{broth}, wherein the volume of broth includes the volume of the cells and the cell medium) during this time period. In some embodiments, the amount of isoprene is between about 2 to about 5,000 mg/L_{broth}, such as between about 2 to about 100 mg/L_{broth}, about 100 to about 500 mg/L_{broth}, about 500 to about 1,000 mg/L_{broth}, about 1,000 to about 2,000 mg/L_{broth}, or about 2,000 to about 5,000 mg/L_{broth}. In some embodiments, the amount of isoprene is between about 20 to about 5,000 mg/L_{broth}, about 100 to about 5,000 mg/L_{broth}, about 200 to about 2,000 mg/L_{broth}, about 200 to about 1,000 mg/L_{broth}, about 300 to about 1,000 mg/L_{broth}, or about 400 to about 1,000 mg/L_{broth}.

[0337] In some embodiments, the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%) over a certain time period (such as greater than or about 5, 10, 15, 20, 25, 30, 40, 50 or 60 hours), and the cells convert greater than or about 0.0015, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, or 8.0% of the carbon in the cell culture medium into isoprene during this time period. In some embodiments, the percent conversion of carbon into isoprene is between such as about 0.002 to about 4.0%, about 0.002 to about 3.0%, about 0.002 to about 2.0%, about 0.002 to about 1.6%, about 0.002 to about 0.005%, about 0.005 to about 0.01%, about 0.01 to about 0.05%, about 0.05 to about 0.15%, 0.15 to about 0.2%, about 0.2 to about 0.3%, about 0.3 to about 0.5%, about 0.5 to about 0.8%, about 0.8 to about 1.0%, or about 1.0 to about 1.6%. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about 0.4%, 0.002 to about 0.16%, 0.04 to about 0.16%, about 0.005 to about 0.3%, about 0.01 to about 0.3%, or about 0.05 to about 0.3%.

[0338] In some embodiments, isoprene is only produced in stationary phase. In some embodiments, isoprene is produced in both the growth phase and stationary phase. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of isoprene produced during the growth phase for the same length of time. In various embodiments, greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99% or more of the total amount of isoprene that is produced (such as the production of isoprene during a fermentation for a certain amount of time, such as 20 hours) is produced while the cells are in stationary phase. In various embodiments, greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99% or more of the total amount of isoprene that is produced (such as the production of isoprene during a fermentation for a certain amount of time, such as 20 hours) is produced while the cells divide slowly or not at all such that the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%). In some embodiments, isoprene is only produced in the growth phase.

[0339] In some embodiments, one or more isoprene synthase nucleic acid, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, and/or IDI nucleic acid are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more isoprene synthase nucleic acid, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, and/or IDI nucleic acid may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more isoprene synthase nucleic acid, iron-sulfur cluster-interacting redox nucleic acid, DXP pathway nucleic acid, DXP pathway associated nucleic acid, and/or IDI nucleic acid are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

Production of Isoprene within Safe Operating Ranges

[0340] The invention provides, *inter alia*, compositions and methods for increasing the production of isoprene from cultured cells. The production of isoprene within safe operating levels according to its flammability characteristics simplifies the design and construction of commercial facilities, vastly improves the ability to operate safely, and limits the potential for fires to occur. In particular, the optimal ranges for the production of isoprene are within the safe zone, *i.e.*, the nonflammable range of isoprene concentrations. In one such aspect, the invention features a method for the production of isoprene within the nonflammable range of isoprene concentrations (outside the flammability envelope of isoprene).

[0341] Thus, computer modeling and experimental testing were used to determine the flammability limits of isoprene (such as isoprene in the presence of O_2, N_2, CO_2, or any combination of two or more of the foregoing gases) in order to ensure process safety. The flammability envelope is characterized by the lower flammability limit (LFL), the upper flammability limit (UFL), the limiting oxygen concentration (LOC), and the limiting temperature. For a system to be flammable, a minimum amount of fuel (such as isoprene) must be in the presence of a minimum amount of oxidant, typically oxygen.

The LFL is the minimum amount of isoprene that must be present to sustain burning, while the UFL is the maximum amount of isoprene that can be present. Above this limit, the mixture is fuel rich and the fraction of oxygen is too low to have a flammable mixture. The LOC indicates the minimum fraction of oxygen that must also be present to have a flammable mixture. The limiting temperature is based on the flash point of isoprene and is that lowest temperature at which combustion of isoprene can propagate. These limits are specific to the concentration of isoprene, type and concentration of oxidant, inerts present in the system, temperature, and pressure of the system. Compositions that fall within the limits of the flammability envelope propagate combustion and require additional safety precautions in both the design and operation of process equipment.

[0342] The following conditions were tested using computer simulation and mathematical analysis and experimental testing. If desired, other conditions (such as other temperature, pressure, and permanent gas compositions) may be tested using the methods described herein to determine the LFL, UFL, and LOC concentrations.

(1) Computer simulation and mathematical analysis

Test Suite 1:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

Test Suite 2:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

Saturated with H_2O

Test Suite 3:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

CO_2: 5 wt% - 30 wt%

(2) Experimental testing for final determination of flammability limits

Test Suite 1:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

Test Suite 2:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

Saturated with H_2O

[0343] Simulation software was used to give an estimate of the flammability characteristics of the system for several different testing conditions. CO_2 showed no significant affect on the system's flammability limits. Test suites 1 and 2 were confirmed by experimental testing. The modeling results were in-line with the experimental test results. Only slight variations were found with the addition of water.

[0344] The LOC was determined to be 9.5 vol% for an isoprene, O_2, N_2, and CO_2 mixture at 40°C and 1 atmosphere. The addition of up to 30% CO_2 did not significantly affect the flammability characteristics of an isoprene, O_2, and N_2 mixture. Only slight variations in flammability characteristics were shown between a dry and water saturated isoprene, O_2, and N_2 system. The limiting temperature is about -54 °C. Temperatures below about -54 °C are too low to propagate combustion of isoprene.

[0345] In some embodiments, the LFL of isoprene ranges from about 1.5 vol.% to about 2.0 vol%, and the UFL of isoprene ranges from about 2.0 vol.% to about 12.0

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

[0346] In some embodiments, isoprene is produced in the presence of less than about 9.5 vol% oxygen (that is, below the LOC required to have a flammable mixture of isoprene). In some embodiments in which isoprene is produced in the presence of greater than or about 9.5 vol% oxygen, the isoprene concentration is below the LFL (such as below about 1.5 vol.%). For example, the amount of isoprene can be kept below the LFL by diluting the isoprene composition with an inert gas (*e.g.*, by continuously or periodically adding an inert gas such as nitrogen to keep the isoprene composition below the LFL). In some embodiments in which isoprene is produced in the presence of greater than or about 9.5 vol% oxygen, the isoprene concentration is above the UFL (such as above about 12 vol.%). For example, the amount of isoprene can be kept above the UFL by using a system (such as any of the cell culture systems described herein) that produces isoprene at a concentration above the UFL. If desired, a relatively low level of oxygen can be used so that the UFL is also relatively low. In this case, a lower isoprene concentration is needed to remain above the UFL.

[0347] In some embodiments in which isoprene is produced in the presence of greater than or about 9.5 vol% oxygen, the isoprene concentration is within the flammability envelope (such as between the LFL and the UFL). In some embodiments when the isoprene concentration may fall within the flammability envelope, one or more steps are performed to reduce the probability of a fire or explosion. For example, one or more sources of ignition (such as any materials that may generate a spark) can be avoided. In some embodiments, one or more steps are performed to reduce the amount of time that the concentration of isoprene remains within the flammability envelope. In some embodiments, a sensor is used to detect when the concentration of isoprene is close to or within the flammability envelope. If desired, the concentration of isoprene can be

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

[0348] In some embodiments, the amount of flammable volatiles other than isoprene (such as one or more sugars) is at least about 2, 5, 10, 50, 75, or 100-fold less than the amount of isoprene produced. In some embodiments, the portion of the gas phase other than isoprene gas comprises between about 0% to about 100% (volume) oxygen, such as between about 0% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 90% to about 90%, or about 90% to about 100% (volume) oxygen. In some embodiments, the portion of the gas phase other than isoprene gas comprises between about 0% to about 99% (volume) nitrogen, such as between about 0% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 90% to about 90%, or about 90% to about 99% (volume) nitrogen.

[0349] In some embodiments, the portion of the gas phase other than isoprene gas comprises between about 1% to about 50% (volume) CO_2, such as between about 1% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, or about 40% to about 50% (volume) CO_2.

[0350] In some embodiments, an isoprene composition also contains ethanol. For example, ethanol may be used for extractive distillation of isoprene, resulting in compositions (such as intermediate product streams) that include both ethanol and isoprene. Desirably, the amount of ethanol is outside the flammability envelope for

ethanol. The LOC of ethanol is about 8.7 vol%, and the LFL for ethanol is about 3.3 vol% at standard conditions, such as about 1 atmosphere and about 60 °F (NFPA 69 *Standard on Explosion Prevention Systems*, 2008 edition, which is hereby incorporated by reference in its entirety, particularly with respect to LOC, LFL, and UFL values). In some embodiments, compositions that include isoprene and ethanol are produced in the presence of less than the LOC required to have a flammable mixture of ethanol (such as less than about 8.7% vol%). In some embodiments in which compositions that include isoprene and ethanol are produced in the presence of greater than or about the LOC required to have a flammable mixture of ethanol, the ethanol concentration is below the LFL (such as less than about 3.3 vol.%).

[0351] In various embodiments, the amount of oxidant (such as oxygen) is below the LOC of any fuel in the system (such as isoprene or ethanol). In various embodiments, the amount of oxidant (such as oxygen) is less than about 60, 40, 30, 20, 10, or 5% of the LOC of isoprene or ethanol. In various embodiments, the amount of oxidant (such as oxygen) is less than the LOC of isoprene or ethanol by at least 2, 4, 5, or more absolute percentage points (vol %). In particular embodiments, the amount of oxygen is at least 2 absolute percentage points (vol %) less than the LOC of isoprene or ethanol (such as an oxygen concentration of less than 7.5 vol% when the LOC of isoprene is 9.5 vol%). In various embodiments, the amount of fuel (such as isoprene or ethanol) is less than or about 25, 20, 15, 10, or 5% of the LFL for that fuel.

Exemplary Production of Isoprene

[0352] The invention provides, *inter alia*, compositions and methods for increasing the production of isoprene from cultured cells using various DXP pathway enzymes in combination with iron-sulfur cluster-interacting redox genes or polypeptides and isoprene synthase genes or polypeptides, optionally with IDI and DXP pathway associated genes and polypeptides. 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.

[0353] By "peak specific productivity" is meant the maximum amount of isoprene produced per cell during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak specific productivity time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per cell is at a maximum. The specific productivity is determined by dividing the total productivity by the amount of cells, as determined by optical density at 600nm (OD600). In some embodiments, the isoprene amount is measured at the peak specific productivity time point. In some embodiments, the peak specific productivity for the cells is about any of the isoprene amounts per cell disclosed herein.

[0354] 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.

[0355] By "relative detector response" refers to the ratio between the detector response (such as the GC/MS area) for one compound (such as isoprene) to the detector response (such as the GC/MS area) of one or more compounds (such as all C5 hydrocarbons). The detector response may be measured as described herein, such as the GC/MS analysis performed with an Agilent 6890 GC/MS system fitted with an Agilent HP-5MS GC/MS column (30 m x 250 μm; 0.25 μm film thickness). If desired, the relative detector response can be converted to a weight percentage using the response factors for each of

the compounds. This response factor is a measure of how much signal is generated for a given amount of a particular compound (that is, how sensitive the detector is to a particular compound). This response factor can be used as a correction factor to convert the relative detector response to a weight percentage when the detector has different sensitivities to the compounds being compared. Alternatively, the weight percentage can be approximated by assuming that the response factors are the same for the compounds being compared. Thus, the weight percentage can be assumed to be approximately the same as the relative detector response.

[0356] In some embodiments, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr). In some embodiments, the amount of isoprene is between about 2 to about 5,000 nmole/g_{wcm}/hr, such as between about 2 to about 100 nmole/g_{wcm}/hr, about 100 to about 500 nmole/g_{wcm}/hr, about 150 to about 500 nmole/g_{wcm} /hr, about 500 to about 1,000 nmole/g_{wcm}/hr, about 1,000 to about 2,000 nmole/g_{wcm}/hr, or about 2,000 to about 5,000 nmole/g_{wcm}/hr. In some embodiments, the amount of isoprene is between about 20 to about 5,000 nmole/g_{wcm}/hr, about 100 to about 5,000 nmole/g_{wcm}/hr, about 200 to about 2,000 nmole/g_{wcm}/hr, about 200 to about 1,000 nmole/g_{wcm}/hr, about 300 to about 1,000 nmole/g_{wcm}/hr, or about 400 to about 1,000 nmole/g_{wcm}/hr.

[0357] The amount of isoprene in units of nmole/g_{wcm}/hr can be measured as disclosed in U.S. Patent No. 5,849,970, which is hereby incorporated by reference in its entirety, particularly with respect to the measurement of isoprene production. For example, two mL of headspace (*e.g.*, headspace from a culture such as 2 mL of culture cultured in sealed vials at 32°C with shaking at 200 rpm for approximately 3 hours) are analyzed for isoprene using a standard gas chromatography system, such as a system operated isothermally (85°C) with an n-octane/porasil C column (Alltech Associates, Inc., Deerfield, Ill.) and coupled to a RGD2 mercuric oxide reduction gas detector (Trace Analytical, Menlo Park, CA) (*see*, for example, Greenberg et al, *Atmos. Environ.* 27A: 2689-2692, 1993; Silver *et al.*, *Plant Physiol.* 97:1588-1591, 1991, which are each hereby

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

[0358] 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).

[0359] 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}.

[0360] In some embodiments, the cells in culture produce at least about 2 g/ L_{broth}, at least about 2.1 g/ L_{broth}, at least about 2.2 g/ L_{broth}, at least about 2.3 g/ L_{broth}, at least about 2.4 g/ L_{broth}, at least about 2.5 g/ L_{broth}, at least about 2.6 g/ L_{broth}, at least about 2.7 g/ L_{broth}, at least about 2.8 g/ L_{broth}, at least about 2.9 g/ L_{broth}, at least about 3.0 g/ L_{broth}, at least about 3.2 g/ L_{broth}, at least about 3.5 g/ L_{broth}, at least about 3.7 g/ L_{broth}, or at least about 4.0 g/ L_{broth}.

[0361] 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.

[0362] 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}.

[0363] In some embodiments, the cells in culture convert greater than or about 0.0015, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, or 8.0% of the carbon in the cell culture medium into isoprene. In some embodiments, the percent conversion of carbon into isoprene is between such as about 0.002 to about 4.0%, about 0.002 to about 3.0%, about 0.002 to about 2.0%, about 0.002 to about 1.6%, about 0.002 to about 0.005%, about 0.005 to about 0.01%, about 0.01 to about 0.05%, about 0.05 to about 0.15%, 0.15 to about 0.2%, about 0.2 to about 0.3%, about 0.3 to about 0.5%, about 0.5 to about 0.8%, about 0.8 to about 1.0%, or about 1.0 to about 1.6%. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about 0.4%, 0.002 to about 0.16%, 0.04 to about 0.16%, about 0.005 to about 0.3%, about 0.01 to about 0.3%, or about 0.05 to about 0.3%.

[0364] 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

[0365] 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%

[0366] 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.

[0367] One skilled in the art can readily convert the rates of isoprene production or amount of isoprene produced into any other units. Exemplary equations are listed below for interconverting between units.

Units for Rate of Isoprene production (total and specific)

Equation 3

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

Equation 4

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

Equation 5

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

Equation 6

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

Equation 7

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

Units for Titer (total and specific)

Equation 8

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

Equation 9

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

[0368] 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

[0369] If desired, Equation 11 can be used to convert between units of ppm and ug/L. In particular, "ppm" means parts per million defined in terms of ug/g (w/w). Concentrations of gases can also be expressed on a volumetric basis using "ppmv" (parts per million by volume), defined in terms of uL/L (vol/vol). Conversion of ug/L to ppm (*e.g.*, ug of analyte per g of gas) can be performed by determining the mass per L of off-gas (*i.e.*, the density of the gas). For example, a liter of air at standard temperature and pressure (STP; 101.3 kPa(1 bar) and 273.15K) has a density of approximately 1.29 g/L. Thus, a concentration of 1 ppm (ug/g) equals 1.29 ug/L at STP (equation 11). The conversion of ppm (ug/g) to ug/L is a function of both pressure, temperature, and overall composition of the off-gas.

Equation 11

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

[0370] Conversion of ug/L to ppmv (*e.g.*, uL of analyte per L of gas) can be performed using the Universal Gas Law (equation 12). For example, an off-gas concentration of 1000 ug/L_{gas} corresponds to 14.7 umol/L_{gas}. The universal gas constant is 0.082057 L.atm $K^{-1}mol^{-1}$, so using equation 12, the volume occupied by 14.7 umol of HG at STP is equal to 0.329 mL. Therefore, the concentration of 1000 ug/L HG is equal to 329 ppmv or 0.0329% (v/v) at STP.

Equation 12

[0371] PV = nRT, where "P" is pressure, "V" is volume, "n" is moles of gas, "R" is the Universal gas constant, and "T" is temperature in Kelvin.

[0372] The amount of impurities in isoprene compositions are typically measured herein on a weight per volume (w/v) basis in units such as ug/L. If desired, measurements in units of ug/L can be converted to units of mg/m^3 using equation 13.

Equation 13

1 ug/L = 1 mg/m^3

[0373] 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.

[0374] 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 DXP 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.

[0375] In some embodiments, the isoprene composition comprises greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has a relative detector response of greater than or about 99.90, 99.91, 99.92, 99.93, 99.94, 99.95, 99.96, 99.97, 99.98, 99.99, or 100% for isoprene compared to the detector response for all C5 hydrocarbons in the composition. In some embodiments, the isoprene composition comprises between about 99.90 to about 99.92, about 99.92 to about 99.94, about 99.94 to about 99.96, about 99.96 to about 99.98, about 99.98 to 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition.

[0376] In some embodiments, the isoprene composition comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such as 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has a relative detector response of less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for C5 hydrocarbons other than isoprene compared to the detector response for all C5 hydrocarbons in the composition. In some embodiments, the composition has a relative detector response of less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne compared to the detector response for all C5 hydrocarbons in the composition. In some embodiments, the isoprene composition comprises between about 0.02 to about 0.04%, about 0.04 to about

0.06%, about 0.06 to 0.08%, about 0.08 to 0.10%, or about 0.10 to about 0.12% C5 hydrocarbons other than isoprene (such as 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the composition.

[0377] In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In some embodiments, the isoprene composition comprises between about 0.005 to about 50, such as about 0.01 to about 10, about 0.01 to about 5, about 0.01 to about 1, about 0.01 to about 0.5, or about 0.01 to about 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a hydrocarbon other than isoprene (such as 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne). In some embodiments, the isoprene composition comprises between about 0.005 to about 50, such as about 0.01 to about 10, about 0.01 to about 5, about 0.01 to about 1, about 0.01 to about 0.5, or about 0.01 to about 0.005 ug/L of a hydrocarbon other than isoprene. In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a protein or fatty acid (such as a protein or fatty acid that is naturally associated with natural rubber).

[0378] In some embodiments, the isoprene composition comprises less than or about 10, 5, 1, 0.8, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of alpha acetylenes, piperylenes, acetonitrile, or 1,3-cyclopentadiene. In some embodiments, the isoprene composition comprises less than or about 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of sulfur or allenes. In some embodiments, the isoprene composition comprises less than or about 30, 20, 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of all acetylenes (such as pentyne-1, butyne-2, 2MB1-3yne, and 1-pentyne-4yne). In some embodiments, the isoprene composition

comprises less than or about 2000, 1000, 500, 200, 100, 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of isoprene dimers, such as cyclic isoprene dimmers (*e.g.*, cyclic C10 compounds derived from the dimerization of two isoprene units).

[0379] In some embodiments, the composition comprises greater than about 2 mg of isoprene, such as greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 mg of isoprene. In some embodiments, the composition comprises greater than or about 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 g of isoprene. In some embodiments, the amount of isoprene in the composition is between about 2 to about 5,000 mg, such as between about 2 to about 100 mg, about 100 to about 500 mg, about 500 to about 1,000 mg, about 1,000 to about 2,000 mg, or about 2,000 to about 5,000 mg. In some embodiments, the amount of isoprene in the composition is between about 20 to about 5,000 mg, about 100 to about 5,000 mg, about 200 to about 2,000 mg, about 200 to about 1,000 mg, about 300 to about 1,000 mg, or about 400 to about 1,000 mg. In some embodiments, greater than or about 20, 25, 30, 40, 50, 60, 70, 80, 90, or 95% by weight of the volatile organic fraction of the composition is isoprene.

[0380] In some embodiments, the composition includes ethanol. In some embodiments, the composition includes between about 75 to about 90% by weight of ethanol, such as between about 75 to about 80%, about 80 to about 85%, or about 85 to about 90% by weight of ethanol. In some embodiments in which the composition includes ethanol, the composition also includes between about 4 to about 15% by weight of isoprene, such as between about 4 to about 8%, about 8 to about 12%, or about 12 to about 15% by weight of isoprene.

Exemplary Isoprene Purification Methods

[0381] In some embodiments, any of the methods described herein further include recovering the isoprene. For example, the isoprene produced using the compositions and methods of the invention can be recovered using standard techniques, such as gas stripping, membrane enhanced separation, fractionation, adsorption/desorption, pervaporation, thermal or vacuum desorption of isoprene from a solid phase, or extraction of isoprene immobilized or absorbed to a solid phase with a solvent (see, for example,

U.S. Patent Nos. 4,703,007 and 4,570,029, which are each hereby incorporated by reference in their entireties, particularly with respect to isoprene recovery and purification methods). In one embodiment, the isoprene is recovered by absorption stripping (*see, e.g.*, U.S. Appl. 61/288,142). In particular, embodiments, extractive distillation with an alcohol (such as ethanol, methanol, propanol, or a combination thereof) is used to recover the isoprene. In some embodiments, the recovery of isoprene involves the isolation of isoprene in a liquid form (such as a neat solution of isoprene or a solution of isoprene in a solvent). Gas stripping involves the removal of isoprene vapor from the fermentation off-gas stream in a continuous manner. Such removal can be achieved in several different ways including, but not limited to, adsorption to a solid phase, partition into a liquid phase, or direct condensation (such as condensation due to exposure to a condensation coil or do to an increase in pressure). In some embodiments, membrane enrichment of a dilute isoprene vapor stream above the dew point of the vapor resulting in the condensation of liquid isoprene. In some embodiments, the isoprene is compressed and condensed.

[0382] 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. In one embodiment, the isoprene is recovered by using absorption stripping as described in U.S. Provisional Appl. No. 61/288,142.

[0383] 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.

[0384] In some embodiments, at least a portion of the gas phase remaining after one or more recovery steps for the removal of isoprene is recycled by introducing the gas phase into a cell culture system (such as a fermentor) for the production of isoprene.

[0385] In some embodiments, any of the methods described herein further include polymerizing the isoprene. For example, standard methods can be used to polymerize the purified isoprene to form *cis*-polyisoprene or other down stream products using standard methods. Accordingly, the invention also features a tire comprising polyisoprene, such as *cis*-1,4- polyisoprene and/or *trans*-1,4- polyisoprene made from any of the isoprene compositions disclosed herein.

EXAMPLES

[0386] The examples, which are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way, also describe and detail aspects and embodiments of the invention discussed above. Unless indicated otherwise, temperature is in degrees Centigrade and pressure is at or near atmospheric. The foregoing examples and detailed description are offered by way of illustration and not by way of limitation. All publications, patent applications, and patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or patent were specifically and individually indicated to be incorporated by reference. In particular, all publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention. Although the foregoing invention

has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

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

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

[0387] 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).

[0388] 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).

[0389] The kudzu isoprene synthase gene was also cloned into the low copy number plasmid pCL1920. Primers were used to amplify the kudzu isoprene synthase gene from pTrcKudzu described above. The forward primer added a *Hin*dIII site and an *E. coli* consensus RBS to the 5' end. The *Pst*I cloning site was already present in pTrcKudzu just 3' of the stop codon so the reverse primer was constructed such that the final PCR product includes the *Pst*I site. The sequences of the primers were: HindIII-rbs-Kudzu F: 5'-CATATGAAAGCTTGTATCGATTAAATAAGGAGGAATAAACC (SEQ ID NO:6) and BamH1-Kudzu R:

[0390] 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 *Hin*dIII and *Pst*I and ligated into pCL1920 which had also been digested with *Hin*dIII and *Pst*I. The ligation mix was transformed into *E. coli* Top10. Several transformants were checked by sequencing. The resulting plasmid was designated pCL-lac-Kudzu (Figures 6 and 7).

II. Determination of isoprene production

[0391] 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).

[0392] 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).

[0393] The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (30 m x 0.25 mm; 0.25 μm film thickness) was used for separation of analytes. The sampler was set up to inject 500 μL of headspace gas. The GC/MS method utilized helium as the carrier gas at a flow of 1 ml/min. The injection port was held at 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 2000 μg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method.

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

[0394] The vectors described above were introduced to *E. coli* strain BL21 (Novagen) to produce strains BL21/ptrcKudzu, BL21/pCL-lac-Kudzu and BL21/pETHisKudzu. The strains were spread for isolation onto LA (Luria agar) + carbenicillin (50 μg/ml) and incubated overnight at 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) + 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

[0395] 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, $ZnSO_4$ * $7H_2O$ 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, then q.s. to volume and filter sterilized with a 0.22 μ filter.

[0396] 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

[0397] 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 26 and 27), was verified by sequencing.

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

[0398] 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

[0399] 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

[0400] 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:29)

CF 07-43 (-) Fuse aprE promoter to Kudzu ispS
5'- ATTGAGAAGAGGTCGCACACACTCTTTACCCTCTCCTTTTA (SEQ ID NO:30)

b) Amplification of the isoprene synthase gene

[0401] 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:31)

CF 07-45 (-) Fuse the 3' end of kudzu isoprene synthase gene to the terminator
5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:32)

c) Amplification of the transcription terminator

[0402] 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:33)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:34)

[0403] 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:32)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:34)

[0404] 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:35)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:34)

[0405] The fusion PCR fragment was purified using a Qiagen kit and digested with the restriction enzymes *Mfe*I and BamHI. 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.

[0406] The ligation mix was transformed into *E. coli* Top 10 cells and colonies were selected on LA+50 carbenicillin plates. A total of six colonies were chosen and grown overnight in LB+50 carbenicillin and then plasmids were isolated using a Qiagen kit. The plasmids were digested with *Eco*RI and *Bam*HI to check for inserts and three of the correct plasmids were sent in for sequencing with the following primers:

CF 149 (+) EcoRI start of *aprE* promoter
5'- GACATGAATTCCTCCATTTTCTTCTGC (SEQ ID NO:36)

CF 847 (+) Sequence in pXX 049 (end of *aprE* promoter)
5'- AGGAGAGGGTAAAGAGTGAG (SEQ ID NO:37)

CF 07-45 (-) Fuse the 3' end of kudzu isoprene synthase to the terminator
5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:32)

CF 07-48 (+) Sequencing primer for kudzu isoprene synthase
5'- CTTTTCCATCACCCACCTGAAG (SEQ ID NO:38)

CF 07-49 (+) Sequencing in kudzu isoprene synthase
5'- GGCGAAATGGTCCAACAACAAAATTATC (SEQ ID NO:39)

[0407] The plasmid designated pBS Kudzu #2 (Figures 44 and 12) was correct by sequencing and was transformed into BG 3594 comK, a *Bacillus subtilis* host strain. Selection was done on LA + 5 chloramphenicol plates. A transformant was chosen and struck to single colonies on LA + 5 chloramphenicol, then grown in LB+5 chloramphenicol until it reached an OD_{600} of 1.5. It was stored frozen in a vial at -80° C in the presence of glycerol. The resulting strain was designated CF 443.

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

[0408] Overnight cultures were inoculated with a single colony of CF 443 from a LA + Chloramphenicol (Cm, 25 μg/ml). Cultures were grown in LB + Cm at 37° C with shaking at 200 rpm. These overnight cultures (1 ml) were used to inoculate 250 ml baffled shake flasks containing 25 ml Grants II media and chloramphenicol at a final concentration of 25 μg/ml. Grants II Media recipe was 10 g soytone, 3 ml 1M K_2HPO_4, 75 g glucose, 3.6 g urea, 100 ml 10X MOPS, q.s. to 1 L with H_2O, pH 7.2; 10X MOPS recipe was 83.72 g MOPS, 7.17 g tricine, 12 g KOH pellets, 10 ml 0.276M K_2SO_4 solution, 10 ml 0.528M $MgCl_2$ solution, 29.22 g NaCl, 100 ml 100X micronutrients, q.s. to 1 L with H_2O; and 100X micronutrients recipe was 1.47 g $CaCl_2*2H_2O$, 0.4 g $FeSO_4*7H_20$, 0.1 g $MnSO_4*H_20$, 0.1 g $ZnSO_4*H_2O$, 0.05 g $CuCl_2*2H_2O$, 0.1 g $CoCl_2*6H_2O$, 0.1 g $Na_2MoO_4*2H_2O$, q.s. to 1 L with H_2O. Shake flasks were incubated

at 37° C and samples were taken at 18, 24, and 44 hours. At 18 hours the headspaces of CF443 and the control strain were sampled. This represented 18 hours of accumulation of isoprene. The amount of isoprene was determined by gas chromatography as described in Example 1. Production of isoprene was enhanced significantly by expressing recombinant isoprene synthase (Figure 11).

III. Production of isoprene by CF443 in 14 L fermentation

[0409] 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 45A and 45B.

IV. Integration of the kudzu isoprene synthase (ispS) in *B. subtilis*.

[0410] 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*

[0411] 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.

[0412] The PCR product was then cloned using the TOPO pENTR/D-TOPO Cloning Kit following manufacturer's protocol: 1 µl PCR reaction, 1 µl Salt solution, 1 µl TOPO pENTR/D-TOPO vector and 3 µl water in a total reaction volume of 6 µl. The reaction was incubated at room temperature for 5 minutes. One microliter of TOPO reaction was transformed into TOP10 chemically competent *E. coli* cells. The transformants were

selected on LA + 50 μg/ml kanamycin plates. Several colonies were picked and each was inoculated into a 5 ml tube containing LB + 50 μg/ml kanamycin and the cultures grown overnight at 37° C with shaking at 200 rpm. Plasmids were isolated from the overnight culture tubes using QIAprep Spin Miniprep Kit, following manufacturer's protocol. Several plasmids were sequenced to verify that the DNA sequence was correct.

[0413] A single pENTR/D-TOPO plasmid, encoding a *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, was used for Gateway Cloning into a custom-made pTrex3g vector. Construction of pTrex3g is described in WO 2005/001036 A2. The reaction was performed following manufacturer's protocol for the Gateway LR Clonase II Enzyme Mix Kit (Invitrogen): 1 μl *Y. lipolytica* codon-optimized kudzu isoprene synthase gene pENTR/D-TOPO donor vector, 1 μl pTrex3g destination vector, 6 μl TE buffer, pH 8.0 in a total reaction volume of 8 μl. The reaction was incubated at room temperature for 1 hour and then 1 μl proteinase K solution was added and the incubation continued at 37° C for 10 minutes. Then 1 μl of reaction was transformed into TOP10 chemically competent *E. coli* cells. The transformants were selected on LA + 50 μg/ml carbenicillin plates. Several colonies were picked and each was inoculated into a 5 ml tube containing LB + 50 μlg/ml carbenicillin and the cultures were grown overnight at 37° C with shaking at 200 rpm. Plasmids were isolated from the overnight culture tubes using QIAprep Spin Miniprep Kit (Qiagen, Inc.), following manufacturer's protocol. Several plasmids were sequenced to verify that the DNA sequence was correct.

[0414] 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*

[0415] 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*.

[0416] 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.

[0417] 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:40)

ICL1 5
5'- GCAGGTGGGAAACTATGCACTCC (SEQ ID NO:41)

XPR 3
5'- CCTGAATTCTGTTGGATTGGAGGATTGGATAGTGGG (SEQ ID NO:42)

XPR 5

5'- GGTGTCGACGTACGGTCGAGCTTATTGACC (SEQ ID NO:43)

XPRT3

5'- GGTGGGCCCGCATTTTGCCACCTACAAGCCAG (SEQ ID NO:44)

XPRT 5

5'- GGTGAATTCTAGAGGATCCCAACGCTGTTGCCTACAACGG (SEQ ID NO:45)

Y18S3

5'- GGTGCGGCCGCTGTCTGGACCTGGTGAGTTTCCCCG (SEQ ID NO:46)

Y18S 5

5'- GGTGGGCCCATTAAATCAGTTATCGTTTATTTGATAG (SEQ ID NO:47)

YURA3

5'- GGTGACCAGCAAGTCCATGGGTGGTTTGATCATGG (SEQ ID NO:48)

YURA 50

5'- GGTGCGGCCGCCTTTGGAGTACGACTCCAACTATG (SEQ ID NO:49)

YURA 51

5'- GCGGCCGCAGACTAAATTTATTTCAGTCTCC (SEQ ID NO:50)

[0418] 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.

[0419] 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).

[0420] 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.*

[0421] 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.

[0422] 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

[0423] 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 *Hin*dIII and *Pvu*I, and end-filled. A transformant carrying a plasmid conferring kanamycin resistance (pTrcKudzuKan) was selected on LA containing kanamycin 50 µg/ml.

ii) Construction of pTrcKudzu yIDI Kan

[0424] pTrcKudzuKan was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *idi* from *S. cerevisiae* with a synthetic

RBS. The primers for PCR were NsiI-YIDI 1 F 5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC (SEQ ID NO: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 28 and 29).

iii) Construction of pTrcKudzu DXS Kan

[0425] Plasmid pTrcKudzuKan was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *dxs* from *E. coli* with a synthetic RBS. The primers for PCR were MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCA AATACCCG (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 30 and 31).

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

[0426] pTrcKudzu-yIDI(kan) was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *E. coli dxs* with a synthetic RBS (primers MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCA AATACCCG (SEQ ID NO:18) and MCM14 5'-CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:19); template TOP10 cells) which had been digested with *Nsi*I and *Pst*I and gel purified. The final plasmid was called pTrcKudzu-yIDI-dxs (kan) (Figures 21 and 22).

v) Construction of pCL PtrcKudzu

[0427] 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 32-35).

vi) Construction of pCL PtrcKudzu yIDI

[0428] 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 36 and 37).

vii) Construction of pCL PtrcKudzu DXS

[0429] The *Nsi*I-*Pst*I digested, gel purified, DXS PCR amplicon from (iii) above was ligated into pCL PtrcKudzu (A3) which had been digested with *Pst*I, treated with SAP, and heat killed. The ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 μg/ml. Several clones were isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu DXS (Figures 38 and 39).

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

[0430] 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.

[0431] Plasmid pTrcKudzu-yIDI-dxs (kan) was introduced into *E. coli* strain BL21 by transformation. The resulting strain BL21/pTrc Kudzu IDI DXS was grown overnight in LB containing kanamycin (50 µg/ml) at 20° C and used to inoculate shake flasks of TM3 (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) containing 1% glucose. Flasks were incubated at 30° C until an OD_{600} of 0.8 was reached, and then induced with 400 µM IPTG. Samples were taken at various times after induction and the amount of isoprene in the head space was measured as described in Example 1. Results are shown in Figure 23H.

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

[0432] The strain BL21 pTrcKudzuIDIDXS was tested for the ability to generate isoprene from three types of biomass; bagasse, corn stover and soft wood pulp with glucose as a control. Hydrolysates of the biomass were prepared by enzymatic hydrolysis (Brown, L and Torget, R., 1996, NREL standard assay method Lap-009 "Enzymatic Saccharification of Lignocellulosic Biomass") and used at a dilution based upon glucose equivalents. In this example, glucose equivalents were equal to 1% glucose. A single colony from a plate freshly transformed cells of BL21 (DE3) pTrcKudzu yIDI DXS (kan) was used to inoculate 5 ml of LB plus kanamycin (50 µg/ml). The culture was incubated overnight at 25° C with shaking. The following day the overnight culture was diluted to an OD_{600} of 0.05 in 25 ml of TM3 + 0.2% YE + 1% feedstock. The feedstock was corn stover, bagasse, or softwood pulp. Glucose was used as a positive control and no glucose was used as a negative control. Cultures were incubated at 30° C with shaking at 180 rpm. The culture was monitored for OD_{600} and when it reached an OD_{600} of ~0.8, cultures were analyzed at 1 and 3 hours for isoprene production as described in Example 1. Cultures are not induced. All cultures containing added feedstock produce isoprene

equivalent to those of the glucose positive control. Experiments were done in duplicate and are shown in Figure 40.

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

[0433] A single colony from a plate freshly transformed cells of BL21 (λDE3)/pTrcKudzu yIDI DXS (kan) was used to inoculate 5 mL of LB + kanamycin (50 μg/ml). The culture was incubated overnight at 25° C with shaking. The following day the overnight culture was diluted to an OD_{600} of 0.05 in 25 ml of TM3 + 0.2% YE + 1% feedstock. Feedstock was glucose, inverted glucose or corn stover. The invert sugar feedstock (Danisco Invert Sugar) was prepared by enzymatically treating sucrose syrup. AFEX corn stover was prepared as described below (Part V). The cells were grown at 30° C and the first sample was measured when the cultures reached an OD_{600} ~0.8-1.0 (0 hour). The cultures were analyzed for growth as measured by OD_{600} and for isoprene production as in Example 1 at 0, 1 and 3 hours. Results are shown in Figure 41.

V. Preparation of hydrolysate from AFEX pretreated corn stover

[0434] 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

[0435] 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 42A). 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 42B). The amount of isoprene produced was linearly proportional to the amount of fed yeast extract (Figure 42C).

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

[0436] A 500 liter fermentation of *E. coli* cells with a kudzu isoprene synthase, *S. cerevisiae* IDI, and *E. coli* DXS nucleic acids (*E. coli* BL21 (λDE3) pTrc Kudzu dxs yidi) was used to produce isoprene. The levels of isoprene varied from 50 to 300 μg/L over a time period of 15 hours. On the basis of the average isoprene concentrations, the average flow through the device and the extent of isoprene breakthrough, the amount of isoprene collected was calculated to be approximately 17 g.

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

Medium Recipe (per liter fermentation medium):

[0437] 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:

[0438] 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.

[0439] 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.

[0440] 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 43A. 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 43B). 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 43C.

Example 8: Overexpression of *flavodoxin I (fldA)* increase isoprene production in a strain expressing over-expressing *E. coli dxs*, Saccharomyces *idi*, and kudzu isoprene synthase

[0441] BL21 (DE3) strain harboring pTrcKudzuDXSyIDI produced more isoprene under non-inducing conditions compared to IPTG induction conditions, and was observed to accumulate HMBPP ((E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate), the substrate of HDS (GcpE or IspG). Using the BL21 (DE3) strain harboring pTrcKudzuDXSyIDI as the parental host strain, the introduction of an additional plasmid-born copy of the Kudzu isoprene synthase gene alone and in combination with the *fldA*

gene encoding flavodoxin I were assessed for the effects on isoprene production by the strains under non-inducing conditions relative to the empty vector control strain.

[0442] These experiments investigated whether an additional copy of the isoprene synthase improves isoprene production under non-inducing conditions in the BL21 (DE3) strain harboring the pTrcKudzuDXSyIDI construct. Under the non-inducing conditions, isoprene synthase may be limiting and an additional copy of the Kudzu enzyme may be able to improve the specific productivity of isoprene generation by the strain. The experiments also investigated whether other factor(s) contributed to the modest level of isoprene produced by the strain and whether a plasmid-born copy of *fldA* could increase isoprene production by the BL21 (DE3) strain that harbors the pTrcKudzuDXSyIDI construct under non-inducing conditions. The flavodoxin I encoded by *fldA* was intended to be expressed ectopically from the pTrcHgSfldA/pBAD33 construct at a level surpassing that generated from the endogenous *fldA* locus. An increased amount of flavodoxin I may increase the activity demonstrated by the DXP pathway enzymes GcpE (HDS or IspG) and LytB (HDR or IspH) *in vivo*, as was previously seen *in vitro* (Seemann, M. *et al. Agnew. Chem. Int. Ed.*, 41: 4337-4339, 2002; Wolff, M. *et al. FEBS Letters*, 541: 115-120, 2003), and possibly improve carbon flux to isoprene synthesis in the strain of interest over that of the comparable pTrcKudzuDXSyIDI-containing BL21 (DE3) control strain.

[0443] Bacterial transformation and molecular biology techniques were performed using standard protocols (Sambrook *et al*), which is hereby incorporated by reference in its entirety, particularly with respect to bacterial transformation. The *E. coli* strains BL21 (DE3) and TOP10 were obtained from Invitrogen. TOP10 cells were used during the preparation of the pTrcHgS/pBAD33 and pTrcHgSfldA/pBAD33 constructs described below. Vector constructs were moved via chemical transformation into the BL21 (DE3) strain for the subsequent assessment of isoprene production.

Constructs

Forward primer
Name: 5' fldA NsiI SpeI rbs

Sequence: GG ATGCAT ACTAGT TTCA AGAGG TATTTCACTC ATG (SEQ ID NO:54)
Features: NsiI SpeI rbs start

A G
Region homologous to MG1655 *fldA* locus

Primers were purchased from Integrated DNA Technologies (Coralville, Iowa). PCR reactions were performed with Herculase II Fusion (Stratagene) according to manufacturer's specifications.

Reverse primer
Name: 3' fldA PstI stop
Sequence: ATC CTGCAG TCA GGCATTGAGAATTTCGTC (SEQ ID NO:55)

Features: PstI stop

T C
Region homologous to MG1655 *fldA* locus

[0444] Primers were purchased from Integrated DNA Technologies (Coralville, Iowa). PCR reactions were performed with Herculase II Fusion (Stratagene) according to manufacturer's specifications.

[0445] *E. coli* 12 MG1655 (world wide web at genome.wisc.edu/resources/strains.htm) was the source of genomic template used to amplify the *fldA* locus; cells were added directly to the PCR reaction using a sterile toothpick.

[0446] The *fldA* PCR product was cleaned utilizing the MinElute PCR Purification Kit (Qiagen). pBAD33 is described, for example, in Luz-Maria, G. *et al.*, *J. Bacteriology*, 77: 4121-4130, 1995, which is hereby incorporated by reference in its entirety, particularly with respect to pBAD33. pTrcKudzu, and pTrcKudzuDXSyIDI kan, were described, for example, in US Appl. No.: 12/335,071 and PCT/US2008/086809, which are hereby incorporated by reference in their entireties, particularly with respect to Examples 1 and 7.

[0447] pTrcHgS/pBAD33was constructed here by cloning the SspI – PstI (1934 bp) fragment containing the Trc promoter region, rbs, and the coding sequence of the Kudzu isoprene synthase derived from pTrcKudzu into the SmaI – PstI sites of pBAD33.

[0448] pTrcHgSfldA/pBAD33 was constructed here. The NsiI – PstI (1471 bp) digested PCR amplified *fldA* fragment encompassing 22 bp upstream of the *fldA* start, including the endogenous rbs, through the stop codon of the *fldA* gene was cloned into the PstI site located just downstream of the isoprene synthase open reading frame in pTrcHgS/pBAD33.

[0449] Constructs were verified by sequencing that was performed by Sequetech (Mountain View, California).

Culture conditions

[0450] Bacteria were grown at 25 °C and 30 °C on LB 1.5% agar plates and in TM3 liquid media (*see* description of TM3, for example, US Appl. No.: 12/335,071 and PCT/US2008/086809, which are hereby incorporated by reference in their entireties, particularly with respect to TM3 liquid media) supplemented to a final concentration with 0.1% yeast extract and 1.0% glucose. When appropriate, kanamycin (Kan) and/or chloramphenicol (Cmp) were added to the growth media at 50 µg/ml and 10 µg/ml, respectively; pTrc-based constructs encode Kan^R and pBAD33-based constructs encode Cmp^R. Bacterial growth was monitored by optical density measured at 600 nm.

Assessment of isoprene production

[0451] Headspace assay for isoprene production was described in Example 1. The specific productivity of each strain was reported as µg/L·OD·hour; note ratio of 1900 µl headspace:100 µl broth in assay vials. Graphs depicting the growth rate and specific productivity of each strain were generated using Microsoft Office Excel 2003 software.

Construction of BL21 (DE3) strains and assessment of the isoprene production

[0452] The following BL21 (DE3) strains were constructed and assessed for the production of isoprene relative to one another: BL21 (DE3) harboring the pTrcKudzuDXSyIDI vector and either 1) empty pBAD33 vector (also referred to as "empty vector"); 2) pTrcHgS/pBAD33 construct (also referred to as "HgS"), or 3) pTrcHgSfldA/pBAD33 construct (as referred to as "HgS-FldA").

[0453] All three BL21 (DE3) test strains harbor Kan^R and Cmp^R and were grown under appropriate selection for both plasmid constructs. The empty vector strain represented the parental control strain; the HgS strain represented the parental strain harboring an addition plasmid-born copy of the Kudzu isoprene synthase gene; the HgS-FldA strain represented the parental strain harboring the addition plasmid-born copies of flavodoxin I and isoprene synthase genes.

[0454] The bacteria strains were grown overnight shaking (250 rpm) at 25 °C in 10 ml of supplemented TM3 media containing antibiotics; here and for the following experiments 50 μg/ml of kanamycin and 10 μg/ml of chloramphenicol were present in the growth media. The cultures were then diluted into fresh supplemented TM3 media containing antibiotics to an optical density at 600 nm of approximately 0.05 and allowed to grow shaking (250 rpm) at 30°C in 12.5-25 ml of supplemented TM3 media containing antibiotics in 250 ml Erlenmeyer flasks. Strains were typically assessed for isoprene production once the optical density at 600 nm of the culture reached 0.4. In the most densely sampled experiments, once isoprene measurements commenced the isoprene production for each culture was monitored in 45 min. intervals. The results from two independent experiments depicting growth rate and specific productivity of isoprene generation for the empty vector (control), HgS, and HgS-FldA strains are shown in the Figures 46A-46D. The strains were grown under non-inducing conditions; meaning that IPTG-induced expression from the Trc promoter regulated gene constructs was not performed. All plasmid-born genes of interest in the experiments described here were governed by the IPTG-inducible Trc promoter. The Trc promoter is well known in the art to be active in the absence of the IPTG inducer.

[0455] Under the non-inducing conditions tested, the results obtained from the isoprene headspace assays performed on the empty vector, HgS, and HgS-FldA strains indicate that an additional copy of fldA present on the pTrcHgSfldA/pBAD33 construct substantially increases isoprene production in the HgS-FldA strain over that produced by both the HgS and empty vector control strains. The HgS-FldA strain was observed to exhibit increased specific productivity of isoprene generation ranging from 1.5- to 1.9-fold and 1.3- to 1.8-fold higher than the control strain over a 3.75-hour and 2.5-hour time course, respectively, during two independent experiments. The observed effect on isoprene production appears to be specific to the presence of the *fldA*-containing construct, as the HgS strain produces comparable levels of isoprene under the non-inducing conditions to that produced by the empty vector control strain.

Example 9: Expression of alternative ispG (gcpE or HDS) and ispH (lytB or HDR) and their corresponding reducing shuttle system, from *Thermosynechococcus elongatus* BP-1 in an isoprene-producing *E. coli* to improve isoprene production

[0456] In this example, we demonstrated that the ferredoxin/ferredoxin-NADP oxidoreductase/NADPH reducing system together with the GcpE and LytB enzymes from *T. elongates* improve isoprene production in *E. coli* BL21(DE3).

[0457] *T.* elongatus, like *E. coli*, synthesizes isoprenoids via the DXP pathway, but does not harbor any genes coding for a flavodoxin protein. It was previously shown that the plant GcpE enzyme is a ferredoxin-dependent enzyme, and that flavodoxin could not support the enzymatic conversion of cMEPP (ME-CPP) into HDMAPP (HMBPP) by this enzyme (*see* Seemann *et al.*, *FEBS Lett.*, 580(6):1547-52 (2006), which is hereby incorporated by reference in its entirety). It was also demonstrated *in vitro* that GcpE of *T. elongatus* together with PetF (ferredoxin), Pet H (ferredoxin-$NADP^+$ oxidoreductase), and NADPH could convert cMEPP into HDMAPP (Okada and Hase, *J Biol Chem*, 280(21):20627-9 (2005)), which is hereby incorporated by reference in its entirety). With the lack of other small electron carrier proteins besides ferredoxin in the genome, it is likely that LytB of *T. elongatus* also utilizes the same reducing shuttle system as GcpE.

[0458] Demonstration of increased isoprene production and elevated cMEPP levels in REM23-26 by overexpression of GcpE , PetF, and PetH from T. elongatus BP-1

[0459] We have previously demonstrated that increased expression of *dxs* increases flux through the DXP pathway in *E. coli.* Isoprene-producing strains (REM19-22) harboring increased and varied levels of *dxs* expression were constructed by integrating the GI 1.X-promoter series immediately upstream of the *dxs* locus within the *E. coli* BL21(DE3) genome. Subsequently, the test set of strains, REM23-26 were created by transformation with plasmids expressing the *T. elongatus* GcpE and its corresponding reducing shuttle system encoded by *petF* and *petH.* The parental and test strains were evaluated for growth, isoprene production, and the presence of DXP pathway metabolites. The results are presented in Figures 47-49.

Construction of MCM16 MCM640, MCM639, MCM641, and the parental strains to REM19-22

[0460] The GI 1.X-promoter insertions and subsequent loopout of the antibiotic resistance markers described in this example were carried out using the Red/ET system from Gene Bridges GmbH according to the manufacturer's instructions. The strain BL21(DE3) (Invitrogen) was used.

[0461] Primer Sequences

[0462] MCM319: 5'-
ctctctttcggcaacagtcgtaactcctgggtggagtcgaccagtgccagggtcgggtatttggcaatatcaaaactcatatattcc
accagctatttgttagtgaataaaagtggttgaattatttgctcaggatgtggcatNgtcaagggctaatacgactcactatagggc
tc (SEQ ID NO:57).

[0463] degenerate N base: A base yields GI 1.6-, T base yields GI 1.5-, G base yields GI1.2-, and C base yields GI 1.0-promoter.

[0464] MCM320: 5'-
tcgatacctcggcactggaagcgctagcggactacatcatccagcgtaataaataaacaataagtatta
ataggcccctgaattaaccctcactaaagggcgg (SEQ ID NO: 58).

[0465] MCM327: 5'- TTGTAGACATAGTGCAGCGCCA (SEQ ID NO: 59).

[0466] GB-DW: 5'-aaagaccgaccaagcgacgtctga (SEQ ID NO: 60).

[0467] Strategy for Creating the MCM638-641 Strains

[0468] The strategy for inserting the GI1.X-promoter series in front of *dxs* is shown in Figure 50. The antibiotic resistance cassette GB-NeoR was amplified by PCR using primer sets MCM319/ MCM320. The primers contain 50 bases of homology to the region immediately 5' to the *dxs* coding region to allow recombination at the specific locus upon electroporation of the PCR product in the presence of the pRed-ET plasmid.

[0469] Amplification of the Deletion Cassettes

[0470] To amplify the GB-NeoR cassette for inserting the GI 1.X-promoters immediately upstream of the *dxs* locus the following PCR reactions were set up:

1ul (100ng GB-NeoR)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) MCM319
1.25ul primer (10uM) MCM320
35 ul diH2O

[0471] + 1ul of *HerculaseII fusion* from Stratagene

[0472] Cycle Parameter

[0473] 95°C x 2 minutes, [95°C x 20 seconds, 55°C x 20 seconds, 72°C x 50 seconds] x 30 cycles; 72°C x 3 minutes, 4°C until cool (BioRadPCR machine).

[0474] The resulting PCR fragments were separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits according to manufacturer's instructions. The resulting stock was GB-NeoR-GI 1.X-dxs fragment.

[0475] Integration of GB-NeoR- GI 1.X-dxs PCR product into BL21(DE3)/pRed-ET Strain

[0476] The pRed-ET vector (Gene Bridges kit) was transformed into BL21(DE3) by electroporation resulting in strain MCM327 (BL21(DE3)/pRed-ET). Approximately 500 ng of the GB-NeoR-GI 1.x-dxs PCR fragment was electroporated into MCM327. The transformants were recovered in L Broth for 1 hour with shaking at 200 rpm at 37°C and then plated on L agar containing kanamycin (10ug/ml). Kanamycin resistant colonies were analyzed by PCR for the presence of the GB-NeoR cassette and the GI 1.X-promoters using primers GB-DW/ MCM327. The PCR fragments from a number of transformants (MCM617-625) were sequenced using the MCM327 and GB-DW primers (Quintara; Berkeley, CA) and the various GI 1.X-*dxs* strains of interest identified. The correct strains were designated MCM617 (FRT-neo-FRT-GI 1.0-dxs), MCM618 (FRT-neo-FRT-GI 1.5-dxs), MCM623 (FRT-neo-FRT-GI 1.2-dxs), and MCM625 (FRT-neo-FRT-GI 1.6-dxs). The kanamycin resistance cassette was looped out of the strains using pCP20 from the RED/ET kit according to the manufacturer's instructions. Transformants were verified by loss of resistance to kanamycin (10ug/ml) and PCR demonstrating loopout of the GB-NeoR cassette. The resulting strains were designated MCM638 (BL21(DE3) GI 1.0-dxs), MCM639 (BL21(DE3) GI 1.5-dxs), MCM640 (BL21(DE3) GI 1.2-dxs) and MCM641 (BL21(DE3) GI 1.6-dxs).

Construction of the parental strains REM19-22 from MCM638, MCM640, MCM639, and MCM641, respectively

[0477] The construction of the T7-MEARR alba/pBBR1MCS-5 described in this example was carried out using standard molecular biology techniques (Sambrook *et al.*, 1989, which is hereby incorporated by reference in its entirety). The pBBR1MCS-5 plasmid has been previously described (Kovach *et al.*, *Biotechniques*, 16(5):800-2 (1994), which is hereby incorporated by reference in its entirety, particularly with respect to cloning of the pBBR1MCS). A picture illustrating the resulting plasmid construct is shown in Figure 51. The MCM638-641 strains were used for the transformations described here.

Primer Sequences

5' KpnI to lacI MEARR T7 frag: 5'-GCTGGGTACCCTGCCCGCTTTCCAG

TCGGGAAACCT (SEQ ID NO:61)

3' SpeI to T7 terminator MEARR T7 frag: 5'-TAGAACTAGTCAAAAAACCCC TCAAGACCCGTTTAG (SEQ ID NO:62)

M13 Forward (-20): 5'-GTAAAACGACGGCCAGT (SEQ ID NO:63)

EL-1000: 5'-GCACTGTCTTTCCGTCTGCTGC (SEQ ID NO:64)

A-rev: 5'-CTCGTACAGGCTCAGGATAG (SEQ ID NO:65)

A-rev2: 5'-TTACGTCCCAACGCTCAACT (SEQ ID NO:66)

Strategy for creating the REM19-22 strains

[0478] Electroporation of T7-MEARR alba/pBBR1MCS-5 into strains MCM638-641. The vector construct harboring the T7 polymerase governed MEARR alba allele, MD09-173 (BL21(DE3)pLysS, pET24a-P.alba (MEA) Untagged (pDu39)), was used as the PCR template.

[0479] Amplification of the T7-MEARR alba fragment

To amplify the T7-MEARR alba fragment for cloning into the pBBR1MCS-5 plasmid the following PCR reaction was performed:
1ul (approx. 120ng MDO9-173)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' KpnI to lacI MEARR T7 frag
1.25ul primer (10uM) 3' SpeI to T7 terminator MEARR T7 frag
35 ul diH2O
[0480] + 1ul of *HerculaseII fusion* from Stratagene.

Cycle parameter:
95°C x 2 minutes, [95°C x 30 seconds, 63°C x 30 seconds, 72°C x 3 minutes] x 29 cycles; 72°C x 5 minutes,
4°C until cool (Biometra T3000 Combi Thermocycler).

[0481] The resulting PCR fragment was separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits (Qiagen) according to manufacturer's instructions. The resulting stock was T7-MEARR alba fragment.

Cloning of the T7-MEARR alba fragment into pBBR1MCS-5

[0482] Approximately 600ng of the T7-MEARR alba fragment and 200ng of the pBBR1MCS-5 plasmid were digested with *Kpn*I and *Spe*I (Roche) according to the manufacturer's specifications. The digests were subsequently combined and cleaned using the Qiagen QiaQuick Gel Extraction Kit. Approximately a fourth to a third of the cleaned cut DNA was ligated using T4 DNA Ligase (New England Biolabs) according to the manufacturer's suggested protocol. Chemically competent TOP10 cells (Invitrogen) was transformed with the ligation reaction using a standard heat-shock protocol (*See, e.g.*, Sambrook et al., 1989, which is hereby incorporated by reference in its entirety), recovered in L broth for 1 hour at 37°C and then plated on L agar containing gentamycin (10ug/ml) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-GAL at 40ug/ml; Sigma). White, gentamycin resistant colonies were selected, grown overnight in L broth containing gentamycin (10ug/ml), and harvested for plasmid preparation the following day. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit and first analyzed by restriction enzyme digestion and electrophoresis (as described above) for the putative presence of the T7-MEARR alba fragment. Plasmid preparations of interest were sequenced (Sequetech; Mountain View, CA) using primers M13 Forward (-20), EL-1000, A-rev, and A-rev2, and the correct T7-MEARR alba/pBBR1MCS-5 clone identified.

Transformation of T7-MEARR alba/pBBR1MCS-5 into MCM638-641

[0483] To build the isoprene-producing strains REM19-22 the T7-MEARR alba/pBBR1MCS-5 plasmid was transformed by electroporation into MCM638-641. Transformants were recovered in L broth and plated on L agar containing gentamycin (10ug/ml). The resulting strains were designated as such: REM19 (MCM638 / T7-MEARR alba/pBBR1MCS-5), REM20 (MCM640 / T7-MEARR alba/pBBR1MCS-5), REM21 (MCM639 / T7-MEARR alba/pBBR1MCS-5), and REM22 (MCM641 / T7-MEARR alba/pBBR1MCS-5).

Construction of the Test Strains REM23-26

[0484] REM23-26 were constructed by transformation of the Ptac-gcpE-petF-petH/pK184 construct into MCM638, MCM640, MCM639, and MCM641. The plasmid Ptac-gcpE-petF-petH/pK184 described in this example was synthesized by Gene Oracle, Inc. (Mountain View, CA) with codon optimization of *gcpE, petF,* and *petH* for expression in *E. coli.* The Ptac promoter and *aspA* terminator sequences have been previously described (Genbank accession # E02927 and CP001164 , respectively). The pK184 cloning vector has been described, for example, by Jobling and Holmes, *Nucleic Acids Res.* 18(17):5315-6 (1990), which is hereby incorporated by reference in its entirety, particularly with respect to the pK184 cloning vector. A picture illustrating the resulting plasmid construct is shown in Figure 52. The REM19-22 strains were used for the transformations described herein.

Strategy for Creating the REM23-26 Strains

[0485] Electroporation of Ptac-gcpE-petF-petH/pK184 into strains REM19-22. A plasmid preparation of Ptac-gcpE-petF-petH/pK184 was provided by Gene Oracle, Inc.

Transformation of Ptac-gcpE-petF-petH/pK184 into REM19-22

[0486] To build the isoprene-producing test strains, REM23-26, the Ptac-gcpE-petF-petH/pK184 plasmid was transformed by electroporation into REM19-22. Transformants were recovered in L broth and plated on L agar containing kanamycin (10ug/ml) and gentamycin (10ug/ml). The resulting strains were designated as such: REM23 (REM19 / Ptac-gcpE-petF-petH/pK184), REM24 (REM20 / Ptac-gcpE-petF-petH/pK184), REM25 (REM21 / Ptac-gcpE-petF-petH/pK184), and REM26 (REM22 / Ptac-gcpE-petF-petH/pK184).

Analysis of REM19-26 for growth, isoprene production, and DXP metabolite generation

[0487] The parental strains REM19-22 were compared against the test strains REM23-26 in a shake flask isoprene headspace assay as well as in a DXP metabolite determination study. The benefits of expressing the *T. elongatus* GcpE enzyme on DXP metabolite generation and isoprene production from the *E. coli* host is illustrated in Figures 47 and 48.

Growth

[0488] Strains REM19-26 were grown at 30°C in TM3 liquid media (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) supplemented to a final concentration with 0.1% yeast extract and 1.0% glucose and including kanamycin (10ug/ml) and gentamycin (10ug/ml). Growth was monitored periodically by recording each of the culture's optical density measured at 600nm using an Eppendorf Biophotometer spectrometer (Eppendorf).

Isoprene Production

[0489] Isoprene production was analyzed using a headspace assay. 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. 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. The specific productivity of each strain is reported as ug/L OD Hr. Note, ratio of 1900ul headspace:100ul broth in assay vials for 30 min. incubation results in the

following conversion of isoprene ug/L of culture to specific productivity: (isoprene/L determined by GC-MS) X (38)/(OD 600nm of the culture).

DXP metabolite accumulation

[0490] The DXP metabolites of the isoprene-producing parental and test strains, REM19-22 and REM23-26, respectively, described above and depicted in Figure 48 were isolated and quantified as follows:

Metabolite quantification

[0491] Cell metabolism was rapidly inactivated by withdrawing 3.5 mL of the culture into a tube filled with 3.5 mL of dry ice-cold methanol. Cell debris was pelleted by centrifugation and the supernatant was loaded onto Strata-X-AW anion exchange column (Phenomenex) containing 30 mg of sorbent. The pellet was re-extracted twice, first with 3 mL of 50% MetOH containing 1 mM NH_4HCO_3 buffer (pH=7.0) and then with 3 mL of 75% MetOH/ 1 mM NH_4HCO_3 buffer (pH=7.0). After each extraction, cell debris was pelleted by centrifugation and the supernatants were consecutively loaded onto the same anion exchange column. During the extraction and centrifugation steps the samples were kept at below +4°C. Prior to metabolite elution, the anion exchange columns were washed with water and methanol (1 mL of each) and the analytes were eluted by adding 0.35 mL of concentrated NH_4OH/methanol (1:14, v/v) and then 0.35 mL of concentrated NH_4OH/water/methanol (1:2:12, v/v/v) mixtures. The eluant was neutralized with 30 µL of glacial acetic acid and cleared by centrifugation in a microcentrifuge.

Metabolite quantification

[0492] Metabolites were analyzed using a Thermo Scientific TSQ Quantum Access mass spectrometer (Thermo Electron Corporation, San Jose, CA). All system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). For the LC-ESI –MS/MS method, a chiral Nucleodex ß-OH 5µM HPLC column (100 x 2 mm, Macherey-Nagel, Germany) equipped with a CC 8/4 Nucleodex beta-OH guard cartridge was eluted with a mobile

phase gradient shown in Table 1 (flow rate of 0.4 mL/min). The sample injection volume was 10 µL.

Table 1. HPLC gradient used to elute metabolites.

Time, min	Mobile phase, %		
	A (water)	B (100 mM ammonium bicarbonate, pH=8.0)	C (acetonitrile)
0.0	0.0	20.0	80.0
0.5	15.0	5.0	80.0
4.5	37.5	12.5	50.0
6.5	37.5	12.5	50.0
7.0	49.5	0.5	50.0
12.0	34.9	0.1	65.0
12.5	0.0	20.0	80.0
13.0	0.0	20.0	80.0

[0493] Mass detection was carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 245.0 for IPP and DMAPP, 381.1 for FPP, 213.0 for DXP, 215.0 for MEP, 260.0 for HDMAPP, and 277.0 for cMEPP. Concentrations of metabolites were determined based on the integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0). Calibration curves obtained by injection of corresponding standards purchased from Echelon Biosciences Inc. Intracellular concentrations of metabolites were calculated based on the assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 µL.

Demonstration of increased isoprene production in REM31 and REM29 by overexpression of GcpE, LytB PetF and PetH of T. elongatus BP-1

[0494] We have demonstrated that increased expression of *dxs* permits increased flux through the DXP pathway within *E. coli,* while the additional overexpression of an *idi* gene increases the production of downstream isoprenoids significantly. To demonstrate the benefits of expressing the non-flavodoxin-dependent GcpE and LytB enzymes on carbon flux through the endogenous *E. coli* DXP pathway to isoprene synthesis, *E. coli*

BL21(DE3) isoprene-producing strains with constitutive expression of *dxs* and the yeast IDI enzyme were constructed. The BL21(DE3) GI 1.6-dxs strain MCM641 is described above. The construction of the vector construct harboring the yeast IDI enzyme, pDU9-pET-16b rev-yIDI, is described herein. The T7-(-3) alba/pBBR1MCS-5 and T7-MTE alba/pBBR1MCS-5 *P. alba* isoprene synthase-containing constructs are described below. A set of parental isoprene-producing, IDI-overexpressing strains derived from MCM641 were created (REM H76 and REMH86) to compare to the newly generated test set of strains (REM31 and REM29) which harbor the *T. elongatus* GcpE, LytB, and their corresponding reducing shuttle system (described below). The parental and test strains were evaluated for growth, isoprene production, and the presence of DXP pathway metabolites. The results are depicted in Figure 49.

[0495] Construction of pDU-9

[0496] The IPP isomerase from *Saccharomyces cerevisiae* (yIDI) was cloned into the vector pET16b (Invitrogen). The primer set Hg-yIDI-R2/ Hg-yIDI-F2 was used for PCR with the template DNA pTrcKudzu yIDI Kan. The PCR cycle conditions:

PCR reaction

1ul of template (pMVK1- Fernando's template)
5ul of 10X PfuII Ultra buffer
1ul of dNTP
1ul of primer (50uM) Hg-MVK-F2-NdeI
1ul of primer (50uM) Hg-yIDI-R2
40 ul of DiH2O
+ 1ul of *Pfu Ultra*II Fusion DNA Polymerase from Stratagene

Cycle Parameter:
(95°C 2min., 95°C 20sec., 55°C 20sec., 72°C 21sec., 29X, 72C 3min., 4°C until cool, use Eppendorf Mastercycler Gradient Machine)

[0497] The PCR product was purified using the QiaQuick PCR purification kit according to the manufacturer's suggestion. An aliquot of 5uL purified of the PCR product was ligated to Invitrogen pET-16b Vector that was previously digested with *Nde*I-SAP (Shrimp Alkaline Phosphatase) treated using T4 ligase enzyme (NEB). The ligation was carried out overnight at16°C.

[0498] 5uL of overnight ligation mixture was introduced into Invitrogen TOP10 cells and transformants were selected on L agar containing Carbenicillin (50 ug/ml) incubated at 37°C. Plasmids from transformants were isolated using QiaQuick spin miniprep kit. The insert is sequenced with T7 promoter and T7 terminator (Use Quintara Bio Sequencing Service). The resulting plasmid r is called pDu-9.

[0499] Once the sequence is verified, 1 ul of plasmid pDu-9 was transformed into BL21(DE3) pLysS hst strain according to manufacturer's protocol. Transformants are selected on L agar containing Carbenicillin (50 ug/ml) plate and incubated at 37°C.

Primer sequences
Hg-yIDI-R2 5'...cagcagcagGGATCCgacgcgttgttatagca (SEQ ID NO:111)
Hg-yIDI-F2 5'...cagcagcagCATATGactgccgacaacaatag (SEQ ID NO:112)
[0500]

Construction of REMD76 (MCM641 / pDU9-pET-16b rev-yIDI), REMH76 and REMH86 (REMD76 / T7-(-3) alba/pBBR1MCS-5 and REMD76 / T7-MTE alba/pBBR1MCS-5, respectively)

Strategy for creating the REMD76
[0501] pDU9-pET-16b rev-yIDI was electroporated into MCM641.

Transformation of pDU9-pET-16b rev-yIDI into MCM641

[0502] To build the BL21(DE3) GI 1.6-dxs yIDI-overexpressing strain REMD76, the pDU9-pET-16b rev-yIDI plasmid expressing a yeast IDI (yIDI) allele was transformed by electroporation into MCM641. Transformants were recovered in L broth and plated on L agar containing carbinicillin (50ug/ml). A carbinicillin resistant colony was selected and designated REMD76.

Generation of the parental strains REMH76 and REMH86 (REMD76 / T7-(-3) alba/pBBR1MCS-5 and REMD76 / T7-MTE alba/pBBR1MCS-5, respectively

[0503] The construction of the T7-(-3) alba/pBBR1MCS-5 and T7-MTE alba/pBBR1MCS-5 constructs described in this example were carried out using standard molecular biology techniques (*ee, e.g.*, Sambrook *et al.*, 1989). The pBBR1MCS-5

plasmid has been previously described *(see, Kovach et al.,* Biotechniques, 16(5):800-2 (1994), which is hereby incorporated by reference in its entirety, particularly with respect to the pBBR1MCS-5 plasmid). The pictures illustrating the resulting plasmid constructs are shown in Figure 53. The REMD76 strain was used for the transformations described herein.

[0504] Strategy for creating the REMH76 and REMH86 strains

[0505] Electroporation of T7-(-3) alba/pBBR1MCS-5 and T7-MTE alba/pBBR1MCS-5 into strain REMD76. The vector constructs harboring the T7 polymerase governed (-3) and MTE alba alleles, pDU47-3-pET24a-P.alba (-3) and pDU42 pET24a-P.alba-MTE untagged, were used as the PCR templates.

[0506] Primer Sequences

5' KpnI to lacI MEARR T7 frag: 5'-GCTGGGTACCCTGCCCGCTTTCCAG TCGGGAAACCT (SEQ ID NO:67)

3' SpeI to T7 terminator MEARR T7 frag: 5'-TAGAACTAGTCAAAAAACCCC TCAAGACCCGTTTAG (SEQ ID NO:68)

M13 Forward (-20): 5'-GTAAAACGACGGCCAGT (SEQ ID NO:69)

EL-1000: 5'-GCACTGTCTTTCCGTCTGCTGC (SEQ ID NO:70)

A-rev: 5'-CTCGTACAGGCTCAGGATAG (SEQ ID NO:71)

A-rev2: 5'-TTACGTCCCAACGCTCAACT (SEQ ID NO:72)

Amplification of the T7-(-3) and T7-MTE alba fragments

[0507] To amplify the T7-(-3) and T7-MTE alba fragments for cloning into the pBBR1MCS-5 plasmid the following PCR reactions were performed: 1ul (approx. 100ng pDU47-3-pET24a-P.alba (-3) or pDU42 pET24a-P.alba-MTE untagged)

10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' KpnI to lacI MEARR T7 frag
1.25ul primer (10uM) 3' SpeI to T7 terminator MEARR T7 frag
35 ul diH2O

+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2 minutes, [95°C x 30 seconds, 63°C x 30 seconds, 72°C x 3 minutes.] x 29 cycles; 72°C x 5 minutes,
4°C until cool (Biometra T3000 Combi Thermocycler)

[0508] The resulting PCR fragments were separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits according to manufacturer's instructions. The resulting stocks were T7-(-3) alba fragment and T7-MTE alba fragment.

Cloning of the T7-(-3) alba and T7-MTE alba fragments into pBBR1MCS-5

[0509] Approximately 600ng of the T7-(-3) alba fragment or T7-MTE alba fragment and 200ng of the pBBR1MCS-5 plasmid were digested with KpnI and SpeI from Roche according to the manufacturer's specifications. The digests were subsequently combined and cleaned using the Qiagen QiaQuick Gel Extraction Kit. Approximately a fourth to a third of the cleaned cut DNA was ligated using T4 DNA Ligase from New England Biolabs according to the manufacturer's suggested protocol.

[0510] Chemically competent TOP10 cells (Invitrogen) were transformed with the ligation reaction using a standard heat-shock protocol (Sambrook *et al.*, 1989, which is hereby incorporated by reference in its entirety), recovered in L broth for 1 hour at 37°C and then plated on L agar containing gentamycin (10ug/ml) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-GAL at 40ug/ml; Sigma). White gentamycin resistant colonies were selected, grown overnight in L broth containing gentamycin (10ug/ml), and harvested for plasmid preparation the following day. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit and first analyzed by restriction digest and electrophoresis (as described above) for the putative presence of the T7-(-3) alba fragment or T7-MTE alba fragment. Plasmid preparations of interest identified were sequenced (Sequetech; Mountain View, CA) using primers M13 Forward (-20), EL-1000, A-rev, and A-rev2, and the correct T7-(-3) alba/pBBR1MCS-5 and T7-MTE alba/pBBR1MCS-5 clones identified.

Construction of the test strains REM31 and REM29

[0511] To create strains REM31 and 29 the plasmid Ptac-gcpE-lytB-petF-petH/pK184 was transformed into REMH76 and REMH86. The synthesis and codon optimization for *E. coli* of the Ptac-gcpE-lytB-petF-petH/pK184 described in this example was performed by Gene Oracle, Inc. (Mopuntain View, CA). The Ptac promoter and *aspA* terminator sequences have been previously described (Genbank accession # E02927 and CP001164 , respectively) and were also constructed synthetically. The pK184 cloning vector has been described previously (*see*, Jobling and Holmes, *Nucleic Acids Res.* 18(17):5315-6 (1990), which is hereby incorporated by reference in its entirety, particularly with respect to the pK184 cloning vector). A picture illustrating the resulting plasmid construct is shown in Figure 54. The REMH76 and REMH86 strains were used for the transformations described herein.

Strategy for creating the REM31 and REM29 strains

[0512] Electroporation of Ptac-gcpE-lytB-petF-petH/pK184 into strains REMH76 and REMH86 strains: A plasmid preparation of Ptac-gcpE-lytB-petF-petH/pK184 was provided by Gene Oracle, Inc.

[0513] Transformation of Ptac-gcpE-lytB-petF-petH/pK184 into REMH76 and REMH86

[0514] To build the isoprene-producing test strains (REM31 and REM29) which harbor the *T. elognatus* GcpE and LytB enzymes to assess against the parental strains (REMH76 and REMH86) for benefits in DXP pathway flux and isoprene production, the Ptac-gcpE-lytB-petF-petH/pK184 plasmid was transformed by electroporation into REMH76 and REMH86. Transformants were recovered in L broth and plated on L agar containing carbinicillin (50ug/ml), kanamycin (10ug/ml), and gentamycin (10ug/ml). The resulting strains are designated as such: REM31 (REMH76 / Ptac-gcpE-lytB-petF-petH/pK184) and REM29 (REMH86 / Ptac-gcpE-lytB-petF-petH/pK184).

Comparing REMH76 and REMH86 to REM31 and REM29 for growth and isoprene production

[0515] The parental strains REMH76 and REMH86 were compared against the test strains REM31 and REM29, respectively, in a shake flask isoprene headspace assay as well as in a DXP metabolite determination study. The benefit of expressing the *T. elongatus* GcpE and LytB enzymes on isoprene production from the *E. coli* host is illustrated in Figure 48.

[0516] Growth

[0517] Parental strains REMH76 and REMH86 and test strains REM31 and REM29 were grown at 30°C in TM3 liquid media (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) supplemented to a final concentration with 0.1% yeast extract and 1.0% glucose and including carbinicillin, (50ug/ml) kanamycin (10ug/ml) and gentamycin (10ug/ml). Growth was monitored periodically by recording each of the culture's optical density measured at 600nm using an Eppendorf Biophotometer spectrometer (Eppendorf).

[0518] Isoprene Production

[0519] Isoprene production was analyzed using a headspace assay. 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. 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.

[0520] The specific productivity of each strain was reported as ug/L OD Hr. Ratio of 1900ul headspace:100ul broth in assay vials for 30 min. incubation resulted in the following conversion of isopreneug/L of culture to specific productivity: (isoprene/L determined by GC-MS) X (38)/(OD 600nm of the culture).

[0521] DXP metabolite accumulation

[0522] The DXP metabolites of the isoprene-producing parental (REMH76 and REMH86) and test strains (REM31 and REM29) described above were isolated and quantified as described below. The resulting data is discussed in the legend to Figure 48.

[0523] Metabolite extraction

[0524] Cell metabolism was rapidly inactivated by withdrawing 3.5 mL of the culture into a tube filled with 3.5 mL of dry ice-cold methanol. Cell debris was pelleted by centrifugation and the supernatant was loaded onto Strata-X-AW anion exchange column (Phenomenex) containing 30 mg of sorbent. The pellet was re-extracted twice, first with 3 mL of 50% MetOH containing 1 mM NH_4HCO_3 buffer (pH=7.0) and then with 3 mL of 75% MetOH/ 1 mM NH_4HCO_3 buffer (pH=7.0). After each extraction, cell debris was pelleted by centrifugation and the supernatants were consecutively loaded onto the same anion exchange column. During the extraction and centrifugation steps the samples were kept at below +4°C. Prior to metabolite elution, the anion exchange columns were washed with water and methanol (1 mL of each) and the analytes were eluted by adding 0.35 mL of concentrated NH_4OH/methanol (1:14, v/v) and then 0.35 mL of concentrated NH_4OH/water/methanol (1:2:12, v/v/v) mixtures. The eluant was neutralized with 30 μL of glacial acetic acid and cleared by centrifugation in a microcentrifuge.

[0525] Metabolite quantification

[0526] Metabolites were analyzed using a Thermo Scientific TSQ Quantum Access mass spectrometer (Thermo Electron Corporation, San Jose, CA). All system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). For the LC-ESI –MS/MS method, a chiral Nucleodex ß-OH 5µM HPLC column (100 x 2 mm, Macherey-Nagel, Germany) equipped with a CC 8/4 Nucleodex beta-OH guard cartridge was eluted with a mobile phase gradient shown in Table 1 (flow rate of 0.4 mL/min). The sample injection volume was 10 µL.

[0527] Mass detection was carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 245.0 for IPP and DMAPP, 381.1 for FPP, 213.0 for DXP, 215.0 for MEP, 260.0 for HDMAPP, and 277.0 for cMEPP. Concentrations of metabolites were determined based on the integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0). Calibration curves obtained by injection of corresponding standards purchased from Echelon Biosciences Inc. Intracellular concentrations of metabolites were calculated based on the assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 µL.

Example 10: Deletion of iscR in E. coli BL21(DE3) genotype to improve isoprene production

[0528] Previous studies suggest that repair of damaged Fe-S centers and the turnover or regeneration of active 4Fe-4S centers within GcpE is partially contributable to the perceived bottleneck in DXP-mediated isoprenoid biosynthesis at the catalytic step carried out by GcpE. Increased levels of the related enzyme LytB have been obtained from *E. coli* engineered to overexpress the *isc* operon (Gräwert *et al., J Am Chem Soc.* 126(40):12847-55 (2004), which is hereby incorporated by reference in its entirety). The enzymes encoded by the *E. coli isc* operon have been shown to play a role in Fe-S cluster biogenesis and maintenance (Tokumoto and Takahashi, *J. Biochem.,* 130: 63-71 (2001); Djaman *et al., J. of Biol. Chem.*, 279(43):44590-44599 (2004), which are each hereby incorporated by reference in their entireties). An alternative approach to overexpressing

the *isc* operon in *E. coli* to generate increased levels of active 4Fe-4S cluster containing enzymes such as GcpE and LytB is to remove the IscR transcriptional repressor that inhibits expression of the *isc* operon (Schwartz *et al.*, *PNAS,* 98(26):14751-3 (2001), which is hereby incorporated by reference in its entirety). Such an approach was recently proved successful for a group expressing Clostridial hydrogenase in *E. coli* BL21(DE3) (Akhtar and Jones, *Appl. Microbiol. Biotechnol.* 78(5):853-62 (2008), which is hereby incorporated by reference in its entirety).

[0529] In this example, we demonstrated that the removal of *iscR* from the *E. coli* BL21(DE3) genome significantly improves isoprene production over that produced from the corresponding wild-type strain.

Deletion of iscR from BL21(DE3)/pRed/ET

[0530] The gene deletions and subsequent loopout of the antibiotic resistance markers described in this example were carried out using the Red/ET system from Gene Bridges GmbH according to the manufacturer's instructions. The strain BL21(DE3) (Invitrogen) was used.

[0531] Primer sequences used

top iscR deletion: 5'-GGGCGAGTTTGAGGTGAAGTAAGACATGAGACTGACA TCTGAACCCTCACTAAAGGGCGGCCGC (SEQ ID NO:80)

bottom iscR deletion: 5'-TTCTTTTTATTAAGCGCGTAACTTAACGTCGATCGC GTCTTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCTTACGCCCCGCC CTGCCACTCATCGCA (SEQ ID NO:81)

5' screen up of up iscR: 5'-AGCCAGGAGTTGAATATCCTG (SEQ ID NO:82)

3' down of down iscR: 5'-TGATGGACACGAGGATGGTGT (SEQ ID NO:83)

[0532] Strategy for creating the deletion strains

[0533] The strategy for the deletion of *iscR* is shown in Figure 61. The antibiotic resistance cassette GB-CmR was amplified by PCR using primer sets top iscR deletion/ bottom iscR deletion for deletion of the *iscR* locus. The primers contain 50 bases of

homology to the region flanking the *iscR* gene to allow recombination at the specific locus upon electroporation of the PCR product in the presence of the pRed-ET plasmid.

[0534] Amplification of the deletion cassettes

To amplify the GB-CmR cassette for deletion of *iscR* the following PCR reactions were set up:
1ul (100ng GB-CmR)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) top iscR deletion
1.25ul primer (10uM) bottom iscR deletion
35 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 63°C x 30sec., 72°C x 3 min] x 29 cycles; 72°C x 5min, 4°C until cool (Biometra T3000 Combi Thermocycler).

[0535] The resulting PCR fragment was separated on a 1.2% E-gel (Invitrogen) to verify successful amplification, and purified using QIAquick PCR Purification kit according to manufacturer's instructions. The resulting stock was designated GB-CmR-*iscR* fragment.

Integration of GB-CmR- iscR product into the BL21(DE3) genome

[0536] The pRed-ET vector (Gene Bridges) was transformed into electrocompetent BL21(DE3) (Invitrogen) by electroporation resulting in strain BL21(DE3)/pRed-ET. Approximately 500ng of GB-CmR- *iscR* PCR fragment was electroporated into BL21(DE3)/pRed-ET. The transformants were recovered in L Broth for 1 hour at 37°C and then plated on L agar containing chloramphenical (10ug/ml). Chloramphenicol resistant colonies were analyzed by PCR for the replacement of the *iscR* by the GB-CmR-*iscR* fragment using primers 5' screen up of up iscR/3' screen down of down iscR. The correct strain was designated REM14::CMP. The chloramphenicol resistance cassette was looped out of the strain using pCP20 from the RED/ET kit according to the manufacturer's instructions. Transformants were verified by loss of resistance to chloramphenicol (10ug/ml) and PCR demonstrating loopout of the GB-CmR cassette. The resulting strain was designated REM14.

[0537] Creation of strains REM65-1 and REM4, the parental strains to REM12 and REM13

[0538] The wild-type BL21(DE3) (Invitrogen) and *ΔiscR* strain REM14 were transformed with the T7-MEARR alba/pBBR1MCS-5 construct to create the isoprene-producing strains REM65-1 and REM4 strains, respectively. A picture of the isoprene synthase containing vector, T7-MEARR alba/pBBR1MCS-5, is shown in Figure 61. The construction of T7-MEARR alba/pBBR1MCS-5 is described in the Example: Expression of alternative *ispG* (*gcpE*) and *ispH* (*lytB*) and their corresponding reducing shuttle system, from *Thermosynechococcus elongatus* BP-1 in an isoprene-producing *E.* coli to improve isoprene production.

Transformation of T7-MEARR alba/pBBR1MCS-5 into BL21(DE3) and REM14

[0539] To build the isoprene-producing strains REM65-1 and REM4 strains, the T7-MEARR alba/pBBR1MCS-5 plasmid was transformed by electroporation into BL21(DE3) (Invitrogen) and REM14. Transformants were recovered in L broth and plated on L agar containing gentamycin (10ug/ml). The resulting strains are designated as such: REM65-1 (BL21(DE3)/T7-MEARR alba/pBBR1MCS-5 and REM4 (REM14/T7-MEARR alba/pBBR1MCS-5).

[0540] Construction of the test strains REM12 and REM13

[0541] The entire DXP pathway from *E. coli* was synthesized by DNA2.0 (Menlo Park, CA) and cloned into pET24a (see Figure 63).

[0542] To build the higher flux DXP pathway isoprene-producing REM12 and REM13 strains, the DXP operon pET24a plasmid was transformed by electroporation into REM65-1 and REM4. A picture of the DXP pathway enzyme containing vector, DXP operon pET24a plasmid, is shown in Figure 63.

[0543] Transformation of DXP operon pET24a into REM65-1 and REM4

[0544] To build the test strains REM12 and REM13 strains, the DXP operon pET24a plasmid was transformed by electroporation into REM65-1 and REM4. Transformants

were recovered in L broth and plated on L agar containing gentamycin (10ug/ml) and kanamycin (10ug/ml). The resulting strains are designated as such: REM12 (REM65-1/DXP operon pET24a) and REM13 (REM4//DXP operon pET24a).

[0545] Analysis of REM12 and REM13 for growth and isoprene production

[0546] The wild-type strain REM12 and otherwise isogenic *ΔiscR* strain REM13 were compared in a shake flask isoprene headspace assay. The benefits on isoprene production and effect on growth rate the loss of *iscR* causes in the *E. coli* host are illustrated in Figure 60.

[0547] Growth

[0548] Strains REM12 and REM13 were grown at 30°C in TM3 liquid media (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) supplemented to a final concentration with 0.1% yeast extract and 1.0% glucose and including kanamycin (10ug/ml) and gentamycin (10ug/ml). Growth was monitored periodically by recording each of the culture's optical density measured at 600nm using an Eppendorf Biophotometer spectrometer (Eppendorf). 50uM isopropyl ß-D-1-thiogalactopyranoside (IPTG) was added to the cultures to induce expression of the isoprene synthase and DXP enzymes harbored by the strains at time zero, as indicated in the legend to Figure 60.

[0549] Isoprene production

[0550] Isoprene production was analyzed using a headspace assay. 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. 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. The specific productivity of each strain is reported as ug/L OD Hr. Note, ratio of 1900ul headspace:100ul broth in assay vials for 30 min. incubation results in the following conversion of isopreneug/L of culture to specific productivity: (isoprene/L determined by GC-MS) X (38)/(OD 600nm of the culture).

Example 11: Evaluation of alternative *ispG* (*gcpE*) and *ispH* (*lytB*) alleles from different organisms by complementation of *ΔispG* and/or *ΔispH* strains of BL21(DE3)PL.2 *mKKDyI*::FRT

[0551] We constructed an *E. coli* strain expressing the lower mevalonic acid pathway (mevalonate kinase, phosphomevalonate kinase, diphosphomevalonte decarboxylase and IPP isomerase from yeast) as a base strain for testing the functionality of DXP pathway enzymes from heterologous organisms. This strain produces IPP and DMAPP from the lower mevalonate pathway if it is grown in the presence of mevalonate. Deletions of enzymes of the DXP pathway can be rescued by growing the stain in the presence of mevalonate. Therefore, functionality of heterologous DXP pathway genes can be expressed in the *E. coli* containing the lower MVA pathway and looking for growth in the absence of mevalonate.

[0552] Construction of MD09-170 (BL21(DE3)PL .2 mKKDyI ::FRT

Primer Sequences
MCM 161: 5'-CACCATGGTATCCTGTTCTGCG (SEQ ID NO:84)
MCM162: 5'-TTAATCTACTTTCAGACCTTGC (SEQ ID NO:85)
MCM143: 5'-aggaggtggtctcaaATGACTGCCGACAACAATAGTA (SEQ ID NO:86)

MCM144: 5'-aggaggtggtctcagcgctctgcagTTATAGCATTCTATGAATTTGCCTG (SEQ ID NO:87)

[0553] A P1 phage lysate was generated from MCM521 (BL21 neo-PL.2-mKKDyI) and transduced into BL21(DE3) (according to Procedure 12-Genetic Transduction Using P1vir protocol). The transductants were selected on L agar plates containing kanamycin (20 ug/ml), with incubation at 37°C overnight. Four colonies were verified by PCR to be correct transductants. One of these colonies was selected and designated MD09-169 (BL21(DE3)PL .2 *mKKDyI* ::Kan). The kanamycin resistance marker was looped out of this strain using pCP20 from the Red/ET system from Gene Bridges GmbH according to the manufacturer's instructions. The correct loopout was confirmed by testing for sensitivity to kanamycin (20 ug/ml) and then loss of the kanamycin resistance cassette was verified by PCR. The correct strain was designated MD09-170 (BL21(DE3)PL .2 *mKKDyI* ::FRT).

[0554] Deletion of ispG and ispH from MD09-170

[0555] The gene deletions and subsequent loopout of the antibiotic resistance markers described in this example were carried out using the Red/ET system from Gene Bridges GmbH according to the manufacturer's instructions. The strain MD09-170 was used.

Primer sequences used

MQ09-18F
5'-
GAACAATCACCGGCGCAGTAACAGACGGGTAACGCGGGAGATTTTTCATGaatt aaccctcactaaagggcgg (SEQ ID NO:88)

MQ09-18R 5'-
CGGGAAGCGAGGCGCTTCCCATCACGTTATTATTTTTCAACCTGCTGAACTAA TACGACTCACTATAGGGCTCG (SEQ ID NO:89)

MQ09-19F 5'-
TTTTGATATTGAAGTGCTGGAAATCGATCCGGCACTGGAGGCGTAACATGaatta accctcactaaagggcgg (SEQ ID NO:90)

MQ09-19R 5'-
ATTTTCGCATAACTTAGGCTGCTAATGACTTAATCGACTTCACGAATATCTAA TACGACTCACTATAGGGCTCG (SEQ ID NO:91)

MQ09-20F 5'-cggcgcagtaacagacgggtaacgcgggagatttttcatg (SEQ ID NO:92)
MQ09-20R 5'-cgcttcccatcacgttattatttttcaacctgctgaac (SEQ ID NO:93)
MQ09-21F 5'-gaagtgctggaaatcgatccggcactggaggcgtaacatg (SEQ ID NO:94)
MQ09-21R 5'-cttaggctgctaatgacttaatcgacttcacgaatatc (SEQ ID NO:95)

Strategy for creating the deletion strains

[0556] The strategy for the deletion of ispG and ispH is shown in Figure 66. The antibiotic resistance cassette GB-CmR was amplified by PCR using primer sets MQ09-18F/ MQ09-18R or MQ09-19F/ MQ09-19R for deletion of ispG or ispH respectively. The primers contain 50 bases of homology to the region flanking the ispG or ispH genes to allow recombination at the specific locus upon electroporation of the PCR product in the presence of the pRed-ET plasmid.

Amplification of the deletion cassettes

[0557] To amplify the GB-CmR cassette for deletion of *ispG* or *ispH* the following PCR reactions were set up:

2ul (100ng GB-CmR)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) MQ09-18F/19F
1.25ul primer (10uM) MQ09-18R/19R
2ul DMSO
32 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 20sec., 55°C x 20sec., 72°C x 50sec] x 29 cycles; 72°C x 3min,
[0558] 4°C until cool (Eppendorf Mastercycler PCR machine)

[0559] The resulting PCR fragments were separated on a 1.2% E-gel (Invitrogen), and purified using the Qiagen QiaQuick Gel Extraction and QIAquick PCR Purification kits according to manufacturer's instructions. The resulting stocks were: GB-CmR- *ispG* fragment (1.593 kb)~180ng/ul, and GB-CmR- *ispH* fragment (1.593 kb)~165ng/ul.

[0560] Integration of GB-CmR- ispG or GB-CmR- ispH PCR products into MD09-170/pRed-ET Strain

[0561] The pRed-ET vector (Gene Bridges kit) was transformed into MD09-170 by electroporation resulting in strain MD09-170/pRed-ET. Approximately 300-500 ng of GB-CmR- *ispG* or GB-CmR- *ispH* PCR fragments were electroporated into MD09-170/pRed-ET. The transformants were recovered in L Broth containing 500uM mevalonic acid (Sigma) for 1 hour at 37°C and then plated on L agar containing chloramphenical (5 ug/ml) and mevalonic acid (MVA) (500uM). Chloramphenicol resistant colonies were analyzed by PCR for the presence of the GB-CmR cassette and the absence of the *ispG* or *ispH* genes using primers MQ09-20F/ MQ09-20R or MQ09-21F/ MQ09-21R respectively. The correct strains were designated MD09-209(BL21(DE3)PL.2 mKKDyI::FRT-*ΔispG::Cm*) and MD09-210 (BL21(DE3)PL.2 mKKDyI::FRT-*ΔispH::Cm*). The chloramphenicol resistance cassette was looped out of both strains using pCP20 from the RED/ET kit according to the manufacturer's instructions. Transformants were verified by loss of resistance to chloramphenicol (5ug/ml) and PCR demonstrating loopout of the GB-CmR cassette.

[0562] The resulting strains were designated MD09-219 (BL21(DE3)PL.2 *mKKDyI*::FRT-ΔispG::FRT) and MD09-220 (BL21(DE3)PL.2 *mKKDyI*::FRT-ΔispH::FRT).

[0563] Complementation of MD09-219 and MD09-220 with alleles from Thermosynechococcus elongatus BP-1

[0564] To test the functionality of the *gcpE* and *lytB* genes (annotated) from *T. elongates*, the following plasmids expressing these constructs or *gcpE* and *lytB* from *E. coli* were transformed by electroporation into MD09-219 and MD09-220:

1. *E.coli*: GI 1.6-gcpE-lytB-yidi/pCR-Blunt II-TOPO (Kan) (positive control)
2. *T. elong*: Ptac-gcpE-petF-petH/pK184 (Kan)

[0565] 3. *T. elong*: Ptac-gcpE-lytB-petF-petH/pK184 (Kan)

[0566] Transformants from 1. (*E.coli*) were recovered in L broth containing MVA (500uM) and plated on L agar containing kanamycin (50 ug/ml). The resulting strain is designated MD09-219/ GI1.6-gcpE-lytB-yidi/pCRII-TOPO (Kan).

[0567] Transformants from 2. (*T. elong*) or 3. (*T. elong*) were recovered in L broth containing MVA (500uM) and IPTG (200uM) and then plated on L agar containing on kanamycin (50 ug/ml) and IPTG (200uM). The resulting strains were designated MD09-219/ Ptac-gcpE-petF-petH/pK184 (Kan) and MD09-219/ Ptac-gcpE-lytB-petF-petH/pK184 (Kan) respectively.

[0568] Several transformants were obtained on all of the plates suggesting that the *T. elongatus gcpE* and *lytB* were functional in *E. coli*. To confirm this, transformants were grown in L broth containing kanamycin (50 ug/ml) with and without IPTG (200 uM).

[0569] Construction of GI 1.6-gcpE-lytB-yidi/pCR-Blunt II-TOPO

[0570] The construction of the GI 1.6-gcpE-lytB-yidi/pCR-Blunt II-TOPO described in this example was carried out using standard molecular biology techniques (*see,* for example, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to cloning techniques). A picture illustrating the resulting plasmid construct is shown in Figure 67. The MD09-219 and MD09-220 strains were used for the transformations described herein.

Primer sequences

[0571] 5' EcoRI-GI 1.X-BamHI gcpE DXP oper: 5'-GAG GAA TTC GCG AGC CGT CAC GCC CTT GAC NAT GCC ACA TCC TGA GCA AAT AAT TCA ACC ACT AAA CAA ATC AAC CGC GTT TCC CGG AGG TAA CCG GAT CCA AGG AGA TAT ACC ATG CAT AAC CAG GCT CCA ATT CAA CGT AGA (SEQ ID NO:96)

[0572] 3' PstI idi DXP operon: 5'- ATA TCC TGC AGT TAT AGC ATT CTA TGA ATT TGC CTG TC (SEQ ID NO:97)

[0573] M13 Forward (-20): 5'-GTAAAACGACGGCCAGT (SEQ ID NO:98)

[0574] M13 Reverse (-27): 5'-CAGGAAACAGCTATGAC (SEQ ID NO:99)

[0575] degenerate N base: A base yields GI 1.6-, T base yields GI 1.5-, G base yields GI1.2-, and C base yields GI 1.0-promoter

[0576] Strategy for constructing GI 1.6-gcpE-lytB-yidi/pCR-Blunt II-TOPO

[0577] The vector construct harboring the T7 polymerase governed synthetic DXP operon, DXP operon pET24a, was used as the PCR template..

Amplification of the GI 1.6-gcpE-lytB-yidi fragment

To amplify the GI 1.6-gcpE-lytB-yidi fragment (among the other GI 1.X- possibilities) for cloning into the pCR-Blunt II-TOPO vector the following PCR reaction was performed:
1ul (approx. 100ng DXP operon pET24a)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' EcoRI-GI 1.X-BamHI gcpE DXP oper
1.25ul primer (10uM) 3' PstI idi DXP operon
35 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 63°C x 30sec., 72°C x 3.5 min.] x 29 cycles; 72°C x 5min, 4°C until cool (Biometra T3000 Combi Thermocycler).

[0578] The resulting PCR fragment was separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits (Qiagen) according to manufacturer's instructions. The resulting stock was GI 1.X-gcpE-lytB-yidi fragments.

[0579] Cloning of the GI 1.6-gcpE-lytB-yidi fragment into pCR-Blunt II-TOPO

[0580] The GI 1.X-gcpE-lytB-yidi fragments were cloned into pCR-Blunt II-TOPO using Invitrogen's Zero Blunt® TOPO® PCR Cloning Kit using the suggested protocol. Chemically competent TOP10 cells (Invitrogen) were transformed with 2ul of the ligation reaction using a standard heat-shock protocol, and recovered in L broth for 1 hour at 37°C and then plated on L agar containing kanamycin (10ug/ml). Resulting colonies were selected, grown overnight in L broth containing kanamycin (10ug/ml), and harvested for plasmid preparation the following day. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit. A number of plasmid preparations were sequenced (Quintara; Mountain View, CA) using primers M13 Forward (-20) and M13

Reverse (-27) and the correct GI 1.6-gcpE-lytB-yidi/pCR-Blunt II-TOPO clone identified.

Example 12: Improving isoprene production in *E. coli* by deregulating glucose uptake

[0581] In *Escherichia coli*, glucose is transported using the phosphoenolpyruvate transport system (PTSglc), which consists of PtsHICRR and the transporter PtsG (*see* Tchieu *et al.*, *J. Mol. Microbiol. Biotechnol.* 3(3):329-46 (2001), which is hereby incorporated by reference in its entirety). Glucose is phosphorylated as it is transported into the cell, with the phosphate originating from phosphoenol pyruvate. The resulting glucose-6-phosphate is metabolized via glycolysis regenerating the PEP. Glucose transport continues through exponential growth but is down-regulated as cells enter stationary phase. For commercial purposes it is desirable to maximize production time and yield of the desired molecule, which is difficult to achieve if the feedstock transporter is downregulated. To solve this problem, the PTSglc system is deleted by deleting *ptsHIcrr*, and in some embodiments, *ptsG*, and constitutively express *galP* and *glk*, encoding the galactose permease and glucokinase respectively. The galactose permease transports glucose without phosphorylation so it is necessary to express the glucokinase (*see* US Patent Application No. 20050079617, which is hereby incorporated by reference in its entirety).

[0582] The *ptsHIcrr* operon is deleted in BL21using the Red/ET system from Gene Bridges. Electrocompetent BL21 (Invitrogen) are transformed with the pRed/ET plasmid and the resulting cells are made electrocompetent by washing 3-4 x in ice cold dH_2O. The GB-cmR cassette is amplified using forward and reverse primers have at least 50 bases of homology to the regions immediately upstream of *ptsH* or immediately downstream of *crr*. The resulting PCR product is used to transform BL21/pRED and transformants are plated on MacConkey agar containing glucose (1%) and chloramphenical (5 ug/ml). Transformants that grown and are white in color will be the correct genotype. The *ptsHIcrr* knockout is transduced into the desired isoprene-producing hosts using P1 transduction.

[0583] The Ptrc-galP-cat and Ptrc-glk-cat cassettes are amplified by PCR from strains KLpts::gal-trc::Cm or KLgalPglk-trc-cat S (*see* U.S. Patent Application No. 20050079617, which is hereby incorporated by reference in its entirety) with at least 50 base pairs (bp) of homology on the 5' and 3' ends to allow homologous recombination into BL21 with either the DXP or the MVA or both pathways and isoprene synthase (example *ispS* from *P. alba* or a variant thereof) expressed and the *ptsHIcrr* and/or *ptsG* deleted. The desired strain is made competent and transformed with the pRed/ET plasmid, and after being made competent, the new strain is transformed with the galP-trc-cat cassette. Transformants are selected on MacConkey agar containing 1% glucose and chloramphenicol (5 ug/ml). Colonies which are slightly pink have the correct genotype. The CAT markers in these cassettes are flanked by *loxP* sites and can be looped out by standard methods (Palmeros *et al.*, Gene 18;247(1-2):255-64 (2000)) which is hereby incorporated by reference in its entirety). The strain expressing *galP* from Ptrc is then transformed with the glk-trc-cat cassette and transformants are select on MacConkey agar containing 1% glucose and chloramphenicol (5 ug/ml). Colonies which are deep red in color are the correct colonies.

[0584] The resulting strains have the full MVA pathway, with or without the DXP pathway constitutively expressed, an isoprene synthase (example *P. alba IspS* or a variant thereof), a deletion of the *ptsHIcrr* and/or *ptsG*, and constitutive expression of the galactose permease and glucokinase. To demonstrate that isoprene production is enhanced and/or prolonged in these strains compared to the parent which transports glucose via the PTSglc system, the strains are tested in shake flask (TM3 containing 1% glucose, 0.1% yeast extract), microfermentor (TM3 containing 1% glucose, 0.1% yeast extract), and in 14-Liter fermentation. These strains are also tested using pretreated and saccharified biomass, for example corn fiber, corn stover, switch grass, forage sorghum, softwood pulp, hardwood pulp or other suitable biomass.

[0585] Isoprene production is enhanced and/or prolonged in the strains with *ptsHIcrr* and/or *ptsG* deletion and constitutive expression of the galactose permease and glucokinase compared to the compared to the parent strains without the deletion of

ptsHIcrr and/or *ptsG* and constitutive expression of the galactose permease and glucokinase.

Example 13: Expression of monoterpene and sesquiterpene synthases in combination with the expression of isoprene synthase increases the specific productivity of isoprene in *E. coli*.

[0586] Isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) are biosynthesized by the DXP pathway (also called the non-mevalonate pathway and MEP pathway) in *E. coli*. IPP and DMAPP can be condensed to form geranyl pyrophosphate (GPP) and subsequently farnesyl pyrophosphate (FPP) by farnesene synthase (IspA). FPP can be converted to octaprenyl pyrophosphate (OPP) and undecaprenyl pyrophosphate (UPP) by extension of FPP with IPP. These products serve a variety of functions in *E. coli* including prenylation of tRNA (protein synthesis component) with DMAPP, formation of quinones (respiratory chain component) with OPP, and peptidoglycan formation (cell wall component) with UPP.

[0587] The products of the DXP pathway may be regulated by the production of IPP and DMAPP. Accordingly, the example shows that the introduction of a terpene synthase that utilizes downstream products of the DXP pathway in combination with isoprene synthase in *E. coli* results in increased flux through the DXP pathway and increased specific productivity of isoprene.

[0588] Methods

Strain Construction
The following strains are constructed.

Ocimene synthase, farnesene synthase and artemesinin synthase are cloned into pTrchis2A plasmids to give pTrcFPP, pTrcAS, or pTrcOS. Isoprene synthase (for example IspS from P. alba or variants thereof) is cloned into pBBR under control of the Ptrc promoter to give pBBRPtrcalba .

Strain set 1) BL21GI1.6yIDI/pBBRPtrcalba itself or combined with PtrcFPP or pTrcAS or pTrcOS.

Strain set 2) BL21GI1.6yIDIGI1.6DXS/pBBRPtrcalba itself or combined with PtrcFPP or pTrcAS or pTrcOS.

[0589] The strains in strain set 1) or 2) are grown in shake flask or in the microfermentor in TM3 containing 0.1% yeast extract and 1% glucose. The specific productivity of isoprene is measured over time.

[0590] The specific productivity of isoprene from strains in strain set 1) are compared. The specific productivity of isoprene in the strains containing FPP, OS, or AS is higher than in the strain without FPP, OS, or AS.

[0591] The specific productivity of isoprene from strains in strain set 2) are compared. The specific productivity of isoprene in the strains containing FPP, OS, or AS is higher than in the strain without FPP, OS, or AS.

Example 14: Deletion or reduction of carbon into thiamine and pyridoxine paths for relief of inhibition

[0592] 1-deoxy-D-xylulose-5-phosphate (DXP) is a substrate in three essential anabolic pathways in *E. coli*, namely isoprenoids, thiamine and pyridoxal synthesis. In order to avoid any feedback regulation from thiamine or pyridoxal pathways, which could then decrease the flux in the DXP pathway for isoprenoid production, we build strains mutated in the thiamine and/or pyridoxal pathways.

[0593] A: Construction of an *E. coli* strain deleted in the thiamine synthesis pathway

[0594] Several enzymes are involved in the biosynthesis of thiamine from DXP. ThiG and ThiH combine to form a complex containing an iron-sulfur cluster (Leonardi *et al. FEBS Lett.* 539(1-3):95-9 (2003), PMID: 12650933, which is hereby incorporated by reference in its entirety). Together, they are required for the synthesis of 4-methyl-5-(ß-hydroxyethyl)thiazole phosphate, which is the rate-limiting step in thiamine synthesis (Leonardi *et al. J Biol. Chem.* 279(17):17054-62 (2004), PMID: 14757766; Vander et al., J. Bacteriol. 175(4):982-92 (1993), PMID: 8432721; which are hereby incorporated by reference in their entireties). Since it is in the rate-limiting step, and it is the first enzyme after 1-deoxy-D-xylulose-5-phosphate, thiG was chosen as the gene to be deleted.

[0595] A PCR product was obtained using primers GB400thiGF (caggagccagaacgcaactgc (SEQ ID NO:100) and GB400thiGR (CACTTTCGCCTGATGTTCACC (SEQ ID NO:101), and genomic DNA of strain JW5549 from the Keio collection (Baba *et al.*, *Mol. Syst. Biol.* 2006.008 (2006), which is hereby incorporated by reference in its entirety). The PCR product contains a kanamycin cassette replacing most of the thiG gene and around 400 bp flanking regions of both sides of the thiG gene.

[0596] A BL21(DE3) thiG::Kan mutant is then obtained by Red/ET recombineering (Gene Bridges, Dresden, Germany) using the PCR product mentioned above. It is proven correct by amplification and sequencing. The strain is named CMP179.

[0597] B: Construction of an E. coli strain deleted in the pyridoxal synthesis pathway

[0598] PdxJ catalyses the formation of pyridoxine-5-phosphate (precursor of pyridoxal-5-phosphate then pyridoxal) from 1-deoxy-D-xylulose-5-phosphate and 1-amino-propan-2-one-3-phosphate. The latter is produced by a sequence of reactions coming from erythrose-4-phsophate, the first one catalyzed by D-erythrose 4-phosphate dehydrogenase (epd). Thus both *pdxJ* and *epd* are good candidates for deleting the production of pyridoxal. However, epd has been reported not to be required for glycolysis or for synthesis of pyridoxal (Seta *et al.*, *J. Bacteriol.* 179(16):5218-21 (1997), which is hereby incorporated by reference in its entirety). Thus, *pdxJ* is chosen as the target for mutation.

[0599] A PCR product is obtained using primers GB400pdxJF (CAT TCA GTC TCT TGC AGG GGT C (SEQ ID NO:102) and GB400pdxJR (gcatagtgccgctcatctgcc (SEQ ID NO:103)), and genomic DNA of strain JW2548 from the Keio collection (Baba *et al.* 2006). The PCR product contains a kanamycin cassette replacing most of the pdxJ gene and around 400 bp flanking regions of both sides of the pdxJ gene.

[0600] A BL21(DE3) pdxJ::Kan mutant is then obtained by Red/ET recombineering (Gene Bridges, Dresden, Germany) using the PCR product mentioned above. It is proven correct by amplification and sequencing. The strain is named CMP180.

[0601] C: Construction of an E. coli strain deleted in the thiamine and pyridoxal synthesis pathways

[0602] The kanamycin cassette is removed from CMP179 and/or CMP180 by Flp-mediated excision, using plasmid 706-Flp from Gene Bridges (Dresden, Germany). Then the PCR product described in section A is used to mutate BL21(DE3) pdxJ through Red/ET recombineering.

D: Production of isoprene via the DXP pathway, in a thiG and/or a pdxJ mutant

[0603] The effect of the thiG, pdxJ or thiG pdxJ mutations on the production of isoprene through the DXP pathway is assessed in different constructs enhancing DXP pathway flux and expressing IspS (isoprene synthase) from *Populus alba*, such as MCM597 (BL21(DE3)pLysS pET24(MEA)alba-DXS-yIDI) or MCM719 (BL21 gi1.6-yIDI gi1.6-dxs, pTrc(MEA)alba)).

[0604] Strains are grown overnight at 30° C, 200 RPM, in HM1 medium (Table 2) plus appropriate antibiotics. The morning after, they are resuspended to an OD = 0.2 in fresh HM1 medium + appropriate antibiotics. Flasks are incubated at 30°C, 200 RPM, and regularly sampled for OD and isoprene productivity.

Table 2: HM1 medium composition

Compounds	Concentration (g/L)
K2HPO4	13.6
KH2PO4	13.6
MgSO4 * 7H2O	2
Citric Acid Monohydrate	2
Ferric Ammonium Citrate	0.3
(NH4)2SO4	3.2
Trace metal solution	1 ml

[0605] Specific productivity (ug isoprene/OD.h) is increased when strains MCM597 or MCM719 contains thiG, pdxJ, or thiG pdxJ mutations.

Example 15: Balancing Pyruvate and G-3-P (glyceraldehyde-3-phosphate) to increase isoprene production

[0606] Flux to the DXP pathway may be positively (more flux) effected to increase isoprene production by maximizing the balance between the two precursors required for the DXP pathway, pyruvate and G-3-P (glyceraldehyde-3-phosphate). Accordingly, adjusting the expression level of enzymes that determine flux into glycolysis, into the pentose phosphate pathway (PPP) and into the Entner-Doudoroff (ED) pathway (Figure 68). In Sections B-D, flux of pyruvate and G-3-P are affected simultaneously. Optimal balance of the two precursors to the DXP pathway may also be achieved by redirecting flux with the effect of elevating or lowering pyruvate or G-3-P separately. Section E demonstrates this approach with the coexpression of the mevalonate pathway. In addition it is proposed that desired flux balance can be achieved by choice of feed stock, *e.g.*, feeding a mixture of glucose + gluconic acid; Section A shows this approach. A combination of these approaches may prove to be additive in achieving precursor balance and maximize yield of isoprene; this is tested in Section F.

[0607] Section A

[0608] Cells that have been constructed by procedures known to practitioners of the art and as exemplified in this application to overexpress the DXP pathway or wild type cells are fed with various carbon sources, but more specifically cells are fed glucose plus gluconic acid or gluconic acid alone. The culture is sampled and analyzed for improved evolution of isoprene. This analysis is accomplished by monitoring the head space of the culture with a mass spectrometer either continuously or at specific time points during the cultivation of cells with different concentrations of the carbon sources.

[0609] Section B

[0610] Cells in Section A harboring the overexpressed DXP pathway or wild type cells are genetically engineered to overexpress glucose-6-phosphate dehydrogenase to redirect flux to PPP and ED. Effect and benefit of these mutations can be assessed by measuring isoprene specific productivity.

[0611] Section C

[0612] Cells in Section A harboring the overexpressed DXP pathway or wild type cells are genetically engineered to limit expression of glucose-6-phosphate isomerase to redirect flux to PPP and ED. Effect and benefit of these mutations can be assessed by measuring isoprene specific productivity.

[0613] Section D

[0614] Cells in Section A harboring the overexpressed DXP pathway or wild type cells are genetically engineered to limit expression of Gluconate-6-phosphate dehydrogenase (*gnd*) to limit flux to pentose phosphate and maximize flux to ED. Effect and benefit of these mutations can be assessed by measuring isoprene specific productivity.

[0615] Section E

[0616] In this section, the DXP precursor pyruvate is adjusted by the level of expression of the mevalonic acid pathway for which pyruvate is the sole precursor. Cells are constructed to overexpress the DXP pathway enzymes as well as the mevalonic acid pathway enzymes and expression of both pathways is adjusted, by choosing the appropriate promoter strengths, such that pyruvate flux is balanced with G-3-P flux and neither precursor accumulates in the cell. Similar, approaches in the presence of zwf, gnd, and pgi mutations, singly or in all possible combination, have potential for improved performance.

[0617] Section F

[0618] The strains created in Sections B-E, are combined for potential additivity. Combination of zwf and gnd in a overexpressed DXP pathway strain is tested for improved performance of the strain. Similarly, the combination of pgi and gnd is envisaged to provide similar results.

Example 16: Improved carbon flux through the DXP pathway in strains containing PDH E1 E636Q subunit variants

[0619] This example describes methods for the construction of E. coli BL21 strains containing pyruvate dehydrogenase E1 subunit (PDH) variants that increase carbon flux

through the DXP pathway. In particular, these strains contain a mutant aceE gene, encoding for a PDH variant with an E636Q point mutation which possesses a reduced activity (26% of wild-type PDH activity) for the conversion of pyruvate to acetyl-CoA. In addition, the PDH E636Q variant is thought to have a dxs-like activity that results in the production of 1-deoxyxylulose-5-phosphate (DXP) from the aldol condensation of pyruvate and glyceraldehyde-3-phosphate. The carboligase activity of the pyruvate dehydrogenase E1 E636Q mutant has been reported by Nemeria et al. (J. Biol. Chem., 280(22), 21473–21482 (2005), which is hereby incorporated by reference in its entirety).The net effect is increased carbon flux into the DXP pathway, and reduced carbon flux to acetyl–CoA relative to strains containing wild-type PDH E1 activity.

[0620] The construction of E. coli BL21 strains containing the PDH E1 E636Q mutant was as described by Sauret-Güeto et al. (FEBS Lett., 580, 736–740 (2006)), which is hereby incorporated by reference in its entirety. Briefly, the chromosomal copy of the dxs gene is disrupted by the insertion of a chloramphenicol acetyl transferase (CAT) containing cassette into the dxs locus of an E. coli BL21 strain that contains one or more plasmids encoding a heterologous mevalonic acid pathway (MVA). The resulting E. coli BL21 MVA+ (dxs::CAT) strain requires mevalonic acid for normal growth. When the strain is cultured in the absence of mevalonic acid, a suppressor mutation aceE gene arises at a low to moderate frequency that rescues the surviving clones from the otherwise lethal dxs- phenotype. Sequencing of the aceE gene and associated promoter region is performed in order to confirm the presence of the missense mutation that results in the PDH E636Q mutant.

[0621] The resulting E. coli BL21 dxs::CAT PDH E1 E636Q MVA+ strain is complemented with one or more functional copies of the dxs gene derived from E. coli or from a heterologous source as described herein. The resulting strains exhibits improved flux into the DXP pathway relative to strains that do not possess the PDH E1 E636Q variant.

[0622] Additionally, the resulting E. coli BL21 dxs::CAT PDH E1 E636Q MVA+ strain can be further complemented with one or more functional copies of a DXP

pathway gene, a DXP pathway associated gene, an iron-sulfur cluster-interacting redox gene (e.g., fldA or fpr), and/or an IDI gene derived from E. coli or from a heterologous source as described herein.

[0623] The strains can also be transformed with one or more copies of genes encoding isoprene synthases, for example IspS from P. alba or variants thereof as described herein. These strains produce isoprene by both the DXP and MVA pathways where a greater proportion of isoprene is derived from the DXP pathway relative to the MVA pathway, as compared to strains that do not possess the PDH E636Q variant. The ratio the DXP to MVA carbon flux is determined using isotope-labeling techniques known to those skilled in the art.

[0624] The strains can be optionally cured of the MVA pathway encoding plasmids (e.g., CHL18 or any other MVA pathway strains as described in U.S. Patent Application Nos: 61/097,186, 61/097,189, and 61/125,336, which are each hereby incorporated by reference in their entireties) if desired using techniques known to those skilled in the art.

Example 17: Mutation of CRP increases flux to the DXP pathway and increases the production of isoprene

[0625] Catabolite repression, in which the transcription of sensitive operons is reduced by certain carbon sources, could be a major restriction to flux in the DXP pathway, thereby reducing the amount of isoprene which could be produced.

[0626] A CRP (cAMP Receptor Protein)-delete mutant is available from the Keio collection and could easily be assessed for the production of isoprene through the DXP pathway. Impact of its global transcriptional regulation has been studied (Perrenoud and Sauer, *J. Bact.* 187:3171-3179 (2005). which is hereby incorporated by reference in its entirety). Other types of CRP mutants could also be beneficial to the process. One such example is the CRP mutant described by Eppler and Boos (Eppler and Boos, *Mol. Microbiol.* 33:1221-1231 (1999), which is hereby incorporated by reference in its entirety). CRP* is a cAMP-independent CRP variant.

[0627] A: Construction of an isoprene-producing Crp* mutant of E. coli

[0628] CRP* mutation is introduced by P1 transduction (lysate prepared from *E. coli* strain ET25 (to be obtained from W. Boos)) in an isoprene-producing strain, such as MCM597 (BL21(DE3)pLysS pET24(MEA)alba-DXS-yIDI) or MCM719 (BL21 gi1.6-yIDI gi1.6-dxs, pTrc(MEA)alba)) to form strains CMP220 and CMP221 respectively.

[0629] B: Production of isoprene in a Crp* mutant of E. coli, via the DXP pathway

[0630] Strains CMP220 and CMP221, and strains MCM597 and MCM719, are grown overnight at 30 C, 200 RPM, in HM1 medium (Table 3) plus appropriate antibiotics + 10 g/L glucose + 1 g/L yeast extract. The morning after, they are resuspended to an OD = 0.2 in fresh HM1 medium + appropriate antibiotics + 5 g/L glucose + 1 g/L yeast extract. Flasks are incubated at 30° C, 200 RPM, and regularly sampled for OD_{600} and isoprene productivity.

Table 3: HM1 medium composition

Compounds	Concentration (g/L)
K2HPO4	13.6
KH2PO4	13.6
MgSO4 * 7H2O	2
Citric Acid Monohydrate	2
Ferric Ammonium Citrate	0.3
(NH4)2SO4	3.2
Trace metal solution	1 ml

[0631] Specific productivity (ug isoprene/OD.h) is increased in strains CMP220 and CMP221 in comparison to strains MCM597 or MCM719.

[0632] C: Production of isoprene in a Crp* mutant of E. coli, via the DXP pathway, when the strain is grown on a glucose/xylose mixture

[0633] Pretreated biomass samples contain a mixture of glucose, xylose and acetate as the main components. Xylose consumption by *E. coli* is usually prevented in the presence of glucose. The CRP* mutation should be helpful to enhance glucose and xylose coconsumption (Cirino et al. biotech. Bioeng. 95:1167-1176 (2006), which is hereby incorporated by reference in its entirety).

[0634] Strains CMP220 and CMP221, and strains MCM597 and MCM719, are grown overnight at 30°C, 200 RPM, in HM1 medium (Table 3) plus appropriate antibiotics + 10 g/L glucose + 1 g/L yeast extract. The morning after, they are resuspended to an OD_{600} = 0.2 in fresh HM1 medium + appropriate antibiotics + 2.5 g/L xylose and 2.5 g/L glucose + 1 g/L yeast extract. Flasks are incubated at 30 °C, 200 RPM, and regularly sampled for OD_{600}, isoprene productivity and carbohydrate concentration. Carbohydrate concentration is determined by HPLC (Ion exclusion column Aminex HPX-87H, 300 mm X 7.8 mm, 0.005 M H2SO4, 0.6 mL/min as the mobile phase).

[0635] While strains MCM597 and MCM719 show a diauxic growth curve, co-consumption of xylose and glucose is increased in strains CMP220 and CMP221. This allows the fermentation to be completed in a shorter time.

Example 18: Increased isoprene production in an E. coli strain with LytBG120D mutation

[0636] The primary issues of this concept involve the biochemical determination of the mutant DXP pathway enzyme LytBG120D and whether or not the anticipated function of the LytBG10D enzyme can help serve a relevant aspect of our target DXP pathway strain to be used for BioIsoprene production. In this example, the desired DXP pathway strain is to produce a majority (if not as close to all as possible) of isoprene via the dimethylallyl pyrophosphate (DMAPP) molecule derived directly from the LytBG120D catalysis of *(E)*-4-hydroxy-3-methylbutyl-2-enyl pyrophosphate (HMBPP); as opposed to DMAPP generated via the IDI enzyme, which isomerizes isopentenyl pyrophosphate (IPP) into DMAPP.

[0637] The wild-type LytB of *E. coli* and the LytB enzyme common to a number of other organisms, including plants and algae as well as other bacteria, have been reported to produce both DMAPP and IPP in ratios typically ranging from 1:4 to 1:6 (DMAPP:IPP). The work by Kia-Joo Puan *et al.* (*FEBS Letters*, 579:3802-3806 (2005), which is hereby incorporated by reference in its entirety) provides *in vivo* data that supports the hypothesis that the LytBG120D mutant enzyme can produce DMAPP, but can not generate sufficient levels of IPP to support the viability of an *E. coli* deficient for

IDI. No i*n vitro* data supporting the suggested activity for LytBG120D has been introduced to the field yet.

[0638] Currently, isoprenoid production systems derive the majority of their products from IPP. If the LytBG120D is determined to solely generate DMAPP or a majority of DMAPP relative to IPP, then the use of the *lytBG120D* allele in a DXP pathway-mediated isoprene production strain may allow the unique generation of an isoprenoid product that is derived almost entirely from DMAPP.

[0639] The *lytBG12D* is generated via PCR-based methods using *E. coli* MG1655 as a template and cloned into an expression vector (pET-15b). For comparison, the wild-type *lytB* is cloned into the same pET-15b expression vector backbone. Each construct is moved into BL21(DE3), or a comparable expression host, once the sequence of the construct has been verified. From the expression strains, LytB and LytBG120D is produced and subsequently purified using standard affinity purification procedures. The protein may need to be reconstituted under anaerobic conditions prior to activity assessment (protocols exist in the literature) for robust enzymatic function to be determined. LytB is a 4Fe-4S cluster containing enzyme and is known to be sensitive to oxygen. Alternatively, LytB and LytBG120D may be able to be assayed directly from cell lysates prior to purification if sufficient activity of each enzyme can be supported under those conditions and if an absence of significant Idi activity can be achieved. Expression of each enzyme is determined and quantified by gel electrophoresis and/or immuno-blot. Activity assays are described in the literature, but briefly may include incubation of each enzyme (purified or contained within a cell extract) in a previously described buffer including the substrate HMBPP and in the absence of Idi activity. After a defined time(s) the ratio of DMAPP to IPP is determined using HPLC methods. The resulting data are the first *in vitro* results for LytBG120D available to us.

[0640] If LytBG120D is found to solely produce DMAPP, or at least produce DMAPP in vast abundance to IPP, then the use of the *lytBG120D* allele is incorporated in the DXP pathway isoprene production strains. Initially, this is accomplished by overexpressing the *lytBG120D* gene relative to the wild-type allele under isoprene-production phases

within a host background that supports carbon flux through the DXP pathway to isoprene synthase. As a control to assess, any benefits specific to generating increased DMAPP levels relative to IPP that are expected to accompany the overexpression of *lytBG120D*, a similar strain overexpressing the wild-type *lytB* gene is also constructed and assessed. The levels of DMAPP and IPP generated by these strains, as well as isoprene and other downstream isoprenoids, are determined by HPLC and/or GC-MS methods.

[0641] Our past findings indicate that increased IPP levels are not tolerated well by *E. coli*. Further more, we have seen that increased IPP levels accompanied by a significantly active Idi result in the synthesis of larger downstream isoprenoid products, which also cause a significant decrease in viability. Because LytB produces a majority of IPP to DMAPP, and because the endogenous IdI activity of *E. coli* is minimal, and because DMAPP is the substrate for isoprene synthase, our current DXP system relies on the use of an IdI derived from yeast. The use of LytBG120 in a DXP production strain removes the dependence our current system has on the yeast IdI (if LytBG120D is determined to produce mostly DMAPP). The use of LytBG120 is also expected to reduce the levels of downstream isoprenoid synthesis since IPP, the major subunit of larger isoprenoids, is not abundantly available.

Example 19: Host change for relief of endogenous regulation of DXP pathway

[0642] The DXP pathway, required for isoprenoids production in most Prokaryotes, is a strongly regulated pathway. Indeed, it is essential but also needed in small amount, as it diverts carbon from the central metabolism intermediates glyceraldehyde-3-P and pyruvate. As such, it might be difficult to escape regulation when working with endogenous genes.

[0643] A solution to this problem may be to express the whole DXP pathway from one organism into another host organism, the latter organism being close or far on the phylogenetic tree. These host organisms include, but not limited to industrial organisms, such as *Escherichia coli*, *Pseudomonas fluorescens*, *Zymomonas mobilis*, *Bacillus sp.*, *Saccharomyces cerevisiae*, *Clostridium sp.*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae*. The fact that all the genes involved in the pathway are cloned

from one organism guarantees that the enzymes produced by those genes can work together to produce the end product DMAPP.

[0644] A: Construction of a DXP pathway-expressing plasmid by cloning E. coli DXP genes

[0645] A Ptrc promoter, PCR-amplified from plasmid pTrcHis2A (Invitrogen, Carlsbad, CA) is cloned into pBBR1-MCS4 plasmid (Kovach et al, Gene, 166:175-176 (1995), which is hereby incoporated by reference in its entirety) multiple cloning site, leaving a PstI site downstream of the promoter. This plasmid is named pBBR4Ptrc. *E. coli* genes yajP (dxs), ispC (dxr), ispD, ispE, ispF, gcpE, lytB and idi are amplified from genomic DNA of *E. coli* MG1655 with primers containing an NsiI site and a RBS on the upstream primer, and a PstI site on the downstream primer. Genes are added one by one to the plasmid. Restriction digestion is used to check and select clones with the right orientation. Alternatively, a terminator is introduced after ispF and a new promoter (e.g. Ptrc) has been introduced in front of an operon constituted from gcpE, lytB and idi. The plasmid thus generated is named pBBR4PtrcDXPc and pBBR4PtrcDXPc2.

[0646] B: Construction of a codon-optimized DXP pathway-expressing plasmid by synthetic DNA synthesis

[0647] A synthetic operon similar to the one described above is designed and ordered, codon-optimized for Pseudomonas *fluorescens*, from GeneArt (Regensburg, Germany). It is subcloned in plasmid pBBR4Ptrc to generate plasmid pBBR4PtrcDXPa.

[0648] C: Expression of E. coli DXP pathway in Pseudomonas fluorescens, and its effect on isoprene production

[0649] An ispS (isoprene synthase from Populus) gene codon optimized for *Pseudomonas* (see other Pseudomonas patent example) is cloned into plasmid pHRP309 (gentamycin resistant) (Parales and Harwood, Gene 133:23-30 (1993), which is hereby incorporated by reference in it entirety), and transformed by biparental mating into *Pseudomonas fluorescens* ATCC 13525. Plasmids pBBR4PtrcDXPc, PBBR4PtrcDXPc2 and pBBR4PtrcDXPa are transformed in *E. coli* S17-1 by electroporation and selection of

transformants on LB + kanamycin 50 ug/ml. The plasmids are then transformed into *Pseudomonas fluorescens* with IspS-expressing pHRP309 by biparental mating and selection on M9 medium + 16 mM sodium citrate + kanamycin 50 µg/ml + gentamycin 50 ug/ml, to form strain CMP222, CMP223 and CMP224 respectively.

[0650] When strains CMP222, CMP223 and CMP224 are grown in HM1 medium + 10 g/L glucose, isoprene specific productivity is higher than for the *Pseudomonas* fluorescens strain devoid of the DXP pathway-expressing plasmids.

Example 20: Identification of compounds affecting production of isoprene via the DXP pathway

[0651] Isoprene production and growth by a strain of *E. coli* that over-expresses DXP pathway enzymes and isoprene synthase was investigated using 96-well microtiter plates with a range of different carbon, nitrogen or phosphate sources. A number of compounds that affected production of isoprene to a significant degree either positively or negatively were surprisingly identified. Compounds positively or negatively affecting the specific productivity of isoprene may help identify metabolic pathways that affect isoprene production. Such pathways may be implicated directly in the production of isoprene or they may have regulatory roles. The identified compounds or metabolic pathways may be modified for example by genetic modification to optimize the production of isoprene. The identified carbon, nitrogen or phosphate sources may also be supplemented directly to the media for increased production of isoprene.

[0652] Experimental procedure:

TM3 Media Recipe (per liter fermentation media):

[0653] K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2g, 1000X 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. Before use, $MgSO_4$ * $7H_2O$ 2 g, yeast extract 0.2 g was added to the media. Carbon source was added to a final concentration of 0.5% if needed. Required antibiotics were added after sterilization and pH adjustment.

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

[0654] Citric Acids * H_2O 40g, $MnSO_4$ * H2O 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 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 0.22 micron filter.

Strain:
MCM597

(i) Construction of MCM597 (BL21(DE3) pLysS pet24(MEA)albadxsyIDI

Construction of pDU-39

[0655] Primer sequences:

[0656] Alba TRC(MEA)-NdeI-F

[0657] 5'-gaaactgaaaccCATATGgaagctcgtcgttctgc (SEQ ID NO:104)

[0658] Alba FLTRC (-) TEV-R

[0659] 5'-cccgcgcttaCTCGAGgcgttcaaacggcagaatcggttcagtg (SEQ ID NO:105)

[0660] A truncated version of the Populus alba isoprene synthase was created by amplifying the gene using the primer set Alba TRC(MEA)-NdeI-F/Alba FLTRC(-) TER-R and the template pET24 alba HGS (described in Example 10, U.S. Patent Application No. 12/335,071, which is hereby incorporated in its entirety). The PCR reaction was set up as follows:

1ul (pET24a-*P.alba*)
5ul 10X PfuUltraII Fusion buffer
1ul dNTP's (10 mM)
1ul primer (50uM) Set #1 forward
1ul primer (50uM) Set #1 reverse
41 ul diH2O
+ 1ul of *PfuUltra* II Fusion DNA Polymerase from Stratagene

Cycle Parameter:
95°C 1min. [95°C 30sec., 55°C 20sec., 72°C 25sec] x 29 cycles, 72°C 3min,
4°C until cool, (Eppendorf Mastercycler)

[0661] The PCR products were digested with *Nde*I-*Xho*I restriction endonucleases (Roche) and gel purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. An aliquot of 3 ul of the purified product was ligated using T4 ligase (New England BioLabs) to pET-24a vector (Invitrogen) that was previously digested with *Nde*I-*Xho*I, gel purified and treated with Shrimp Alkaline Phosphatase (SAP, Roche). The ligation was carried out overnight at16°C.

[0662] An aliquot of 5 uL of the overnight ligation mixture was transformed into TOP10 cells (Invitrogen) and transformants were selected on L agar containing kanamycin (50 ug/ml) at 37°C overnight.

[0663] Plasmids were isolated from a few of the transformants using the QiaQuick Spin Kit (Qiagen) according to the manufacturer's instructions. The insert was verified by digestion *Nde*I-*Xho*I restriction endonucleases and the clones were sequenced with the commercially available T7 promoter and T7 terminator (Quintara Bio Sequencing Service, Berkeley, CA).

[0664] The correct plasmid was designated pDu-39 (Figure 69)

[0665] Construction of MCM597

[0666] Primer Sequences

MCM270 5'-GATCGGATCCATTCGCCCTTAGGAGGTAAA (SEQ ID NO:106)

MCM271 5'-GATCGCGGCCGCCAGCTGCAGGACGCGTTGTTATAGCATT (SEQ ID NO:107)

[0667] The DXS-yIDI genes were amplified by PCR using primers MCM270/MCM271 and the template pMCM72 (described in Example 7 U.S. Patent Application No. 12/335,071, which is hereby incorporated by reference in its entirety). Two identical PCR reactions were set up according to the manufacturer's protocol for Herculase II Fusion (Stratagene). 35uL water, 10 uL buffer, 1.25 uL each primer, 0.5uL dNTPs, 1uL polymerase. Reactions were cycled: 95C, 2:00; (95C 0:15, 55C 0:15, 72C 1:45)x30; 72C 3:00, 4C until cold.

[0668] The resulting PCR fragment was digested with *Bam*HI and *Not*I (Roche), and then ligated using Roche Rapid Ligation Kit into pDu39 that had been digested with the same restriction endonucleases. The ligation reaction was set up in 10uL containing 5uL Buffer 1, 1 uL vector, 3 uL insert and 1 uL ligase and incubated for 1 hour at room temperature. An aliquot of 5 uL was transformed into *E. coli* Top10 chemically competent cells (Invitrogen). Transformants were selected on L agar containing kanamycin (50 ug/ml) at 37°C overnight. Plasmids were purified from a few transformants and screened for the presence of insert using Herculase II Fusion (Stratagene). 17.5uL water, 5 uL buffer, 0.625 uL each primer, 0.25uL dNTPs, 0.5uL polymerase. Reactions were cycled: 95C, 2:00; (95C 0:15, 52C 0:15, 72C 0:45)x30; 72C 3:00, 4C until cold. Clones with a PCR product near 1.5kbp were sequenced (Quintara Biosciences, Berkeley CA). A correct plasmid was designated MCM596. The plasmid was then transformed into electrocompetent BL21(DE3)pLysS cells (Invitrogen) and transformants were selected on L agar containing kanamycin (50 ug/ml) and chloramphenicol (35 ug/mL). One colony was selected and designated MCM597.

[0669] Experimental Protocol

[0670] An inoculum of the *E. coli* strain MCM597 over-expressing the DXP pathway enzymes *dxs* from *E. coli* and *idi* from *Saccharomyzes cereviciae* and the isoprene synthase *ispS* from *Populus alba* was taken from a frozen vial and streaked onto an LB broth agar plate (with antibiotics) and incubated at 30°C overnight. A single colony was inoculated into TM3 media containing glucose as the only carbon source and grown overnight at 30°C. The overnight cultures were washed by centrifugation and resuspended into fresh TM3 media containing no glucose or yeast extract. The bacteria were then diluted into 20 mL of TM3 media to reach an optical density of 0.05 measured at 600nm. For experiments testing the effect of different nitrogen sources using the Biolog PM3B microtiter plates (Biolog, USA), the bacteria were diluted into media containing 0.5% glucose and no yeast extract . For experiments testing the effect of different carbon sources using the Biolog PM1 and PM2A microtiter plates (Biolog, USA), the bacteria were diluted into media containing 0.2% yeast extract and either no or 0.5% glucose. A total of 120 μL of culture was dispensed into each well of the Biolog

plates and the plate was incubated on an orbital shaker (250 rpm) at 30°C. The optical density was measured in the wells at 600 nm using a 384 well microtiter plate reader (Molecular Devices, Spectramax Plus 384) in the beginning of the experiment and every hour thereafter to follow growth of the bacteria. None of the compounds in the biolog plates were found to interfere with the optical density measurement at 600 nm. After four to six hours of growth, the optical density was measured again and two times 50 μL was transferred to two 96 well quartz glass blocks (Zinsser, Germany) and sealed with Biomek aluminum foil tape lids (Beckman Coulter, USA). The glass blocks were shaken at 450 rpm at 30°C for 30 minutes and then heat treated for 12 minutes at 70°C. The produced isoprene was measured using a GC-MS (GC 7889A and MSD 5975C, Agilent Technologies, USA). To account for differences from glass block to glass block, the isoprene measurement was normalized to the block average. The specific isoprene productivity was calculated by dividing the isoprene production with the optical density for each well. Each Biolog experiment was performed in duplicate. Statistical analysis (students T-test) was used to identify compounds in the microtiter plates that affected specific isoprene productivity with statistical significance ($p<0.1$).

[0671] Results

Nitrogen sources affecting isoprene production:

[0672] When *E. coli* harboring the DXP pathway and isoprene synthase was grown on 0.5% glucose as the sole carbon source in media lacking yeast extract, a number of nitrogen containing compounds were found to either positively or negatively affect the production of isoprene through the DXP pathway. The PM3B plates from Biolog were used for these experiments. Statistical analysis was used to identify compounds that most significantly affect isoprene production (Table 4). The addition of nitrite, nitrate, ammonia and urea did not significantly change the specific isoprene production, suggesting the bacteria were not directly lacking nitrogen in the fermentation media. Compounds increasing the specific production of isoprene surprisingly include L-glutamic acid, L-aspartic acid, the purines inosine and guanosine, L-threonine, L-serine, L-tryptophan and L-asparagine. Compounds negatively affecting specific isoprene

productivity particularly include adenine, and L-methionine, and L-tyrosine among others. Some of these compounds are involved in purine and thiamine biosynthesis, which are related to the DXP pathway, and may as such play important roles in the regulation of the DXP pathway.

Carbon sources affecting isoprene production during growth on glucose:

[0673] When *E. coli* harboring the DXP pathway and isoprene synthase was grown on 0.5% glucose in fermentation media containing 0.2% yeast extract, a range of carbon sources were found to affect the specific productivity of isoprene through the DXP pathway to a surprisingly high degree. The PM1 and PM2A carbon source plates from Biolog were used for these experiments. Statistical analysis was used to identify compounds that most significantly affect isoprene production (Table 5). Compounds most significantly increasing specific productivity of isoprene include, but are not limited to, phenylethylamine, propionic acid, D-galacturonic acid, inosine, L-galactonic acid-γ-lactone, D-psicose, glucuronamide, 2-aminoethanol, D-cellobiose, sucrose, mucic acid, L-malic acid, L-phenylalanine, 2,3-butanediol, L-ornithine, D-gluconic acid, D-glucosaminic acid, D-mannose. It is to be expected that the addition of these compounds to glucose fed fermentations would increase the specific productivity of isoprene. A range of other compounds were found to negatively affect specific productivity (Table 5). These effects may be caused by regulatory roles of the compounds or associated metabolic pathways, making these pathways interesting for genetic modification.

Identification of carbon sources useful for the production of isoprene:

[0674] When *E. coli* harboring the DXP pathway and isoprene synthase was grown in media containing 0.2% yeast extract in micro titer plates containing a range of different carbon sources, it was possibly to identify carbon sources that lead to the production of isoprene with a surprisingly high specific productivity. The PM1 carbon source plate from Biolog was used for these experiments. A range of compounds that lead to a very high specific isoprene productivity is shown in Table 6. Compounds most significantly increasing specific productivity of isoprene include, but is not limited to, D-galacturonic acid, D-trehalose, N-acetyl-D-glucosamine, D-mannitol, D-fructose, D-glucose-6-

phosphate, a-D-glucose. The final optical densities of the cultures grown on the different compounds are shown in Table 6. Some of these carbon sources may be used for the production of isoprene.

Table 4: Nitrogen sources affecting specific production of isoprene through the DXP pathway in *E. coli*. Only compounds affecting the specific isoprene production with statistical significance ($p<0.1$) are shown. Nitrate, nitrite, ammonia and urea have been included to illustrate that the addition of general nitrogen sources does not affect specific productivity of isoprene in the fermentation media (marked with grey).

Compound	Isoprene production normalized to negative control	P-value (T-test)
L-Glutamic Acid	2.13	0.003
Gly-Gln	1.80	0.008
Gly-Glu	1.48	0.008
Ala-Gln	1.46	0.045
Ala-Glu	1.45	0.030
L-Aspartic Acid	1.42	0.007
d-Amino-N-Valeric Acid	1.40	0.012
Inosine	1.37	0.013
Guanosine	1.33	0.023
Gly-Asn	1.26	0.092
L-Threonine	1.22	0.022
Ethanolamine	1.22	0.055
L-Serine	1.21	0.059
L-Tryptophan	1.21	0.071
Ala-Asp	1.20	0.056
L-Asparagine	1.16	0.023
Nitrate	1.08	0.360
Nitrite	1.06	0.110
Ammonia	1.01	0.837
Negative Control	1.00	1.000
Urea	0.99	0.919
D-Alanine	0.84	0.046
N-Phthaloyl-L-Glutamic Acid	0.81	0.021
N-Acetyl-D-Mannosamine	0.81	0.091
Histamine	0.80	0.036
D-Valine	0.80	0.061
Tyramine	0.76	0.037
Ala-Thr	0.72	0.013
ß-Phenylethylamine	0.70	0.028
L-Tyrosine	0.69	0.012
Gly-Met	0.61	0.011
D,L-a-Amino-N-Butyric Acid	0.60	0.012
Hyroxylamine	0.60	0.010
L-Methionine	0.56	0.003
Met-Ala	0.55	0.007
a-Amino-N-Valeric Acid	0.52	0.004
Adenine	0.22	0.000

Table 5: Carbon sources affecting specific production of isoprene through the DXP pathway in *E. coli* during growth on glucose. All carbon sources are normalized to the negative control that was only fed glucose. Only compounds affecting the specific

isoprene production with statistical significance ($p<0.1$) are shown. Negative control is marked with grey.

Compound	Isoprene production normalized to negative control	P-value (T-test)
Phenylethylamine	1.74	0.014
Propionic Acid	1.67	0.010
D-Galacturonic Acid	1.60	0.056
Inosine	1.60	0.015
L-Galactonic Acid-γ-Lactone	1.55	0.059
D-Psicose	1.53	0.055
Glucuronamide	1.50	0.093
2-Aminoethanol	1.38	0.042
D-Cellobiose	1.38	0.044
Sucrose	1.37	0.080
Mucic Acid	1.35	0.095
L-Malic Acid	1.28	0.086
L-Phenylalanine	1.23	0.004
2,3-Butanediol	1.22	0.044
L-Ornithine	1.21	0.010
D-Gluconic Acid	1.17	0.035
D-Threonine	1.15	0.032
D-Lactic Acid Methyl Ester	1.15	0.011
Chondroitin Sulfate C	1.15	0.035
L-Arginine	1.15	0.099
Salicin	1.13	0.063
M-Inositol	1.13	0.033
D-Glucosaminic Acid	1.13	0.002
D-Mannose	1.11	0.036
Negative Control	1.00	1.000
Turanose	0.94	0.042
ß-D-Allose	0.92	0.100
L-Isoleucine	0.90	0.040
Sedoheptulosan	0.89	0.049
D-Tagatose	0.87	0.090
L-Arabitol	0.85	0.090
D,L-Malic Acid	0.82	0.031
L-Arabinose	0.82	0.090
a-Methyl-D-Glucoside	0.82	0.068
Stachyose	0.82	0.033
D-Glucose-6-Phosphate	0.81	0.041
D-Ribose	0.74	0.007
D-Galactose	0.72	0.011
Lactitol	0.70	0.031
ß-Methyl-D-Galactoside	0.70	0.011
ß-Methyl-D-Xyloside	0.68	0.085
a-Methyl-D-Galactoside	0.62	0.062
2,3-Butanone	0.51	0.013
D-Melibiose	0.49	0.001
D-Raffinose	0.45	0.001
4-Hydroxy Benzoic Acid	0.41	0.005
Sorbic Acid	0.40	0.052
Capric Acid	0.35	0.008
Dihydroxy Acetone	0.22	0.002
2-Deoxy-D-Ribose	0.20	0.002
2-Hydroxy Benzoic Acid	0.18	0.000
Caproic Acid	0.18	0.001

Table 6: Carbon sources leading to a high specific production of isoprene in *E. coli* that over-expresses enzymes from the DXP pathway and isoprene synthase. The specific

isoprene productivity was normalized to α-D-glucose. The final optical density (OD600) of the cultures is also shown in the table, indicating the growth of *E. coli* on the specific carbon sources. The negative control was not fed any carbon source and is marked with grey.

Compound	Isoprene production normalized to a-D-Glucose	Growth OD600
D-Galacturonic Acid	1.36	0.217
D-Trehalose	1.31	0.243
N-Acetyl-DGlucosamine	1.17	0.283
D-Mannitol	1.16	0.270
D-Fructose	1.09	0.250
D-Glucose-6-Phosphate	1.09	0.299
a-D-Glucose	1.00	0.279
D-Gluconic Acid	1.00	0.276
Methyl Pyruvate	0.99	0.213
Pyruvic Acid	0.95	0.211
Inosine	0.93	0.191
L-Serine	0.92	0.213
D-Serine	0.90	0.221
Adenosine	0.88	0.187
L-Glutamic Acid	0.78	0.194
a-D-Lactose	0.75	0.198
Thymidine	0.66	0.202
D-Fructose-6-Phosphate	0.66	0.172
Mucic Acid	0.62	0.167
2-Deoxy Adenosine	0.57	0.160
Dulcitol	0.53	0.182
D-Glucose-1-Phosphate	0.49	0.167
m-Hydroxy Phenyl Acetic Acid	0.48	0.170
Propionic Acid	0.35	0.131
Sucrose	0.31	0.147
M-Tartaric Acid	0.24	0.144
Negative Control	0.17	0.145

Example 21: Increased expression of *fpr* improves isoprene production

[0675] In this example, we demonstrate an increase in activity of the GcpE and LytB enzymes of the DXP pathway by providing more of an essential auxiliary factor, Fpr, which has been shown to positively influence their *in vitro* and *in vivo* activities (Seemann, M. *et al.* Agnew. *Chem. Int. Ed.*, 41: 4337-4339 (2002); Wolff, M. *et al. FEBS Letters*, 541: 115-120 (2003), which are hereby incorporated by reference in their entireties). Fpr provides the necessary electrons derived from NADPH via *FldA* for GcpE and LytB to perform their catalytic functions (reviewed in report by L. A. Furgerson, *The mevalonate-independent Pathway to Isoprenoid Compounds: Discovery, Elucidation, and Reaction Mechanisms*, published February 13, 2006, which is hereby incorporated by reference in its entirety).

[0676] The expression of *fpr* (encoding flavodoxin/ferredoxin NADPH-oxidoreductase) is increased in an engineered, isoprene producing strain of E. coli. Our previously tested higher DXP flux strains produce only modest isoprene levels, and are observed to accumulate significant levels of both cMEPP, 2-*C*-methyl-D-erythritol-2, 4-cyclodiphosphate, and HMBPP, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate. The cMEPP and HMBPP DXP intermediates are the substrates of GcpE and LytB, respectively. The increased amount of Fpr may increase the activity demonstrated by the DXP pathway enzymes GcpE and LytB resulting in improved carbon flux to isoprene synthesis in the strain of interest over that of the comparable BL21 (DE3) control strain producing only endogenous levels of Fpr. The improved flux is demonstrated by an increase in isoprene titer.

[0677] The flavodoxin/ferredoxin NADPH-oxidoreductase encoded by *fpr* is intended to be expressed at increased levels from the *E. coli* chromosome by incorporating a constitutive highly active GI 1.6-promoter in front of the *fpr* open-reading frame, while replacing the endogenous promoter sequence. Alternatively, *fpr* can be expressed ectopically from a multi-copy vector construct. For either method, our goal is to express and accumulate Fpr at a level surpassing that generated from the endogenous *fldA* locus. Our preliminary qRT-PCR results suggest GI 1.6-*fpr* generates more *fpr*-transcript than the endogenous locus, and will likely accumulate more Fpr than the control as a result of the increased level of *fpr*-message. This is confirmed by immuno-blot once we receive the antibodies to Fpr.

[0678] Using a BL21(DE3) high DXP flux strain as the parental host strain, the introduction of the up-regulated *fpr* locus is assessed for the effects on isoprene production relative to the control strains. In addition, metabolite studies on the DXP intermediates provides insight into the beneficial affects of increased Fpr levels on GcpE and LytB activities.

[0679] Initially, the following BL21 (DE3) test strain is constructed and assessed for growth and the production of isoprene relative to the control: BL21 (DE3) GI 1.6-*dxs* GI 1.6-*fpr* T7-MEARR alba/pBBR1MCS-5. This strain is compared to the parental control

strain (BL21 (DE3) GI 1.6-*dxs* T7-MEARR alba/pBBR1MCS-5) for growth, isoprene production, and DXP metabolite accumulation.

[0680] Growth

[0681] Strains are grown at 30°C in TM3 liquid media (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) supplemented to a final concentration with 0.1% yeast extract and 1.0% glucose and including the appropriate antibiotics. Growth is monitored periodically by recording each of the culture's optical density measured at 600nm using an Eppendorf Biophotometer spectrometer (Eppendorf).

[0682] Isoprene production

[0683] Isoprene production is analyzed using a headspace assay. For the shake flask cultures, one ml of a culture is transferred from shake flasks to 20 ml CTC headspace vials (Agilent vial cat# 5188 2753; cap cat# 5188 2759). The cap is screwed on tightly and the vials incubated at the equivalent temperature with shaking at 250 rpm. After 30 minutes the vials are removed from the incubator and analyzed. The analysis is performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (30 m x 0.25 mm; 0.25 μm film thickness) is used for separation of analytes. The sampler is set up to inject 500 μL of headspace gas. The GC/MS method utilized helium as the carrier gas at a flow of 1 ml/minutes The injection port is held at 250° C with a split ratio of 50:1. The oven temperature is held at 37° C for the 2 minute duration of the analysis. The Agilent 5793N mass selective detector is run in single ion monitoring (SIM) mode on m/z 67. The detector 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) is observed to elute at 1.78 minutes. A calibration table is 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 is estimated to be 50 to 100 ng/L using this method. The specific productivity of each strain is reported as ug/L OD Hr. Ratio of 1900ul

headspace:100ul broth in assay vials for 30 min. incubation results in the following conversion of isopreneug/L of culture to specific productivity: (isoprene/L determined by GC-MS) X (38)/(OD 600nm of the culture).

[0684] DXP metabolite accumulation

[0685] The DXP metabolites of the isoprene-producing parental and test strains will be isolated and quantified as follows:

[0686] Metabolite extraction

[0687] Cell metabolism is rapidly inactivated by withdrawing 3.5 mL of the culture into a tube filled with 3.5 mL of dry ice-cold methanol. Cell debris is pelleted by centrifugation and the supernatant is loaded onto Strata-X-AW anion exchange column (Phenomenex) containing 30 mg of sorbent. The pellet is re-extracted twice, first with 3 mL of 50% MetOH containing 1 mM NH_4HCO_3 buffer (pH=7.0) and then with 3 mL of 75% MetOH/ 1 mM NH_4HCO_3 buffer (pH=7.0). After each extraction, cell debris is pelleted by centrifugation and the supernatants are consecutively loaded onto the same anion exchange column. During the extraction and centrifugation steps the samples are kept at below +4°C. Prior to metabolite elution, the anion exchange columns are washed with water and methanol (1 mL of each) and the analytes were eluted by adding 0.35 mL of concentrated NH_4OH/methanol (1:14, v/v) and then 0.35 mL of concentrated NH_4OH/water/methanol (1:2:12, v/v/v) mixtures. The eluant is neutralized with 30 µL of glacial acetic acid and cleared by centrifugation in a microcentrifuge.

[0688] Metabolite quantification

[0689] Metabolites are analyzed using a Thermo Scientific TSQ Quantum Access mass spectrometer (Thermo Electron Corporation, San Jose, CA). All system control, data acquisition, and mass spectral data evaluation are performed using XCalibur and LCQuan software (Thermo Electron Corp). For the LC-ESI –MS/MS method, a chiral Nucleodex ß-OH 5µM HPLC column (100 x 2 mm, Macherey-Nagel, Germany) equipped with a CC 8/4 Nucleodex beta-OH guard cartridge is eluted with a mobile phase gradient shown in Table 7 (flow rate of 0.4 mL/min). The sample injection volume was 10 µL.

Table 7. HPLC gradient used to elute metabolites.

Time, min	Mobile phase, %		
	A (water)	B (100 mM ammonium bicarbonate, pH=8.0)	C (acetonitrile)
0.0	0.0	20.0	80.0
0.5	15.0	5.0	80.0
4.5	37.5	12.5	50.0
6.5	37.5	12.5	50.0
7.0	49.5	0.5	50.0
12.0	34.9	0.1	65.0
12.5	0.0	20.0	80.0
13.0	0.0	20.0	80.0

[0690] Mass detection is carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions are selected to detect the metabolites of interest in SRM mode: 245.0 for IPP and DMAPP, 381.1 for FPP, 213.0 for DXP, 215.0 for MEP, 260.0 for HDMAPP, and 277.0 for cMEPP. Concentrations of metabolites are determined based on the integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0). Calibration curves obtained by injection of corresponding standards purchased from Echelon Biosciences Inc. Intracellular concentrations of metabolites are calculated based on the assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 μL

Example 22: Improved carbon flux into the DXP pathway using a heterologous DXS

[0691] Living organisms synthesize isoprenoids via two distinct pathways: the mevalonate (MVA) pathway and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. MEP pathway starts from 1-deoxy-D-xylulose 5-phosphate (DXP), which is synthesized by condensation of pyruvate and glyceraldehyde-3-phosphate. This reaction is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS). In some bacteria, including *E.coli,* DXP serves not only as a precursor of isoprenoids but is also used for biosynthesis of two important cofactors: thiamine (vitamin B1) and pyridoxol phosphate (vitamin B6).

[0692] The rate of isoprenoid synthesis in *E.coli* is regulated at the level of DXS. One of the mechanisms of this regulation may involve feedback inhibition of DXS activity by metabolites downstream the MEP pathway or/and intermediates of vitamin B1/B6 biosynthesis. Accordingly, the overall flux into the MEP pathway may be increased in *E.coli* by expressing an enzyme from a different organism that is not subject to inhibition by downstream products. Heterologous DXS may also be superior to the native *E.coli* DXS due lower K_m or higher K_{cat} values with respect to pyruvate or glyceraldehyde-3-phosphate. Earlier studies have shown that a single Y392F substitution in the DXS of *E.coli* results in two-fold increase in the activity of the enzyme *in vitro*, although catalytic properties of the modified enzyme have not been studied in detail.

[0693] The choice of the sources of DXS for heterologous expression in *E.coli* can be based on the following considerations (see Table 8). First, organisms which have genome coding for several *dxs* isogenes can be selected. These organisms include plants (different forms of DXS in plants are classified as DXS1 and DXS2), and bacteria (e.g. species of *Streptomyces)* having two or more *dxs* isogenes. Second, bacteria in which isoprenoids are synthesized via both the MEP (or DXP) pathway and the MVA pathway can be selected. Third, bacteria, which synthesize isoprenoids via the MVA pathway but contain a copy of the *dxs* gene in their genome specifically needed to make the vitamin cofactors. The DXS sequence this group of microorganisms is characterized by a significantly shorter loop corresponding to the amino acids 203-242 of *E.coli* DXS sequence (Fig. 74).

[0694] In one set of the experiment, DXS from a variety of organisms (examples are listed in Table 8) is introduced into *E.coli* cells over-expressing plant isoprene synthase and isopentenyl-diphosphate delta-isomerase (IDI). (IDI activity in *E.coli* is normally very low; therefore enhanced expression of this enzyme is necessary to provide efficient conversion of isopentenyl-diphosphate into dimethylallyl-diphosphate, the substrate of isoprene synthase.). The resulting strains are tested for isoprene production and accumulation of DXP pathway intermediates, including but not limited to DXP, MEP, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol, 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate and 1-hydroxy-2-

methyl-2-butenyl 4-diphosphate, and compared to the control strain containing native *E. coli* DXS expressed in the same context as in the tested mutants. Increased concentrations of DXP intermediates and/or elevated rate of isoprene evolution in mutants containing heterologous DXSs indicated that the enzyme from the particular organism has higher activity in *E.coli* and is not subject to feedback inhibition by accumulated products.

[0695] In another set of experiments, a set of mutants over-expressing either heterologous *dxs* genes or *dxs* from *E. coli* (the control) are introduced into the background *E. coli* strain containing plant isoprene synthase, IDI, and several enzymes of MVA pathway allowing that strain to synthesize excessive amounts of isoprenoids when grown in the media containing exogenous MVA. These strains are tested for the accumulation of the intermediates specific to the DXP pathway. As in the previous case, increased concentrations of DXP intermediates compared to the control showed that DXS from specific organisms have higher activity in *E.coli* than the native enzyme and is not subject to feedback inhibition by isopentenyl-diphosphate and/or downstream isoprenoid products. To verify that a particular mutant have an improved rate of the isoprene production specifically due to the modified DXS, isoprene production rate is measured in cells grown on ^{13}C-uniformly labeled glucose in the presence of non-labeled MVA. In this case, ^{13}C composition of isoprene analyzed by mass spectrometry unequivocally indicated that this compound is synthesized via the DXP pathway from the labeled glucose, not from exogenous non-labeled MVA.

[0696] In a third set of experiments, experiments are performed to demonstrate that substitution of the tyrosine at position 392 of *E.coli* DXS for phenylalanine results in higher flux rate into the DXP pathway compared to the wild type enzyme. For this experiment the wild-type and the mutated DXS are over-expressed in an *E.coli* strain containing plant isoprene synthase and IDI. The two strains are compared for isoprene production rate and accumulation of DXP pathway intermediates. Increased concentrations of DXP intermediates and/or elevated rate of isoprene evolution in the strain bearing the superior properties of the engineered enzyme demonstrated the superior attributes of the mutant enzyme.

Table 8. Examples of organisms have kinetic properties of DXSs different from that of *E.coli*.

Organism	Reason
Myxococcus xanthus DK 1622 *Gramella forsetii* KT0803 *Flavobacterium johnsoniae* UW101 *Lactobacillus johnsonii* NCC 533 *Lactobacillus gasseri* ATCC 33323, *Lactococcus lactis* subsp. *lactis* Il1403	DXS is needed to synthesize vitamin cofactor(s); isoprenoids are made via the MVA pathway
Listeria monocytogenes EGD-e *Lactobacillus plantarum* *Streptomyces griseolosporeus* MF730-N6	Both MVA and DXP pathways are present in these organisms
Streptomyces hygroscopicus NRRL 3418 *Streptomyces spheroides* NCIMB 11891 *Streptomyces spheroides* NCIMB 11891 *Streptomyces griseolosporeus* MF730-N6 *Streptomyces coelicolor* *Streptomyces griseolosporeus* MF730-N6	Organisms have multiple copies of DXS
DXS type1 and DXS type 2 from higher plants	

Example 23: The Identification of combinations of genes, gene expression or mutations that increase flux through the DXP pathway

[0697] Populations of cells with a high degree of genotypic diversity are generated to identify combinations of genes, gene expression or mutations that increase flux through the DXP pathway. Three different methods are used in this example. First, combinations of genes, either endogenous to *E. coli* or from heterologous organisms, are assembled using the Multisite Gateway (Invitrogen) procedure and introduced into the *E. coli* screening strain. Second, libraries of genomic DNA, either from *E. coli* or heterologous organisms, are generated and introduced in the *E. coli* screening strain. Third, transposons that can result in either gene disruption or activation due to an internal promoter that is directed towards the inverted repeat of the transposable element are introduced.

[0698] A. The Multisite gateway (Invitrogen) procedure for generating synthetic operons

[0699] Genes either endogenous to *E. coli* or from heterologous organisms are assembled into synthetic operons that are subsequently screened for increased flux through the DXP pathway and resulting isoprene production. The Multisite Gateway (Invitrogen) kit provides for a maximum of four discrete DNA "elements" that can be assembled together into one operon. Four genes are individually cloned into pENTR vectors, according to the manufacturer's protocol. For example, the last two genes in the DXP pathway, *ispG* and *ispH* are amplified by PCR with appropriate att recombination sites (according to manufacturer's protocol) and variable RBS (*see* Yarchuk *et al.*, J. of Mol. Biol., 226(3):581-596 (1992), which is hereby incorporated by reference in its entirety) to generate plasmid pools with varying expression levels of each gene. The same procedure is applied to the electron carrier genes *fldA* and *fpr*, and the four resulting plasmid pools are recombined together onto Gateway destination vectors (pDEST-14 (Invitrogen), pET54-DEST or pCOLA-2-DEST (Novagen)) according to the manufacturer's protocol. The resulting plasmids harbor four gene operons with varying expression levels of each ORF. The pooled destination vectors are then introduced into *E. coli* strains by selecting for antibiotic resistance markers (kanamycin or ampicillin) and resulting pools are screened by GC-MS (described below).

[0700] B. Generation of genomic libraries

[0701] Genomic DNA either endogenous to *E. coli* or from heterologous organisms is cloned into the pSMART LCKan vector (Lucigen) according to the manufacturer's recommended protocol (*see* Lynch *et al.*, Nat. Methods, 4(1):87-93 (2007), which is hereby incorporated by reference in its entirety). DNA from *E. coli* BL21 and K12 strains, *B. subtilis, Lb. plantarum, Lb. sakei, P. citrea, S. coelicolor, S. spheroides, L. monocytogenes, A. tumefaciens, S. meliloti,* and *C. jejuni* is used to generate libraries. The genomic DNA inserts of up to 20 kb in size are then introduced into *E. coli* strains for screening. Positive transformants are selected for by introduction of antibiotic resistance (kanamycin), pooled, and screened by GC-MS.

[0702] C. Transposon mutagenesis and gene activation

[0703] A transposon that can both inactive genes by disruption of the ORF and also drive expression of proximal genes due to an endogenous promoter in the transposable element is introduced into *E. coli* for screening. The custom transposon is generated by inserting either a constitutive or inducible promoter into the MCS of the EZ-Tn5 transposon construction vectors (Epicentre). Examples of internal promoters include PT7, Ptrc, Ptac, Pbad, Plac, PL (phage lambda), the gi series, and Ptet. These promoters are cloned into the transposable element, and the resulting custom transposon is introduced into *E. coli.* Strains harboring transposon insertions are identified by antibiotic resistance, pooled, and subjected to screening by GC-MS.

[0704] E. coli strains and screening

[0705] Plasmid pools or transposons are introduced into different *E. coli* strains for screening. Positive transformants are identified by antibiotic resistance markers (typically Kan or Amp) located on the plasmid or within the transposable element. Strains include: A strain harboring a plasmid carrying dxs, dxr, idi, and IspS (isoprene synthase) under control of the T7 promoter; a strain harboring integrated and constitutively expressed dxs, dxr, and idi with ispS also integrated or expressed from a plasmid; a strain expressing the entire DXP operon under the control of the T7 Promoter; any strain harboring the current best conformation of DXP pathway genes for isoprene production, yet still displays clear accumulation of DXP pathway metabolites (*e.g.* HDMAPP). Individual transformants are pooled (in groups of 100 to 1000 individuals per pool) and screened via GC-MS in a 96-well glass block. The analysis is performed (for the 2 mL and 96-well plate methods) using an Agilent 6890 GC/MS system interfaced with a 5973 MS Leap CTC CombiPAL autosampler operating in headspace mode. An Agilent HP-5 (5% Phenyl Methyl Siloxane (15m x 0.25 mm x 0.25 uM)) column is used for separation of analytes. The sampler is set up to inject 100 µL of headspace gas. The GC/MS method utilizes helium as the carrier gas at a flow of 1 ml/min. The injection port is held at 250 °C with a split ratio of 50:1. The oven temperature is held at 37 °C for the 2 min duration of the analysis. The Agilent 5793N mass selective detector is run in single ion monitoring (SIM) mode on mass 67. The detector is switched off from 0.00 to 0.44 minutes to allow the elution of permanent gases

and on 0.44 mins to 0.60 mins. Under these conditions isoprene (2-methyl-1,3-butadiene) is observed to elute at 0.49 minutes. A calibration table is used to quantify the absolute amount of isoprene and was found to be linear from 0 μg/L to 5600 mg/L (using calibration gas). Positive pools are then re-assayed to confirm any positive effect on isoprene production. The individual plasmids or constructs in strains or pools which display increased isoprene production are identified to determine the precise nature of positive influence on DXP pathway flux.

[0706] Genes of organisms examined

[0707] Genes including, but not limiting to, the following organisms are examined: Arabidopsis thaliana, Zea mays, Campylobacter jejuni, Sinorhizobium meliloti, Helicobacter pylori Agrobacterium tumefaciens, Deinococcus radiodurans, Bacillus subtilis, Pantoea citrea, Listeria monocytogenes, Lactobacillus spp., and Streptomyces spp.

Materials

Multisite Gateway kit (Invitrogen)
Lucigen (Clonesmart Cloning Kits) – library construction
EZ-Tn5 System (EpiCentre) – gene disruption/activation

Plasmids

pET -PT7-driven full DXP pathway plasmid
pET -PT7 driven dxs, dxr. idi, ispS
pET – best conformation of DXP pathway genes for isoprene production
pBBR – PT7 or Ptrc ispS
pET-54-DEST, pCOLA-DEST vectors (Novagen)
pDEST14, pDEST15 (Invitrogen)

Example 24: Increased isoprene production in REMG39 by overexpression of GcpE, LytB PetF and PetH of *T. elongatus* BP-1 within CMP272

[0708] This example provides further demonstration of increased isoprene production in REMG39 by overexpression of GcpE, LytB PetF and PetH of T. elongatus BP-1 within CMP272, a BL21 derived host.

[0709] As described and shown *infra*, increased expression of both *dxs* and yeast *idi* allow increased flux through the endogenous DXP pathway of *E. coli*. Previous work by the field (see, for example, Chao et al., *Biotechnol Prog.*,18(2):394-400 (2002) and Zhang et al., *Protein Expression and Purification*, 29(1): 132-139 (May 2003)) has lead to the conclusion that T7-based expression systems are unstable and their behavior not entirely predictable when subjected to 14-L fermentation conditions. The CMP271 and subsequent CMP272 strain were constructed to: (1) replace our current T7-governed plasmid-based expression of yeast *idi* with expression originating from the chromosome; permitting the use of a non-T7 based expression strain for DXP-mediated isoprene production and/or (2) introduce the genomically encoded locus harboring the genes for the lower MVA pathway enzymes and yeast IDI to provide sufficient levels of yeast IDI for maximal flux to Isoprene Synthase.

[0710] The CMP271 strain was made into an isoprene generating strain by the addition of pDW33, harboring a *P. alba* isoprene synthase allele, via electroporation, and subsequently yielding strain CMP272.

[0711] The CMP272 strain serves as the baseline host in which isoprene production has been successfully improved by the addition of the *T. elogatus* IspG (GcpE) and IspH (LytB) encoding genes along with their putative reducing shuttle system (PetF and PetH). The construct harboring the *T. elongatus* genes, Ptac-gcpE-lytB-petF-petH/pK184, has been described *infra* in the example utilizing *T. elongatus*. The parental CMP272 and test strain REMG39 were evaluate for growth, isoprene production, metabolite profile, and product yield on carbon under 14-L fermentation conditions described below. The results are depicted in Figure 77.

A. Construction of strains CMP271, CMP272, and REMG39

[0712] The GI 1.X-promoter insertions and subsequent loopout of the antibiotic resistance markers described in this example were carried out using the Red/ET system from Gene Bridges GmbH according to the manufacturer's instructions. The strain BL21

(Novagene) was used. P1 lysate preparations and transductions were performed as previously described (Thomason *et al.*, 2007).

[0713] Primers

[0714] MQ09-10F- 5'
ggttaatcatttcactcttcaattatctataatgatgagtgatcagaattacatgtgagaaattaattaaccctcac

taaagggcggccgcgaa

[0715] MQ09-10R-
5'
atattccaccagctatttgttagtgaataaaagtggttgaattatttgctcaggatgtggcatNgtcaagggctaatacgactcacta
tagggctcgagg

* for the case of GI1.6 N=T in the primer sequence above.

[0716] MQ09-11F-

5' gcccttgacNatgccacatcctgagcaaataattcaaccacttttattcactaacaaatagctggtggaatata
tgactgccgacaacaatagtatgccc
* for the case of GI1.6 N=A in the primer sequence above.

[0717] MQ09-11R-

5' gatgcgtccagtaaaataagcattacgttatgctcataaccccggcaaatgtcggggttttttatagcattctatgaatttg
top Gb's CMP 5' ACTGAAACGTTTTCATCGCTC

[0718] MQ09-12R- 5' gatgcgtccagtaaaataagcattacgttatgctc

[0719] galMR 5' gtcaggctggaatactcttcg

[0720] galMF 5' gacgctttcgccaagtcagg

[0721] The strategy for inserting the GI1.X-yidi series into the *E. coli idi* locus using the Gene Bridges GmbH methods is illustrated in Figure 77. The antibiotic resistance cassette GB-CMP containing fragment (Frag A) was amplified by PCR using primer sets MQ09-10F/MQ09-10R. The GI1.X-yidi containing fragment (Frag B) was amplified by PCR using primer sets MQ09-11F/MQ09-11R. The GB-CMP-GI1.X-yidi fragment was ultimately generated using the primers MQ09-10F and MQ09-11R. The MQ09-10F and MQ09-11R primers each contain at least 50 bases of homology to the *E. coli idi* locus which allow recombination at the specific sites upon electroporation of the PCR product in the presence of the pRed-ET plasmid.

Amplification of the GB-CMP-GI1.X-yidi fragment

PCR Reaction for GB-CmR (Frag A)
2ul (100ng GB-CmR)
10ul HerculaseII Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) MQ09-10F
1.25ul primer (10uM) MQ09-10R
2ul DMSO
32 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

PCR Reaction for GB-CmR (Frag B)
2ul (100ng GB-CmR)
10ul HerculaseII Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) MQ09-11F
1.25ul primer (10uM) MQ09-11R
2ul DMSO
32 ul diH2O

+ 1ul of *HerculaseII fusion* from Stratagene

PCR Reaction for GB-CmR (Frag A+B)

1ul (Frag A)

1ul (Frag B)

10ul HerculaseII Buffer

0.5ul dNTP's (100 mM)

1.25ul primer (10uM) MQ09-10F``

1.25ul primer (10uM) MQ09-11R

2ul DMSO

32 ul diH2O

+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:

Frag A

(95°C 2min., 95°C 20sec., 55°C 20sec., 72°C 1min., 29X, 72°C 3min,

4°C until cool, use Eppendorf Mastercycler)

Frag B

(95°C 2min., 95°C 20sec., 55°C 20sec., 72°C 35sec., 29X, 72°C 3min,

4°C until cool, use Eppendorf Mastercycler)

Frag A &B

(95°C 2min., 95°C 20sec., 55°C 20sec., 72°C 1.2min., 29X, 72°C 3min,

4°C until cool, use Eppendorf Mastercycler)

[0722] The resulting PCR fragments Frag A, B, and A+B were separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits according to manufacturer's instructions. The purifed stocks of Frag A and Frag B were used in the Frag A +B PCR reaction described above. The resulting purified stock of Frag A+B is referred to as GB-CMP-GI1.X-yidi.

Amplification of the galM *locus of CMP263*

[0723] One colony of CMP263 was stirred in 30 uL H_2O and then heated to 95°C for 5 min. The resulting solution was spun down to pellet debris and 2 uL of the supernatant was used as the template in the following PCR reaction:

2 ul colony in H_2O (see above)
5 ul *Herculase* Buffer
1 ul dNTP's (100 mM)
1 ul galMF primer (10uM)
1 ul galMR primer (10uM)
39.5 ul H_2O
+ 0.5 ul of *Herculase Enhanced DNA* polymerase from Stratagene

Cycle Parameter:
95°C x 2 min., [95°C x 30sec., 52°C x 30sec., 72°C x 60sec] x 30 cycles; 72°C x 7min, 4°C until cool (PCRExpress Thermocycler from ThermoHybaid).

[0724] The size of the resulting PCR fragment was determined on a 0.8% E-gel (Invitrogen), using DNA Molecular Weight X (Roche) as a ladder; a corresponding PCR product was not obtained from BL21 cells, as expected for the negative control.

Integration of GB-CMP GI 1.X-yidi PCR product into BL21/pRed-ET Strain

[0725] The pRed-ET vector (Gene Bridges kit) was transformed into BL21 by electroporation using the BIO RAD Gene Pulser system and a transformation protocol suggested by the manufacturer (BIO RAD) resulting in strain MD08-114 (BL21/pRed-ET). Approximately 400ug of the purified GB-CMP GI 1.X-yidi PCR fragment was electroporated into MD08-114. The transformants were recovered in L Broth and then plated on L agar containing chloramphenicol (5ug/ml). Chloramphenicol resistant colonies were analyzed by PCR for the presence of the GB-CMP GI 1.X-yidi sequence at the desired locus using the top Gb's CMP and MQ09-12R primers. The PCR fragments from a number of transformants were sequenced using the MQ09-12R and top GB's

CMP primers (Quintara; Albany, CA) and the various GI1.X-*yidi* strains of interest identified. One chloramphenicol resistant clone harboring the GI1.6-yidi locus (BL21 FRT-CmR-FRT GI1.6(A)-*yidi*) was chosen and designated MD09-211.

B. Strategy for creating the CMP271 strain

[0726] The GI1.6-dxs::kan locus of strain MCM625, described in Example 9, was introduced into MD09-211 via P1-mediated transduction and the resulting kanamycin and chloramphenicol resistant strain named MD09-221. The antibiotic resistance markers of strain MD09-221 were looped out using pCP20 from the pRed-ET kit according to the manufacturer's instructions (GeneBridges). Transformants of interest were verified by the loss of resistance to chloramphenicol (5ug/ml) and kanamycin (50ug/ml); one chloramphenicol and kanamycin sensitive clone was chosen and designated MCM710. The FRT-Neo-FRT PL.2 mKKDyI locus (harboring an additional copy of the yeast *idi*gene) of strain MCM521, described in US Appl. No. 61/289,959, was moved into MCM710 by P1-mediated transduction. One kanamycin resistant clone was chosen and designated MCM783. MCM783 was transduced with a P1 lysate of *E. coli* K-12 MG1655, and selected on M9 medium (Na2HPO4 6 g/L, KH2PO4 3 g/L, NaCl 0.5 g/L, NH4Cl 0.5 g/L, 0.1 mM CaCl2, 2mM MgSO4) + 0.4% w/v galactose. One galactose ultizing clone was chosen and designated CMP263. The presence of the *galM* locus within the 17,257 bp of MG1655 that is not endogenous to BL21, but was now harbored by CMP263, was verified by PCR using the primer set galMF/galMR; this PCR reaction is described above. The kanmycin resistance marker within strain CMP263 was looped out using Gene Bridges GmbH methods. One kanamycin sensitive clone was chosen and designated CMP271.

C. Strategy for creating the CMP272 strain

[0727] Electroporation of pDW33 into strain CMP271 was done using the BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). The vector construct harbors the PTrc-governed MEARR *P. alba* allele encoding a truncated form of Isoprene Synthase. The template for pDW33 construction, EWL230, has been described in US. Publ. No.

2009/0203102 and WO 2009/076676. A picture of the pDW33 vector map is presented in Figure 78.

Construction of pDW33

[0728] pDW33 was constructed in order to generate an isoprene producing *Escherichia coli* strain harboring the truncated version of *P. alba* isoprene synthase (the MEA variant) under control of the Ptrc promoter.

Construction of Strain DW194:

The plasmid harboring truncated *P. alba* isoprene synthase (IspS) was constructed by Quikchange PCR mutagenesis (Stratagene – see Table below for primer sequences) upon the template EWL230 (aka pTrc-P. alba). PCR reaction and cycling parameters are described below. The PCR product was visualized by gel electrophoresis (E-gel, Invitrogen), and then treated with 1 µl DpnI restriction endonuclease (Roche) for three hours at 37°C. Ten µl of the PCR product was then de-salted using a microdialysis membrane (MilliPore) and transformed into electrocompetent *E. coli* strain MCM531 (previously described) using standard molecular biology techniques. Cells were recovered in one ml of LB medium for 1.5 hours at 30°C, plated onto LB solid agar plates containing 50 µg/ml carbenicillin and 5 mM mevalonic acid, and then incubated overnight at 37°C. The next day, positive colonies (of strain DW194, see below) were selected for growth and plasmid purification (Qiagen), and ultimately confirmed by DNA sequencing (Quintara) with the primers listed below. The final plasmid, pDW33, carries the open reading frame encoding the truncated version (MEA) of IspS.

Primers:

QC EWL244 MEA F	gaggaataaaccatggaagctcgtcgttct
QC EWL244 MEA R	agaacgacgagcttccatggtttattcctc
EL-1006	gacagcttatcatcgactgcacg
EL-1000	gcactgtctttccgtctgctgc
A-rev	ctcgtacaggctcaggatag
A-rev-2	ttacgtcccaacgctcaact
QB1493	cttcggcaacgcatggaaat
MCM66 (aka pTrc Reverse)	ccaggcaaattctgttttatcag

Strains:

Strain	Background	Plasmid	Resistance	Genotype
DW194	MCM531	pDW33	Carb	BL21 (Novagen) PL.2mKKDyI, + pTrc-P. alba(MEA)

QuikChange PCR Reaction:

1 ul plasmid EWL230 (aka pTrc P. alba)

5 ul 10X PfuUltra HF buffer

1 ul dNTPs (100 mM)

1 ul (50uM) QC EWL244 MEA F

1 ul (50uM) QC EWL244 MEA R

2 ul DMSO

39 ul diH2O

1 ul PfuUltra HF Polymerase (Stratagene)

PCR Cycling Parameters:

1. 95°C 1 min.
2. 95°C 30 sec.
3. 55°C 1 min.
4. 68°C 6 min.
5. Go to step 2 – 18 cycles
6. 4°C

Sequence of truncated *P. alba* IspS (MEA)

mearrsanyepnswdydyllssdtdesievykdkakkleaevrreinnekaefltllelidnvqrlglgyrfesdirgaldrfvs
sggfdavtktslhgtalsfrllrqhgfevsqeafsgfkdqngnflenlkedikailslyeasflalegenildeakvfaishlkelse
ekigkelaeqvnhalelplhrrtqrleavwsieayrkkedanqvllelaildynmiqsvyqrdlretsrwwrrvglatklhfar
drliesfywavgvafepqysdcrnsvakmfsfvtiiddiydvygtldelelftdaverwdvnaindlpdymklcflalyntin

eiaydnlkdkgenilpyltkawadlcnaflqeakwlynkstptfddyfgnawksssgplqlvfayfavvqnikkeeienlqk
yhdtisrpshifrlcndlasasaeiargetansvscymrtkgiseelatesvmnlidetwkkmnkeklggslfakpfvetainl
arqshctyhngdahtspdeltrkrvlsvitepilpfer

Sequence of pDW33:

gtttgacagcttatcatcgactgcacggtgcaccaatgcttctggcgtcaggcagccatcggaagctgtggtatggctgtgcagg
tcgtaaatcactgcataattcgtgtcgctcaaggcgcactcccgttctggataatgtttttgcgccgacatcataacggttctggca
aatattctgaaatgagctgttgacaattaatcatccggctcgtataatgtgtggaattgtgagcggataacaatttcacacaggaaac
agcgccgctgagaaaaagcgaagcggcactgctctttaacaatttatcagacaatctgtgtgggcactcgaccggaattatcgat
taactttattattaaaaattaaagaggtatatattaatgtatcgattaaataaggaggaataaaccatggaagctcgtcgttctgcgaa
ctacgaacctaacagctgggactatgattacctgctgtcctccgacacggacgagtccatcgaagtatacaaagacaaagcgaa
aaagctggaagccgaagttcgtcgcgagattaataacgaaaaagcagaatttctgaccctgctggaactgattgacaacgtcca
gcgcctgggcctgggttaccgtttcgagtctgatatccgtggtgcgctggatcgcttcgtttcctccggcggcttcgatgcggtaa
ccaagacttccctgcacggtacggcactgtctttccgtctgctgcgtcaacacggttttgaggtttctcaggaagcgttcagcggct
tcaaagaccaaaacggcaacttcctggagaacctgaaggaagatatcaaagctatcctgagcctgtacgaggccagcttcctgg
ctctggaaggcgaaaacatcctggacgaggcgaaggttttcgcaatctctcatctgaaagaactgtctgaagaaaagatcggtaa
agagctggcagaacaggtgaaccatgcactggaactgccactgcatcgccgtactcagcgtctggaagcagtatggtctatcga
ggcctaccgtaaaaaggaggacgcgaatcaggttctgctggagctggcaattctggattacaacatgatccagtctgtataccag
cgtgatctgcgtgaaacgtcccgttggtggcgtcgtgtgggtctggcgaccaaactgcactttgctcgtgaccgcctgattgaga
gcttctactgggccgtgggtgtagcattcgaaccgcaatactccgactgccgtaactccgtcgcaaaaatgttttctttcgtaaccat
tatcgacgatatctacgatgtatacggcaccctggacgaactggagctgtttactgatgcagttgagcgttgggacgtaaacgcc
atcaacgacctgccggattacatgaaactgtgctttctggctctgtataacactattaacgaaatcgcctacgacaacctgaaagat
aaaggtgagaacatcctgccgtatctgaccaaagcctgggctgacctgtgcaacgctttcctgcaagaagccaagtggctgtac
aacaaatctactccgacctttgacgactacttcggcaacgcatggaaatcctcttctggcccgctgcaactggtgttcgcttacttc
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cagtgagcgcaacgcaattaatgtgagttagcgcgaattgatctg

Transformation of pDW33 into CMP271

[0729] This step was done to build the isoprene-producing strain CMP272 the pDW33 plasmid was transformed by electroporation into CMP271. Transformants were recovered in L broth and plated on L agar containing carbenicillin (50ug/ml). The resulting strain was designated as CMP272.

D. Strategy for creating the REMG39 strain

[0730] Electroporation of Ptac-gcpE-lytB-petF-petH/pK184 into strain CMP272 was performed using the BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). A plasmid preparation of Ptac-gcpE-lytB-petF-petH/pK184 was provided by Gene Oracle, Inc. Ptac-gcpE-lytB-petF-petH/pK184 has been described *infra* (*see, e.g.,* Example 11).

Transformation of Ptac-gcpE-lytB-petF-petH/pK184 into CMP272

[0731] To build the REMG39 test strain, Ptac-gcpE-lytB-petF-petH/pK184 was transformed by electroporation into CMP272. Transformants were recovered in L broth and plated on L agar containing carbenicillin (50ug/ml) and kanamycin (50ug/ml). The resulting strain was designated as REMG39.

E. Comparing CMP272 to REMG39 for growth, isoprene production, DXP metabolite profile, and product yield on carbon during 14-L fermentation

[0732] The parental strain CMP272 was compared to the test strain REMG39 under 14-L fermentation conditions. The benefit of the *T. elongatus* IspG (GcpE) and IspH (LytB) activities on isoprene production and overall flux through the otherwise endogenous DXP pathway of *E. coli* is illustrated in Figure 79 and Figure 80A-B, respectively. Expression of the *T. elongatus* genes improved isoprene production approximately 2.7-fold over that of the parental strain CMP272. Despite the higher levels of cMEPP observed for the REM G39 strain during the initial 10 hour period, the REMG39 strain accumulated reduced levels of the cMEPP intermediate during the later portion of the fermentation compared to the parental strain, an observation that is correlated with increased specific productivity during post-exponential and maximal CER growth (see Figure 3B-D).

F. *Large scale fermentation of strain CMP272*

[0733] Isoprene production from *E. coli* expressing genes from the DXP pathway and isoprene synthase, grown in fed-batch culture at the 15-L scale.

[0734] Medium Recipe (per liter fermentation medium):

[0735] K2HPO4 7.5 g, MgSO4 * 7H2O 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in Di H2O. This solution was heat sterilized (123°C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Mercury Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

[0736] 1000X Modified Trace Metal Solution (per liter):

[0737] Citric Acids * H2O 40 g, MnSO4 * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

[0738] Mercury Vitamin Solution (per liter):

[0739] Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, D-pantothenic acid 4.8 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

[0740] Feed solution (per kilogram):

[0741] Glucose 0.57 kg, Di H2O 0.38 kg, K2HPO4 7.5 g, and 100% Foamblast 10 g. All components were mixed together and autoclaved. Mercury Vitamin Solution 6.7 mL was added after the solution had cooled to 25°C.

[0742] Fermentation was performed in a 15-L bioreactor with *E. coli* BL21 cells overexpressing the first enzyme in the dxp pathway(GI1.6-dxs), the last enzyme in the DXP pathway (GI1.6y-IDI), the lower MVA pathway (PL.2-mKKDyI) and truncated isoprene synthase from *P. alba* (pDW33) and containing a restored 17,257 bp chromosomal *galM*-containing region derived from MG1655 (strain name CMP272). 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 for the bioreactor. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0743] The feed solution was fed at an exponential rate until a top feed rate of 4.8 g/min was reached. After this time, the glucose feed was fed to meet metabolic demands at rates less than or equal to 4.8 g/min. The total amount of glucose delivered to the bioreactor during the 45 hr fermentation was 5.6 kg. Induction was achieved by adding

isopropyl-beta-D-1-thiogalactopyranoside (IPTG). A single shot of IPTG was added to the tank to bring the concentration to 200 uM when the cells were at an OD of 8.

[0744] The isoprene level in the off-gas from the bioreactors was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 0.97 g/L at 45 hr.

[0745] Equation for calculating Isoprene Titer: $\int$(Instantaneous isoprene production rate, g/L/hr)dt from t = 0 to 45 hrs [=] g/L broth

[0746] Equation for calculating Specific Productivity levels: (mg isoprene$_t$ – mg isoprene$_{to}$) / [(OD550$_t$ * L broth$_t$ - OD550$_{to}$ * L broth$_{to}$) / (2.7 OD*L / g cell)]/ (t – t$_0$) [=] mg isoprene/g cell/hr

G. *Large scale fermentation of strain REMG39*

[0747] Isoprene production from *E. coli* expressing genes from the DXP pathway and isoprene synthase, grown in fed-batch culture at the 15-L scale.

[0748] Medium Recipe (per liter fermentation medium):

[0749] K2HPO4 7.5 g, MgSO4 * 7H2O 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 Di H2O. This solution was heat sterilized (123°C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Mercury Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

[0750] 1000X Modified Trace Metal Solution (per liter):

[0751] Citric Acids * H2O 40 g, MnSO4 * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

[0752] Mercury Vitamin Solution (per liter):

[0753] Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, D-pantothenic acid 4.8 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

[0754] Feed solution (per kilogram):

[0755] Glucose 0.57 kg, Di H2O 0.38 kg, K2HPO4 7.5 g, and 100% Foamblast 10 g. All components were mixed together and autoclaved. Macro Salt Solution 3.4 mL, 1000X Modified Trace Metal Solution 0.8 ml, and Mercury Vitamin Solution 6.7 mL were added after the solution had cooled to 25°C.

[0756] Macro Salt Solution (per liter):

[0757] MgSO4 * 7H2O 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

[0758] Fermentation was performed in a 15-L bioreactor with *E. coli* BL21 cells overexpressing the first enzyme in the dxp pathway(GI1.6-dxs), the last enzyme in the DXP pathway (GI1.6-yIDI), the lower MVA pathway (PL.2-mKKDyI), various other genes from the DXP pathway of *T. elongatus* (Ptac-gcpE-lytB-petF-petH/pK184), and truncated isoprene synthase from *P. alba* (pDW33) and containing a restored 17,257 bp chromosomal *galM*-containing region derived from MG1655 (strain name REMG39). 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 for the bioreactor. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0759] The feed solution was fed at an exponential rate until a top feed rate of 4.8 g/min was reached. After this time, the glucose feed was fed to meet metabolic demands

at rates less than or equal to 4.8 g/min. The total amount of glucose delivered to the bioreactor during the 56 hr fermentation was 7.0 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). A single shot of IPTG was added to the tank to bring the concentration to 300 uM when the cells were at an OD of 5. After a run time of 36 h, whole broth, including cell mass, was drawn off periodically to prevent overflow of the bioreactor.

[0760] The isoprene level in the off-gas from the bioreactors was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 2.7 g/L at 56 hr.

[0761] Equation for calculating Isoprene Titer: $\int$(Instantaneous isoprene production rate, g/L/hr)dt from t = 0 to 56 hrs [=] g/L broth

[0762] Equation for calculating Specific Productivity levels: (mg isoprene$_t$ – mg isoprene$_{to}$) / [(OD550$_t$ * L broth$_t$ - OD550$_{to}$ * L broth$_{to}$) / (2.7 OD*L / g cell)]/ (t – t$_0$) [=] mg isoprene/g cell/hr

Example 25: DXP metabolite determination

A. Metabolite extraction: processing 14-L fermentor samples.

[0763] Cell metabolism was rapidly inactivated by withdrawing several milliliters of the fermentor culture into a pre-weighed tube filled with 9.0 mL of dry ice-cold methanol. The resulting sample was weighed again to calculate the amount of withdrawn cell culture and then put to -80°C for storage until further analysis. In order to extract metabolites, 500 µL of methanol-quenched fermentation sample was spun down by centrifugation for 4 min at 4500x g, at -9°C. The pellet was then re-extracted twice, first with 350 µL of 85 % methanol buffered with 5 mM ammonium acetate in water (pH=7.0) and then with 350 µL of 50% methanol in the ammonium acetate buffer. After each extraction, cell debris was pelleted by centrifugation and all three supernatants were pooled together for further analysis.

[0764] Metabolite quantitation

[0765] Extracted metabolites were analyzed by LC-ESI –MS/MS on a Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA). The system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). LC separation was done on a Synergi 45μM Hydro-RP HPLC column (150 x 2 mm, Phenomenex, USA) at a flow rate of 0.4 mL/min and the column temperature of 40°C. The LC gradient was t = 0 min, 12% B; t = 5 min, 12% B; t = 9 min, 23% B; t = 20 min, 99% B; t = 23 min, 99% B; t = 24 min, 12% B; t = 29 min, 12% B, where solvent A was 10 mM tributylamine/15 mM acetic acid in water and solvent B was LCMS-grade methanol. The sample injection volume was 10 μL.

[0766] Mass detection was carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 213.0 for DXP, 215.0 for MEP, 245.0 for IPP and DMAPP, 260.0 for HDMAPP, and 277.0 for cMEPP, 381.1 for FPP, 520.1 for CDP-ME, 600.0 for CDP-MEP. Concentrations of metabolites were determined based on integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0) using calibration curves obtained by injection of corresponding standards (Echelon Biosciences Inc). The concentration of CDP-MEP was expressed in arbitrary units because of the unavailability of commercial standard. Intracellular concentrations of metabolites were calculated based on a standard assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 μL.

Example 26: Result of Increased Activity of IspG

[0767] This example demonstrates that increased activity of IspG can be detrimental to isoprene production when it occurs in the absence of increased FldA expression.

[0768] Data obtained using 14-L REMG39 indicates that despite the increased production of isoprene in REMG39, the strain is still limited for IspG activity; this is suggested by the approx. 19mM cMEPP level the REMG39 strain maintains across the majority of the fermentation (see Figure 80B). One way to improve IspG activity is to increase its expression, as was observed for strain REM E7_12 (Figure 85B). However,

increasing IspG activity in the test strain REM E7_12 compared to the parental strain CMP272 proved to be detrimental to isoprene production (Figure 9A). An alternative method to increase the IspG activity generated from the CMP272 strain background is to increase *fldA* expression (test strain REM C9_12; Figure 85B). The largest benefit determined at small scale that increased both the increased IspG activity and endogenous IspH activity as well as improved isoprene production from the CMP272 background was to co-overexpress *fldA* and *ispG* (test strain REM D6_12; Figure 85).

A. Construction of test strains REM C9_12, REM D6_12, and REM E7_12

[0769] The construction of GI1.6 fldA/pCL, GI1.6 fldA-ispG/pCL, and GI1.6 ispG/pCL were done using standard molecular biology techniques (Sambrook *et al.*, 1989). The pCL1920 (pCL) cloning vector has been described in publications, *see, e.g.,* Lerner, C.G. et al., *Nucleic Acids Research*, Vol. 18: 4631(1990). Figure 82-84 depict the resulting plasmid constructs. The CMP272 strain was used for the transformations described below.

[0770] Chromosomal DNA from strain REM I6_4 was used as a PCR template for the generation of the PCR fragment harboring GI1.6 fldA, which was used to create GI1.6 fldA/pCL. Generation of strain REM I6_4 is described below. The DNA ultimately derived from the DXP operon pET24a plasmid (*see, e.g.,* Example 11) was used as the PCR template for both the generation of the PCR fragments harboring ispG and GI1.6 ispG, which were used to create GI1.6 fldA-ispG/pCL and GI1.6 ispG/pCL, respectively. The The DXP operon pET24a plasmid and GI1.6 gcpE-lytB-yidi pCR Blunt II TOPO vector PCR templates utilized have been described previously (*see, e.g.,* Example 11).

B. The generation of REM I6_4, the precursor to GI1.6 fldA/pCL

[0771] The GI 1.X-promoter insertions and subsequent loopout of the antibiotic resistance markers described in this example were carried out using the Red/ET system from Gene Bridges GmbH according to the manufacturer's instructions. The strain BL21(DE3) (Invitrogen) was used. . The BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD) was used for the electroporations described.

[0772] Primers

[0773] fldA confirm-F 5' tgattccgcaagactgcctgt

[0774] fldA confirm-R 5' ttcggtattaccggtgtcgct

[0775] fldA cmpGI1.X-F 5' ctatgattgc ctttatccgt gggcaatttt ccacccccat aattaaccctcactaaagggcggccgc

[0776] fldA cmpGI1.X-R 5' aagatgccagtgatagccatgagtgaaataacctcttgaa ggttacctccgggaaacgcggttgatttgtttagtggttgaattatttgctcaggatgtggcatngtcaagggcgtgacggctcgc taatacgactcactatagggctcgag
* for the case of GI1.6 fldA N=T in the primer sequence above.

[0777] top Gb's CMP 5' actgaaacgttttcatcgctc

[0778] bottom Pgb2 5' ggtttagttcctcaccttgtc

[0779] The GI1.X promoters introduced upstream of the endogenous *fldA* coding region using the Gene Bridges GmbH methods are illustrated in Figure 81. The antibiotic resistance cassette GB-CMP was amplified by PCR using primer sets fldA cmpGI1.X-F/fldA cmpGI1.X-R. The primers contain 40 bases of homology to the region immediately 5' to the *fldA* coding region to allow recombination at the specific locus upon electroporation of the PCR product in the presence of the pRed-ET plasmid. The FRT "scar" sequences remaining after Flipase-mediated excision of the antibiotic markers are also depicted in the figure.

Amplification of the GB-CmpR-fldA fragment

[0780] To amplify the GB-CmpR cassette for inserting the GI 1.X-promoters immediately upstream of the *fldA* locus the following PCR reaction was set up:

1ul template (100ng GB-CmpR)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) fldA cmpGI1.X-F

1.25ul primer (10uM) fldA cmpGI1.X-R

35 ul diH2O

+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:

95°C x 2min., (95°C x 30sec., 63°C x 30sec., 72°C x 2 min.) x 29 cycles; 72°C x 5 min., 4°C until cool (Biometra T3000 Combi Thermocycler)

[0781] The resulting PCR fragments were separated on a 0.8% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits (Qiagen) according to manufacturer's instructions. The resulting stock was GB-CmpR-GI 1.X-fldA fragment.

Integration of GB-CmpR- GI 1.X-fldA PCR product into BL21(DE3)/pRed-ET Strain

[0782] The pRed-ET vector (Gene Bridges kit) was transformed into BL21(DE3) by electroporation resulting in strain DW30 (BL21(DE3)/pRed-ET). The purified GB-CmpR-GI 1.X-fldA PCR fragment was electroporated into DW30. The transformants were recovered in L Broth and then plated on L agar containing chloramphenicol (10ug/ml). Chloramphenicol resistant colonies were analyzed by PCR for the presence of the GB-CmpR cassette and the GI 1.X-promoters using primers fldA confirm-F, fldA confirm-R, top GB's CMP, and bottom Pgb2. The PCR fragments from a number of transformants were sequenced using the fldA confirm-R and top GB's CMP primers (Sequetech; Mountain View, CA) and the various GI 1.X-*fldA* strains of interest identified. The chloramphenicol resistant strain, BL21(DE3) CMP::GI1.6 *fldA*, was designated REM I6_4.

C. Strategy for creating REM C9_12

[0783] Electroporation of GI1.6 fldA/pCL into CMP272 was performed using the BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). Cells of the strain REM I6_4 encoding GI1.6 *fldA* were used as the PCR template for vector construction.

Primers Sequences

[0784] 5' SalI GI1.X- 5' cgag gtcgac gcgagccgtcacgcccttgac

[0785] 3' NruI/SacII fldA stop – 5' gctc tcgcga gagc ccgcgg tcaggcattgagaatttcgtcgag

[0786] M13 (-20) 5' GTAAAACGACGGCCAGT

[0787] M13 reverse 5' CAGGAAACAGCTATGAC

Amplification of the GI1.6 fldA fragment

[0788] To amplify the GI1.6 fldA fragment for inserting the GI1.6 *fldA* fragment into pCL the following PCR reaction was set up:

1ul template (approx. 1ul volume of I6_4 cells)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' SalI GI1.X
1.25ul primer (10uM) 3' NruI/SacII fldA stop
35 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 60°C x 30sec., 72°C x 3 min.] x 29 cycles; 72°C x 5 min., 4°C until cool (Biometra T3000 Combi Thermocycler)

[0789] The resulting PCR fragment was separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits according to manufacturer's instructions. The resulting stock was GI 1.6-fldA fragment.

Cloning of the GI1.6 fldA fragment into pCL

[0790] Approximately 600ng of the GI1.6 fldA fragment was digested with *Sal*I (Roche) according to the manufacturer's specifications and approx. 200ng of the pCL

plasmid was digested with *Sal*I and *Sma*I (Roche) according to the manufacturer's specifications. The digests were subsequently combined and cleaned using the Qiagen QiaQuick Gel Extraction Kit. Approximately one half of the cleaned cut DNA was ligated using T4 DNA Ligase from New England Biolabs according to the manufacturer's suggested protocol. Chemically competent TOP10 cells (Invitrogen) were transformed with the ligation reaction using a standard heat-shock protocol (Sambrook *et al.*, 1989), recovered in L broth for 1 hour at 37°C and then plated on L agar containing spectinomycin (50ug/ml) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-GAL at 40ug/ml; Sigma). White, spectinomycin resistant colonies were selected, grown overnight in L broth containing spectinomycin (50ug/ml), and harvested for subsequent plasmid preparation. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit. Plasmid preparations of interest were sequenced (Sequetech; Mountain View, CA) using primers M13 (-20) and M13 Reverse, and the correct GI1.6 fldA/pCL clone identified, which has been designated as strain REM A1_11 (TOP10 w/ GI1.6 fldA/pCL; 5' Sal I-3' SacII/NruI uncut (blunt 3') end PCR fragment into 5' SalI -3'Sma I of pCL). A picture of the GI1.6 fldA/pCL vector map is presented in Figure 82.

Transformation of GI1.6 fldA/pCL into CMP271

[0791] To build the isoprene producing test strain REM C9_12, the GI1.6 fldA/pCL plasmid was transformed by electroporation into CMP272. Transformants were recovered in L broth and plated on L agar containing spectinomycin (50ug/ml) and carbenicillin (50ug/ml). The resulting strain was designated REM C9_12.

Strategy for creating REM D6_12

[0792] Electroporation of GI1.6 fldA-ispG/pCL into CMP272 was performed using the BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). The DXP operon pET24a plasmid was used as the PCR template for vector construction.

Primers Sequences

[0793] 5' SacII Ec ispG w/ rbs - 5' tcca ccgcgg gctc gaa ggag atatacc atg cat aac cag gct cca att caa

[0794] 3' NruI Ec ispG stop – 5' gctc tcgcga tta ttt ttc aac ctg ctg aac gtc

[0795] M13For – 5' gttgtaaaacgacggccagt

[0796] 5' BamHI Ec ispG w/ rbs – 5' tacg ggatcc atttga ggag taagcc atg cat aac cag gct cca att caa

[0797] 3' SacI Ec ispG w/ stop – 5' gctg gagctc cac tta ttt ttc aac ctg ctg aac gtc

[0798] pRA42 -5' gatgatcaacatgacgcatggc

[0799] pRA43 -5' cattccgatccgtattggcg

Amplification of the5' SacII- ispG-3' NruI fragment

[0800] To amplify the ispG fragment for inserting into GI1.6 fldA/pCL the following PCR reaction was set up:

1ul template (approx. 1ul volume of I6_4 cells)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' SacII Ec ispG w/ rbs
1.25ul primer (10uM) 3' NruI Ec ispG stop
35 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 60°C x 30sec., 72°C x 2 min.] x 29 cycles; 72°C x 5 min., 4°C until cool (Biometra T3000 Combi Thermocycler)

[0801] The resulting PCR fragment was separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits according to manufacturer's instructions. The resulting stock was 5' SacII-ispG-3' NruI fragment.

Cloning of the GI1.6 fldA fragment into pCL

[0802] Approximately 600ng of the 5' SacII-ispG-3' NruI fragment was digested with Sac II (New England BioLabs) according to the manufacturer's specifications and approx. 200ng of the GI1.6 fldA/CL plasmid was digested with SacII and NruI (New England BioLabs) according to the manufacturer's specifications. The digests were subsequently combined and cleaned using the Qiagen QiaQuick Gel Extraction Kit. Approximately one half of the cleaned cut DNA was ligated using T4 DNA Ligase(New England Biolabs) according to the manufacturer's suggested protocol. Chemically competent TOP10 cells (Invitrogen) were transformed with the ligation reaction using a standard heat-shock protocol (Sambrook *et al.*, 1989), recovered in L broth for 1 hour at 37°C and then plated on L agar containing spectinomycin (50ug/ml). Some spectinomycin resistant colonies were selected, grown overnight in L broth containing spectinomycin (50ug/ml), and harvested for subsequent plasmid preparation. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit. Plasmid preparations of interest were sequenced (Sequetech; Mountain View, CA) using primers 5' SacII Ec ispG, 3' NruI Ec ispG stop , M13For, 5' BamHI Ec ispG w/ rbs , 3' SacI Ec ispG w/ stop, pRA42, and pRA43 and the correct GI1.6fldA-ispG/pCL clone identified, which has been designated as strain REM D9_11 (TOP10 w/ GI1.6 fldA-ispG/pCL; 5' Sac II -3' NruI uncut (blunt 3' end) PCR fragment into 5' SacII -3'NruI of pCL). A picture of the GI1.6 fldA-ispG/pCL vector map is presented in Figure 83.

Transformation of GI1.6 fldA-ispG/pCL into CMP271

[0803] To build the isoprene producing test strain REM D6_12, the GI1.6 fldA-ispG/pCL plasmid was transformed by electroporation into CMP272. Transformants were recovered in L broth and plated on L agar containing spectinomycin (50ug/ml) and carbenicillin (50ug/ml). The resulting strain was designated REM D6_12.

E. Strategy for creating REM E7_12

[0804] Electroporation of GI1.6 ispG/pCL into CMP272 was performed using the BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). The GI1.6 gcpE-lytB-yidi pCR Blunt II TOPO vector was used as the PCR template for vector construction.

Primers Sequences

[0805] 5' SalI GI1.X- 5' cgag gtcgac gcgagccgtcacgcccttgac

[0806] 3' SacI Ec ispG w/ stop – 5' gctg gagctc cac tta ttt ttc aac ctg ctg aac gtc

[0807] M13For 5' gttgtaaaacgacggccagt

[0808] M13Rev 5' tcacacaggaaacagctatga

Amplification of the GI1.6 ispG fragment

[0809] To amplify the GI1.6 ispG fragment for inserting into pCL the following PCR reaction was set up:

1ul template (approx. 1ul volume of I6_4 cells)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' SalI GI1.X
1.25ul primer (10uM) 3' SacI Ec ispG w/ stop
35 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 60°C x 30sec., 72°C x 2 min.] x 29 cycles; 72°C x 5 min., 4°C until cool (Biometra T3000 Combi Thermocycler)

[0810] The resulting PCR fragment was separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits according to manufacturer's instructions. The resulting stock was GI1.6 ispG fragment.

Cloning of the GI1.6 ispG fragment into pCL

[0811] Approximately 600ng of the GI1.6 ispG fragment and 200ng of the pCL vector were digested with SalI and SacI (Roche) according to the manufacturer's specifications. The digests were subsequently combined and cleaned using the Qiagen QiaQuick Gel

Extraction Kit. Approximately one half of the cleaned cut DNA was ligated using T4 DNA Ligase (New England Biolabs) according to the manufacturer's suggested protocol. Chemically competent TOP10 cells (Invitrogen) were transformed with the ligation reaction using a standard heat-shock protocol (Sambrook *et al.*, 1989), recovered in L broth for 1 hour at 37°C and then plated on L agar containing spectinomycin (50ug/ml) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-GAL at 40ug/ml; Sigma). White spectinomycin resistant colonies were selected, grown overnight in L broth containing spectinomycin (50ug/ml), and harvested for subsequent plasmid preparation. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit. Plasmid preparations of interest were sequenced (Sequetech; Mountain View, CA) using primers 3' SacI Ec ispG w/ stop, M13For, and M13 Rev and the correct GI1.6 ispG/pCL clone identified, which has been designated as strain REM H5_11 (TOP10 w/ GI1.6 ispG/pCL; 5' SalI -3' SacI PCR fragment into 5' SalI -3'SacI of pCL). A picture of the GI1.6 ispG/pCL vector map is presented in Figure 84.

[0812] Transformation of GI1.6 ispG/pCL into CMP271

[0813] To build the isoprene producing test strain REM E7_12, the GI1.6 ispG/pCL plasmid was transformed by electroporation into CMP272. Transformants were recovered in L broth and plated on L agar containing spectinomycin (50ug/ml) and carbenicillin (50ug/ml). The resulting strain was designated REM E7_12.

F. Analysis of test strains REM C9_12, REM D6_12, and REM E7_12 and the parental strain CMP272 for growth, isoprene production, and DXP metabolite accumulation.

[0814] The parental strain CMP272 was compared against the test strains (REM C9_12, REM D6_12, and REM E7_12) in a shake flask assay as well as in a DXP metabolite determination study Figure 85A and Figure 85B, respectively. The detriment, approximately 20% decrease in isoprene production, of expressing *ispG* alone within the CMP272 background (strain REM E7_12) is shown in figure 85A. The increased benefit on isoprene production in small scale of co-expressing *fldA* along with *ispG* in comparison to expressing either *fldA* or *ispG* alone from the CMP272 host is also

depicted in Figure 85A. A 1.4-fold improvement in isoprene production was observed for the REM D6_12 strain relative to the parental control strain CMP272. The benefit of increasing the level of *fldA* expression on endogenous levels of *E. coli* IspG and IspH activity in strain REM C9_12 as well as improving the activity of IspH within the *ispG*-overexpressing strain REM D6_12 is indicated by the metabolite profile described in Figure 85B. More specifically, the additional FldA in strain REM C9_12 decreased the levels of both the IspG and IspH substrates, cMEPP and HDMAPP, respectively, relative to the parental strain CMP272 (cMEPP, 17% decrease; HDMAPP, 16% decrease); while the additional FldA within the co (*fldA* and *ispG*) -overexpression strain REM D6_12 compared to the REM E7_12 strain overexpressing *ispG* alone was seen to decrease HDMAPP roughly 4.3-fold.

Growth

[0815] Strains CMP272, REM C9_12, REM D6_12, and REM E7_12 were grown as 2-5 ml cultures at 30°C in TM3 liquid media (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) supplemented to a final concentration with 0.1% yeast extract and 1.0% glucose and including spectinomycin (50ug/ml) and carbenicillin (50ug/ml). Induction of LacI-regulated gene expression was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG) to a concentration of 600 uM. Growth was monitored periodically by recording each of the culture's optical density measured at 600nm using an Eppendorf Biophotometer spectrometer (Eppendorf).

Isoprene production

[0816] Isoprene production was anlayzed using a headspace assay. For the shake flask cultures, 200 ul of a culture was transferred from shake flasks to 2 ml CTC headspace vials (SUN-SRI 2mL HS vials, VWR# 66020-950, and caps, VWR# 66008-170). 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. The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An

Agilent HP-5MS GC/MS column (15 m x 0.25 mm; 0.25 μm film thickness) was used for separation of analytes. The sampler was set up to inject 100 μL of headspace gas. The GC/MS method 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 0.6 minute, the duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 67. The detector was switched off from 0 to 0.42 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at approx. 0.49 minutes. A calibration table was used to quantify the absolute amount of isoprene and was found to be linear from 1 μg/L to 5000 μg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method. The specific productivity of each strain is reported as ug/L OD Hr. Note, ratio of 1900ul headspace:100ul broth in assay vials for 30 min. incubation results in the following conversion of isoprene ug/L of culture to specific productivity: (isoprene/L determined by GC-MS) X (38)/(OD 600nm of the culture).

DXP metabolite accumulation

[0817] The DXP metabolites of the isoprene-producing parental and test strains, CMP272 and REM C9_12, REM D6_12, and REM E7_12, respectively, that are described above and depicted in Figure 85B were isolated and quantified as follows:

Metabolite extraction: processing samples from small-scale experiments.

[0818] To measure accumulation of metabolites in small-scale experiments 0.4 to 1.5 mL of cell culture was centrifuged for 3 min at 7500x g, at -9°C. Immediately after centrifugation the supernatant was aspirated to a clean tube for analysis of excreted metabolites and 100 μL of dry ice-cold methanol was added to pelleted cells. The resulting samples were then stored at -80°C until further processing.

[0819] To determine concentrations of excreted metabolites, 500 μL of methanol was added to 300 μL of the supernatant and the resulting mixture was centrifuged for 10 min at 20000x g at 4°C to remove insoluble material before the LCMS analysis.

[0820] For metabolites extraction from the pellet (further referred as intracellular metabolites), 10 μL of water was added to methanol-containing samples, the pellet was

resuspended in the resulting methanol/water mix and cell debris were spun down by 4-min centrifugation at 4500x g. The pellet was re-extracted two more times, first with 100 µL of 75% methanol buffered with 1 mM ammonium acetate in water (pH=8.0), then with 90 µL of 50 % methanol in the ammonium acetate buffer. After each extraction, cell debris was pelleted by centrifugation and the supernatants from all three extractions were combined and analyzed by LCMS. During the extraction procedure, samples were kept on ice or in a refrigerated centrifuge whenever possible to minimize metabolites degradation.

Metabolite quantitation

[0821] Extracted metabolites were analyzed by LC-ESI –MS/MS on a Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA). The system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). LC separation was done on a Synergi 45µM Hydro-RP HPLC column (150 x 2 mm, Phenomenex, USA) at a flow rate of 0.4 mL/min and the column temperature of 40°C. The LC gradient was t = 0 min, 12% B; t = 5 min, 12% B; t = 9 min, 23% B; t = 20 min, 99% B; t = 23 min, 99% B; t = 24 min, 12% B; t = 29 min, 12% B, where solvent A was 10 mM tributylamine/15 mM acetic acid in water and solvent B was LCMS-grade methanol. The sample injection volume was 10 µL.

[0822] Mass detection was carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 213.0 for DXP, 215.0 for MEP, 245.0 for IPP and DMAPP, 260.0 for HDMAPP, and 277.0 for cMEPP, 381.1 for FPP, 520.1 for CDP-ME, 600.0 for CDP-MEP. Concentrations of metabolites were determined based on integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0) using calibration curves obtained by injection of corresponding standards (Echelon Biosciences Inc). The concentration of CDP-MEP was expressed in arbitrary units because of the unavailability of commercial standard. Intracellular concentrations of metabolites were calculated based on a standard assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 µL.

Example 27: Effects of Increased Activity of IspG

[0823] This example demonstrates that increased activity of IspG can be detrimental to isoprene production as a result of insufficient IspH activity within strain REM G4_11.

[0824] As described in the example above, increased expression of *fldA* alone or in combination with *ispG* within the CMP272 strain background improved isoprene production (Figure 85). These learnings were applied to strain REMG39, as the overall goal was to improve IspG activity within this (benchmark) strain background. To reiterate, the REMG39 strain exhibited characteristics perceived to reflect a bottleneck at the point of IspG activity in flux through the DXP pathway toward isoprene production (see 14-L REMG39 example). In Figure 86A, the benefit of increasing IspG activity within the REM G4_11 strain at small scale is made apparent (35% increase in isoprene production over the parental control;) however, as shown in Figure 79 and 80, this benefit did not translate to the large scale fermentation. Results of the large scale fermentation presented in Figure 80 indicate that increased IspH activity is required by the REM G4_11 strain; this is suggested by the high (>15mM) HDMAPP levels observed during exponential phase growth of REM G4_1 (Figure 80C).

A. Construction of test strains REM G2_11 and REM G4_11

[0825] To further improve the IspG activity generated by the REMG39 strain background, the vector constructs GI1.6 fldA/pCL and GI1.6 fldA-ispG/pCL were introduced into the strain, subsequently generating the test strains REM G2_11 and REM G4_11, respectively.

B. Strategy for creating REM G2_11 and REM G4_11

[0826] Electroporation of GI1.6 fldA/pCL and GI1.6 fldA-ispG/pCL into REMG39 was performed using the BIO RAD Gene Pulser system and a transformation protocol suggested by the manufacturer (BIO RAD). Plasmid preparations of GI1.6 fldA/pCL, generated from strain REM A1_11, and GI1.6 fldA-ispG/pCL, generated from strain REM D9_11, were used; these strains and constructs are described above.

Transformation of GI1.6 fldA/pCL and GI1.6 fldA-ispG/pCL into CMP271

[0827] To build the isoprene producing test strains REM G2_11 and REM G4_11, the GI1.6 fldA/pCL and GI1.6 fldA-ispG/pCL plasmids were transformed, separately, by electroporation into REMG39. Transformants were recovered in L broth and plated on L agar containing spectinomycin (50ug/ml), kanamycin (50ug/ml), and carbenicillin (50ug/ml). The resulting strains were designated REM G2_11 and REM G4_11, respectively.

C. Analysis of test strains REM G2_11, REM G4_11, and the parental strain REMG39 for growth, isoprene production, and DXP metabolite accumulation.

[0828] The parental strain REMG39 was compared against the test strains (REM G2_11, REM and REM G4_11) in a shake flask assay as well as in a DXP metabolite determination study. The increase in isoprene production provided by the presence of GI1.6 fldA/pCL and GI1.6 fldA-ispG/pCL within the REMG39 background is depicted in Figure 86A. The test strain REM G4_11 produced approximately 1.35-fold more isoprene than the parental control strain REMG39 at the 3.5 hour time point, where REM G2_11 generated approximately 1.25-fold more isoprene than the parental control at the 3.5 hour time point. As seen in Figure 86B, both of the test strains, REM G2_11 and REM G4_11, were found to accumulate less of the IspG substrate, cMEPP, than the parental strain REMG39 at the 3.5 hour time point (REMG2_11 had approx. 66% of the parental control cMEPP level; and REM G4_11 had approx. 9% of the parental control cMEPP level). The REM G4_11 strain did however accumulate a 5.4-fold higher level of HDMAPP, the substrate of IspH, than both the parental control and test strain REM G2_11 (Figure 86B).

Growth

[0829] Strains REMG39, REM G2_11, and REM G4_11 were grown at 30°C as 2-5 ml cultures in TM3 liquid media (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) supplemented to a final concentration with 0.1% yeast extract and 1.0% glucose and including spectinomycin (50ug/ml) and carbenicillin (50ug/ml). Induction of LacI-regulated gene expression was achieved by adding isopropyl-beta-D-1-

thiogalactopyranoside (IPTG) to a concentration of 400 uM. Growth was monitored periodically by recording each of the culture's optical density measured at 600nm using an Eppendorf Biophotometer spectrometer (Eppendorf).

Isoprene production

[0830] Isoprene production was anlayzed using a headspace assay. For the shake flask cultures, 200 ul of a culture was transferred from shake flasks to 2 ml CTC headspace vials (SUN-SRI 2mL HS vials, VWR# 66020-950, and caps, VWR# 66008-170). 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. The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (15 m x 0.25 mm; 0.25 µm film thickness) was used for separation of analytes. The sampler was set up to inject 100 µL of headspace gas. The GC/MS method 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 0.6 minute, the duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 67. The detector was switched off from 0 to 0.42 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at approx. 0.49 minutes. A calibration table was used to quantify the absolute amount of isoprene and was found to be linear from 1 µg/L to 5000 µg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method. The specific productivity of each strain is reported as ug/L OD Hr. Note, ratio of 1900ul headspace:100ul broth in assay vials for 30 min. incubation results in the following conversion of isoprene ug/L of culture to specific productivity: (isoprene/L determined by GC-MS) X (38)/(OD 600nm of the culture).

DXP metabolite accumulation

[0831] The DXP metabolites of the isoprene-producing parental and test strains, REMG39 and REM G2_11 and REM G4_11, respectively, that are described above and depicted in Figure 10B were isolated and quantified as follows:

Metabolite extraction: processing samples from small-scale experiments.

[0832] To measure accumulation of metabolites in small-scale experiments 0.4 to 1.5 mL of cell culture was centrifuged for 3 min at 7500x g, at -9°C. Immediately after centrifugation the supernatant was aspirated to a clean tube for analysis of excreted metabolites and 100 µL of dry ice-cold methanol was added to pelleted cells. The resulting samples were then stored at -80°C until further processing.

[0833] To determine concentrations of excreted metabolites, 500 µL of methanol was added to 300 µL of the supernatant and the resulting mixture was centrifuged for 10 min at 20000x g at 4°C to remove insoluble material before the LCMS analysis.

[0834] For metabolites extraction from the pellet (further referred as intracellular metabolites), 10 µL of water was added to methanol-containing samples, the pellet was resuspended in the resulting methanol/water mix and cell debris were spun down by 4-min centrifugation at 4500x g. The pellet was re-extracted two more times, first with 100 µL of 75% methanol buffered with 1 mM ammonium acetate in water (pH=8.0), then with 90 µL of 50 % methanol in the ammonium acetate buffer. After each extraction, cell debris was pelleted by centrifugation and the supernatants from all three extractions were combined and analyzed by LCMS. During the extraction procedure, samples were kept on ice or in a refrigerated centrifuge whenever possible to minimize metabolites degradation.

[0835] Metabolite quantitation

[0836] Extracted metabolites were analyzed by LC-ESI –MS/MS on a Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA). The system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). LC separation was done on a Synergi 45µM Hydro-RP HPLC column (150 x 2 mm, Phenomenex, USA) at a flow rate of 0.4 mL/min and the column temperature of 40°C. The LC gradient was t = 0 min, 12% B; t = 5 min, 12% B; t = 9 min, 23% B; t = 20 min, 99% B; t = 23 min, 99% B; t = 24 min, 12% B; t = 29 min, 12% B, where solvent A was 10 mM tributylamine/15 mM

acetic acid in water and solvent B was LCMS-grade methanol. The sample injection volume was 10 µL.

[0837] Mass detection was carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 213.0 for DXP, 215.0 for MEP, 245.0 for IPP and DMAPP, 260.0 for HDMAPP, and 277.0 for cMEPP, 381.1 for FPP, 520.1 for CDP-ME, 600.0 for CDP-MEP. Concentrations of metabolites were determined based on integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0) using calibration curves obtained by injection of corresponding standards (Echelon Biosciences Inc). The concentration of CDP-MEP was expressed in arbitrary units because of the unavailability of commercial standard. Intracellular concentrations of metabolites were calculated based on a standard assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 µL.

D. Analysis of test strain REM G4_11 for growth, isoprene production, and DXP metabolite accumulation at large scale.

[0838] The increased HDMAPP present in the REM G4_11 cells was higher than the parental control strain; however, the averaged 0.63 mM HDMAPP intracellular concentration measured in the REM G4_11 cells was significantly less than the >10mM intracellular HDMAPP level that has been correlated with poor cell growth and reduced isoprene production (see Figure 10B). However, surprisingly strain REM G4_11 performed less well and produced roughly 3-fold less isoprene than the parental control at the 14-L fermentor scale (Figure 3). The moderate accumulation of HDMAPP observed to occur in the REM G4_11 cells at small scale was found to be exaggerated under large scale fermentation conditions, reaching intracellular HDMAPP levels >20 mM (Figure 4C). The decrease in cMEPP and corresponding increase in HDMAPP observed for the REM G4_11 strain relative to the parental control strain REMG39 strongly suggests that:

1) IspG activity has been improved within the REM G4_11 strain.
2) a bottleneck in DXP flux now occurs at the point of IspH activity in the REM G4_11 strain.

E. *Large scale fermentation of strain REM G4_11*

[0839] The large scale fermentation of the parental strain REMG39 is described above. Isoprene production from *E. coli* expressing genes from the DXP pathway and isoprene synthase, grown in fed-batch culture at the 15-L scale.

Medium Recipe (per liter fermentation medium):

[0840] K2HPO4 7.5 g, MgSO4 * 7H2O 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 Di H2O. This solution was heat sterilized (123°C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Mercury Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution (per liter):

[0841] Citric Acids * H2O 40 g, MnSO4 * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

Mercury Vitamin Solution (per liter):

[0842] Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, D-pantothenic acid 4.8 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

Feed solution (per kilogram):

[0843] Glucose 0.57 kg, Di H2O 0.38 kg, K2HPO4 7.5 g, and 100% Foamblast 10 g. All components were mixed together and autoclaved. Macro Salt Solution 3.4 mL, 1000X Modified Trace Metal Solution 0.8 ml, and Mercury Vitamin Solution 6.7 mL were added after the solution had cooled to 25°C.

Macro Salt Solution (per liter):

[0844] MgSO4 * 7H2O 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

[0845] Fermentation was performed in a 15-L bioreactor with *E. coli* BL21 cells overexpressing the first enzyme in the dxp pathway(GI1.6-dxs), the last enzyme in the DXP pathway (GI1.6-yIDI), the lower MVA pathway (PL.2-mKKDyI), various other genes from the DXP pathway of *T. elongatus* (Ptac-gcpE-lytB-petF-petH/pK184), the *E. coli ispG* and *fldA* genes (GI1.6 fldA-ispG/pCL), and truncated isoprene synthase from *P. alba* (pDW33) and containing a restored 17,257 bp chromosomal *galM*-containing region derived from MG1655 (strain name REM G4_11). 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 for the bioreactor. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0846] The feed solution was fed at an exponential rate until a top feed rate of 4.9 g/min was reached. After this time the glucose feed was fed to meet metabolic demands a rates less than or equal to 4.9 g/min. The total amount of glucose delivered to the bioreactor during the 44 hr fermentation was 3.0 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The inital IPTG concentration when the tank was first inoculated was 50 uM. Shots of 50 uM were added over the next five hours to bring the IPTG concentration to 350 uM when the cells were at an OD_{550} of 10.

[0847] The isoprene level in the off-gas from the bioreactors was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 0.98 g/L at 44 hours.

[0848] Equation for calculating Isoprene Titer: $\int$(Instantaneous isoprene production rate, g/L/hr)dt from t = 0 to 44 hrs [=] g/L broth

[0849] Equation for calculating Specific Productivity levels: (mg isoprene$_t$ – mg isoprene$_{to}$) / [(OD550$_t$ * L broth$_t$ - OD550$_{to}$ * L broth$_{to}$) / (2.7 OD*L / g cell)]/ (t – t$_0$) [=] mg isoprene/g cell/hr

Example 28: DXP metabolite determination

A. Metabolite extraction: processing 14-L fermentor samples.

[0850] Cell metabolism was rapidly inactivated by withdrawing several milliliters of the fermentor culture into a pre-weighted tube filled with 9.0 mL of dry ice-cold methanol. The resulting sample was weighted again to calculate the amount of withdrawn cell culture and then put to -80°C for storage until further analysis. In order to extract metabolites, 500 µL of methanol-quenched fermentation sample was spun down by centrifugation for 4 min at 4500x g, at -9°C. The pellet was then re-extracted twice, first with 350 µL of 85 % methanol buffered with 5 mM ammonium acetate in water (pH=7.0) and then with 350 µL of 50% methanol in the ammonium acetate buffer. After each extraction, cell debris was pelleted by centrifugation and all three supernatants were pooled together for further analysis.

B. Metabolite quantitation

[0851] Extracted metabolites were analyzed by LC-ESI –MS/MS on a Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA). The system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). LC separation was done on a Synergi 45µM Hydro-RP HPLC column (150 x 2 mm, Phenomenex, USA) at a flow rate of 0.4 mL/min and the column temperature of 40°C. The LC gradient was t = 0 min, 12% B; t = 5 min, 12% B; t = 9 min, 23% B; t = 20 min, 99% B; t = 23 min, 99% B; t = 24 min, 12% B; t = 29 min, 12% B, where solvent A was 10 mM tributylamine/15 mM acetic acid in water and solvent B was LCMS-grade methanol. The sample injection volume was 10 µL.

[0852] Mass detection was carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 213.0 for DXP, 215.0 for MEP, 245.0 for IPP and DMAPP,

260.0 for HDMAPP, and 277.0 for cMEPP, 381.1 for FPP, 520.1 for CDP-ME, 600.0 for CDP-MEP. Concentrations of metabolites were determined based on integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0) using calibration curves obtained by injection of corresponding standards (Echelon Biosciences Inc). The concentration of CDP-MEP was expressed in arbitrary units because of the unavailability of commercial standard. Intracellular concentrations of metabolites were calculated based on a standard assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 μL.

Example 29: Increased isoprene production by expression of the IspH enzyme and coincident demonstration of maintained accumulation of higher DXP metabolite levels

[0853] This example demonstrates increased isoprene production by expression of the IspH enzyme from *Anabaena* sp. PCC7120 in strain REM H8_12 and coincident demonstration of maintained accumulation of higher DXP metabolite levels in the REM H8_12 strain exhibiting increased IspG activity.

[0854] Data in the above example(s) generated with test strain REM G4_11 indicates that increased IspG activity within an enhanced DXP fluxing strain needs to be balanced by sufficient IspH activity in order to avoid high levels of HDMAPP accumulation during 14-L fermentation. Intracellular levels of HDMAPP, the substrate for IspH, in excess of 10mM have been correlated in both small scale and large scale experiments with poor cell growth, reduced flux through the DXP pathway, and subsequently reduced isoprene generation from isoprene production strains. Therefore, increased IspH activity within an enhanced DXP pathway strain (REM I7_11; described below) was achieved by over-expressing the *ispH* allele of *Anabaena* sp. PCC7120, generating test strain REM H8_12. Demonstrated in Figure 89 is the small scale benefit increased IspH activity, provided by expression of the IpsH of *Anabaena* sp. PCC7120, has on isoprene production by test strain REM H8_12. At 14-L scale, the test strain REM H8_12 produced the highest (2.6 g/L) isoprene titer recorded for a strain exhibiting the enhanced IspG activity provided by GI1.6 fldA-ispG/pCL (Figure 90A). Furthermore, unlike the REM G4_11 strain at the

14-L scale, strain REM H8_12 is able to maintain flux through the DXP pathway, as indicated by the maintained accumulation of the MEPP and cMEPP intermediates (compare Figure 80C to Figure 90C).

A. Construction of test strain REM H8_12, and the parental strain REM I7_11.

[0855] REM I7_11 and REM H8_12 are derivatives of WW119. This strain was constructed by electoporation of Strain WW103 with plasmid pDW33 (see Example 30 for construction of WW119). WW119 exhibits improved DXP-flux, but generates similar isoprene levels to that of the previous parental strain CMP272; this is potentially due to a bottleneck in flux at the point of IspG. WW119 harbors two improvements over the CMP272 strain. These beneficial modifications include increased *dxs* expression and increased *dxr* expression and are described *infra*. REM I7_11 was generated by introducing GI1.6 fldA-ispG/pCL into WW119 and REM H8_12 was made by moving Ptac Anabaena ispH aspA term/pEWL454 into REM I7_11; both plasmids were incorporated into their corresponding host strain via electroporation transformation methods.

Primers

[0856] 5' AseI F- pgl pET-15b 5' cagtct ATTAAT atgAAGCAAACAGTTTATATC

[0857] 3' BamHI R- pgl pET-15b 5' TAGCAGCC GGATCC TTAGTGTGCGTTAACCACCAC

[0858] EL-1098: 5' TAACTTTAAGGAGGTATACATATGGAGCTCACGCGTGCGGCCGC

CTCGAGCTGCAGTACAAATAAAAAAGGCACGTCAG

[0859] EL-1099: 5' GGATCCGTAATCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACAC ATTATACGAGCCGATGATTAATTGTCAACAGAATTCCTTTC CAGTCGGGAAACCTGTCG

[0860] EL-1100 : 5' CGTCGTTTTACAACGTCGTG

[0861] EL-1101 : 5' GAACTCCAAGACGAGGCAGC

[0862] EL-1102 : 5' GTGATATTGCTGAAGAGCTTGG

[0863] EL-1103 : 5' GGACTCAAGACGATAGTTACC

[0864] EL-1104 : 5' CACGACAGGTTTCCCGACTGG

[0865] EL-1150 5' GAGCGCCCAATACGCAAACC

[0866] Neo.21 5' GGCGATAGAAGGCGATGC

Amplification of the pgl *locus of REM I1_9*

[0867] To verify/amplify the *pgl* locus of REM I1_9 the following PCR reaction was set up:

1ul template (approx. 1ul volume of I1_9 cells)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' AseI F- pgl pET-15b
1.25ul primer (10uM) 3' BamHI R- pgl pET-15b
35 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 55°C x 30sec., 72°C x 2 min.] x 29 cycles; 72°C x 5 min., 4°C until cool (Biometra T3000 Combi Thermocycler)

[0868] The resulting PCR fragment was separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification. A *pgl*+ verified clone was selected as REM I1_9

Amplification of the pEWL454 fragment

[0869] To generate pEWL454 the following PCR reaction was set up:

1ul template (approx. 1ul volume of pK184 w/ aspA term vector (Gene Oracle, Inc.))
5ul 10X *Pfu Ultra II Fusion* DNA polymerase
2.5ul dNTP's (10 mM)
1.0 primer (10uM) EL-1098
1.0 primer (10uM) EL-1099
39.5 ul diH2O
+ 1ul of *Pfu Ultra II Fusion* DNA polymerase from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 60°C x 30sec., 72°C x 52 sec.] x 29 cycles; 72°C x 3 min., 4°C until cool (MJ Research PTC-200 Peltier Thermal Cycler)

[0870] The resulting PCR fragment was separated on a 1.2% E-gel (Invitrogen) for verification of successful amplification.

Strain REM I1_9 description

[0871] The strain REM I1_9 was used to clone the *Anabaena* sp. PCC7120 *ispH* allele, which had been codon optimized for expression in *E. coli* (provided by Gene Oracle, Inc.). Surprisingly Gene Oracle, Inc. was unable to provide an *E. coli* strain harboring the desired clone. Therefore, strain REM I1_9 was used as a host to obtain the Ptac Anabaena ispH aspA term/pEWL454 clone of interest using a survival based strategy.

[0872] Strain REM I1_9 is derived from MD09-220 (BL21(DE3)PL.2 *mKKDyI*::FRT-ΔispH::FRT) and has been described previously. The FRT-neo-FRT-GI1.6-*dxs* locus of strain MCM625 was transduced into the genome of MD09-220 via standard P1 lystate / P1 transduction protocol (Thomason *et al*,. 2007) and the resulting kanamycin resistant strain named REM C5_9. Using Gene Bridge's GmbH methods the antibiotic marker was looped out, generating strain REM H5_9. Subsequently, the *pgl* and *galP* region of MG1655 was transduced into strain REM H5_9 using standard P1 lystate / P1 transduction protocol (Thomason *et al*,. 2007), and the cells selected for growth on M9 agar (Na2HPO4 6 g/L, KH2PO4 3 g/L, NaCl 0.5 g/L, NH4Cl 0.5 g/L, 0.1 mM CaCl2, 2mM MgSO4, 1.5% agar) containing 0.4% w/v galactose and 500uM mevalonic acid.

The presence of the *pgl* locus in the galactose-utlizing, mevalonic acid-dependent, kanamycin sensitive cells was verified by PCR (see above) and one clone selected as REM I1_9 (BL21(DE3) PL.2 *mKKDyI*::FRT-ΔispH::FRT pgl^+ FRT::GI1.6-dxs).

Cloning of the Anabaena sp. PCC7120 ispH allele *into pEWL454*

[0873] Approximately 90ng of a precut 5' BamHI – 3' PstI purified DNA fragment harboring the *Anabaena* sp. PCC7120 *ispH* allele codon optimized for expression in *E. coli* (provided by Gene Oracle, Inc.) was ligated to precut 5' BamHI – 3' PstI purified DNA vector backbone pEWL454 (provided by Gene Oracle, Inc.), harboring the tac promoter and aspA terminator sequences separated by a multiple cloning site (MCS) within a pK184 (Jobling and Holmes, 1990) derived plasmid, using T4 DNA Ligase (New England Biolabs) according to the manufacturer's suggested protocol. The aspA terminator sequences present in pEWL454 were synthesized by Gene Oracle, Inc. Using the PCR method outlined above, the lac promoter sequence present in pK184 was removed and the tac promoter and MCS harbored within pEWL454 was inserted using the oligos detailed above (Integrated DNA Technologies). The resulting PCR fragment was ligated using T4 DNA Ligase from New England Biolabs according to the manufacturer's suggested protocol. Chemically competent TOP10 cells (Invitrogen) were transformed with the ligation reaction using a standard heat-shock protocol (Sambrook *et al.*, 1989), recovered in L broth for 1 hour at 37°C and then plated on L agar containing kanamycin (50ug/ml). A kanamycin resistant clone was selected, grown overnight in L broth containing kanamycin (50ug/ml), and harvested for subsequent plasmid preparation. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit. Plasmid preparations of interest were sequenced (Quintara; Albany, CA) using primers EL-1100, EL-1101, EL-1102, EL-1103, and EL-1104 and the correct pEWL454 clone identified, which has been designated as strain EWL454 (TOP10 w/ pEWL454; pK184-derived cloning vector harboring Ptac-RBS-NdeI-SacI-MluI-NotI-XhoI-PstI-aspA terminator). A picture illustrating pEWL454 is shown in Figure 87.

[0874] Water-washed REM I1_9 cells were transformed with the ligation reaction via electroporation using the BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). The cells

were recovered in L broth plus 500uM mevalonic acid (available commercially, for example, Sigma-Aldrich) for 1 hour at 37°C and then plated on L agar containing kanamycin (50ug/ml). Kanamycin resistant colonies that grew in the absence of mevalonic acid were selected, grown overnight in L broth containing kanamycin (50ug/ml), and harvested for subsequent plasmid preparation; the presence of the *Anabaena ispH* allele relieved the cell's dependence on mevalonic acid for growth. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit. Plasmid preparations of interest were sequenced (Sequetech; Mountain View, CA) using primers EL-1105 and Neo.21 and the correct Ptac Anabaena ispH aspA term/pEWL454 clone identified, which has been designated as strain REM F5_12 (REM I1_9 w/ Ptac Anabaena ispH aspA term/pEWL454; 5' BamHI -3' PstI synthetic fragment into 5' BamHI -3' PstI of pEWL454). A picture of the resulting Ptac Anabaena ispH aspA term/pEWL454 construct is shown in Figure 88.

B. Strategy for creating REM I7_11 and REM H8_12

[0875] REM I7_11 was constructed by transformation of GI1.6 fldA-ispG/pCL into WW119. The transformation was performed by electroporation using a BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). A plasmid preparation of GI1.6 fldA-ispG/pCL, generated from strain REM D9_11, was used; this strain and corresponding plasmid construct are described *infra*.

[0876] REM H8_12 was constructed by transformation of Ptac Anabaena ispH aspA term/pEWL454. The transformation was performed by electroporation using a BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). A plasmid preparation of Ptac Anabaena ispH aspA term/pEWL454 was made from strain REM F5_12.

Transformation of GI1.6 fldA-ispG/pCL into WW119 and Ptac Anabaena ispH aspA term/pEWL454 into REM I7_11

[0877] To build the *isoprene* producing parental strain, REM I7_11, from which the test strain REM H8_12 is derived, the GI1.6 fldA-ispG/pCL plasmid was transformed by

electroporation into WW119. Transformants were recovered in L broth and plated on L agar containing spectinomycin (50ug/ml) and carbenicillin (50ug/ml). The resulting strain was designated REM I7_11.

[0878] REM I7_11 was then transformed by electroporation with Ptac Anabaena ispH aspA term/pEWL454. Transformants were recovered in L broth and plated on L agar containing spectinomycin (50ug/ml), kanamycin (50ug/ml), and carbenicillin (50ug/ml). The resulting strain was designated REM H8_12.

C. Analysis of test strain REM H8_12 and the parental strain REM I7_11 for growth, isoprene production, and DXP metabolite accumulation at small scale.

[0879] The parental strain REM I7_11 was compared against the test strain REM H8_12 in a shake flask assay as well as in a DXP metabolite determination study. The increased benefit on isoprene production of the REM H8_12 strain harboring the Ptac Anabaena ispH aspA term/pEWL454 construct over the parental control strain REM I7_11 is depicted in Figure 89. The increased IspH activity present in the REM H8_12 strain compared to the parent strain REM I7_11 is reflected by the averaged 10-fold decrease in HDMAPP across the 3 hour and 3.75 hour time points (Figure 89). This elevated IspH activity provided by expression of the *Anabaena* sp. PCC7120 *ispH* allele permitted a 2.1 to 3.2-fold increase in isoprene production from the REM H8_12 test strain over the parental control (Figure 89). The REM H8_12 test strain also grew moderately better (approx. 20% faster) than the parental strain REM I7_11.

Growth

[0880] Strains REM I7_11 and REM H8_12 were grown at 30°C in 2-5 ml cultures of TM3 liquid media (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) supplemented to a final concentration with 0.1% yeast extract and 1.0% glucose and including spectinomycin (50ug/ml) and carbenicillin (50ug/ml).). Induction of LacI-regulated gene expression was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG) to a concentration of 500 uM.Growth was monitored

periodically by recording each of the culture's optical density measured at 600nm using an Eppendorf Biophotometer spectrometer (Eppendorf).

Isoprene production

[0881] Isoprene production was analyzed using a headspace assay. For the shake flask cultures, 200 ul of a culture was transferred from shake flasks to 2 ml CTC headspace vials (SUN-SRI 2mL HS vials, VWR# 66020-950, and caps, VWR# 66008-170). 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. The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (15 m x 0.25 mm; 0.25 µm film thickness) was used for separation of analytes. The sampler was set up to inject 100 µL of headspace gas. The GC/MS method 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 0.6 minute, the duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 67. The detector was switched off from 0 to 0.42 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at approx. 0.49 minutes. A calibration table was used to quantify the absolute amount of isoprene and was found to be linear from 1 µg/L to 5000 µg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method. The specific productivity of each strain is reported as ug/L OD Hr. Note, ratio of 1900ul headspace:100ul broth in assay vials for 30 min. incubation results in the following conversion of isoprene ug/L of culture to specific productivity: (isoprene/L determined by GC-MS) X (38)/(OD 600nm of the culture).

DXP metabolite accumulation

[0882] The DXP metabolites of the isoprene-producing parental strain REM I7_11 and test strain REM H8_12 that are described above and depicted in Figure 89 were isolated and quantified as follows:

Metabolite extraction: processing samples from small-scale experiments.

[0883] To measure accumulation of metabolites in small-scale experiments 0.4 to 1.5 mL of cell culture was centrifuged for 3 min at 7500x g, at -9°C. Immediately after centrifugation the supernatant was aspirated to a clean tube for analysis of excreted metabolites and 100 µL of dry ice-cold methanol was added to pelleted cells. The resulting samples were then stored at -80°C until further processing.

[0884] To determine concentrations of excreted metabolites, 500 µL of methanol was added to 300 µL of the supernatant and the resulting mixture was centrifuged for 10 min at 20000x g at 4°C to remove insoluble material before the LCMS analysis.

[0885] For metabolites extraction from the pellet (further referred as intracellular metabolites), 10 µL of water was added to methanol-containing samples, the pellet was resuspended in the resulting methanol/water mix and cell debris were spun down by 4-min centrifugation at 4500x g. The pellet was re-extracted two more times, first with 100 µL of 75% methanol buffered with 1 mM ammonium acetate in water (pH=8.0), then with 90 µL of 50 % methanol in the ammonium acetate buffer. After each extraction, cell debris was pelleted by centrifugation and the supernatants from all three extractions were combined and analyzed by LCMS. During the extraction procedure, samples were kept on ice or in a refrigerated centrifuge whenever possible to minimize metabolites degradation.

Metabolite quantitation

[0886] Extracted metabolites were analyzed by LC-ESI –MS/MS on a Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA). The system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). LC separation was done on a Synergi 45µM Hydro-RP HPLC column (150 x 2 mm, Phenomenex, USA) at a flow rate of 0.4 mL/min and the column temperature of 40°C. The LC gradient was t = 0 min, 12% B; t = 5 min, 12% B; t = 9 min, 23% B; t = 20 min, 99% B; t = 23 min, 99% B; t = 24 min, 12% B; t = 29 min, 12% B, where solvent A was 10 mM tributylamine/15 mM acetic acid in water and solvent B was LCMS-grade methanol. The sample injection volume was 10 µL.

[0887] Mass detection was carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 213.0 for DXP, 215.0 for MEP, 245.0 for IPP and DMAPP, 260.0 for HDMAPP, and 277.0 for cMEPP, 381.1 for FPP, 520.1 for CDP-ME, 600.0 for CDP-MEP. Concentrations of metabolites were determined based on integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0) using calibration curves obtained by injection of corresponding standards (Echelon Biosciences Inc). The concentration of CDP-MEP was expressed in arbitrary units because of the unavailability of commercial standard. Intracellular concentrations of metabolites were calculated based on a standard assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 µL.

D. Analysis of test strain REM H8_12 for growth, isoprene production, and DXP metabolite accumulation at 14-L fermentation scale.

[0888] REM_H8_12 produced 2.6 g/L isoprene in 14-L fermentation (Figure 14A). In addition to increased isoprene, the REM H8_12 test strain maintained roughly 2-fold higher levels of the MEP metabolite (product of DXR) and greater than 15-fold higher levels of cMEPP (substrate for IspG) across the entire 14-L fermentation than previously observed for the GI1.6 fldA-ispG/pCL containing strain REM G4_11 (compare Figure 80C to Figure 90C).

E. Large scale fermentation of strain REM H8_12

[0889] Isoprene production from *E. coli* expressing genes from the DXP pathway and isoprene synthase, grown in fed-batch culture at the 15-L scale.

1000X Modified Trace Metal Solution (per liter):

[0890] Citric Acids * H2O 40 g, MnSO4 * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

Mercury Vitamin Solution (per liter):

[0891] Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, D-pantothenic acid 4.8 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

Feed solution (per kilogram):

[0892] Glucose 0.57 kg, Di H2O 0.38 kg, K2HPO4 7.5 g, and 100% Foamblast 10 g. All components were mixed together and autoclaved. Macro Salt Solution 3.4 mL, 1000X Modified Trace Metal Solution 0.8 ml, and Mercury Vitamin Solution 6.7 mL were added after the solution had cooled to 25°C.

Macro Salt Solution (per liter):

[0893] MgSO4 * 7H2O 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

[0894] Fermentation was performed in a 15-L bioreactor with *E. coli* BL21 cells overexpressing the first enzyme in the dxp pathway(GI1.6-dxs), the last enzyme in the DXP pathway (GI1.6-yIDI), the lower MVA pathway (PL.2-mKKDyI), various other genes from the DXP pathway of *T. elongatus* (Ptac-gcpE-lytB-petF-petH/pK184), the *E. coli ispG* and *fldA* genes (GI1.6 fldA-ispG/pCL), and truncated isoprene synthase from *P. alba* (pDW33) and containing a restored 17,257 bp chromosomal *galM*-containing region derived from MG1655 (strain name REM H8_12). 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 for the bioreactor. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0895] The feed solution was fed at an exponential rate until a top feed rate of 5.8 g/min was reached. After this time, the glucose feed was fed to meet metabolic demands at rates less than or equal to 5.8 g/min. The total amount of glucose delivered to the

bioreactor during the 44 hr fermentation was 4.4 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). A single shot of IPTG was added to the tank to bring the concentration to 300 uM when the cells were at an OD_{550} of 7.

[0896] The isoprene level in the off-gas from the bioreactors was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 2.6 g/L at 44 hr.

[0897] Equation for calculating Isoprene Titer: $\int$(Instantaneous isoprene production rate, g/L/hr)dt from t = 0 to 84 hrs [=] g/L broth

F. DXP metabolite determination

Metabolite extraction: processing 14-L fermentor samples.

[0898] Cell metabolism was rapidly inactivated by withdrawing several milliliters of the fermentor culture into a pre-weighted tube filled with 9.0 mL of dry ice-cold methanol. The resulting sample was weighted again to calculate the amount of withdrawn cell culture and then put to -80°C for storage until further analysis. In order to extract metabolites, 500 µL of methanol-quenched fermentation sample was spun down by centrifugation for 4 min at 4500x g, at -9°C. The pellet was then re-extracted twice, first with 350 µL of 85 % methanol buffered with 5 mM ammonium acetate in water (pH=7.0) and then with 350 µL of 50% methanol in the ammonium acetate buffer. After each extraction, cell debris was pelleted by centrifugation and all three supernatants were pooled together for further analysis.

Metabolite quantitation

[0899] Extracted metabolites were analyzed by LC-ESI –MS/MS on a Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA). The system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). LC separation was done on a Synergi 45µM Hydro-RP HPLC column (150 x 2 mm, Phenomenex, USA) at a flow rate of 0.4 mL/min and the column temperature of 40°C. The LC gradient was t = 0 min, 12% B; t = 5 min, 12% B; t = 9 min, 23% B; t = 20 min, 99% B; t = 23 min, 99% B; t = 24 min, 12% B; t = 29 min, 12% B, where solvent A was 10 mM tributylamine/15 mM

acetic acid in water and solvent B was LCMS-grade methanol. The sample injection volume was 10 µL.

[0900] Mass detection was carried out using electrospray ionization in the negative mode. The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 213.0 for DXP, 215.0 for MEP, 245.0 for IPP and DMAPP, 260.0 for HDMAPP, and 277.0 for cMEPP, 381.1 for FPP, 520.1 for CDP-ME, 600.0 for CDP-MEP. Concentrations of metabolites were determined based on integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0) using calibration curves obtained by injection of corresponding standards (Echelon Biosciences Inc). The concentration of CDP-MEP was expressed in arbitrary units because of the unavailability of commercial standard. Intracellular concentrations of metabolites were calculated based on a standard assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 µL.

Example 30: Discovery of apparent biochemical feedback inhibition of Dxr and alleviation of negative effects thereof

[0901] We made the surprising observation that in a DXP strain production of isoprene was shut off while cells were still in a vigorous growth phase. In addition these cells also accumulate1-deoxyxylulose-5-phosphate, the substrate for Dxr. Without being bound by theory, one possible hypothesis to explain this observation is that the pathway is subject to regulation either at the genetic level or at the biochemical level. Jawaid et. al., *PLoS One*, 4(12):e8288 (2009) reported that a fraction of Dxr protein from *Francisella tularensis* was phosphorylated at ser177 when overexpressed in E. coli. This phosphorylation was presumed to inactivate the protein based on the observation that the mutations S177D and S177E led to inactive protein. We subsequently showed that purified Dxr from *E. coli* is inactivated when incubated with dimethylallyl diphosphate (DMAPP) or 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP). Further, an *E. coli* strain with a genetically modified deoxyxylulose phosphate (DXP) pathway was shown to accumulate DMAPP and/or HMBPP to levels higher than that observed in wild type. Without being bound by theory, based on the result of *in vitro* inactivation of Dxr

and i*n vivo* metabolite accumulation observed in the engineered DXP pathway strain, we postulate that the shut down of the pathway and the accumulation of 1-deoxyxylulose-5-phosphate is due to the *in vivo* inactivation of Dxr in the engineered strain. We discovered that shut down of the pathway in engineered strains is prevented by rebalancing pathway enzymes and maintaining levels of HDMAPP and DMAPP at concentrations below 1 to 2 mM DMAPP and 1 to 2 mM HDMAPP. These observations are exemplified in Figure 90. Figure 90A shows the isoprene production for strain REM H8_12,a strain with an improved DXP pathway as judged by sustained isoprene production and reaching a titer of 2.6 g/L, compared to REMG4_11 a less well balanced DXP pathway strain. Growth for REM H8_12 is shown in panel B of figure 90, while the growth of REMG4_11 is shown in Figure 79C (grey triangles). Corresponding metabolite levels for REM H8_12 are shown in Figure 90C. By 8 hours the HDMAPP levels are below 1 to 2 mM and isoprene production is maintained for a period of 30 hours or more (Figure 90A open squares). In comparison Figure 80C shows the metabolite levels for REM G4_11 . The HDMAPP levels are significantly above 1 to 2 mM for a period of 10-12 hours and isoprene production is maintained only for about 10 to 15 hours, 15 to 20 hours short of expectation (Figure 90A open circles). The final titer of this strain was 0.98 g/L.

A. Methods

[0902] Strains description

[0903] REM I7_11 – This strain arose from the modification of CMP271 detailed *infra*. CMP271 was transduced with P1 lysate MCM754, obtained as described below, harboring a modified PL.6 promoter (DNA seq.#1) replacing the native promoter in front of the *dxs* gene.

[0904] FRT-neo-FRT PL.x(trimmed) integrated at dxs.gb DNA seq.#1 sequence includes upstream FRT to and including ATG of dxs

[0905] cgcgaagttcctattctctagaaagtataggaacttcattctaccgggtaggggaggcgcttttcccaaggcagtct ggagcatgcgctttagcagccccgctgggcacttggcgctacacaagtggcctctggcctcgcacacattccacatccaccggt aggcgccaaccggctccgttctttggtggccccttcgcgccaccttccactcctccctagtcaggaagttcccccccgccccgc

agctcgcgtcgtgcaggacgtgacaaatggaagtagcacgtctcactagtctcgtgcagatggacagcaccgctgagcaatgg
aagcgggtaggcctttggggcagcggccaatagcagctttgctccttcgctttctgggctcagaggctgggaaggggtgggtcc
gggggcgggctcaggggcgggctcaggggcggggcgggcgcccgaaggtcctccggaggcccggcattctgcacgcttc
aaaagcgcacgtctgccgcgctgttctcctcttcctcatctccgggcctttcgacctgcagcagcacgtgttgacaattaatcatcg
gcatagtatatcggcatagtataatacgacaaggtgaggaactaaaccatgggatcggccattgaacaagatggattgcacgca
ggttctccggccgcttgggtggagaggctattcggctatgactgggcacaacagacgatcggctgctctgatgccgccgtgttc
cggctgtcagcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatgaactgcaggacgaggcagc
gcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgttgtcactgaagcgggaagggactggctgc
tattgggcgaagtgccggggcaggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcg
gcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcggatgg
aagccggtcttgtcgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggc
gcgcatgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgcttttct
ggattcatcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctgaagagcttg
gcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgac
gagttcttctgagcgggactctggggttcgaataaagaccgaccaagcgacgtctgagagctccctggcgaattcggtaccaat
aaaagagctttatttttcatgatctgtgtgttggtttttgtgtgcggcgcggaagttcctattctctagaaagtataggaacttcctcgag
ccctatagtgagtcgtattaagataaccatctgcggtgataaattatctctggcggtgttgacntaaataccactggcggtgatactg
agcacatcagcaggacgcactgcaaaggaggtaaaaaaacatg

[0906] Looping out the associated antibiotic marker according to Gene Bridges instructions yielded strain WW102. This strain was additionally transduced with P1 lysate MCM755 harboring a promoter named gi1.6 (DNA seq.#2).

[0907] FRT-neo-FRT-gi1.x-dxr region BL21.gb DNA seq#2; sequence includes upstream FRT to and including ATG of dxr

[0908] actaaagggcggccgcgaagttcctattctctagaaagtataggaacttcattctaccgggtaggggaggcgctttt
cccaaggcagtctggagcatgcgctttagcagccccgctgggcacttggcgctacacaagtggcctctggcctcgcacacattc
cacatccaccggtaggcgccaaccggctccgttctttggtggcccccttcgcgccaccttccactcctccccctagtcaggaagttc
ccccccgccccgcagctcgcgtcgtgcaggacgtgacaaatggaagtagcacgtctcactagtctcgtgcagatggacagcac
cgctgagcaatggaagcgggtaggcctttggggcagcggccaatagcagctttgctccttcgctttctgggctcagaggctggg
aaggggtgggtccgggggcgggctcaggggcgggctcaggggcggggcgggcgcccgaaggtcctccggaggcccgg

cattctgcacgcttcaaaagcgcacgtctgccgcgctgttctcctcttcctcatctccgggcctttcgacctgcagcagcacgtgtt
gacaattaatcatcggcatagtatatcggcatagtataatacgacaaggtgaggaactaaaccatgggatcggccattgaacaag
atggattgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactgggcacaacagacgatcggctgctctg
atgccgccgtgttccggctgtcagcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatgaactgca
ggacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgttgtcactgaagcgggga
agggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatgg
ctgatgcaatgcggcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagca
cgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgc
caggctcaaggcgcgcatgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaa
atggccgcttttctggattcatcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatatt
gctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttct
atcgccttcttgacgagttcttctgagcgggactctggggttcgaataaagaccgaccaagcgacgtctgagagctccctggcga
attcggtaccaataaaagagctttattttcatgatctgtgtgttggtttttgtgtgcggcgcggaagttcctattctctagaaagtatag
gaacttcctcgagccctatagtgagtcgtattagcccttgacnatgccacatcctgagcaaataattcaaccacttttattcactaac
aaatagctggtggaatatatg

[0909] This promoter was targeted to replace the native promoter of the *dxr* gene. Looping out the antibiotic marker according to Gene Bridges instructions yielded strain WW103. Strain WW103 was transformed by electroporation with plasmid pDW33 (Example 24 Part C) providing ispS, the isoprene synthase expression cassette and the resultant strain is designated WW119.

B. Detailed Strain construction protocols

Construction of Strain CMP271

Construction of Strain

[0910] Construction of P1 lysates MCM754 and MCM755 are detailed below:

Primers (provided by Integrated DNA Technologies; Coralville, Iowa USA)

	5'-
MCM3	tcgatacctcggcactggaagcgctagcggactacatcatccagcgtaataaataaacaataa
20	gtattaataggcccctgaattaaccctcactaaagggcgg
MCM3	5'-

21	tgttcgggattatggcgcaccacgtccagcgtgctgcaaccaatcgagccggtcgagcccag
	aatggtgagttgcttcatatattccaccagctatttgttagtgaataaaagtggttgaattatttgctc
	aggatgtggcatNgtcaagggctaatacgactcactatagggctcg
	5'-
MCM3	acaaaaacgccgctcagtagatccttgcggatcggctggcggcgttttgctttttattctgtctca
37	actctggatgtttcaattaaccctcactaaagggcgg
	5'-
	aacagtcgtaactcctgggtggagtcgaccagtgccagggtcgggtatttggcaatatcaaaa
	ctcatgttttttacctcctttgcagtgcgtcctgctgatgtgctcagtatcaccgccagtggtattta
MCM3	Ngtcaacaccgccagagataatttatcaccgcagatggttatcttaatacgactcactataggg
47	ctcg
MCM3	
27	5'-ttgtagacatagtgcagcgcca
MCM3	
30	5'-ccctgttgctgtagcatcgttt
GB-	
DW	5'-aaagaccgaccaagcgacgtctga

C. Creation of Amplicon for Promoter Integration

PL.6(trim)-*dxs*

[0911] PCR reactions were carried out in quadruplicate using the Herculase II Fusion Kit (Stratagene).

35uL ddH_2O
10uL 5x buffer
1.25uL 10uM primer MCM320, (gel purified)
1.25uL 10uM primer MCM347, (gel purified)
0.5uL dNTPs
1uL polymerase
1uL FRT-PGK-gb2-neo-FRT template DNA, GeneBridges Cat. No. K006

[0912] Reactions were cycled as follows:

95Cx 2 min followed by (95Cx 15 sec; 55Cx 15 sec; 72Cx 1 min) x30 cycles
72C x 3 min 30 sec 4C until cold.

gi1.6-dxr

[0913] Four PCR reactions were carried out in using the Herculase II Fusion Kit (Stratagene). Reactions varied by the presence or absence of 2uL DMSO and an annealing temperature of 55C or 60C.

35uL ddH_2O
10uL 5x buffer
1.25uL 10uM primer MCM321, IDT (gel purified)
1.25uL 10uM primer MCM337, IDT (gel purified)
0.5uL dNTPs
1uL polymerase
1uL FRT-PGK-gb2-neo-FRT template DNA, GeneBridges Cat. No. K006
+/- 2uL DMSO

[0914] Reactions were cycled as follows:

95Cx 2 min followed by (95Cx 20 sec; 55C or 60C x 20 sec; 72Cx 1 min) x30 cycles
72C x 3 min; 4C until

[0915] For each amplicon, four reactions were pooled and purified using a QIAquick PCR Purification kit (Qiagen) PCR column, eluting in 30uL EB.

D. Integration of Amplicon onto Chromosome

[0916] Strain MCM327 (BL21) carrying pRedET-carb (GeneBridges) was grown in L broth (LB) containing carbenicillin (50 ug/ml) at 30C overnight and then diluted 1:100 into fresh LB + carb50 and cultured at 30C for 2hr. 130uL of 10% arabinose was added and cells cultured at 37C for approximately 2 hours. Cells were prepared for electroporation by washing 3x in one half culture volume iced ddH_2O and resuspended in one tenth culture volume of the same. 100uL of cell suspension was combined with 3uL DNA amplicon in a 2mm electroporation cuvette, electroporated at 25uFD, 200ohms,

2.5kV, (Gene Pulser MXcell; BioRad) and immediately quenched with 500uL LB. Cells were recovered shaking at 37C for 1-3hrs and then transformants selected overnight on L agar (LA) plates containing kanamycin (10 ug/ml) at 37C.

[0917] Single colonies arising from transformations with each DNA amplicon were patched to LA + kan50 and grown overnight at 37C. Clones were inoculated into 5mL LB + kan10, grown to an OD_{600} ~1 and then frozen by mixing 1mL 50% glycerol and 0.5mL culture, placing on dry ice until solid, and then storing at -80C. These manipulations resulted in strain MCM754 [PL.6(trim) dxs] and strain MCM755 (gi1.6 dxr).

[0918] The integrated promoters were amplified for sequencing by colony PCR using the Herculase II Fusion kit (Stratagene).

35uL ddH_2O
10uL 5x buffer
1.25uL 10uM primers GB-DW
1.25uL 10uM primer MCM327 (dxs) or MCM330 (dxr)
0.5uL dNTPs
1uL polymerase
Colony scraping

[0919] Reactions were cycled as follows:
95C for 2 min ; (95C for 20 sec; 55C for 20 sec; 72C for 30 sec) x 30 cycles; 72C 3 min; 4C until cold

[0920] PCR products were sequenced (Quintara Biosciences) following treatment by ExoSAP.

[0921] P1 lysate MCM754, containing PL.6-dxs, was sequenced with primers GB-DW and MCM327

[0922] 5'-
Aaagaccgaccaagcgacgtctgagagctccctggcgaattcggtaccaataaaagagctttattttcatgatctgtgtgttggttt
ttgtgtgcggcgcggaagttcctattctctagaaagtataggaacttcctcgagccctatagtgagtcgtattaagataaccatctgc

ggtgataaattatctctggcggtgttgacataaataccactggcggtgatactgagcacatcagcaggacgcactgcaaaggag gtaaaaaaacatgagttttgatattgccaaatacccgaccctggcactggtcgactccacccaggagttacgactgtt

[0923] P1 lysate MCM755, containing gi1.6-dxr, sequenced with primers GB-DW and MCM330

5'-
aaagaccgaccaagcgacgtctgagagctccctggcgaattcggtaccaataaaagagctttattttcatgatctgtgtgttggtttt tgtgtgcggcgcggaagttcctattctctagaaagtataggaacttcctcgagccctatagtgagtcgtattagcccttgacaatgc cacatcctgagcaaataattcaaccacttttattcactaacaaatagctggtggaatatatgaagcaactcaccattctgggctcgac cggctcgattggttgcagcacgctggacgtggtgcgccataatcccgaacacttccgcgtagttgcgctggtggcaggcaaaaa tgtcactcgcatggtagaacagtgcctggaattctctccccgctatgccgtaatggacgatgaagcgagtgcgaaacttcttaaaa cgatgctacagcaacaggg

[0924] E. Preparation of P1 Lysates from strains MCM754 and MCM755.

[0925] 100uL of respective overnight cultures (LB + kan10) were diluted into 10mL LB + 0.2% glucose + 5mM CaCl2, and grown with shaking at 250rpm, 37C. After 30min., 100uL of a generic P1 lysate from MG1655 was added and the culture returned to the shaker for ~3 hours. The lysed culture was transferred to a 15mL tube, 200uL chloroform added, and it was vortexed for 30sec. The sample was centrifuged at 4500g for 10min and then the aqueous supernatant transferred to a fresh 15mL tube. 200uL chloroform was added and the lysate stored at 4C.

F. Cloning and purification of the enzyme.

[0926] Dxr from E. coli was cloned and purified by methods well known to those of skill in the art. The gene was inserted into the pET15b vector as described by the vendor to include a N-terimal His tag sequence (Invitrogen, Carlsbad, CA). A BL21(λDE3) E. coli culture harboring the plasmid and expressing the protein was harvested, the cell pellet lysed in a French pressure cell and protein was purified using a Ni-NTA column following the protocol recommended by the manufacturer (GE Healthcare, Pittsburg, PA).

G. Dxr inactivation by incubation with DMAPP and HDMAPP.

[0927] The purified protein, 5 uM, was incubated at several concentration of DMAPP or HMBPP (Echelon Bioscience, Salt Lake City, Utah) in buffer consisting of 100 mM Tris, 100 mM NaCl pH 8, 5 mM $MgCl_2$, 0.2 mM NADPH, 0.2 mM DXP, and 250 nM DXR. D at 37°C for two hour in a total volume of 50 uL. Dxr activity was measured periodically according to standard assay, *see, e.g.*, Koppisch et al, *Biochemistry*, 41:236-43 (2002) with a 20-fold dilution of the inactivation reaction mixture. Control incubations and assays of the enzyme were conducted under similar conditions in the absence of DMAPP or HMBPP in the inactivation reaction. Where appropriate additional control activity assays were conducted in the presence of a 20-fold diluted concentration of inactivators (DMAPP or HMBPP). A larger aliquot of enzyme (about 400 ug) was inactivated similarly with DMAPP for analysis by mass spectroscopy to verify the anticipated amino acid residue modification. As shown in Figure 92 enzyme activity declined during the inactivation incubation and yielding an inactivation half-life of 0.72 hours.

Example 31: Co-expression of DXP and MVA pathways for the production of isoprene in *E. coli*

[0928] Comparison of the energetics and carbon utilization efficiency for the DXP pathway and the MVA pathway reveal that the DXP pathway is more efficient in carbon utilization but less efficient in redox balance than the MVA pathway. When glucose is the carbon source stoichiometric yield on carbon of the DXP pathway is about 85% (grams of isoprene produced per grams of glucose utilized). The energy balance of the DXP pathway is less efficient when compared to the MVA pathway. For DXP glucose to isoprene suffers a shortage of 3 moles of NAD(P)H per mole of isoprene formed and is minus 2 moles of ATP. For the similar comparison of glucose to isoprene via MVA this pathway produces an excess of 4 moles of NAD(P)H; ATP is balanced, however, the carbon utilization efficiency is only about 55%. Without being bound by theory, a more balanced and more efficient production host can be made by combining the two pathways in a single host to optimize redox chemistry and efficiency of carbon utilization.

[0929] In this example, we provide evidence consistent with that combination of the two pathways in a single host can be established in practice. Combination of the two pathways should lead to an improved process. A series of cultures comprising two strains, REM H8_12 and REM I7_11, described above, were set up in a 48-deep-well plate (cat# P-5ML-48-C-S Axygen Scientific, California, USA) with each well providing a 2 mL culture. The media, named TM3, is described below. The two strains were grown overnight at 30 degrees Celsius at 250 rpm in TM3 medium supplemented with 1% glucose and 0.1% yeast extract. In the morning, the two strains were inoculated into the 48-deep well block in replicate. The TM3 medium was supplemented with 1% [U-^{13}C]-glucose and 0.1% yeast extract. The cultures were shaken at 30 degrees C at 600 rpm (Shel-Lab Inc. Model SI6R Refrigerated Shaking Incubator; Oregon, USA). Culture OD was determined after two hours and then at timed intervals out to 4.25 hours. The cultures were induced at two hours of growth by the addition of 400 uM IPTG. After one hour of induction the cultures of each strain also received from 0 to 8 mM (R)-mevalonic acid [cat#; Sigma M4667]. At timed intervals a 100 uL aliquot of each culture was transferred to a 98-deep well glass block (cat# 3600600 Zinsser; North America) which was immediately sealed with an impermeable adhesive aluminum film and incubated for 30 minutes with shaking at 450 rmp on an Eppendorf thermomixer (Eppendorf; North America.). The cultures were killed by heating at 70 degrees C for 7 minutes on a second Eppendorf thermomixer. The glass block was transferred to an Agilent 6890 GC attached to an Agilent 5973 MS and outfitted with a LEAP CTC CombiPAL autosampler for head space analysis. The column was an Agilent HP-5 (5% Phenyl Methyl Siloxane (15m x 0.25mm x 0.25um)). A 100 uL gas volume was injected on the column. Other conditions were as follows. Oven Temperature: 37C (held isothermal for 0.6 mins); Carrier Gas: Helium (flow - 1mL/min), split ratio of 50:1 at 250°C on the injection port; Single Ion Monitoring mode (SIM) on mass 67 or 73; Detector off: 0.00 min - 0.42 mins; Dectector on: 0.42 mins - 0.60 mins; elution time for Isoprene (2-methyl-1,3 butadiene) was ~0.49 min for a total analysis time of 0.6 mins. Calibration of the instrument was performed by methods well known to those of skill in the art.

[0930] Isoprene head space measurements were normalized by culture OD_{600} to yield a measure of specific isoprene production in units of ug/L/H/OD. All reactions were

followed for 4 hours. Figure 92A and B show the results for this experiment. Isoprene is simultaneously produced from [U-^{13}C]-glucose (Figure 92 panel B) as well as from mevalonic acid (Figure 92 panel A). The data indicate that the isoprene produced from [U-^{13}C]-glucose by the two strains is independent of isoprene produced by mevalonate. Panel B of Figure 92 further shows that the specific productivity of isoprene from [U-^{13}C]-glucose is the same for both strains at mevalonate concentrations ranging from 0 to 8 mM. These measurements were made at m/z of 73 indicative of [U-^{13}C]-glucose utilization. At the same time, the isoprene specific productivity increased with increasing mevalonic acid concentration over the same concentration range. This measurement was made at m/z of 67 indicative of mevalonate (all ^{12}C) utilization. The overall conclusion of this experiment is that isoprene produced by the DXP pathway is not affected by isoprene produced from mevalonic acid by the lower MVA pathway.

[0931] TM3 (per liter fermentation medium):

[0932] 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, yeast extract 1.0g, 1000X Modified Trace Metal stock solution 1 ml. All of the components were added together and dissolved in Di H2O. The pH is adjusted to 6.8 with NH_4OH and the solution is filter sterilized over a 0.22 micron membrane. Antibiotics were added post-sterile as needed. U-^{13}C-Glucose and [R]-mevalonic acid were added post sterile as indicated.

1000X Modified Trace Metal Stock Solution (per liter):

[0933] Citric Acids * H2O 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 Di H_2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

Example 32 Demonstration of isoprene generated by strain REM A2_17 via dual isoprenoid biosynthetic pathways

[0934] Described here is the construction of an isoprene producing *E. coli* strain that harbors both an exogenous MVA isoprenoid biosynthetic pathway and an enhanced DXP biosynthetic pathway. Data presented here indicates that isoprene produced by strain REM A2_17 is derived from both types of isoprenoid biosynthetic pathways simultaneously. For this particular example, roughly 3:2 to 1:1 MVA-flux:DXP-flux contributions to isoprene production were observed; see Figure 96-102.

Construction of strain REM A2_17

[0935] The genomic insertions described in this example were carried out using the Red/ET system from Gene Bridges GmbH according to the manufacturer's instructions. The strain BL21 (Novagene) was used. P1 lysate preparations and transductions were performed as previously described (Thomason *et al.,* 2007). The pBBR1MCS-5 vector has been described (Kovach et al., 1994) as have vector constructs MCM82, pMCM296, pDW34, pDW33, GI1.6 fldA-ispG/pCL, and Ptac Anabaena ispH aspA term/pEWL454 (see, e.g., Example 29 above and WO 2009/076676). MCM82 contains the pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS). The Trc promoter, Trp promoter and aspA terminator sequences were obtained from the information provided by NCBI (http://www.ncbi.nlm.nih.gov/) and EcoCyc (http://ecocyc.org/).

Construction of pDW15 (Ptrc-upper MVA pathway on pBBR1MCS-5)

[0936] To insert the upper MVA pathway onto the pBBR1MCS-5 vector, the entire expression cassette containing Ptrc, mvaE, mvaS, and the rrn terminator was amplified by PCR from MCM82 using the primers Upper5'XhoI and Upper3'XbaI. See below for PCR primer sequences (Table 9), reaction and cycling parameters. The approximately 4.2 kb PCR product was confirmed by gel electrophoresis (E-Gel, Invitrogen) and then purified using QiaQuick purification columns (Qiagen) according to the manufacturers recommended protocol. Purified PCR product and the pBBR1MCS-5 vector were then treated with XbaI and XhoI restriction endonucleases overnight at 37°C. See below for

reaction conditions. The next day, reactions were heated to 65°C to deactivate restriction enzymes prior to ligation. Ligation reactions (see below for conditions) were carried out at 4°C overnight. Approximately 5 µl of the ligation reactions were transformed into chemically competent *E. coli* TOP10 cells (Invitrogen) according to the manufacturer's recommended protocol, recovered at 37°C in LB for 1 hour, and then plated onto LB plates containing X-gal and Gentamicin at 10 µg/ml. Colonies displaying no β-galactosidase activity were selected for further analysis by PCR using primers M13 Reverse and MCM163 to confirm the presence of the insert. The plasmid from one of these colonies was purified (Qiagen) and completely sequenced (Quintara Biosciences, see Table 9 for primer sequences) to verify that it contained the complete upper MVA pathway expression cassette in the correct orientation. The sequence and map of pDW15 is listed below and in Figure 93, respectively.

PCR Reaction and Cycling parameters:

1 µl MCM82 (approx. 30 ng)
10 µl 5X Herculase Buffer (Stratagene)
0.5 µl dNTPs (100 mM)
1 µl Upper5'XhoI (20 uM)
1 µl Upper3'XbaI (20 uM)
35.5 µl diH2O
1 µl Herculase DNA Polymerase (Stratagene)

1. 95°C 4min.
2. 95°C 20 min, 52°C 20sec., 72°C 4 min., 5X
3. 95°C 20 min, 55°C 20sec., 72°C 4 min., 25X
4. 72°C 10 min,
5. 4°C until cool

DNA Digestion:

6 µl diH2O
2 µl 10X Buffer H (Roche)
10 µl DNA (pBBR1MCS-5 or PCR insert)
1 µl XhoI (Roche)
1ul XbaI (Roche)

1. 37°C overnight
2. 65°C 20 min (heat kill)

Ligation:

2 µl diH20
1 µl 10X ligase buffer (NEB)
1 µl T4 DNA ligase (NEB)
2 µl vector (pBBR1MCS-5)
4 µl insert (upper MVA expression cassette)

1. 4°C overnight
2. microdialyze (Millipore) and transform into competent *E. coli* (Invitrogen)

Table 9. PCR and Sequencing Primers

Upper5'XhoI	atgctcgagctgttgacaattaatcatccggctc
Upper3'XbaI	cgatctagaaaggcccagtctttcgactgagcc
MCM163	
CF07-58	atgaaaacagtagttattattgatgc
CF07-59	cttaaatcatttaaaatagc
CF07-82	atgacaattgggattgataaaattag
CF07-86	gaaatagccccattagaagtatc
CF07-87	ttgccaatcatatgattgaaaatc
CF07-88	gctatgcttcattagatccttatcg
CF07-89	gaaacctacatccaatcttttgccc

Sequence of pDW15

accttcgggagcgcctgaagcccgttctggacgccctggggccgttgaatcgggatatgcaggccaaggccgccgcgatcat
caaggccgtgggcgaaaagctgctgacggaacagcgggaagtccagcgccagaaacaggcccagcgccagcaggaacgc
gggcgcgcacatttccccgaaaagtgccacctggcggcgttgtgacaatttaccgaacaactccgcggccgggaagccgatct
cggcttgaacgaattgttaggtggcggtacttgggtcgatatcaaagtgcatcacttcttcccgtatgcccaactttgtatagagag
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gagcagcccgcatggatttgacttggtcagggccgagcctacatgtgcgaatgatgcccatacttgagccacctaactttgtttta
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tgccctacaggccgccgcaggagcgcgtgagatgcgcaagaaggccgatcaagcccaagagacggcccgag

Construction of PTrp mMVK/pDW15

Primers

* primers were modified with 5' phosphorylation

*5' phos Ptrp 5' mMVK 5'-TGGCAAATATTCTGAAATGAGCTGTTGACAATT AATCATCGAACTAGTTAACTAGTACGCAAGTTCACGTAAAAAGGGTATCGAC ATGGTATCCTGTTCTGCGCCGGGTAAGA

*3' phos aspA term 3' mMVK 5'-CAAGAAAAAAGGCACGTCATCTGACGTGCCTT TTTTATTTGT ATTAATCTACTTTCAGACCTTGCTCGGTCGG

5' mMVK seq prim 5'-GATACGTATGTTTCTACCTTC

3' mMVK seq prim 5'-GAAGGTAGAAACATACGTATC

EL1003 5'-GATAGTAACGGCTGCGCTGCTACC

MCM 177 5'-GGGCCCGTTTAAACTTTAACTAGACTTTAATCTACTTTCAGACCTTGC

Amplification of the PTrp mMVK fragment

PCR Reaction for PTrp mMVK
0.5ul vector template pDW34
10ul HerculaseII Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' phos Ptrp 5' mMVK
1.25ul primer (10uM) 3' phos aspA term 3' mMVK
36 ul diH2O
+ 0.5 ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 60°C x 30sec., 72°C x 2 min.] x 29 cycles; 72°C x 5 min., 4°C until cool (Biometra T3000 Combi Thermocycler)

[0937] The resulting PCR fragment was separated on a 0.8% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits (Qiagen) according to manufacturer's instructions. The resulting purified stock is referred to as PTrp mMVK; note the primers used contained 5' phosphorylated ends.

Cloning of the PTrp mMVK fragment into pDW15

[0938] Approximately 500ng of the pDW15 plasmid was digested with SfoI (New England Biolabs) according to the manufacturer's specifications. The SfoI cut vector was then dephosphorylated using rAPpid Alkaline Phosphatase (Roche) according to the manufacturer's suggested protocol. The digested/dephosphorylated DNA was cleaned using the Qiagen QiaQuick Gel Extraction Kit prior to ligation. A portion of the PTrp mMVK fragment (5' ends phosphorylated) was ligated to the cleaned/SfoI cut/dephosphorylated pDW15 plasmid using T4 DNA Ligase from New England Biolabs according to the manufacturer's suggested protocol. Chemically competent TOP10 cells (Invitrogen) were transformed with the ligation reaction using a standard heat-shock protocol (Sambrook *et al.*, 1989), recovered in L broth for 1 hour at 37°C and then plated on L agar containing gentamicin (10ug/ml). Gentamicin resistant colonies were selected, grown overnight in L broth containing gentamicin (10ug/ml), and harvested for subsequent plasmid preparation. Plasmid constructs were isolated using Qiagen Qiaprep Spin Miniprep Kit. Plasmid preparations of interest were sequenced (Sequetech; Mountain View, CA) using primers 5' mMVK seq prim, 3' mMVK seq prim, EL1003, and MCM 177, and the correct PTrp mMVK/pDW15 clone identified; the resulting clone of interest has been designated as strain REM H9_14 (TOP10 w/ PTrp mMVK/pDW15; SfoI site destroyed with PTrp mMVK inserted in the orientation as the Ptrc mvaE-mvaS operon present in the construct; see Figure 94).

Sequence of PTrp mMVK/pDW15

accttcgggagcgcctgaagcccgttctggacgccctggggccgttgaatcgggatatgcaggccaaggccgccgcgatcat
caaggccgtgggcgaaaagctgctgacggaacagcgggaagtccagcgccagaaacaggcccagcgccagcaggaacgc
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Construction of strain MCM928, BL21 t pgl FRT-cmp-FRT-Ptrc-PMK-MVD-yIDI

Construction of Integration construct pMCM900

[0939] The GI1.6 promoter and yeast MVK gene of pMCM296 were replaced with a chloramphenicol resistance cassette and Trc promoter. The cmR resistance cassette-Ptrc fragment was created by amplification from pMCM883 (GeneBridges cmR cassette) using primers MCM127 and MCM375. 2, 50uL reactions were created according the manufacturer's protocol for Herculase II Fusion (Agilent #600679) containing 35uL water, 10uL buffer, 0.5uL dNTPs, 1.25uL each primer at 10uM, 1uL plasmid template, 1uL polymerase. Reactions were cycled as follows: 95°C, 2:00; 30x (95°C, 0:20; 55°C, 0:20; 72°C, 1:00); 72°C, 3:00; 4°C until cold.

[0940] The ~1.6kb amplicon and plasmid pMCM296 (described *infra*) were digested at 37°C for 2 hour in 10uL reactions containing 5uL DNA, 1uL EcoRV, 1uL NotI (amplicon) or 1uL StuI (pMCM296), 1uL Roche Buffer H, and 2uL ddH2O. Reactions were heat-killed at 65°C for 2hr then digested DNA was purified on Qiagen PCR columns and eluted in 30uL EB. The eluted DNAs were ligated 1hr at room temperature in a 10uL Roche Rapid Ligation kit reaction containing 1uL pMCM296, 3uL cut amplicon, 5uL buffer 3, and 1uL ligase. Ligated DNA was transformed into Invitrogen Pir1 chemically competent cells, recovered for 1hr at 37°C, plated on LB/cmp 25ug/mL, then grown overnight at 37°C. The resulting plasmids were purified and sequenced across the promoter region. Clone four was frozen as pMCM900; see Figure 95.

Integration of cmR-Ptrc-KDyI into host BL21 t pgl to create MCM928

[0941] Strain MCM865 is an aliquot of strain MD253 (BL21 t pgl pRedET-carb). MCM865 was grown in LB + carb50 at 30°C overnight and then diluted 1:100 into fresh LB + carb50 and cultured at 30°C for 2hr. 130uL 10% arabinose was added and cells cultured at 37°C for approximately 2 hours. Cells were prepared for electroporation by washing 3x in one half culture volume iced ddH2O and resuspended in one tenth culture volume of the same. 100uL of cell suspension was combined with 1uL pMCM900 DNA in a 2mm electroporation cuvette, electroporated at 25uFD, 200ohms, 2.5kV, and immediately quenched with 500uL LB. Cells were recovered shaking at 37°C for 1-3hrs and then transformants selected overnight on LB cmp5 plates at 37°C.

[0942] After restreaking on LB cmp5, transformants were tested for growth on LB cmp5, LB kan10 and LB carb50. A cmpR/carbS/kanS clone was frozen as MCM928.

Primers

MCM139	ttttgcggccgcaattaaccctcactaaagggcgg
MCM375	gatcgatatccctgcaggaaattgttatccgctcacaattccacacattatacgagccggatgattaattgtcaacagctaatacgactcactatagggctcg

Sequence of pMCM900

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cggagaaacaatcgttgcatacacgtttgatgcaggtccaaatgctgtgttgtactacttagctgaaaatgagtcgaaactctttgcatttatctataa
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tctagtgagacgtcaaatgacgaaagcggagaaacatgtttttctggtcatgatgaggagcaaattaagttaatgaatgaaaattgtattgttttggat
tgggacgataatgctattggtgccggtaccaagaaagtttgtcatttaatggaaaatattgaaaagggtttactacatcgtgcattctccgtctttatttt
caatgaacaaggtgaattacttttacaacaaagagccactgaaaaaataactttccctgatctttggactaacacatgctgctctcatccactatgtat
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agaagatgaaactaagacaaggggtaagtttcacttttaaacagaatccattacatggcaccaagcaatgaaccatggggtgaacatgaaattg
attacatcctattttataagatcaacgctaaagaaaacttgactgtcaacccaaacgtcaatgaagttagagacttcaaatgggtttcaccaaatgatt
tgaaaactatgtttgctgacccaagttacaagtttacgccttggtttaagattatttgcgagaattacttattcaactggtgggagcaattagatgacctt
tctgaagtggaaaatgacaggcaaattcatagaatgctataacaacgcgtctacaaataaaaaaggcacgtcagatgacgtgccttttttcttggg
gcc

Construction of REM H4_15, the parent background of strain REM A2_17

[0943] The chloramphenicol marked PTrc PMK-MVD-yIDI locus of strain MCM928, described above, was introduced into strain WW103 (*see*, e.g., Examples 29 and 30) via P1-mediated transduction. The resulting chloramphenicol resistant strain was named REM H4_15 (BL21 pgl^+ PL.6-dxs, GI1.6-dxr, GI1.6 yIDI, CMP::PTrc PMK-MVD-yIDI).

Strategy for creating REM A2_17

[0944] REM A2_17 was created by subsequent plasmid transformations of pDW33, PTrp mMVK/pDW15, Ptac Anabaena ispH aspA term/pEWL454, and lastly GI1.6 fldA-ispG/pCL initially into strain REM H4_15.

[0945] Water-washed REM H4_15 cells were transformed with pDW33 via electroporation using the BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). The cells were recovered in L broth for 1 hour at 37°C and then plated on L agar containing carbenicillin (50ug/ml). One carbenicillin resistant colony was chosen, named REM A4_16, and subsequently transformed with PTrp mMVK/pDW15 via the method described; in this case L agar containing carbenicillin (50ug/ml) and gentamicin (10ug/ml) was used as a selection, resulting in the carbenicillin and gentamicin resistant strain REM I4_16. Similarly, REM I4_16 was transformed with Ptac Anabaena ispH aspA term/pEWL454 resulting in the carbenicillin (50ug/ml), gentamicin (10ug/ml) and kanamycin (50ug/ml) resistant strain REM C5_16. Lastly, strain REM C5_16 was transformed with GI1.6 fldA-ispG/pCL resulting in the carbenicillin (50ug/ml), gentamicin (10ug/ml) kanamycin (50ug/ml), and spectinomycin (50ug/ml) resistant strain REM A2_17.

Example 33 Analysis of strain REM A2_17 in the presence and absence of fosmidomycin for growth, isoprene production, and DXP and MVA metabolite accumulation using unlabeled, 1-^{13}C labeled, or 3-^{13}C labeled glucose as the sole carbon source

[0946] It was previously determined that the addition of 1mM fosmidomycin to the growth media of an *E.* coli BL21 strain harboring the GI1.6-dxr locus common to the REM A2_17 strain could inhibit isoprene production to an undetectable level. Fosmidomycin inhibits the activity of the DXR enzyme that performs the committed step of the endogenous *E. coli* DXP pathway (Kuzuyama *et al.*, 1998). Furthermore, the addition of 1 mM fosmidomycin to the growth media of a dxr null *E.* coli BL21 strain that harbors the same heterologous MVA isoprenoid biosynthetic pathway enzymes present in REM A2_17 was found to maintain the same level of isoprene production as that grown in the absence of fosmidomycin. This data indicates that the DXR inhibitor (fosmidomycin) does not adversely affect *in vivo* flux through the MVA isoprenoid biosynthetic pathway.

Specific productivity of isoprene generated by REM A2_17 strain.

[0947] 2mM fosmidomycin in combination with 1-^{13}C (Isotec) or 3-^{13}C glucose (Omicron Biochemicals, Inc) was used in small scale headspace assays and corresponding DXP and MVA metabolite determination studies to demonstrate the simultaneous flux to isoprene via the dual MVA and DXP isoprenoid biosynthetic pathways expressed within REM A2_17. See below for the rationale of using 1-^{13}C glucose and 3-^{13}C glucose to generate uniquely labeled isoprene derived from the DXP pathway that can be differentiated from the isoprene generated via the MVA pathway. Shown in Figures 98 and 99 are the results of the headspace assays utilizing the 1-^{13}C and 3-^{13}C labeled glucose which indicate a 57-58% MVA-flux and 42-43% DXP-flux contribution to the isoprene generated by strain REM A2_17, as determined by isoprene specific productivity. These results are nearly identical to that observed in the unlabeled glucose experiment shown in Figure 96 (58% MVA and 41% DXP). Interestingly, the results depicted in Figures 101 and 102 obtained from the GC/MS analysis on the various ^{12}C and ^{13}C isotope ratios present in the isoprene produced by REM A2_17 suggest a 58-62% MVA and 42-38% DXP-flux contribution to the

isoprene generated, respectively. This data is in agreement with that determined by the isoprene specific productivity determination.

[0948] The dual flux of carbon to isoprene down the MVA and DXP isoprenoid biosynthetic pathways harbored by REM A2_17 was further support by use of tryptophan to repress expression of the MVK enzyme common to the MVA pathway (see Figure 94 for an illustration of the PTrp-MVK containing vector). [The tryptophan promoter, PTrp, governs expression of MVK in REM A2_17; the Trp repressor inhibits activity of the Trp promoter when bound to tryptophan; please see information about the trp operon available through EcoCyc (http://ecocyc.org/)]. The data in Figure 98 indicates that the proportion of MVA-flux to isoprene is reduced by approximately 8% when REM A2_17 is grown in the presence of 50uM tryptophan, resulting in a strain with nearly 1:1 MVA-flux:DXP-flux contribution to isoprene.

Accumulation of DXP and MVA pathway metabolites in the REM 8A2_17 strain.

[0949] Figure 97 compares accumulation of DXP and MVA pathway metabolites in the REM A2_17 strain grown in the presence and in the absence of fosmidomycin. Among the metabolites that were detected and quantified by LC-MS/MS were mevalonic acid (the MVA pathway intermediate), DXP, MEP, CDP-ME, cMEPP, HDMAPP (the DXP pathway intermediates), and IPP and DMAPP (intermediates of both DXP and MVA pathways). Growing cells in the presence of fosmidomycin, which inhibits DXP to MEP conversion, caused a significant increase in the DXP concentration and a drop in the concentration of MEP, CDP-ME and cMEPP, but did not change the concentration of MVA. The observed decrease in HDMAPP in fosmidomycin-treated samples was noticeably smaller that the decrease in other DXP pathway metabolites, such as MEP, CDP-ME and cMEPP, presumably due to a poor sensitivity of the LC-MS/MS method to HDMAPP and a large error associated with HDMAPP measurements. The cumulative amount of IPP and DMAPP decreased in the presence of fosmidomycin in average by 55% that correlates with a 41% decrease in the isoprene production rate. Taken altogether these data demonstrate that both DXP and MVA pathways are functional in the REM A2_17 strain and are consistent with the idea that the two pathways are contributing to the isoprene production in cells grown without fosmidomycin.

Rationale for use of labeled glucose to measure contribution of DXP and MVA pathways to isoprene production

[0950] To demonstrate that in the REM A2_17 strain isoprene is produced by the DXP and MVA pathways operating simultaneously, the above strain was grown on glucose containing ^{13}C isotope at specific positions. As illustrated in Figure 100, when cells are grown on 1-^{13}C glucose, it is expected that isoprene molecules synthesized by the MVA route will be more enriched in ^{13}C than the molecules synthesized by the DXP route, whereas when cells are grown on 3-^{13}C glucose, the isoprene molecules synthesized by the MVA route should contain less ^{13}C than the isoprene molecules made by the DXP route because ^{13}C-labeled carbon is released as $^{13}CO_2$ when pyruvate is converted to acetyl-CoA. When both pathways are operating simultaneously, ^{13}C labeling pattern of isoprene emitted by the cells should be represented by superposition of the labeling patterns of isoprene molecules produced by each of the two routes.

Isoprene labeling experiments

[0951] Figure 101 shows calculated relative abundances of cMEPP and isoprene cumomers (cumulative isotopomers) produced by the REM A2_17 strain grown on: A) 1-^{13}C or B) 3-^{13}C glucose. The cumomer abundances of cMEPP and isoprene can be directly compared to each other because both compounds contain five carbon atoms in their molecules, whereas differences in the number of O, P, and H atoms can be neglected due to a very low natural abundance of isotopes other than ^{16}O, ^{31}P, and ^{1}H. The measured distributions of cMEPP cumomer abundances should be equivalent to the cumomer distributions in isoprene made exclusively by the DXP pathway and were clearly different from the calculated distributions of isoprene cumomers for cells grown in the absence of fosmidomycin (compare the amplitudes of "Isoprene (-FM)" and cMEPP (-FM) bars in Figure 101A and 9B) indicating that the DXP and MVA pathways together contribute to the isoprene synthesized by the REM A2_17 strain.

[0952] The distribution of cumomers of isoprene produced exclusively via the MVA pathway by REM A2_17 cells grown on 1-^{13}C or 3-^{13}C glucose was estimated by measuring GC spectra of isoprene emitted in the presence of 2 mM fosmidomycin (Figure 101 and 102 and relative contribution of the DXP and MVA pathways to the total isoprene production was calculated by

superimposing the "Isoprene (+FM)" and cMEPP cumomer spectra with the coefficients f^{MVA} and f^{DXP} to fit the "Isoprene (-FM)" spectra, as described in "Methods" section. Based on these calculations, the relative contribution of the DXP pathway to the total isoprene production was estimated to be 42% and 38% for the experiments with 1-^{13}C and 3-^{13}C glucose, respectively. These numbers are close to the DXP pathway contributions of 42% and 43%, respectively, estimated from the inhibition of total isoprene production rate by fosmidomycin.

Methods

Growth

[0953] Strains REM A2_17 was grown at 34°C in TM3 liquid media (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 $(NH_4)_2SO_4$, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) supplemented to a final concentration with either 1% unlabeled glucose and 0.1% yeast extract (Figure 96 and 97 experiment), or with 1% unlabeled glucose, no yeast extract, and no tryptophan; 1.0% 1-^{13}C glucose (Isotec), no yeast extract, and with or without 50uM tryptophan (Figures 98 and 101), or with 1.0% 3-^{13}C glucose (Omicron Biochemicals, Inc.) and no yeast extract (Figures 99 and 10). All growth media also contained carbenicillin (50ug/ml), gentamicin (10ug/ml) kanamycin (50ug/ml), and spectinomycin (50ug/ml). The culture was induced with 400uM IPTG and later DXP flux inhibited for half of the culture by the addition of 2mM fosmidomycin (Invitrogen). Growth was monitored periodically by recording each of the culture's optical density measured at 600nm using an Eppendorf Biophotometer spectrometer (Eppendorf).

GC measurements of isoprene

[0954] Isoprene production was analyzed using a headspace assay. For the headspace cultures, 100 uL to 200 ul of the cultures was transferred from the shake flasks to 2 ml CTC headspace vials (SUN-SRI 2mL HS vials, VWR# 66020-950, and caps, VWR# 66008-170). The cap was screwed on tightly and the vials incubated at the equivalent temperature with shaking at 250 rpm. After approx. 30 min. to 1 hour the vials were removed from the incubator, heat killed at 70°C for 7 min., and analyzed. The analysis was performed using an Agilent 6890 GC/MS system interfaced with a

CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (15 m x 0.25 mm; 0.25 μm film thickness) was used for separation of analytes. The sampler was set up to inject 100 μL of headspace gas. The GC/MS method utilized helium as the carrier gas at a flow of 1 ml/minute. The injection port was held at 250° C with a split ratio of 50:1. The oven temperature was held at 37° C for 0.6 minute, the duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 67 or in a full scan mode covering m/z from 25 to 80. The detector was switched off from 0 to 0.42 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) standard (SCOTTY® Analyzed Gases) was observed to elute at approx. 0.49 minutes. A calibration table was used to quantify the absolute amount of isoprene and was found to be linear from 1 μg/L to 5000 μg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method. The specific productivity of each strain is reported as ug/L OD Hr. Note, ratio of 1800ul headspace:200ul broth in assay vials for 1 hour incubation results in the following conversion of isoprene ug/L of culture to specific productivity: (isoprene/L determined by GC-MS) X (9)/(OD 600nm of the culture). To quantify the amount of isoprene produced from ^{13}C-labeled glucose, the concentration obtained based on the calibration curve with the non-labeled standard was multiplied by the conversion factor **K** to compensate for isotopic effects. The conversion factors were calculated as

$$\mathbf{K} = (S(\mathbf{A_i}) / \mathbf{A_{67}}) / (S(\mathbf{P_i}) / \mathbf{P_{67}}), \qquad \text{(Eq. 1)}$$

where $\mathbf{A_i}$ are the measured intensities of GC peaks produced by ^{13}C-enriched isoprene and $\mathbf{P_i}$ are the measured intensities of GC peaks produced by the isoprene standard (subscript indices $\mathbf{i} = 60\ldots72$ indicate m/z values of corresponding peaks, which include peaks $\mathbf{A_{67}}$ and $\mathbf{P_{67}}$). For the experiments referred to in this document the conversion factors of 2.901 and 3.369 were applied to no fosmidomycin and to 2 mM fosmidomycin conditions, respectively, for cells grown on 1-^{13}C glucose and the factors of 1.476 and 1.315 were applied to no fosmidomycin and to 2 mM fosmidomycin conditions, respectively, for cells grown on 3-^{13}C glucose.

LC-MS/MS analysis of cellular metabolites

[0955] For metabolite analysis 1.5 to 5 mL of cell culture was spun down by centrifugation and 100 or 150 uL of dry ice-cold methanol was added to pelleted cells after the centrifugation. The resulting samples were then stored at -80°C until further processing. To extract cellular metabolites, 10 or 15 µL of water was added to methanol-containing samples, the pellet was resuspended in the resulting methanol/water mix and then cell debris were spun down for 4-min at 4500x g. The pellet was re-extracted two more times, first with 90 µL of 75% methanol buffered with 1 mM ammonium acetate in water (pH=8.0), then with 100 µL of 50 % methanol in the ammonium acetate buffer. After each extraction, cell debris were spun down by centrifugation and the supernatants from all three extractions were combined. During the extraction procedure, samples were kept on ice or in a refrigerated centrifuge whenever possible to minimize metabolites degradation.

[0956] The extract was analyzed by LC-MS/MS on a TSQ Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA) using electrospray ionization in the negative mode. The system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). LC separation was done on a Synergi 45µM Hydro-RP HPLC column (150 x 2 mm, Phenomenex, USA) at a flow rate of 0.4 mL/min and the column temperature of 40°C. The LC gradient was t = 0 min, 12% B; t = 5 min, 12% B; t = 9 min, 23% B; t = 20 min, 99% B; t = 23 min, 99% B; t = 24 min, 12% B; t = 29 min, 12% B, where solvent A was 10 mM tributylamine/15 mM acetic acid in water and solvent B was LCMS-grade methanol. The sample injection volume was 10 to 25 µL.

[0957] Mass detection was carried out using electrospray ionization in the negative mode. The following MS/MS transitions were chosen to detect the metabolites of interest: 213 → 79 for DXP, 215 → 79 for MEP, 245 → 79 for IPP and DMAPP, 261 → 79 for HDMAPP, 277 → 79 for cMEPP, 520.1 → 79 for CDP-ME, 227→ 79 for MVP, 307→ 79 for MVPP, and 147→59 for MVA. Other mass spec settings were optimized to obtain the highest sensitivity using corresponding standards purchased from Echelon Biosciences Inc. or synthesized in house. To quantify the absolute concentrations of cellular metabolites a calibration table was constructed by injecting the known amounts of these standards. Note that the LC-MS/MS method that was used for

metabolite analysis does not discriminate between structurally similar IPP and DMAPP, therefore their amount in samples was determined as a sum of concentrations of the two compounds.

[0958] Cumomer distribution analysis for cMEPP was done by calculating relative intensities of peaks arising from 277→79, 278→79, 279→79, 280→79, 281→79 and 282→79 MS/MS transitions corresponding to M0, M+1, M+2, M+3, M+4, and M+5 cumomers of this metabolite. In a separate experiment it has been verified that at t ~ 14.3 min (the retention time of cMEPP) extracts from *E.coli* cells grown on a regular glucose do not generate detectable peaks with MS/MS transitions 272→79, 273→79, 274→79, 275→79, 276→79. These control measurements exclude the possibility that compounds potentially co-eluting with cMEPP but having slightly lower molecular weight can contribute to the MS/MS peaks generated by cMEPP when cells are grown on ^{13}C-enriched glucose.

Cumomer analysis of ^{13}C labeled isoprene

[0959] To measure ^{13}C enrichment of isoprene emitted by cells grown on ^{13}C-glucose, GC spectra were monitored from m/z 58 to m/z 68, i.e. over the range of mass to charge ratios that can originate from five-carbon isoprene derivatives. Figure 102 shows typical GC spectra of synthetic isoprene containing the natural abundance of ^{13}C and of isoprene emitted by REM A2_17 strain grown on 3-^{13}C glucose and therefore enriched in ^{13}C.

[0960] The data shown in Figure 102A were used to calculate the theoretical GC spectrum of isoprene containing no ^{13}C isotopes (all-$^{12}C_5$ isoprene) according to the following set of linear equations:

$$\mathbf{P_{60}} = \mathbf{1.00000}*k_{60}$$
$$\mathbf{P_{61}} = \mathbf{0.05561}*k_{60}+\mathbf{1.00000}*k_{61}$$
$$\mathbf{P_{62}} = \mathbf{0.01238}*k_{60}+\mathbf{0.05561}*k_{61}+\mathbf{1.00000}*k_{62}$$
$$\mathbf{P_{63}} = \mathbf{0.01238}*k_{61}+\mathbf{0.05561}*k_{62}+\mathbf{1.00000}*k_{63}$$
$$\mathbf{P_{64}} = \mathbf{0.01238}*k_{62}+\mathbf{0.05561}*k_{63}+\mathbf{1.00000}*k_{64}$$
$$\mathbf{P_{65}} = \mathbf{0.01238}*k_{63}+\mathbf{0.05561}*k_{64}+\mathbf{1.00000}*k_{65}$$
$$\mathbf{P_{66}} = \mathbf{0.01238}*k_{64}+\mathbf{0.05561}*k_{65}+\mathbf{1.00000}*k_{66}$$

$$\mathbf{P_{67}} = \mathbf{0.01238}*k_{65}+\mathbf{0.05561}*k_{66}+\mathbf{1.00000}*k_{67} \quad \text{(Eqs. 2),}$$
$$\mathbf{P_{68}} = \mathbf{0.01238}*k_{66}+\mathbf{0.05561}*k_{67}+\mathbf{1.00000}*k_{68}$$
$$\mathbf{P_{69}} = \mathbf{0.01238}*k_{67}+\mathbf{0.05561}*k_{68}+\mathbf{1.00000}*k_{69}$$
$$\mathbf{P_{70}} = \mathbf{0.01238}*k_{68}+\mathbf{0.05561}*k_{69}+\mathbf{1.00000}*k_{70}$$

[0961] where $\mathbf{P_{60}}$ … $\mathbf{P_{70}}$ are the measured intensities of GC peaks produced by the isoprene standard (subscript indices indicate m/z values of corresponding peaks), k_{60} … k_{70} are the calculated intensities of GC peaks that would be generated by all-$^{12}C_5$ isoprene (subscript indices indicate m/z values of corresponding peaks), and the coefficients 1.00000, 0.05561, and 0.05561 are the estimated relative abundances of three C_5 cumomers containing zero, one or two ^{13}C isotopes per molecule assuming that this C_5 compound has natural abundance of ^{13}C isotope equal to 1.1%. (Note that in our calculations of all-$^{12}C_5$ isoprene spectrum it was assumed that the natural abundance of deuterium is too small to affect the final results). The positive values of k_{60} … k_{70} were obtained using `lsqlin` solver (MATLAB 7.0, MathWorks). The calculated values of k_{69} and k_{70} were effectively zero indicating that GC spectrum of all-$^{12}C_5$ isoprene should not have any peaks with m/z=69 and higher.

[0962] Cumomer distribution analysis of labeled isoprene samples was done according to the following set of linear equations based on the values of k_{62} … k_{68} obtained as described above:

$$\mathbf{A_{67}} = k_{67}*X_{M0} + k_{66}*X_{M+1} + k_{65}*X_{M+2} + k_{64}*X_{M+3} + k_{63}*X_{M+4} + k_{62}*X_{M+5}$$
$$\mathbf{A_{68}} = k_{68}*X_{M0} + k_{67}*X_{M+1} + k_{66}*X_{M+2} + k_{65}*X_{M+3} + k_{64}*X_{M+4} + k_{63}*X_{M+5}$$
$$\mathbf{A_{69}} = k_{68}*X_{M+1} + k_{67}*X_{M+2} + k_{66}*X_{M+3} + k_{65}*X_{M+4} + k_{64}*X_{M+5}$$
$$\mathbf{A_{70}} = k_{68}*X_{M+2} + k_{67}*X_{M+3} + k_{66}*X_{M+4} + k_{65}*X_{M+5} \quad \text{(Eqs. 3),}$$
$$\mathbf{A_{70}} = k_{68}*X_{M+3} + k_{67}*X_{M+4} + k_{66}*X_{M+5}$$
$$\mathbf{A_{71}} = k_{68}*X_{M+4} + k_{67}*X_{M+5}$$
$$\mathbf{A_{72}} = k_{68}*X_{M+5}$$

[0963] where $\mathbf{A_{67}}$-$\mathbf{A_{72}}$ are the measured intensities of GC peaks produced by ^{13}C-enriched isoprene (subscript indices indicate m/z values of corresponding peaks) and X_{M0} … X_{M+5} are the relative abundances of isoprene cumomers having from zero to five ^{13}C atoms (X_{M0} corresponds to

the isoprene molecules in which all carbons atoms are represented by the isotope ^{12}C). The non-negative values of $X_{M0} \ldots X_{M+5}$ were obtained using the `lsqlin` solver (MATLAB 7.0, MathWorks).

Determination of relative contribution of DXP and MVA pathways to the isoprene production

[0964] The relative contribution of DXP and MVA pathways to the total isoprene production (f^{DXP} and f^{MVA}, respectively) was estimated by solving in MATLAB (MathWorks) the following overdetermined system of linear equations:

$$\begin{aligned} X_{MO,\ Isp\text{-}FM} &= f^{DXP} * X_{MO,\ cMEPP} + f^{MVA} * X_{MO,\ Isp+FM} \\ X_{M+1,\ Isp\text{-}FM} &= f^{DXP} * X_{M+1,\ cMEPP} + f^{MVA} * X_{M+1,\ Isp+FM} \\ X_{M+2,\ Isp\text{-}FM} &= f^{DXP} * X_{M+2,\ cMEPP} + f^{MVA} * X_{M+2,\ Isp+FM} \\ X_{M+3,\ Isp\text{-}FM} &= f^{DXP} * X_{M+3,\ cMEPP} + f^{MVA} * X_{M+3,\ Isp+FM} \\ X_{M+4,\ Isp\text{-}FM} &= f^{DXP} * X_{M+4,\ cMEPP} + f^{MVA} * X_{M+4,\ Isp+FM} \end{aligned} \qquad \text{(Eqs. 4),}$$

[0965] where $X_{M0,\ Isp\text{-}FM} \ldots X_{M+4,\ Isp\text{-}FM}$ and $X_{MO,\ Isp+FM} \ldots X_{M+4,\ Isp+FM}$ are the relative abundances of isoprene cumomers containing from zero to four ^{13}C atoms calculated according to Eqs. 3 (subscript indices "Isp+FM" and "Isp-FM" indicate that calculations were done for cells incubated with and without fosmidomycin, respectively), $X_{M0,\ cMEPP} \ldots X_{M+4,\ cMEPP}$ are the relative abundances of corresponding cMEPP cumomers measured by LC-MS/MS as described above.

Example 34 ^{13}C NMR method for the determination of carbon fluxes through the MVA and MEP pathways leading to BioIsoprene™ product

[0966] The relative contributions of the two isoprenoid precursor pathways, the MVA and MEP (DXP) pathways, to isoprene production in a REM A2_17 dual pathway strain were determined by ^{13}C NMR spectroscopy and the resulting information used to calculate the MVA/MEP carbon ratio. Similar techniques have been used to determine the respective contributions of the MVA and MEP pathways to the biosynthesis of polyisoprenoids (Skorupinska-Tudek, K. *et al.* (2008) *J. Biol. Chem.*, **283(30)**, pp. 21024–21035.) and isoprene (Wagner,W.P., Helmig, D. and Fall, R. (2000) *J. Nat. Prod.*, **63**, pp. 37-40). The labeling patterns of isoprene derived from ^{13}C enriched glucose

labels differ according to which pathway was utilized to channel carbon from the substrate to product. These patterns are shown in Figure 100A for a [1-^{13}C]-D-glucose substrate and Figure 100B for a [3-^{13}C]-D-glucose substrate.

[0967] As can be seen from Figure 100A, carbon #3 (C-3) of isoprene is not enriched from either pathway from a [1-^{13}C]-D-glucose substrate, with the extent of ^{13}C-enrichment equal to the natural abundance of 1.1% relative to ^{12}C. In contrast, C-5 is labeled in both cases, thus the enrichment of C-5/C-3 allows the determination of the total extent of ^{13}C-label incorporation. The maximum possible ^{13}C enrichment at C-5 of Biosoprene™ product derived from [1-^{13}C]-D-glucose is 50%, with less if oxidative pentose phosphate pathway is operating at a significant flux relative to glycolysis. The ratio of MVA/MEP pathways is determined by comparing the enrichment of C-1 relative to C-2 and C-4. This is shown in Figure 103.

[0968] In the case where carbon flux though the MVA and MEP pathways is equal (1:1 MVA/MEP ratio), the extent of labeling at C-1 relative to C-2 and C-4 is also equivalent in the Bioisoprene™ product, with a maximum enrichment of 25%. At a MVA/MEP ratio of 9:1, C-1 is only enriched to the extent of 5%, whereas C-2 and C-4 are enriched to a level of 45%.

[0969] A method for the small-scale generation, collection and analysis of ^{13}C-labeled BioIsoprene™ product was developed in order to determine the relative contributions of the MVA and MEP (DXP) pathways to isoprene production in strain REM A2_17. The strain was grown in HM-1 media with [1-^{13}C]-D-glucose (10 mg/mL) as the sole carbon source in a stirred bottle format and the resulting BioIsoprene™ product was adsorbed to a small carbon filter consisting of 200 mg activated carbon (Koby filters, MA) packed into a glass Pasteur pipette with cotton wool (Scheme xx-1). After overnight growth at 34°C, the carbon filter was removed and desorbed directly into a glass NMR tube with $CDCl_3$ (1 mL). A reference spectrum of unlabeled isoprene was obtained by diluting an isoprene standard (5 uL) (Sigma-Aldrich) into 0.75 mL of deuterochloroform (CDCl3) and acquiring a ^{13}C NMR spectrum.

[0970] Relative ^{13}C-enrichment of isoprene at each carbon atom was determined by ^{13}C nuclear magnetic resonance spectroscopy (^{13}C-NMR) by determining the relative intensities of the signals

corresponding to each carbon atom of isoprene and comparing these values to the relative intensities of the carbon signals from unlabeled (natural ^{13}C abundance) isoprene. ^{13}C NMR spectra were obtained on a Varian 500 MHz VNMRS system operating at 125.7 MHz. Acquisition parameters were sw = 30487, at = 1.3 sec, dl = 1, nt = 10000, dn = H1, dm = yyy, dmm = w, dpr = 42, dmf = 12600. ^{13}C signal intensity was determined by peak height and integrated peak area. The ^{13}C-NMR spectrum of unlabeled isoprene (% ^{13}C = 1.1%) is shown in Figure 105. The peak heights of carbons 1-4 are similar, with aliphatic C-5 showing a more intense signal.

[0971] The ^{13}C NMR spectrum of the BioIsoprene™ product derived from dual pathway strain REM A2_17 is shown in Figure 106. The signals for C-1, 2, 4 and 5 are clearly evident, whereas the C-3 signal is equal or less than, or equal to the noise level. The relative peak heights of C-1, C-2 and C-4 indicate that the ratio of MVA/MEP pathway flux is more than 1:1 and less than 2:1. The enrichment of C-1, 2 and 4 relative to C-3 and C-5 indicate that both the MVA and MEP pathways are operating in strain REM A2_17 and contribute to overall carbon flux to isoprene.

Example 35 fkpB-ispH iscR

[0972] In this example, we show that when the promoter PL.6 replaced the native promoter of the operon fkpB-ispH in strain WW119 to create strain REM D8_15, isoprene production drops from ~ 500 to 600 ug/L/H/OD seen in strain WW119 (see Figure 108) to ~ 50 ug/L/H/OD in strain REM D8_15 (see Figure 108). Addition of *ΔispR* to WW119 showed a small decrease in isoprene specific productivity. The result observed for the introduction of PL.6 fkpB-ispH into WW119 was unanticipated. Our hypothesis was that more ispH would yield higher isoprene titer. We further show that when the iron sulfur cluster regulatory gene, *iscR*, is deleted from the latter strain, REM D8_15, to create strain REM D6_15 the ΔiscR mutation substantially restores isoprene production to strain REM D6_15. These observations suggest a beneficial interaction between *ΔiscR* and fkpB-ispH that can improve the process of isoprene production via the DXP pathway.

Construction of strains for this example.

Generation of the PL.6-fkpB locus

[0973] Within the *E. coli* BL21 genome the *ispH* gene is located immediately downstream of the *fkpB* gene which encodes a FKBP-type peptidyl-prolyl cis-trans isomerase. Interestingly, the structure of the *E. coli* IspH enzyme shows that the protein has 2 proline residues that are isomerized (Gräwert, T. *et al.*, 2009). The idea that FkpB could be involved in IspH function may also be reflected by the fact that the *fkpB* and *ispH* orfs are separated by just one nucleotide and together have been shown to be transcribed as the last 2 genes of the *ribF-ileS-lspA-fkpB-ispH* 5-gene operon (see http://ecocyc.org/).

[0974] Further more, BLAST analysis of the 125 bases separating the stop codon of *lspA* and the start codon of *fkpB* revealed a highly conserved sequence that occurs many times throughout the *E. coli* genome. This commonly found sequence is:

AATCGTAG**GCCGGAT**AAGGCGTTTACGCCGC**ATCCGGC**AA

[0975] This sequence harbors characteristics of a transcriptional terminator, which includes the likely formation of a stem loop. The bases with potential of hybridizing together to form the stem loop are highlighted above in bold and underlined text (bold anneals to bold; underlined anneals to underlined). The location of this repeated sequence, in each instance observed, was always found just downstream of the 3' end of a single gene or downstream of the 3' ends of 2 genes transcribed toward one another. The repeated sequence was not found within the coding region of over 40 regions analyzed. Together, this information suggests that the sequence functions as a transcriptional terminator and hints at the possibility of *fkpB* and *ispH* being transcribed as an independent 2-gene operon.

[0976] Our in-house transcriptional analyses of BL21 14-L fermentations show the *ispH* transcript to be present at almost undetectable levels; a result inline with that previously reported in the field. Similarly, the level of IspH protein accumulates to low levels within these and small scale grown cells (for small scale result see Figure 108). Increased expression of endogenous BL21 *ispH* and its effect on isoprene production was an aim of the work described here. The previously described PL.6-promoter is a strong constitutive promoter chosen to up-regulate the expression of *ispH*. Based on the speculation that FkpB and IspH as well as *fkpB* and *ispH* potentially share a functional and a

transcriptional relationship, respectively (described above), the PL.6-promoter was inserted immediately upstream of the *fkpB* orf.

[0977] The PL.6-promoter insertion and subsequent loopout of the chloramphenicol resistance marker described in this example was carried out using the Red/ET system from Gene Bridges GmbH according to the manufacturer's instructions. The strain BL21 (Novagen) was used. P1 lysate preparations and transductions were performed as previously described (Thomason *et al.*, 2007). The BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD) was used for the electroporations described.

Primers

5' CMP::80bp up of fkpB
5'-AGATTGCTGCGAAATCGTAGGCCGGATAAGGCGTTTACGC
CGCATCCGGCAAAAATCCTTAAATATAAGAGCAAACCTGCAA
TTAACCCTCACTAAAGGGCGGCCGC

3' CMP::PL.6-fkpB
5'-AGCGTGAAGTGCACCAGGACGGCGCTATTGCTCTGTACAGATTCAGA
CATGTTTTTACCTCCTTTGCAGTGCGTCCTGCTGATGTGCTCAGTATCA
CCGCCAGTGGTATTTATGTCAACACCGCCAGAGATAATTTATCACCGCA
GATGGTTATCTTAATACGACTCACTATAGGGCTCGAG

5' confirm CMP::80bp up of fkpB
5'-ACGCATCTTA TCCGGCCTACA

3' confirm CMP::PL.6-fkpB
5'-ACCGTTGTTGCGGGTAGACTC

5' primer to PL.6
5'-AGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTG

top Gb's CMP
5'-ACTGAAACGTTTTCATCGCTC

bottom Pgb2
5'-GGTTTAGTTCCTCACCTTGTC

[0978] The PL.6- promoter introduced upstream of the endogenous *fkpB* coding region using the Gene Bridges GmbH methods is illustrated in Figure 107. The antibiotic resistance cassette GB-CMP was amplified by PCR using the primer set 5' CMP::80bp up of fkpB and 3' CMP::PL.6-fkpB. The 5' CMP::80bp up of fkpB primer contains 80 bases of homology to the region immediately 5' to the *fkpB* coding region and the 3' CMP::PL.6-fkpB primer contains 50 bases of homology to the 5' region of the *fkpB* orf (open reading frame) to allow recombination at the specific locus upon electroporation of the PCR product in the presence of the pRed-ET plasmid. The FRT (Flipase recognition target) "scar" sequence remaining after Flipase-mediated excision of the antibiotic marker is also depicted in the figure.

Amplification of the CMP::PL.6 fkpB fragment

To amplify the GB-CmpR cassette for inserting the PL.6-promoter immediately upstream of the *fkpB* locus the following PCR reaction was set up:
1ul template (100ng GB-CmpR)
10ul *HerculaseII* Buffer
0.5ul dNTP's (100 mM)
1.25ul primer (10uM) 5' CMP::80bp up of fkpB
1.25ul primer (10uM) 3' CMP::PL.6-fkpB
35 ul diH2O
+ 1ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 60°C x 30sec., 72°C x 3 min.] x 29 cycles; 72°C x 5 min., 4°C until cool (Biometra T3000 Combi Thermocycler)

[0979] The resulting PCR fragment was separated on a 0.8% E-gel (Invitrogen) for verification of successful amplification, and purified using the QIAquick PCR Purification kits (Qiagen) according to manufacturer's instructions. The resulting stock was CMP::PL.6 fkpB fragment.

Integration of CMP::PL.6 fkpB fragment PCR product into BL21/pRed-ET Strain

[0980] The pRed-ET vector (Gene Bridges kit) was transformed into BL21 (Novagen) by electroporation resulting in strain REM F7_13 (BL21/pRed-ET). The purified CMP::PL.6 fkpB PCR fragment was electroporated into REM F7_13. The transformants were recovered in L Broth

and then plated on L agar containing chloramphenicol (10ug/ml). Chloramphenicol resistant colonies were analyzed by PCR for the presence of the GB-CmpR cassette and the PL.6-promoter upstream of *fkpB* using primers 5' confirm CMP::80bp up of fkpB and bottom Pgb2 as well as 3' confirm CMP::PL.6-fkpB and top Gb's CMP. The PCR fragments from a number of transformants were sequenced using the 3' confirm CMP::PL.6-fkpB and top GB's CMP primers (Sequetech; Mountain View, CA) and PL.6 *fkpB* strain of interest identified. The chloramphenicol resistant strain, BL21 CMP::PL.6 *fkpB*, was designated REM A4_14.

Strategy for creating REM D1_14

Verification of the presence of PL.6 fkpB within REM D1_14

To verify the REM D1_14 strain harbored the PL.6 fkp locus the following PCR reaction was set up:
Approx. 0.5ul cells from a colony
5ul *HerculaseII* Buffer
0.25ul dNTP's (100 mM)
0.625ul primer (10uM) 5' primer to PL.6
0.625ul primer (10uM) 3' confirm CMP::PL.6-fkpB
17.5 ul diH2O
+ 0.5ul of *HerculaseII fusion* from Stratagene

Cycle Parameter:
95°C x 2min., [95°C x 30sec., 60°C x 30sec., 72°C x 2 min.] x 29 cycles; 72°C x 5 min., 4°C until cool (Biometra T3000 Combi Thermocycler)

[0981] The resulting PCR fragment was separated on a 2% E-gel (Invitrogen) for verification of successful amplification.

[0982] The chloramphenicol marked PL.6 *fkpB* locus of strain REM A4_14, described above, was introduced into strain WW103 via P1-mediated transduction. The resulting chloramphenicol resistant strain was named REM A9_14. After Flipase-mediated excision of the antibiotic cassette the resulting chloramphenicol sensitive strain was designated REM D1_14 (BL21 pgl^+ PL.6-dxs, GI1.6-dxr, GI1.6 yIDI, PL.2 lower MVA pathway, CMP::PL.6 fkpB). The presence of the PL.6-promoter upstream of *fkpB* within REM D1_14 was verified by PCR using primers 5' primer to PL.6 and 3' confirm CMP::PL.6-fkpB, which are described above.

Strategy for creating REM A8_15

[0983] The chloramphenicol marked *ΔiscR* locus of strain REM14::CMP, described previously, was introduced into strain WW103 via P1-mediated transduction. The resulting chloramphenicol resistant strain was named REM A5_15. After Flipase-mediated excision of the antibiotic cassette the resulting chloramphenicol sensitive strain was designated REM A8_15 (BL21 pgl^+ PL.6-dxs, GI1.6-dxr, GI1.6 yIDI, PL.2 lower MVA pathway, ΔiscR).

Strategy for creating REM A7_15

[0984] The chloramphenicol marked *ΔiscR* locus of strain REM14::CMP was introduced into strain REM D1_14 via P1-mediated transduction. The resulting chloramphenicol resistant strain was named REM A2_15. After Flipase-mediated excision of the antibiotic cassette the resulting chloramphenicol sensitive strain was designated REM A7_15 (BL21 pgl^+ PL.6-dxs, GI1.6-dxr, GI1.6 yIDI, PL.2 lower MVA pathway, CMP::PL.6 fkpB, ΔiscR).

Verification of increased accumulation of IspH within REM D1_14 and REM A7_15

Western blot method

[0985] REM D1_14, REM A7_15, REM A8_15, and WW103 cells were grown in TM3 medium (1% glucose, 0.1% yeast extract) to limiting OD and cells were harvested by centrifugation and pellets stored at -80 deg until analyzed. For analysis culture pellets were resuspended in 0.05 M sodium phosphate, 0.3 M sodium chloride, 0.02 M imidazole, pH 8 with 0.2 mg/ml DNaseI to 100 OD/ml. Cells were broken by repeated pass through the French Press. 8 ml of each lysate was then clarified by ultracentrifugation at 50,000 rpm for 30 minutes. Soluble material was removed and the insoluble pellet was resuspended in 8 ml of 0.05 M sodium phosphate, 0.3 M sodium chloride, 0.02 M imidazole, pH 8 buffer. Analysis for *E. coli* ispH expression was performed using Nitrocellulose western blot, following transfer and development techniques recommended by Invitrogen as described in iBlot® and WesternBreeze® user manuals. The western blot was probed using primary

polyclonal antibody produced against purified *E. coli* ispH in rabbit by ProSci Inc. The detection used a fluorescent secondary antibody from Invitrogen, Alexa Fluor® 488 goat anti-rabbit IgG (H+L). The raw data is shown in Figure 109. Sample quantitation was performed using ImageQuant 5.2 software and the results are presented in Figure 110.

[0986] The increased expression of *ispH* driven by the PL.6-promoter located upstream of the *fkpB-ispH* 2 gene operon of strains REM D1_14 and REM A7_15 relative to strain REM A8_15 and WW103 was indirectly assessed by measuring the level of IspH accumulation via a Western blot method (see Figure 109 and 110). An approximately 5-fold increase in soluble IspH levels was determined for the PL.6 *fkpB* harboring strains REM D1_14 and REM A7_15 relative to the REM A8_15 and WW103 strains which harbor the endogenous wild type *fkpB-ispH* locus.

Strategy for creating REM D8_15, REM D7_15, and REM D6_15

[0987] Strains WW119, REM D8_15, REM D7_15, and REM D6_15 were created by transforming pDW33 into WW103, REM D1_14, REM A8_15, and REM A7_15, respectively (strains described above).

[0988] Water-washed REM D1_14, REM A8_15, and REM A7_15 cells were transformed with pDW33 via electroporation using the BIO RAD Gene Pulser system (0.1 cm cuvette cat.# 165-2089) and a transformation protocol suggested by the manufacturer (BIO RAD). The cells were recovered in L broth for 1 hour at 37°C and then plated on L agar containing carbenicillin (50ug/ml). One carbenicillin resistant colony was chosen for each strain. The resulting carbenicillin resistant strains were named as such:

[0989] REM D8_15 (BL21 pgl^+ PL.6-dxs, GI1.6-dxr, GI1.6 yIDI, PL.2 lower MVA pathway, PL.6 fkpB, and pDW33);

[0990] REM D7_15 (BL21 pgl^+ PL.6-dxs, GI1.6-dxr, GI1.6 yIDI, PL.2 lower MVA pathway, ΔiscR, and pDW33);

[0991] REM D6_15 15 (BL21 pgl^+ PL.6-dxs, GI1.6-dxr, GI1.6 yIDI, PL.2 lower MVA pathway, PL.6 fkpB, ΔiscR, and pDW33).

Method section for isoprene production and quantitation of ispH

[0992] Isoprene measurements. Cultures to measure isoprene production were set up in a 48-deep-well plate (cat# P-5ML-48-C-S Axygen Scientific, California, USA) with each well providing a 2 mL culture. The culture medium, named TM3, is described below. The strains to be compared were grown o/n at 30 degrees at 250 rpm in TM3 medium supplemented with 1% glucose and 0.1% yeast extract. In the morning the strains were inoculated at 1:100 in quadruplicate sets of wells in the 48-deep well block. The cultures were covered with a "Breath Easier"™ membrane (Electron Microscopy Sciences Cat# 70536-10) and were continuously shaken at at 600 rpm and 30 deg C (Shel-Lab Inc. Model SI6R Refrigerated Shaking Incubator; Oregon, USA). Culture OD was determined after two hours and then at timed intervals out to 6 hours. Induction with IPTG was after two hours of growth by the addition of 50, 100, 200, and 400 uM IPTG to the quadruplicated sets of wells, one through four. At two hours post-induction and hourly thereafter out to six hours these cultures were samples for isoprene production assays as follow: A 100 uL aliquot of each culture was transferred to a 98-deep well glass block (cat# 3600600 Zinsser; North America) which was immediately sealed with an impermeable adhesive aluminum film and incubated for 30 minutes with shaking at 450 rmp on an Eppendorf thermomixer (Eppendorf; North America.). The isoprene assay cultures were killed by heating at 70 deg C for 7 min on a second Eppendorf thermomixer. The glass block was transferred to an Agilent 6890 GC attached to an Agilent 5973 MS and outfitted with a LEAP CTC CombiPAL autosampler for head space analysis. The column was an Agilent HP-5 (5% Phenyl Methyl Siloxane (15m x 0.25mm x 0.25um)). A 100 uL gas volume was injected on the column. Other conditions were as follows. Oven Temperature: 37C (held isothermal for 0.6 mins); Carrier Gas: Helium (flow - 1mL/min), split ratio of 50:1 at 250°C on the injection port; Single Ion Monitoring mode (SIM) on mass 67; Detector off: 0.00 min - 0.42 mins; Dectector on: 0.42 mins - 0.60 mins; elution time for Isoprene (2-methyl-1,3 butadiene) was ~0.49 min for a total analysis time of 0.6 mins. Calibration of the instrument was performed by methods well known to those trained in the art.

[0993] Isoprene head space measurements were normalized by culture OD_{600} to yield a measure of specific isoprene production in units of ug/L/H/OD. All reactions were followed for 4 to 8 hours. The surprising results from this experiment is that when the *ΔiscR* mutation is combined with the chromosomal mutation of PL.6 fkpB-ispH isoprene activity is restored. This result is consistent with that iscR in a background of overexpressed ispH takes on a regulatory role or at least interferes with flux through the DXP pathway. For high flux ispH needs to be overexpressed and under these condition ΔiscR expected to be beneficial for the process.

[0994] Verification of increased ispH expression level by western Blot. The substitution of the PL.6 promoter for the native promoter of the fkpB-ispH operon was expected to raise the level of ispH. This was confirmed in strain REM A7_15, REM D1_14 by comparison to control strains REM A8_15 and WW103 by western Blot with polyclonal antibody prepared against this enzyme as described; the promoter swap resulted in a 5-fold increase of soluble ispH. Cells were grown in TM3 medium (1% glucose, 0.1% yeast extract) to limiting OD and were harvested by centrifugation and the pellets were stored at -80 deg until the next day. For analysis pellets were resuspended in 0.05 M sodium phosphate, 0.3 M sodium chloride, 0.02 M imidazole, pH 8 with 0.2 mg/ml DNaseI to 100 OD/ml. Cells were broken by repeated passage through the French press. Eight ml of each lysate was clarified by ultracentrifugation at 100,000 x g for 30 minutes. Supernatant was removed and the pellet was resuspended in 8 ml of buffer pH8, 0.05 M sodium phosphate, 0.3 M sodium chloride, 0.02 M imidazole. Western blot was performed as described in the users manuals iBlot® and WesternBreeze® (in Vitrogen). The primary polyclonal antibody was against purified *E. coli* IspH overexpressed in *E. coli* and raised in rabbit by ProSci Inc (Poway, CA). For detection a fluorescent secondary antibody from Invitrogen (Alexa Fluor® 488 goat anti-rabbit IgG H+L), was used. The raw data is shown in Figure 109. Sample quantitation was performed using ImageQuant 5.2 software and the results are presented in Figure 110.

TM3 (per liter fermentation medium):

[0995] 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, yeast extract 1.0g, 1000X Modified Trace Metal stock solution 1 ml. All

of the components were added together and dissolved in Di H2O. The pH is adjusted to 6.8 with NH_4OH and the solution is filter sterilized over a 0.22 micron membrane. Glucose was typically added at 1% and yeast extract was typically boosted to 0.1%. Antibiotics were added post-sterile as needed (TM3 medium was sometimes prepared w/o any MgS04 as this Mg++ led to precipitation over time. In this case MgSO4 was added from a sterile 1M solution just prior to use).

1000X Modified Trace Metal Stock Solution (per liter):

[0996] Citric Acids * H2O 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 Di H_2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

Appendix 1

Exemplary 1-deoxy-D-xylulose-5-phosphate synthase nucleic acids and polypeptides

ATH: AT3G21500(DXPS1)
AT4G15560(CLA1) AT5G11380(DXPS3)
OSA: 4338768(Os05g0408900)
4340090(Os06g0142900)
4342614(Os07g0190000)
PPP: PHYPADRAFT_105028(DXS1)
PHYPADRAFT_137710
PHYPADRAFT_175220
PHYPADRAFT_73475
OLU: OSTLU_48774(DXS)
CRE: CHLREDRAFT_196568(DXS1)
CME: CMF089C
PFA: MAL13P1.186
PFD: PFDG_00954
PFH: PFHG_02940
PYO: PY04970
TAN: TA20470
TPV: TP01_0516
ECO: b0420(dxs)
ECJ: JW0410(dxs)
ECD: ECDH10B_0376(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(dxs)
ECM: EcSMS35_0456(dxs)
ECL: EcolC_3213
STY: STY0461(dxs)
STT: t2441(dxs)
SPT: SPA2301(dxs)
SPQ: SPAB_03161
SEC: SC0463(dxs)
SEH: SeHA_C0524(dxs)
SEE: SNSL254_A0469(dxs)
SEW: SeSA_A0482(dxs)
SES: SARI_02505
STM: STM0422(dxs)
YPE: YPO3177(dxs)
YPK: y1008(dxs)
YPM: YP_0754(dxs)
YPA: YPA_2671
YPN: YPN_0911
YPP: YPDSF_2812
YPG: YpAngola_A3074(dxs)
YPS: YPTB0939(dxs)
YPI: YpsIP31758_3112(dxs)
YPY: YPK_3253
YPB: YPTS_0980
YEN: YE3155(b0420)
SFL: SF0357(dxs)
SFX: S0365(dxs)
SFV: SFV_0385(dxs)
SSN: SSON_0397(dxs)
SBO: SBO_0314(dxs)
SBC: SbBS512_E0341(dxs)
SDY: SDY_0310(dxs)
ECA: ECA1131(dxs)
ETA: ETA_25270(dxs)
PLU: plu3887(dxs)
BUC: BU464(dxs)
BAS: BUsg448(dxs)
WBR: WGLp144(dxs)
SGL: SG0656
ENT: Ent638_0887
ESA: ESA_02882
KPN: KPN_00372(dxs)
CKO: CKO_02741
SPE: Spro_1078
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)
HSM: HSM_1383
PMU: PM0532(dxs)
MSU: MS1059(dxs)
APL: APL_0207(dxs)
APJ: APJL_0208(dxs)
APA: APP7_0210
ASU: Asuc_1372
XFA: XF2249
XFT: PD1293(dxs)
XFM: Xfasm12_1447
XFN: XfasM23_1378
XCC: XCC2434(dxs)
XCB: XC_1678
XCV: XCV2764(dxs)
XAC: XAC2565(dxs)
XOO: XOO2017(dxs)
XOM: XOO_1900(XOO1900)
SML: Smlt3355(dxs)
SMT: Smal_2779
VCH: VC0889
VCO: VC0395_A0412(dxs)
VVU: VV1_0315
VVY: VV0868
VPA: VP0686
VFI: VF0711
VHA: VIBHAR_01173
PPR: PBPRA0805
PAE: PA4044(dxs)
PAU: PA14_11550(dxs)
PAP: PSPA7_1057(dxs)
PPU: PP_0527(dxs)
PPF: Pput_0561
PPG: PputGB1_0572
PPW: PputW619_0579
PST: PSPTO_0698(dxs)
PSB: Psyr_0604
PSP: PSPPH_0599(dxs)
PFL: PFL_5510(dxs)
PFO: PflO1_5007
PEN: PSEEN0600(dxs)
PMY: Pmen_3844
PSA: PST_3706(dxs)
CJA: CJA_3336(dxs)
PAR: Psyc_0221(dxs)
PCR: Pcryo_0245
PRW: PsycPRwf_0411
ACI: ACIAD3247(dxs)
ACB: A1S_3106
ABM: ABSDF0389(dxs)
ABY: ABAYE0381
ABC: ACICU_03307
SON: SO_1525(dxs)
SDN: Sden_2571
SFR: Sfri_2790
SAZ: Sama_2436
SBL: Sbal_1357
SBM: Shew185_1343
SBN: Sbal195_1382
SLO: Shew_2771
SPC: Sputcn32_1275
SSE: Ssed_3329
SPL: Spea_2991
SHE: Shewmr4_2731
SHM: Shewmr7_2804
SHN: Shewana3_2901
SHW: Sputw3181_2831
SHL: Shal_3080
SWD: Swoo_3478
ILO: IL2138(dxs)
CPS: CPS_1088(dxs)
PHA: PSHAa2366(dxs)
PAT: Patl_1319
SDE: Sde_3381
MAQ: Maqu_2438
AMC: MADE_01425
PIN: Ping_2240
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)

FTM: FTM_0932(dxs)
FPH: Fphi_1718
NOC: Noc_1743
AEH: Mlg_1381
HHA: Hhal_0983
HCH: HCH_05866(dxs)
CSA: Csal_0099
ABO: ABO_2166(dxs)
MMW: Mmwyl1_1145
AHA: AHA_3321(dxs)
ASA: ASA_0990(dxs)
BCI: BCI_0275(dxs)
RMA: Rmag_0386
VOK: COSY_0360(dxs)
NME: NMB1867(dxs)
NMA: NMA0589(dxs)
NMC: NMC0352(dxs)
NMN: NMCC_0354
NGO: NGO0036
NGK: NGK_0044
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: BMA10229_1706(dxs)
BMN: BMA10247_A0364(dxs)
BXE: Bxe_B2827
BVI: Bcep1808_4257
BUR: Bcep18194_B2211
BCN: Bcen_4486
BCH: Bcen2424_3879
BCM: Bcenmc03_3648
BAM: Bamb_3250
BAC: BamMC406_3776
BMU: Bmul_4820
BMJ: BMULJ_03696(dxs)
BPS: BPSS1762(dxs)
BPM: BURPS1710b_A0842(dxs)
BPL: BURPS1106A_A2392(dxs)
BPD: BURPS668_A2534(dxs)
BTE: BTH_II0614(dxs)
BPH: Bphy_3948
PNU: Pnuc_1704
PNE: Pnec_1422
BPE: BP2798(dxs)
BPA: BPP2464(dxs)
BBR: BB1912(dxs)
BPT: Bpet3060(dxs)
BAV: BAV2177(dxs)
RFR: Rfer_2875
POL: Bpro_1747
PNA: Pnap_1501
AAV: Aave_2015
AJS: Ajs_1038
VEI: Veis_3283
DAC: Daci_2242
MPT: Mpe_A2631
HAR: HEAR0279(dxs)
MMS: mma_0331
LCH: Lcho_3373
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
HPJ: jhp0328(dxs)
HPA: HPAG1_0349
HPS: HPSH_01830
HHE: HH0608(dxs)
HAC: Hac_0968(dxs)
WSU: WS1996
TDN: Suden_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)
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
GUR: Gura_1018 Gura_2175
GLO: Glov_2182 Glov_2235
PCA: Pcar_1667(dxs)
PPD: Ppro_1191 Ppro_2403
DVU: DVU1350(dxs)
DVL: Dvul_1718
DDE: Dde_2200
LIP: LI0408(dsx)
DPS: DP2700
DOL: Dole_1662
ADE: Adeh_1097
AFW: Anae109_1136
MXA: MXAN_4643(dxs)
SAT: SYN_02456
SFU: Sfum_1418
PUB: SAR11_0611(dxs)
MLO: mlr7474
MES: Meso_0735
PLA: Plav_0781
SME: SMc00972(dxs)
SMD: Smed_0492
ATU: Atu0745(dxs)
ATC: AGR_C_1351
RET: RHE_CH00913(dxs)
REC: RHECIAT_CH0001005(dxs)
RLE: RL0973(dxs)
BME: BMEI1498
BMF: BAB1_0462(dxs)
BMB: BruAb1_0458(dxs)
BMC: BAbS19_I04270
BMS: BR0436(dxs)
BMT: BSUIS_A0462(dxs)
BOV: BOV_0443(dxs)
BCS: BCAN_A0440(dxs)
OAN: Oant_0547
BJA: bll2651(dxs)
BRA: BRADO2161(dxs)
BBT: BBta_2479(dxs)
RPA: RPA0952(dxs)
RPB: RPB_4460
RPC: RPC_1149
RPD: RPD_4305
RPE: RPE_1067
RPT: Rpal_1022
NWI: Nwi_0633
NHA: Nham_0778
BHE: BH04350(dxs)
BQU: BQ03540(dxs)
BBK: BARBAKC583_0400(dxs)
BTR: Btr_0649
XAU: Xaut_4733
AZC: AZC_3111
MEX: Mext_1939 Mext_4309
MRD: Mrad2831_3459 Mrad2831_3992
MET: M446_6352 M446_6391
BID: Bind_1811
CCR: CC_2068
CAK: Caul_3314
SIL: SPO0247(dxs)
SIT: TM1040_2920
RSP: RSP_0254(dxsA) RSP_1134(dxs)
RSH: Rsph17029_1897 Rsph17029_2795
RSQ: Rsph17025_2027 Rsph17025_2792
JAN: Jann_0088 Jann_0170
RDE: RD1_0101(dxs) RD1_0548(dxs)
PDE: Pden_0400
DSH: Dshi_3294 Dshi_3526
MMR: Mmar10_0849
HNE: HNE_1838(dxs)
ZMO: ZMO1234(dxs) ZMO1598(dxs)
NAR: Saro_0161
SAL: Sala_2354
SWI: Swit_1461
ELI: ELI_12520
GOX: GOX0252
GBE: GbCGDNIH1_0221
GbCGDNIH1_2404
ACR: Acry_1833
GDI: GDI1860(dxs)
RRU: Rru_A0054 Rru_A2619
MAG: amb2904
MGM: Mmc1_1048
SUS: Acid_1783

SWO: Swol_0582
CSC: Csac_1853
BSU: BSU24270(dxs)
BHA: BH2779
BAN: BA4400(dxs)
BAR: GBAA4400(dxs)
BAA: BA_4853
BAT: BAS4081
BCE: BC4176
BCA: BCE_4249(dxs)
BCZ: BCZK3930(dxs)
BCY: Bcer98_2870
BTK: BT9727_3919(dxs)
BTL: BALH_3785(dxs)
BWE: BcerKBAB4_4029
BLI: BL01523(dxs)
BLD: BLi02598(dxs)
BCL: ABC2462(dxs)
BAY: RBAM_022600
BPU: BPUM_2159
GKA: GK2392
GTN: GTNG_2322
LSP: Bsph_3509
ESI: Exig_0908
LMO: lmo1365(tktB)
LMF: LMOf2365_1382(dxs)
LIN: lin1402
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
LRE: Lreu_0958
LRF: LAR_0902
LFE: LAF_1005
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)
CBL: CLK_1271(dxs)
CBK: CLL_A1441 CLL_A2401(dxs)
CBB: CLD_2756(dxs)
CBF: CLI_1945(dxs)
CBE: Cbei_1706
CKL: CKL_1231(dxs)
CPY: Cphy_2511
AMT: Amet_2508
AOE: Clos_1607
CHY: CHY_1985(dxs)
DSY: DSY2348
DRM: Dred_1078
PTH: PTH_1196(dxs)
DAU: Daud_1027
HMO: HM1_0295(dxs)
TTE: TTE1298(dxs)
TEX: Teth514_1540
TPD: Teth39_1103
MTA: Moth_1511
MPE: MYPE730
MGA: MGA_1268(dxs)
MTU: Rv2682c(dxs1) Rv3379c(dxs2)
MTC: MT2756(dxs)
MRA: MRA_2710(dxs1) MRA_3419(dxs2)
MTF: TBFG_12697 TBFG_13415
MBO: Mb2701c(dxs1) Mb3413c(dxs2)
MBB: BCG_2695c(dxs1) BCG_3450c(dxs2)
MLE: ML1038(dxs)
MPA: MAP2803c(dxs)
MAV: MAV_3577(dxs)
MSM: MSMEG_2776(dxs)
MUL: MUL_3319(dxs1)
MVA: Mvan_2477
MGI: Mflv_3923
MAB: MAB_2990c
MMC: Mmcs_2208

MKM: Mkms_2254
MJL: Mjls_2197
MMI: MMAR_0276(dxs2)
MMAR_2032(dxs1)
CGL: NCgl1827(cgl1902)
CGB: cg2083(dxs)
CGT: cgR_1731
CEF: CE1796
CDI: DIP1397(dxs)
CJK: jk1078(dxs)
CUR: cu0909
NFA: nfa37410(dxs)
RHA: RHA1_ro06843
SCO: SCO6013(SC1C3.01)
SCO6768(SC6A5.17)
SMA: SAV1646(dxs1) SAV2244(dxs2)
SGR: SGR_1495(dxs)
TWH: TWT484
TWS: TW280(Dxs)
LXX: Lxx10450(dxs)
CMI: CMM_1660(dxsA)
AAU: AAur_1790(dxs)
RSA: RSal33209_2392
KRH: KRH_14140(dxs)
PAC: PPA1062
NCA: Noca_2859
TFU: Tfu_1917
FRA: Francci3_1326
FRE: Franean1_5184
FAL: FRAAL2088(dxs)
ACE: Acel_1393
KRA: Krad_1452 Krad_1578
SEN: SACE_1815(dxs)
STP: Strop_1489
SAQ: Sare_1454
BLO: BL1132(dxs)
BLJ: BLD_0889(dxs)
BAD: BAD_0513(dxs)
FNU: FN1208 FN1464
RBA: RB2143(dxs)
OTE: Oter_2780
MIN: Minf_1537(dxs)
AMU: Amuc_0315
CTR: CT331(dxs)
CTA: CTA_0359(dxs)
CTB: CTL0585
CTL: CTLon_0582(dxs)
CMU: TC0608(dxs)
CPN: CPn1060(tktB_2)
CPA: CP0790
CPJ: CPj1060(tktB_2)
CPT: CpB1102
CCA: CCA00304(dxs)
CAB: CAB301(dxs)
CFE: CF0699(dxs)
PCU: pc0619(dxs)
TPA: TP0824
TPP: TPASS_0824(dxs)
TDE: TDE1910(dxs)
LIL: LA3285(dxs)
LIC: LIC10863(dxs)
LBJ: LBJ_0917(dxs)
LBL: LBL_0932(dxs)
LBI: LEPBI_I2605(dxs)
LBF: LBF_2525(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)
SYP: SYNPCC7002_A1172(dxs)
CYA: CYA_1701(dxs)
CYB: CYB_1983(dxs)
TEL: tll0623
MAR: MAE_62650
CYT: cce_1401(dxs)
GVI: gll0194
ANA: alr0599
NPU: Npun_F5466
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
AMR: AM1_5186(dxs)
BTH: BT_1403 BT_4099
BFR: BF0873 BF4306
BFS: BF0796(dxs) BF4114
BVU: BVU_1763 BVU_3090
PGI: PG2217(dxs)
PGN: PGN_2081
PDI: BDI_2664
CHU: CHU_3643(dxs)
GFO: GFO_3470(dxs)
FJO: Fjoh_1523
FPS: FP0279(dxs)
CTE: CT0337(dxs)
CPC: Cpar_1696
CPH: Cpha266_0671
CPB: Cphamn1_1826
PVI: Cvib_0498
PLT: Plut_0450
PPH: Ppha_2222
CTS: Ctha_0174
PAA: Paes_1686
DET: DET0745(dxs)
DEH: cbdb_A720(dxs)
DEB: DehaBAV1_0675
EMI: Emin_0268
DRA: DR_1475
DGE: Dgeo_0994
TTH: TTC1614
TTJ: TTHA0006
AAE: aq_881
HYA: HY04AAS1_1061
SUL: SYO3AOP1_0652
TMA: TM1770
TPT: Tpet_1058
TLE: Tlet_2013
TRQ: TRQ2_1054
TME: Tmel_0252
FNO: Fnod_1517
PMO: Pmob_1001

Exemplary 1-deoxy-D-xylulose-5-phosphate reductoisomerase nucleic acids and polypeptides

ATH: AT5G62790(DXR)
OSA: 4326153(Os01g0106900)
PPP: PHYPADRAFT_127023
PHYPADRAFT_128953
OLU: OSTLU_31255(DXR)
CRE: CHLREDRAFT_196606(DXR1)
CME: CMG148C
PFA: PF14_0641
PFD: PFDG_00980
PYO: PY05578
TAN: TA14290
TPV: TP02_0073
ECO: b0173(dxr)
ECJ: JW0168(dxr)
ECD: ECDH10B_0153(dxr)
ECE: Z0184(yaeM)
ECS: ECs0175
ECI: UTI89_C0188(dxr)
ECP: ECP_0181
ECV: APECO1_1814(dxr)
ECW: EcE24377A_0177(dxr)
ECX: EcHS_A0175(dxr)
ECM: EcSMS35_0184(dxr)
ECL: EcolC_3487
STY: STY0243(dxr)
STT: t0221(dxr)
SPT: SPA0227(dxr)
SPQ: SPAB_00282
SEC: SC0220(dxr)
SEH: SeHA_C0258(dxr)
SEE: SNSL254_A0242(dxr)
SEW: SeSA_A0245(dxr)
SES: SARI_02782
STM: STM0220(dxr)
YPE: YPO1048(dxr)
YPK: y3131
YPM: YP_2802(dxr)
YPA: YPA_0524
YPN: YPN_2952
YPP: YPDSF_1664
YPG: YpAngola_A3431(dxr)
YPS: YPTB2999(dxr)
YPI: YpsIP31758_1017(dxr)
YPY: YPK_1070
YPB: YPTS_3119
YEN: YE3280(b0173)
SFL: SF0163(yaeM)
SFX: S0166(yaeM)
SFV: SFV_0156(yaeM)
SSN: SSON_0185(yaeM)
SBO: SBO_0161(yaeM)
SBC: SbBS512_E0166(dxr)
SDY: SDY_0189(yaeM)
ECA: ECA1035(dxr)
ETA: ETA_08940(dxr)
PLU: plu0676(dxr)
BUC: BU235(dxr)
BAS: BUsg229(dxr)
WBR: WGLp388(yaeM)
SGL: SG1939
ENT: Ent638_0711
ESA: ESA_03169
KPN: KPN_00186(ispC)
CKO: CKO_03194
SPE: Spro_3786
BFL: Bfl275(dxr)
BPN: BPEN_283(dxr)
HIN: HI0807
HIT: NTHI0971(dxr)
HIP: CGSHiEE_08025
HIQ: CGSHiGG_07530
HDU: HD1186(dxr)
HSO: HS_0985(dxr)
HSM: HSM_1463
PMU: PM1988(dxr)
MSU: MS1928(dxr)
APL: APL_0406(dxr)
APJ: APJL_0428(dxr)
APA: APP7_0430
ASU: Asuc_0657
XFA: XF1048
XFT: PD0328(dxr)

XFM: Xfasm12_0359
XFN: XfasM23_0324
XCC: XCC1367(dxr)
XCB: XC_2871
XCV: XCV1472(dxr)
XAC: XAC1415(dxr)
XOO: XOO1970(dxr)
XOM: XOO_1860(XOO1860)
SML: Smlt1500(dxr)
SMT: Smal_1259
VCH: VC2254
VCO: VC0395_A1845(dxr)
VVU: VV1_1866
VVY: VV2551
VPA: VP2312
VFI: VF1956
VHA: VIBHAR_03231
PPR: PBPRA2962
PAE: PA3650(dxr)
PAU: PA14_17130(dxr)
PAP: PSPA7_1489(dxr)
PPU: PP_1597(dxr)
PPF: Pput_4180
PPG: PputGB1_1152
PPW: PputW619_4076
PST: PSPTO_1540(dxr)
PSB: Psyr_1349
PSP: PSPPH_3834(dxr)
PFL: PFL_1182(dxr)
PFO: PflO1_1107
PEN: PSEEN4214(dxr)
PMY: Pmen_3047
PSA: PST_1543(dxr)
CJA: CJA_1118(dxr)
PAR: Psyc_1531(dxr)
PCR: Pcryo_1710
PRW: PsycPRwf_1798
ACI: ACIAD1376(dxr)
ACB: A1S_1971
ABM: ABSDF1684(dxr)
ABY: ABAYE1581
ABC: ACICU_02094
SON: SO_1635(dxr)
SDN: Sden_1560
SFR: Sfri_1276
SAZ: Sama_1145
SBL: Sbal_1456
SBM: Shew185_1451
SBN: Sbal195_1487
SLO: Shew_2629
SPC: Sputcn32_1354
SSE: Ssed_3155
SPL: Spea_2879
SHE: Shewmr4_2635
SHM: Shewmr7_2702
SHN: Shewana3_2809
SHW: Sputw3181_2749
SHL: Shal_2975
SWD: Swoo_3275
ILO: IL0839
CPS: CPS_1559(dxr)
PHA: PSHAa2030(dxr)
PAT: Patl_1255
SDE: Sde_2591
MAQ: Maqu_2542
AMC: MADE_01379
PIN: Ping_2970
MCA: MCA0573(dxr)
FTU: FTT1574c(dxr)
FTF: FTF1574c(dxr)
FTW: FTW_0352(dxr)
FTL: FTL_0534
FTH: FTH_0536(dxr)
FTA: FTA_0567(dxr)
FTN: FTN_1483(dxr)
FTM: FTM_0324(dxr)
FPH: Fphi_1195
NOC: Noc_0814
AEH: Mlg_1857
HHA: Hhal_1460
HCH: HCH_05246(dxr)
CSA: Csal_0569
ABO: ABO_1149(dxr)
MMW: Mmwyl1_1278
AHA: AHA_1179(dxr)
ASA: ASA_3154(dxr)
BCI: BCI_0531(dxr)
RMA: Rmag_0025

VOK: COSY_0025(dxr)
NME: NMB0184(dxr)
NMA: NMA0083(dxr)
NMC: NMC0175(dxr)
NMN: NMCC_1968
NGO: NGO1799
NGK: NGK_2475
CVI: CV_2202(dxr)
RSO: RSc1410(dxr)
REU: Reut_A1875
REH: H16_A2049(dxp)
RME: Rmet_1441
BMA: BMA1549(dxr)
BMV: BMASAVP1_A2050(dxr)
BML: BMA10229_A3261(dxr)
BMN: BMA10247_1322(dxr)
BXE: Bxe_A1688
BVI: Bcep1808_1919
BUR: Bcep18194_A5323
BCN: Bcen_6064
BCH: Bcen2424_2013
BCM: Bcenmc03_2033
BAM: Bamb_2046
BAC: BamMC406_1915
BMU: Bmul_1263
BMJ: BMULJ_01984(dxr)
BPS: BPSL2153(dxr)
BPM: BURPS1710b_2577(dxr)
BPL: BURPS1106A_2487(dxr)
BPD: BURPS668_2431(dxr)
BTE: BTH_I2033(dxr)
BPH: Bphy_1332
PNU: Pnuc_1445
PNE: Pnec_0513
BPE: BP1425(dxr)
BPA: BPP1533(dxr)
BBR: BB2611(dxr)
BPT: Bpet2529(dxr)
BAV: BAV1740(dxr)
RFR: Rfer_1994
POL: Bpro_2689
PNA: Pnap_1764
AAV: Aave_1829
AJS: Ajs_2579
VEI: Veis_1444
DAC: Daci_4942
MPT: Mpe_A1973
HAR: HEAR1341(dxr)
MMS: mma_2052
LCH: Lcho_2844
NEU: NE1712(dxr)
NET: Neut_2029
NMU: Nmul_A0663
EBA: ebA5994(dxr)
AZO: azo1903(dxr)
DAR: Daro_1748
TBD: Tbd_0791
MFA: Mfla_1524
HPY: HP0216
HPJ: jhp0202
HPA: HPAG1_0217
HPS: HPSH_01115
HHE: HH0524(dxr)
HAC: Hac_1502(dxr_fragment_2)
Hac_1503(dxr_fragment_1)
WSU: WS0812
TDN: Suden_0126
CJE: Cj1346c(dxr)
CJR: CJE1535(dxr)
CJJ: CJJ81176_1345(dxr)
CJU: C8J_1262(dxr)
CJD: JJD26997_0364(dxr)
CFF: CFF8240_0210(dxr)
CCV: CCV52592_0594(dxr)
CHA: CHAB381_0121(dxr)
CCO: CCC13826_0420(dxr)
ABU: Abu_0161(dxr)
NIS: NIS_1666(ispC)
SUN: SUN_0144
GSU: GSU1915(dxr)
GME: Gmet_1256
GUR: Gura_3727
GLO: Glov_2714
PCA: Pcar_1915(dxr)
PPD: Ppro_2050
DVU: DVU0866(dxr)
DVL: Dvul_2116
DDE: Dde_1123

LIP: LI0386(dxr)
DPS: DP1160
DOL: Dole_0480
ADE: Adeh_3583
AFW: Anae109_3704
SAT: SYN_00916
SFU: Sfum_1784
WOL: WD0992(dxr)
WBM: Wbm0179
WPI: WP0113(dxr)
AMA: AM743(dxr)
APH: APH_0440(dxr)
ERU: Erum4750(dxr)
ERW: ERWE_CDS_04970(dxr)
ERG: ERGA_CDS_04870(dxr)
ECN: Ecaj_0473
ECH: ECH_0557(dxr)
NSE: NSE_0443(dxr)
PUB: SAR11_0912(yaeM)
PLA: Plav_3190
SME: SMc03105(dxr)
SMD: Smed_2879
ATU: Atu2612(dxr)
ATC: AGR_C_4736
RET: RHE_CH03839(dxr)
REC: RHECIAT_CH0004120(dxr)
RLE: RL4372(dxr)
BJA: bll4855(dxr)
BRA: BRADO4134(dxr)
BBT: BBta_4511(dxr)
RPA: RPA2916(dxr)
RPB: RPB_2822
RPC: RPC_2442
RPD: RPD_2851
RPE: RPE_2559
RPT: Rpal_3262
NWI: Nwi_1853
NHA: Nham_1700
XAU: Xaut_4433
AZC: AZC_1699
MEX: Mext_2083
MRD: Mrad2831_3444
MET: M446_0636
BID: Bind_0297
CCR: CC_1917
CAK: Caul_2799
SIL: SPO1667(dxr)
SIT: TM1040_1410
RSP: RSP_2709(dxr)
RSH: Rsph17029_1366
RSQ: Rsph17025_2149
JAN: Jann_2455
RDE: RD1_2590(dxr)
PDE: Pden_3997
DSH: Dshi_1497
MMR: Mmar10_1386
HNE: HNE_1774(dxr)
ZMO: ZMO1150(dxr)
NAR: Saro_1375
SAL: Sala_1954
SWI: Swit_0466
ELI: ELI_03805
GOX: GOX1816
GBE: GbCGDNIH1_0938
ACR: Acry_2557
GDI: GDI2147(dxr)
RRU: Rru_A1592
MAG: amb2492
MGM: Mmc1_1846
ABA: Acid345_1419
SUS: Acid_7136
SWO: Swol_0889
CSC: Csac_2353
BSU: BSU16550(dxr)
BHA: BH2421
BAN: BA3409(dxr-1) BA3959(dxr-2)
BAR: GBAA3409(dxr-1) GBAA3959(dxr-2)
BAA: BA_4429
BAT: BAS3160 BAS3672
BCE: BC3341 BC3819
BCA: BCE_3862(dxr)
BCZ: BCZK3054(dxr) BCZK3580(dxr)
BCY: Bcer98_2128 Bcer98_2473
BTK: BT9727_3144(dxr) BT9727_3562(dxr)
BTL: BALH_3451
BWE: BcerKBAB4_3082 BcerKBAB4_3644
BLI: BL01237(dxr)
BLD: BLi01876(dxr)

BCL: ABC2236(dxr)
BAY: RBAM_016390
BPU: BPUM_1554
GKA: GK1255
GTN: GTNG_1109
LSP: Bsph_1590
ESI: Exig_1845
LMO: lmo1317
LMF: LMOf2365_1334(dxr)
LIN: lin1354
LWE: lwe1332(dxr)
STH: STH1499(dxr)
CAC: CAC1795
CPE: CPE1694
CPF: CPF_1948(dxr)
CPR: CPR_1666(dxr)
CTC: CTC01268
CNO: NT01CX_2143
CTH: Cthe_0999
CDF: CD2130(dxr)
CBO: CBO2426
CBA: CLB_2290(dxr)
CBH: CLC_2273(dxr)
CBL: CLK_1802(dxr)
CBK: CLL_A1265(dxr)
CBB: CLD_2214(dxr)
CBF: CLI_2482(dxr)
CBE: Cbei_1195
CKL: CKL_1423(dxr)
CPY: Cphy_2622
AMT: Amet_2682
AOE: Clos_1519
CHY: CHY_1778(dxr)
DSY: DSY2539
DRM: Dred_1970
PTH: PTH_1260(dxr)
DAU: Daud_0615
HMO: HM1_2264(dxr)
TTE: TTE1402(dxr)
TEX: Teth514_1654
TPD: Teth39_1218
MTA: Moth_1041
MPE: MYPE1470
MGA: MGA_0787(dxr)
MTU: Rv2870c(dxr)
MTC: MT2938(dxr)
MRA: MRA_2895(dxr)
MTF: TBFG_12886
MBO: Mb2895c(dxr)
MBB: BCG_2892c(dxr)
MLE: ML1583
MPA: MAP2940c
MAV: MAV_3727(dxr)
MSM: MSMEG_2578(dxr)
MUL: MUL_2085(dxr)
MVA: Mvan_2260
MGI: Mflv_4083
MAB: MAB_3171c
MMC: Mmcs_2042
MKM: Mkms_2088
MJL: Mjls_2025
MMI: MMAR_1836(dxr)
CGL: NCgl1940(cgl2016)
CGB: cg2208(dxr)
CGT: cgR_1844
CEF: CE1905
CDI: DIP1500(dxr)
CJK: jk1167(ispC)
CUR: cu0831
NFA: nfa41200(dxr)
RHA: RHA1_ro06588(dxr)
SCO: SCO5694(dxr)
SMA: SAV2563(dxr)
SGR: SGR_1823
TWH: TWT089(dxr)
TWS: TW099(dxr)
LXX: Lxx12180(dxr)
CMI: CMM_2160(dxrA)
ART: Arth_1399
AAU: AAur_1543(dxr)
RSA: RSal33209_0635
KRH: KRH_16160(dxr)
PAC: PPA1510
NCA: Noca_3204
TFU: Tfu_0747
FRA: Francci3_3575
FRE: Franean1_1168
FAL: FRAAL5774(dxr)

ACE: Acel_1524
KRA: Krad_1427 Krad_4655
SEN: SACE_5994(dxr)
STP: Strop_1350
SAQ: Sare_1302
BLO: BL0097(ispC)
BLJ: BLD_0115(dxr)
BAD: BAD_1158(ispC)
RXY: Rxyl_1404
FNU: FN1324
RBA: RB5568(dxr)
OTE: Oter_4632
MIN: Minf_1972(dxr)
AMU: Amuc_1737
CTR: CT071(yaeM)
CTA: CTA_0076(dxr)
CTB: CTL0327
CTL: CTLon_0322(dxr)
CMU: TC0343(dxr)
CPN: CPn0345(yaeM)
CPA: CP0415
CPJ: CPj0344(yaeM)
CPT: CpB0352
CCA: CCA00441(dxr)
CAB: CAB427(dxr)
CFE: CF0566(yaeM)
PCU: pc0260(dxr)
TPA: TP0601
TPP: TPASS_0601(dxr)
TDE: TDE2342(dxr)
LIL: LA3292(dxr)
LIC: LIC10856(dxr)
LBJ: LBJ_0910(dxr)
LBL: LBL_0925(dxr)
LBI: LEPBI_I2611(dxr)
LBF: LBF_2531(dxr)
SYN: sll0019(dxr)
SYW: SYNW0698(dxr)
SYC: syc2498_d(dxr)
SYF: Synpcc7942_1513
SYD: Syncc9605_1970
SYE: Syncc9902_0689
SYG: sync_0920(dxr)
SYR: SynRCC307_1674(dxr)
SYX: SynWH7803_1622(dxr)
SYP: SYNPCC7002_A0818(dxr)
CYA: CYA_0193(dxr)
CYB: CYB_1233(dxr)
TEL: tlr1040
MAR: MAE_50310
CYT: cce_2124(dxr)
GVI: gll2252
ANA: alr4351
NPU: Npun_R5970
AVA: Ava_1300
PMA: Pro1236(dxr)
PMM: PMM1142(dxr)
PMT: PMT1161(dxr)
PMN: PMN2A_0751
PMI: PMT9312_1238
PMB: A9601_13171(dxr)
PMC: P9515_13061(dxr)
PMF: P9303_08651(dxr)
PMG: P9301_13311(dxr)
PMH: P9215_13461
PMJ: P9211_12161
PME: NATL1_15911(dxr)
TER: Tery_0416
AMR: AM1_0563(dxr)
BTH: BT_2002
BFR: BF3699
BFS: BF3492
BVU: BVU_1651
PGI: PG1364(dxr)
PGN: PGN_1151
PDI: BDI_0480
SRU: SRU_1849(dxr)
CHU: CHU_2996(dxr)
CTE: CT0125(dxr)
CPC: Cpar_0071
CCH: Cag_0008
CPH: Cpha266_2680
CPB: Cphamn1_0098
PVI: Cvib_0138
PLT: Plut_0077
PPH: Ppha_0080
CTS: Ctha_1044
PAA: Paes_0121

DET: DET0371(dxr)
DEH: cbdb_A314(dxr)
DEB: DehaBAV1_0353
EMI: Emin_0690
DRA: DR_1508
DGE: Dgeo_1044
TTH: TTC0504
TTJ: TTHA0856
AAE: aq_404
HYA: HY04AAS1_0095
SUL: SYO3AOP1_0479
TMA: TM0889
TPT: Tpet_0038
TLE: Tlet_0658
TRQ: TRQ2_0038
TME: Tmel_0037
FNO: Fnod_0950
PMO: Pmob_1939

Exemplary 4-diphosphocytidyl-2C-methyl-D-erythritol synthase nucleic acids and polypeptides

ATH: AT2G02500(ISPD)
OSA: 4324893(Os01g0887100)
OLU: OSTLU_24843(CMS)
CRE: CHLREDRAFT_196604(CMS)
CME: CMH115C
TAN: TA02505
TPV: TP03_0057
ECO: b2747(ispD)
ECJ: JW2717(ispD)
ECD: ECDH10B_2915(ispD)
ECE: Z4055(ispD)
ECS: ECs3601(ispD)
ECC: c3314(ispD)
ECI: UTI89_C3118(ispD)
ECP: ECP_2729(ispD)
ECV: APECO1_3776(ispD)
ECW: EcE24377A_3048(ispD)
ECX: EcHS_A2885(ispD)
ECM: EcSMS35_2872(ispD)
ECL: EcolC_0965
STY: STY3055(ispD)
STT: t2831(ispD)
SPT: SPA2786(ispD)
SPQ: SPAB_03644
SEC: SC2862(ispD)
SEH: SeHA_C3120(ispD)
SEE: SNSL254_A3136(ispD)
SEW: SeSA_A3081(ispD)
SES: SARI_00026
STM: STM2930(ispD)
YPE: YPO3361(ispD)
YPK: y0828(ispD)
YPM: YP_0326(ispD)
YPA: YPA_2782(ispD)
YPN: YPN_0732(ispD)
YPP: YPDSF_2999(ispD)
YPG: YpAngola_A0964(ispD)
YPS: YPTB0770(ispD)
YPI: YpsIP31758_3299(ispD)
YPY: YPK_3431
YPB: YPTS_0804
YEN: YE0769(ispD)
SFL: SF2770(ispD)
SFX: S2963(ispD)
SFV: SFV_2751(ispD)
SSN: SSON_2895(ispD)
SBO: SBO_2773(ispD)
SBC: SbBS512_E3127(ispD)
SDY: SDY_2946(ispD)
ECA: ECA3535(ispD)
ETA: ETA_27010(ispD)
PLU: plu0713(ispD)
BUC: BU420(ygbP)
BAS: BUsg405(ygbP)
WBR: WGLp532(ygbP)
SGL: SG0526
ENT: Ent638_3218(ispD)
ESA: ESA_00544
KPN: KPN_03109(ispD)
CKO: CKO_04108
SPE: Spro_0826
BPN: BPEN_171(ispD)
HIN: HI0672(ispD)
HIT: NTHI0794(ispD)
HIP: CGSHiEE_08815(ispD)
HIQ: CGSHiGG_06635(ispD)
HDU: HD1329(ispD)
HSO: HS_1496(ispD)
HSM: HSM_0505
PMU: PM1608(ispD)
MSU: MS2275(ispD)
APL: APL_0802(ispD)
APJ: APJL_0807(ispD)
APA: APP7_0861
ASU: Asuc_2032
XFA: XF1293(ispD)
XFT: PD0545(ispD)
XFM: Xfasm12_0618

XFN: XfasM23_0570
XCC: XCC1702(ispD)
XCB: XC_2529(ispD)
XCV: XCV1754(ispD)
XAC: XAC1721(ispD)
XOO: XOO2961(ispD)
XOM: XOO_2812(ispD)
SML: Smlt1717(ispD)
SMT: Smal_1454
VCH: VC0528(ispD)
VCO: VC0395_A0056(ispD)
VVU: VV1_1582(ispD)
VVY: VV2816(ispD)
VPA: VP1320 VP2559(ispD)
VFI: VF2073(ispD)
VHA: VIBHAR_03523
PPR: PBPRA3077
PAE: PA3633(ispD)
PAU: PA14_17340(ispD)
PAP: PSPA7_1506(ispD)
PPU: PP_1614(ispD)
PPF: Pput_4163(ispD)
PPG: PputGB1_1168
PPW: PputW619_4061
PST: PSPTO_1556(ispD)
PSB: Psyr_1365(ispD)
PSP: PSPPH_3818(ispD)
PFL: PFL_1198(ispD)
PFO: Pfl01_1123(ispD)
PEN: PSEEN4198(ispD)
PMY: Pmen_3031(ispD)
PSA: PST_1559(ispD)
CJA: CJA_2223(ispD)
PAR: Psyc_1634
PCR: Pcryo_1868
PRW: PsycPRwf_1662
ACI: ACIAD1999(ispD)
ACB: A1S_1895
ABM: ABSDF2025(ispD)
ABY: ABAYE1672
ABC: ACICU_02004
SON: SO_3438(ispD)
SDN: Sden_1198
SFR: Sfri_1054
SAZ: Sama_1038
SBL: Sbal_3125
SBM: Shew185_3134
SBN: Sbal195_3277
SLO: Shew_1207
SPC: Sputcn32_2755
SSE: Ssed_1292
SPL: Spea_1187
SHE: Shewmr4_1117
SHM: Shewmr7_1188
SHN: Shewana3_1118
SHW: Sputw3181_1257
SHL: Shal_1224
SWD: Swoo_3348
ILO: IL0752(ispD)
CPS: CPS_1072(ispD)
PHA: PSHAa0684(ispD)
PAT: Patl_3857
SDE: Sde_1247
MAQ: Maqu_0923
AMC: MADE_03721
PIN: Ping_0672
MCA: MCA2517(ispD)
FTU: FTT0711(ispD)
FTF: FTF0711(ispD)
FTW: FTW_1530(ispD)
FTL: FTL_1525
FTH: FTH_1475(ispD)
FTA: FTA_1609(ispD)
FTN: FTN_0623(ispD)
FTM: FTM_1371(ispD)
FPH: Fphi_0219
NOC: Noc_0854
AEH: Mlg_1837
HHA: Hhal_1435
HCH: HCH_01869(ispD)
CSA: Csal_2638
ABO: ABO_1166(ispD)
MMW: Mmwyl1_1301
AHA: AHA_0823(ispD)
ASA: ASA_3473(ispD)
BCI: BCI_0211(ispD)
RMA: Rmag_0755
VOK: COSY_0697(ispD)

NME: NMB1513
NMA: NMA1713
NMC: NMC1442
NMN: NMCC_1418
NGO: NGO0972
NGK: NGK_0824
CVI: CV_1258(ispD)
RSO: RSc1643(ispD)
REU: Reut_A1361(ispD)
REH: H16_A1456(ispD)
RME: Rmet_1954(ispD)
BMA: BMA1490(ispD)
BMV: BMASAVP1_A1987(ispD)
BML: BMA10229_A3319(ispD)
BMN: BMA10247_1259(ispD)
BXE: Bxe_A2312(ispD)
BVI: Bcep1808_1870(ispD)
BUR: Bcep18194_A5254(ispD)
BCN: Bcen_6136(ispD)
BCH: Bcen2424_1943(ispD)
BCM: Bcenmc03_1967
BAM: Bamb_1931(ispD)
BAC: BamMC406_1858
BMU: Bmul_1328
BMJ: BMULJ_01918(ispD)
BPS: BPSL2099(ispD)
BPM: BURPS1710b_2512(ispD)
BPL: BURPS1106A_2401(ispD)
BPD: BURPS668_2358(ispD)
BTE: BTH_I2089(ispD)
BPH: Bphy_0998
PNU: Pnuc_0930
PNE: Pnec_0911
BPE: BP0865(ispD)
BPA: BPP3366(ispD)
BBR: BB3817(ispD)
BPT: Bpet1695(ispD)
BAV: BAV1060(ispD)
RFR: Rfer_1332
POL: Bpro_2716
PNA: Pnap_2549
AAV: Aave_1581
AJS: Ajs_3156
VEI: Veis_4360
DAC: Daci_2849
MPT: Mpe_A1570
HAR: HEAR1912(ispD)
MMS: mma_1409
LCH: Lcho_2295
NEU: NE1412
NET: Neut_1525
NMU: Nmul_A2127
EBA: ebA6543(ispD)
AZO: azo1682
DAR: Daro_1973
TBD: Tbd_1003
MFA: Mfla_1116
HPY: HP1020(ispDF)
HPJ: jhp0404(ispDF)
HPA: HPAG1_0427(ispDF)
HHE: HH1582(ispDF)
HAC: Hac_1124(ispDF)
WSU: WS1940(ispDF)
TDN: Suden_1487(ispDF)
CJE: Cj1607(ispDF)
CJR: CJE1779(ispDF)
CJJ: CJJ81176_1594(ispDF)
CFF: CFF8240_0409(ispDF)
GSU: GSU3368(ispD)
GME: Gmet_0060
GUR: Gura_4163
GLO: Glov_0872
PCA: Pcar_0103(ispD)
PPD: Ppro_2969
DVU: DVU1454(ispD)
DVL: Dvul_1625
DDE: Dde_1726
LIP: LI0446
DPS: DP0257
DOL: Dole_2147
ADE: Adeh_1272
SAT: SYN_01401
SFU: Sfum_1637
WOL: WD1143
WBM: Wbm0409
AMA: AM1357(ispD)
APH: APH_1277(ispD)
ERU: Erum1030(ispD)

ERW: ERWE_CDS_01000(ispD)
ERG: ERGA_CDS_00960(ispD)
ECN: Ecaj_0103
ECH: ECH_0157(ispD)
NSE: NSE_0178
PUB: SAR11_0945(ispD)
MLO: mll0395(ispDF)
MES: Meso_1621(ispDF)
SME: SMc01040(ispDF)
ATU: Atu1443(ispF)
ATC: AGR_C_2659
RET: RHE_CH01945(ispDF)
RLE: RL2254(ispDF)
BME: BMEI0863(ispDF)
BMF: BAB1_1143(ispDF)
BMB: BruAb1_1126(ispDF)
BMS: BR1120(ispDF)
BJA: bll4485
BRA: BRADO3869(ispDF)
BBT: BBta_4067(ispDF)
RPA: RPA2590(ispD)
RPB: RPB_2885
RPC: RPC_2575
RPD: RPD_2587
RPE: RPE_2755
NWI: Nwi_1442
NHA: Nham_1834
BHE: BH05820
BQU: BQ04980(ispDF)
BBK: BARBAKC583_0540(ispDF)
BTR: Btr_0870
CCR: CC_1738(ispDF)
SIL: SPO2090(ispDF)
SIT: TM1040_1364
RSP: RSP_2835(ispD)
RSQ: Rsph17025_1485
RDE: RD1_2766(ispD)
PDE: Pden_3667
MMR: Mmar10_1439
HNE: HNE_2014(ispDF)
ZMO: ZMO1128(ispDF)
NAR: Saro_1925(ispDF)
SAL: Sala_1278
ELI: ELI_06290(ispDF)
GOX: GOX1669
GBE: GbCGDNIH1_1019
ACR: Acry_0551
RRU: Rru_A1674
MAG: amb2363
MGM: Mmc1_2672
ABA: Acid345_0188
SWO: Swol_2361
CSC: Csac_2198
BSU: BSU00900(ispD)
BHA: BH0107(ispD)
BAN: BA0084(ispD)
BAR: GBAA0084(ispD)
BAA: BA_0674
BAT: BAS0085(ispD)
BCE: BC0106(ispD)
BCA: BCE_0085(ispD)
BCZ: BCZK0081(ispD)
BCY: Bcer98_0080
BTK: BT9727_0082(ispD)
BTL: BALH_0085(ispD)
BWE: BcerKBAB4_0080
BLI: BL03265(ispD)
BLD: BLi00108(ispD)
BCL: ABC0125(ispD)
BAY: RBAM_001150(yacM)
BPU: BPUM_0075
GKA: GK0081(ispD)
GTN: GTNG_0081(ispD)
LSP: Bsph_4646
ESI: Exig_0071 Exig_0189
SAU: SA0241(ispD) SA0245(ispD)
SAV: SAV0251(ispD) SAV0255(ispD)
SAW: SAHV_0250 SAHV_0254
SAM: MW0227(ispD) MW0231(ispD)
SAR: SAR0246(ispD) SAR0252(ispD)
SAS: SAS0227(ispD) SAS0232(ispD)
SAC: SACOL0236(ispD) SACOL0240(ispD)
SAB: SAB0190 SAB0194(ispD)
SAA: SAUSA300_0245
SAUSA300_0249(ispD)
SAX: USA300HOU_0262(ispD2)
USA300HOU_0266

SAO: SAOUHSC_00220
SAOUHSC_00225(ispD)
SAJ: SaurJH9_0236 SaurJH9_0240(ispD)
SAH: SaurJH1_0242 SaurJH1_0246(ispD)
SAE: NWMN_0185 NWMN_0189(ispD)
SEP: SE0319
SER: SERP0196(ispD)
SSP: SSP0354(ispD)
LMO: lmo0235(ispD) lmo1086(ispD)
LMF: LMOf2365_0247(ispD)
LMOf2365_1100(ispD)
LIN: lin0267(ispD) lin1071(ispD)
LWE: lwe0199(ispD) lwe1061(ispD)
SPN: SP_1271(ispD)
SPR: spr1149(ispD)
SPD: SPD_1127(ispD)
SPV: SPH_1387
SPW: SPCG_1235(ispD)
SPX: SPG_1165
SAG: SAG1417
SAN: gbs1487
SAK: SAK_1452(ispD)
SSA: SSA_2214
SGO: SGO_2017
LPL: lp_1816
LCA: LSEI_1098
EFA: EF2172(ispD)
STH: STH3123
CAC: CAC3184
CPE: CPE2429(ispD)
CPF: CPF_2739(ispD)
CPR: CPR_2426(ispD)
CTC: CTC02626
CNO: NT01CX_1092(ispD)
CTH: Cthe_2941
CDF: CD0047(ispD)
CBO: CBO3504(ispD)
CBA: CLB_3564(ispD)
CBH: CLC_3453(ispD)
CBL: CLK_2951(ispD)
CBK: CLL_A0216(ispD)
CBB: CLD_0997(ispD)
CBF: CLI_3691(ispD)
CBE: Cbei_0129(ispD)
CKL: CKL_0200(ispD)
CPY: Cphy_0353
AMT: Amet_4506
AOE: Clos_0463
CHY: CHY_2342(ispD)
DSY: DSY0443 DSY3011
DRM: Dred_0187
PTH: PTH_0289(ispD)
DAU: Daud_0186
FMA: FMG_1230
TTE: TTE2322(ispD)
TEX: Teth514_0839
TPD: Teth39_0346
MTA: Moth_2487
MPE: MYPE2770
MTU: Rv3582c(ispD)
MTC: MT3688(ispD)
MRA: MRA_3621(ispD)
MTF: TBFG_13615(ispD)
MBO: Mb3613c(ispD)
MBB: BCG_3647c(ispD)
MLE: ML0321(ispD)
MPA: MAP0476(ispD)
MAV: MAV_0571(ispD)
MSM: MSMEG_6076(ispD)
MUL: MUL_4158(ispD)
MVA: Mvan_4129 Mvan_4130
MGI: Mflv_2528 Mflv_2529
MAB: MAB_0569
MMC: Mmcs_4739(ispD)
MKM: Mkms_4825(ispD)
MJL: Mjls_5125(ispD)
MMI: MMAR_5082(ispD)
CGL: NCgl2570(ispD)
CGB: cg2945(ispD)
CGT: cgR_2564(ispD)
CEF: CE2521(ispD)
CDI: DIP1973(ispD)
CJK: jk0308(ispD)
CUR: cu1675
NFA: nfa4360(ispD)
RHA: RHA1_ro04460(ispD)
SCO: SCO4233(ispD)
SMA: SAV3969(mecT)

SGR: SGR_4012
TWH: TWT348(ispDF)
TWS: TW422
LXX: Lxx18250(ispF)
AAU: AAur_0898(ispD)
RSA: RSal33209_0409
KRH: KRH_18710(ispD)
PAC: PPA0353
NCA: Noca_4038
FRA: Francci3_3932 Francci3_4254
FRE: Franean1_0363 Franean1_0798
FAL: FRAAL6243 FRAAL6524(ispD)
ACE: Acel_0080 Acel_1533
KRA: Krad_0899
SEN: SACE_0439(ispD)
STP: Strop_4261
SAQ: Sare_4691
BLO: BL0324(ispD)
BLJ: BLD_1082(ispD)
RXY: Rxyl_2176
FNU: FN1580
RBA: RB9133(ispD)
OTE: Oter_0455 Oter_2440
MIN: Minf_0787(ispD)
AMU: Amuc_0068
CTR: CT462(ispD)
CTA: CTA_0505(ispD)
CTB: CTL0722
CTL: CTLon_0718(ispD)
CMU: TC0747(ispD)
CPN: CPn0579(ispD)
CPA: CP0169(ispD)
CPJ: CPj0579(ispD)
CPT: CpB0603(ispD)
CCA: CCA00162(ispD)
CAB: CAB160(ispD)
CFE: CF0845(ispD)
PCU: pc0327(ispD)
TPA: TP0512
TDE: TDE2291(ispD)
LIL: LA1048(ygbP)
LIC: LIC12617(ispD)
LBJ: LBJ_0280(ispD)
LBL: LBL_2796(ispD)
LBI: LEPBI_I1435(ispD)
LBF: LBF_1381(ispD)
SYN: slr0951
SYW: SYNW1849(ispD)
SYC: syc0848_d(ispD)
SYF: Synpcc7942_0681(ispD)
SYD: Syncc9605_0620(ispD)
SYE: Syncc9902_1742(ispD)
SYG: sync_2140(ispD)
SYR: SynRCC307_0684(ispD)
SYX: SynWH7803_1858(ispD)
SYP: SYNPCC7002_A1905(ispD)
CYA: CYA_1505(ispD)
CYB: CYB_2706(ispD)
TEL: tlr0605
MAR: MAE_45830
CYT: cce_0963(ispD)
GVI: glr2791
ANA: all5167
NPU: Npun_F5020
AVA: Ava_2414(ispD)
PMA: Pro0453(ispD)
PMM: PMM0454(ispD)
PMT: PMT1330(ispD)
PMN: PMN2A_1786(ispD)
PMI: PMT9312_0454(ispD)
PMB: A9601_05101(ispD)
PMC: P9515_05171(ispD)
PMF: P9303_06551(ispD)
PMG: P9301_04791(ispD)
PMH: P9215_05341
PMJ: P9211_04551
PME: NATL1_05091(ispD)
TER: Tery_0609(ispD)
AMR: AM1_3984(ispD)
BTH: BT_2881 BT_3923(ispD)
BFR: BF3962(ispD)
BFS: BF3735(ispD)
BVU: BVU_0472(ispD) BVU_2951
PGI: PG1434(ispD)
PGN: PGN_0841
PDI: BDI_1351 BDI_2700(ispD) BDI_3625
BDI_3828
SRU: SRU_1652

CHU: CHU_3100(ispD)
CTE: CT1317(ispD)
CPC: Cpar_1335
CCH: Cag_0929
CPH: Cpha266_1642
CPB: Cphamn1_1025
PVI: Cvib_1049
PPH: Ppha_1615
CTS: Ctha_2474
PAA: Paes_1464
DET: DET0059(ispD)
DEH: cbdb_A74(ispD)
DEB: DehaBAV1_0053
DRA: DR_2604
DGE: Dgeo_0181
TTH: TTC1815
TTJ: TTHA0171
AAE: aq_1323
HYA: HY04AAS1_1287
SUL: SYO3AOP1_0708
TMA: TM1393
TPT: Tpet_1390
TLE: Tlet_0798
TRQ: TRQ2_1436
TME: Tmel_1925
FNO: Fnod_0183
PMO: Pmob_1218
HMA: rrnAC1932(ispD)
NMR: Nmar_1581

Exemplary 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase nucleic acids and polypeptides

ATH: AT2G26930(ATCDPMEK)
OSA: 4327968(Os01g0802100)
PPP: PHYPADRAFT_190580
OLU: OSTLU_4287(CMK)
CRE: CHLREDRAFT_137673(CMK1)
CME: CMS444C
PFA: PFE0150c
PFD: PFDG_01632
PFH: PFHG_02738
PYO: PY04665
ECO: b1208(ispE)
ECJ: JW1199(ispE)
ECD: ECDH10B_1261(ispE)
ECE: Z1979(ychB)
ECS: ECs1713
ECC: c1666(ispE)
ECI: UTI89_C1402(ychB)
ECP: ECP_1256
ECV: APECO1_324(ychB)
ECW: EcE24377A_1356(ispE)
ECX: EcHS_A1313(ispE)
ECM: EcSMS35_1934(ispE)
ECL: EcolC_2418
STY: STY1905(ipk)
STT: t1097(ipk)
SPT: SPA1094(ipk)
SPQ: SPAB_01449
SEC: SC1773(ipk)
SEH: SeHA_C1975(ispE)
SEE: SNSL254_A1911(ispE)
SEW: SeSA_A1917(ispE)
SES: SARI_01174
STM: STM1779(ipk)
YPE: YPO2014(ipk)
YPK: y2293
YPM: YP_1862(ipk)
YPA: YPA_1398
YPN: YPN_1496
YPP: YPDSF_1104
YPG: YpAngola_A2463(ispE)
YPS: YPTB2002(ipk)
YPI: YpsIP31758_2069(ispE)
YPY: YPK_2182
YPB: YPTS_2060
YEN: YE2434(ipk)
SFL: SF1211(ychB)
SFX: S1295(ychB)
SFV: SFV_1222(ychB)
SSN: SSON_1970(ychB)
SBO: SBO_1859(ychB)
SBC: SbBS512_E1372(ispE)
SDY: SDY_1257(ychB)
ECA: ECA2187(ispE)
ETA: ETA_18820(ispE)
PLU: plu2067(ispE)
BUC: BU170(ychB)
BAS: BUsg164(ipk)
WBR: WGLp348(ychB)
SGL: SG1879
ENT: Ent638_2340
ESA: ESA_01495
KPN: KPN_02237(ispE)
CKO: CKO_01272
SPE: Spro_1987
BFL: Bfl347(ipk)
BPN: BPEN_357(ispE)
HIN: HI1608
HIT: NTHI1434(ispE)
HIP: CGSHiEE_05690
HIQ: CGSHiGG_10080
HDU: HD1628(ispE)
HSO: HS_0997(ispE)
HSM: HSM_1475
PMU: PM0245
MSU: MS1535(ispE)
APL: APL_0776(ispE)
APJ: APJL_0779(ispE)
APA: APP7_0837
ASU: Asuc_1751
XFA: XF2645
XFT: PD2018(ispE)
XFM: Xfasm12_2208

XFN: XfasM23_2119
XCC: XCC0871(ipk)
XCB: XC_3359
XCV: XCV0979(ispE)
XAC: XAC0948(ipk)
XOO: XOO3604(ipk)
XOM: XOO_3406(XOO3406)
SML: Smlt0874(ipk)
SMT: Smal_0725
VCH: VC2182
VCO: VC0395_A1759
VVU: VV1_0256
VVY: VV0928
VPA: VP0740
VFI: VF0765
VHA: VIBHAR_01247
PPR: PBPRA2848
PAE: PA4669(ipk)
PAU: PA14_61750(ipk)
PAP: PSPA7_5318(ispE)
PPU: PP_0723(ipk)
PPF: Pput_0757(ipk)
PPG: PputGB1_0767
PPW: PputW619_4460
PST: PSPTO_1105(ispE)
PSB: Psyr_0945(ipk)
PSP: PSPPH_0993(ipk)
PFL: PFL_5163(ipk)
PFO: Pfl01_4752(ipk)
PEN: PSEEN0858(ipk)
PMY: Pmen_1056(ipk)
PSA: PST_3186(ipk)
CJA: CJA_0646(ispE)
PAR: Psyc_0173(ispE)
PCR: Pcryo_0186
PRW: PsycPRwf_2104
ACI: ACIAD2903(ispE)
ACB: A1S_0834
ABC: ACICU_00788
SON: SO_3836(ispE)
SDN: Sden_0917
SFR: Sfri_0720
SAZ: Sama_2569
SBL: Sbal_0693
SBM: Shew185_3617
SBN: Sbal195_3740
SLO: Shew_2915
SPC: Sputcn32_0798
SSE: Ssed_3462
SPL: Spea_3129
SHE: Shewmr4_3172
SHM: Shewmr7_0794
SHN: Shewana3_0766
SHW: Sputw3181_3377
SHL: Shal_3214
SWD: Swoo_3688
ILO: IL0928(ispE)
CPS: CPS_3556(ispE)
PHA: PSHAa1055(ispE)
PAT: Patl_2566
SDE: Sde_3255
MAQ: Maqu_2364
AMC: MADE_02576
PIN: Ping_0912
MCA: MCA1055(ispE)
FTU: FTT0271(ispE)
FTF: FTF0271(ispE)
FTW: FTW_1830(ispE)
FTL: FTL_0151
FTH: FTH_0144(ispE)
FTA: FTA_0164(ispE)
FTN: FTN_0146(ispE)
FTM: FTM_1592(ispE)
FPH: Fphi_0678
NOC: Noc_0513
AEH: Mlg_0282
HHA: Hhal_0990
HCH: HCH_01727(ispE)
CSA: Csal_1525
ABO: ABO_0519(ispE)
MMW: Mmwyl1_3603
AHA: AHA_3152(ispE)
ASA: ASA_1172(ispE)
BCI: BCI_0292(ispE)
RMA: Rmag_0110
VOK: COSY_0115(ispE)
NME: NMB0874
NMA: NMA1092

NMC: NMC0815
NMN: NMCC_0833
NGO: NGO0440
NGK: NGK_0610
CVI: CV_4059(ispE)
RSO: RSc0396(ipk)
REU: Reut_A0343
REH: H16_A0374
RME: Rmet_0290
CTI: RALTA_A0318(ispE)
BMA: BMA3118(ispE)
BMV: BMASAVP1_A0086(ispE)
BML: BMA10229_A1504(ispE)
BMN: BMA10247_2932(ispE)
BXE: Bxe_A4132
BVI: Bcep1808_2906
BUR: Bcep18194_A6131
BCN: Bcen_2187
BCH: Bcen2424_2801
BCM: Bcenmc03_2812
BAM: Bamb_2861
BAC: BamMC406_2719
BMU: Bmul_0514
BMJ: BMULJ_02745(ispE)
BPS: BPSL0523
BPM: BURPS1710b_0755(ispE)
BPL: BURPS1106A_0587(ispE)
BPD: BURPS668_0571(ispE)
BTE: BTH_I0476(ispE)
BPH: Bphy_0316
PNU: Pnuc_1919
PNE: Pnec_1624
BPE: BP3126(ispE)
BPA: BPP0816(ispE)
BBR: BB0900(ispE)
BPT: Bpet4003(ispE)
BAV: BAV0536(ispE)
RFR: Rfer_1659
POL: Bpro_1294
PNA: Pnap_0900
AAV: Aave_3609
AJS: Ajs_0896
VEI: Veis_0952
DAC: Daci_5432
MPT: Mpe_A3230
HAR: HEAR2892(ispE)
MMS: mma_3127
LCH: Lcho_3497
NEU: NE1827(ipk)
NET: Neut_1139
NMU: Nmul_A0588
EBA: ebA1405(ispE)
AZO: azo0756(ispE)
DAR: Daro_3729
TBD: Tbd_0386
MFA: Mfla_0679
HPY: HP1443
HPJ: jhp1336
HPA: HPAG1_1369
HPS: HPSH_07385
HHE: HH0122
HAC: Hac_0175(ipk)
WSU: WS0881
TDN: Suden_0440
CJE: Cj1104
CJR: CJE1247(ispE)
CJJ: CJJ81176_1122(ispE)
CJU: C8J_1045
CJD: JJD26997_0618(ispE)
CFF: CFF8240_0713
CCV: CCV52592_0696(ispE)
CHA: CHAB381_1110
CCO: CCC13826_0061(ispE)
ABU: Abu_2083(ispE)
NIS: NIS_1475
SUN: SUN_0381
GSU: GSU0660(ispE)
GME: Gmet_2849
GUR: Gura_3683
GLO: Glov_2596
PCA: Pcar_2005(ispE)
PPD: Ppro_0738
DVU: DVU1576(ispE)
DVL: Dvul_1557
DDE: Dde_2125
LIP: LI0735(ychB)
DPS: DP2735
DOL: Dole_2816

ADE: Adeh_0123
AFW: Anae109_0127
SAT: SYN_03046
SFU: Sfum_3651
WOL: WD0360(ispE)
WBM: Wbm0173
WPI: WP0174(ispE)
AMA: AM493(ispE)
APH: APH_0574(ispE)
ERU: Erum3340(ispE)
ERW: ERWE_CDS_03410(ispE)
ERG: ERGA_CDS_03370(ispE)
ECN: Ecaj_0317
ECH: ECH_0757(ispE)
NSE: NSE_0720
PUB: SAR11_0105(ispE)
MLO: mll7422
MES: Meso_0706
PLA: Plav_0721
SME: SMc00862(ipk)
SMD: Smed_0456
ATU: Atu0632(ipk)
ATC: AGR_C_1122
RET: RHE_CH00873(ispE)
REC: RHECIAT_CH0000963(ispE)
RLE: RL0935
BME: BMEI1537
BMF: BAB1_0423(ispE)
BMB: BruAb1_0418(ispE)
BMC: BAbS19_I03890
BMS: BR0394(ispE)
BMT: BSUIS_A0420(ispE)
BOV: BOV_0403(ispE)
BCS: BCAN_A0398(ispE)
OAN: Oant_0512
BJA: blr2526(ipk)
BRA: BRADO2022(ispE)
BBT: BBta_2348(ispE)
RPA: RPA1039(ispE)
RPB: RPB_1086
RPC: RPC_4356
RPD: RPD_1213
RPE: RPE_4419
RPT: Rpal_1231
NWI: Nwi_2593
NHA: Nham_3216
BHE: BH04210(thrB1)
BQU: BQ03230(thrB)
BBK: BARBAKC583_0387(ispE)
BTR: Btr_0633
XAU: Xaut_1381
AZC: AZC_0910
MEX: Mext_3109
MRD: Mrad2831_5351
MET: M446_2748
BID: Bind_0858
CCR: CC_1336
CAK: Caul_2169
SIL: SPO0318(ispE)
SIT: TM1040_3743
RSP: RSP_1779(ispE)
RSH: Rsph17029_0426
RSQ: Rsph17025_2471
JAN: Jann_0486
RDE: RD1_3402(ispE)
PDE: Pden_0423
DSH: Dshi_3073
MMR: Mmar10_2186
HNE: HNE_0676(ispE)
ZMO: ZMO1182(ispE)
NAR: Saro_1782
SAL: Sala_1187
SWI: Swit_4106
ELI: ELI_06920
GOX: GOX1559
GBE: GbCGDNIH1_1848
ACR: Acry_2663
GDI: GDI0728
RRU: Rru_A0263
MAG: amb4435
MGM: Mmc1_0819
ABA: Acid345_4541
SUS: Acid_7097
SWO: Swol_0064
CSC: Csac_2225
BSU: BSU00460(ispE)
BHA: BH0061
BAN: BA0043(ispE)

BAR: GBAA0043(ispE)
BAA: BA_0633
BAT: BAS0044
BCE: BC0050
BCA: BCE_0043(ispE)
BCZ: BCZK0040(ispE)
BCY: Bcer98_0040
BTK: BT9727_0040(ispE)
BTL: BALH_0040(ispE)
BWE: BcerKBAB4_0040
BLI: BL00525(ispE)
BLD: BLi00059(ispE)
BCL: ABC0074(ispE)
BAY: RBAM_000550
BPU: BPUM_0030
OIH: OB0055
GKA: GK0039
GTN: GTNG_0039
LSP: Bsph_0065
ESI: Exig_0038
SAU: SA0453
SAV: SAV0495
SAW: SAHV_0492
SAM: MW0450
SAR: SAR0496
SAS: SAS0452
SAC: SACOL0538(ispE)
SAB: SAB0444
SAA: SAUSA300_0472(ispE)
SAO: SAOUHSC_00466
SAJ: SaurJH9_0516
SAH: SaurJH1_0529
SAE: NWMN_0458
SEP: SE2288
SER: SERP0133(ispE)
SHA: SH2516
SSP: SSP2261
LMO: lmo0190
LMF: LMOf2365_0201(ispE)
LIN: lin0229
LWE: lwe0159(ispE)
SPZ: M5005_Spy_0074 M5005_Spy_0075 M5005_Spy_0076
SPH: MGAS10270_Spy0077 MGAS10270_Spy0078
SPI: MGAS10750_Spy0082 MGAS10750_Spy0083
SPJ: MGAS2096_Spy0077 MGAS2096_Spy0078 MGAS2096_Spy0079
SPK: MGAS9429_Spy0074 MGAS9429_Spy0075 MGAS9429_Spy0076
SPA: M6_Spy0123 M6_Spy0124
SPB: M28_Spy0073 M28_Spy0074
SAG: SAG0153(ispE)
SAN: gbs0149
SAK: SAK_0216(ispE)
SMU: SMU.1996(ipk)
SEZ: Sez_0102(ispE)
LPL: lp_0460(ispE)
LSA: LSA1652(ispE)
LSL: LSL_0234(ispE)
LBR: LVIS_0460
LCA: LSEI_2591
LCB: LCABL_27570(ispE)
LRE: Lreu_0215
LRF: LAR_0206
LFE: LAF_0190
EFA: EF0051(ispE)
STH: STH3246
CAC: CAC2902
CPE: CPE2212(ipk)
CPF: CPF_2476(ipk)
CPR: CPR_2186(ipk)
CTC: CTC00283
CNO: NT01CX_0566(ipk)
CTH: Cthe_2403(ipk)
CDF: CD3566(ipk)
CBO: CBO0121(ipk)
CBA: CLB_0157(ispE)
CBH: CLC_0169(ispE)
CBL: CLK_3296(ispE)
CBK: CLL_A0471(ispE)
CBB: CLD_0665(ispE)
CBF: CLI_0176(ispE)
CBE: Cbei_0394(ipk)
CKL: CKL_3724(ispE)
CPY: Cphy_3793

AMT: Amet_4604
AOE: Clos_0285
CHY: CHY_0188(ispE)
DSY: DSY0148
DRM: Dred_0094
PTH: PTH_0096(ispE)
DAU: Daud_0058
HMO: HM1_0738(ispE)
FMA: FMG_0552
TTE: TTE2559(ispE)
TEX: Teth514_0599
TPD: Teth39_0176
MTA: Moth_0072
MPE: MYPE10380
MGA: MGA_0635
UUR: UU600
MTU: Rv1011(ispE)
MTC: MT1040
MRA: MRA_1020(ispE)
MTF: TBFG_11030
MBO: Mb1038(ispE)
MBB: BCG_1068(ispE)
MLE: ML0242
MPA: MAP0976
MAV: MAV_1149(ispE)
MSM: MSMEG_5436(ispE)
MUL: MUL_4649(ispE)
MVA: Mvan_4799
MGI: Mflv_1934
MAB: MAB_1139
MMC: Mmcs_4262
MKM: Mkms_4348
MJL: Mjls_4641
MMI: MMAR_4477(ispE)
CGL: NCgl0874(cgl0911)
CGB: cg1039
CGT: cgR_1012
CEF: CE0973
CDI: DIP0876
CJK: jk1510(ispE)
CUR: cu0564
NFA: nfa49010(cmeK)
RHA: RHA1_ro05684
SCO: SCO3148(SCE66.27c)
SMA: SAV3586(cmeK)
SGR: SGR_4357
TWH: TWT605(ispE)
TWS: TW159(ispE)
LXX: Lxx17480(ispE)
CMI: CMM_2367(ispE)
AAU: AAur_1338(ispE)
RSA: RSal33209_2993
KRH: KRH_17370(ispE)
PAC: PPA0527
NCA: Noca_3855
TFU: Tfu_0407
FRA: Francci3_3958
FRE: Franean1_0773
FAL: FRAAL6276(ispE)
ACE: Acel_0181
KRA: Krad_1046
SEN: SACE_0807(ispE)
STP: Strop_0783
SAQ: Sare_0727
BLO: BL0656(ispE)
BAD: BAD_1616(ispE)
RXY: Rxyl_0893
FNU: FN0021
RBA: RB10537(ispE)
OTE: Oter_2442
MIN: Minf_1286(ispE)
AMU: Amuc_1195
CTR: CT804(ychB)
CTA: CTA_0876(ispE)
CTB: CTL0173
CTL: CTLon_0174(ispE)
CMU: TC0187
CPJ: CPj0954 CPj0955
CPT: CpB0991 CpB0992
CCA: CCA00815(ispE)
CAB: CAB784
CFE: CF0199(ispE)
PCU: pc1589
TPA: TP0371
TPP: TPASS_0371
TDE: TDE1338(ispE)
LIL: LA3824(ychB)
LIC: LIC10426(ispE)

LBJ: LBJ_2584(ispE)
LBL: LBL_0528(ispE)
LBI: LEPBI_I0238(ispE)
LBF: LBF_0232(ispE)
SYN: sll0711(ipk)
SYW: SYNW1053(ispE)
SYC: syc1203_d(ispE)
SYF: Synpcc7942_0310
SYD: Syncc9605_1188
SYE: Syncc9902_1282
SYG: sync_1593(ispE)
SYR: SynRCC307_1314(ispE)
SYX: SynWH7803_1365(ispE)
SYP: SYNPCC7002_A2416(ispE)
CYA: CYA_0285(ispE)
CYB: CYB_1390(ispE)
TEL: tll0500
MAR: MAE_04520
CYT: cce_1317(ispE)
GVI: gll0102
ANA: alr3230
NPU: Npun_R4911
AVA: Ava_4887
PMA: Pro0764(ispE)
PMM: PMM0932(ispE)
PMT: PMT0620(ispE)
PMN: PMN2A_0279
PMI: PMT9312_0867
PMB: A9601_09281(ispE)
PMC: P9515_10151(ispE)
PMF: P9303_16181(ispE)
PMG: P9301_09261(ispE)
PMH: P9215_09581
PMJ: P9211_07121
PME: NATL1_09481(ispE)
TER: Tery_4700
AMR: AM1_1752(ispE)
BTH: BT_0624
BFR: BF2589
BFS: BF2610
BVU: BVU_3466
PGI: PG0935(ispE)
PGN: PGN_1012
PDI: BDI_0715
SRU: SRU_0689(ispE)
CHU: CHU_1210(ispE)
CTE: CT1495(ispE)
CPC: Cpar_1582
CCH: Cag_1333
CPH: Cpha266_1884
CPB: Cphamn1_0845
PVI: Cvib_1321
PLT: Plut_1496
PPH: Ppha_1063
CTS: Ctha_0721
PAA: Paes_1591
DET: DET0405(ispE)
DEH: cbdb_A356(ispE)
DEB: DehaBAV1_0384
EMI: Emin_0501
DRA: DR_2605
DGE: Dgeo_0180
TTH: TTC1816
TTJ: TTHA0170
AAE: aq_915
HYA: HY04AAS1_1414
SUL: SYO3AOP1_0238
TMA: TM1383
TPT: Tpet_1400
TLE: Tlet_1489
TRQ: TRQ2_1446
TME: Tmel_0318
FNO: Fnod_1663
PMO: Pmob_0160

Exemplary 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase nucleic acids and polypeptides

ATH: AT1G63970(ISPF)
OSA: 4330320(Os02g0680600)
PPP: PHYPADRAFT_150209
OLU: OSTLU_44114(MCS)
CRE: CHLREDRAFT_188593
CME: CMT435C
PFA: PFB0420w
PFH: PFHG_00813
PYO: PY00321
TAN: TA04155
TPV: TP03_0365
TET: TTHERM_01003920
ECO: b2746(ispF)
ECJ: JW2716(ispF)
ECD: ECDH10B_2914(ispF)
ECE: Z4054(ispF)
ECS: ECs3600(ispF)
ECC: c3313(ispF)
ECI: UTI89_C3117(ispF)
ECP: ECP_2728(ispF)
ECV: APECO1_3777(ispF)
ECW: EcE24377A_3047(ispF)
ECX: EcHS_A2884(ispF)
ECM: EcSMS35_2871(ispF)
ECL: EcolC_0966
STY: STY3054(ispF)
STT: t2830(ispF)
SPT: SPA2785(ispF)
SPQ: SPAB_03643
SEC: SC2861(ispF)
SEH: SeHA_C3119(ispF)
SEE: SNSL254_A3135(ispF)
SEW: SeSA_A3080(ispF)
SES: SARI_00027
STM: STM2929(ispF)
YPE: YPO3360(ispF)
YPK: y0829(ispF)
YPM: YP_0327(ispF)
YPA: YPA_2783(ispF)
YPN: YPN_0733(ispF)
YPP: YPDSF_3000(ispF)
YPG: YpAngola_A0963(ispF)
YPS: YPTB0771(ispF)
YPI: YpsIP31758_3298(ispF)
YPY: YPK_3430
YPB: YPTS_0805
YEN: YE0770(ispF)
SFL: SF2769(ispF)
SFX: S2962(ispF)
SFV: SFV_2752(ispF)
SSN: SSON_2894(ispF)
SBO: SBO_2774(ispF)
SBC: SbBS512_E3128(ispF)
SDY: SDY_2945(ispF)
ECA: ECA3534(ispF)
ETA: ETA_27000(ispF)
PLU: plu0714(ispF)
BUC: BU419(ygbB)
BAS: BUsg404(ygbB)
WBR: WGLp531(ygbB)
SGL: SG0527(ispF)
ENT: Ent638_3217(ispF)
ESA: ESA_00545
KPN: KPN_03108(ispF)
CKO: CKO_04107
SPE: Spro_0827
BPN: BPEN_172(ispF)
HIN: HI0671(ispF)
HIT: NTHI0793(ispF)
HIP: CGSHiEE_08820(ispF)
HIQ: CGSHiGG_06630(ispF)
HDU: HD1328(ispF)
HSO: HS_1498(ispF)
HSM: HSM_0503
PMU: PM1609
MSU: MS2274(ispF)
APL: APL_0803(ispF)
APJ: APJL_0808(ispF)

APA: APP7_0862
ASU: Asuc_2031
XFA: XF1294(ispF)
XFT: PD0546(ispF)
XFM: Xfasm12_0619
XFN: XfasM23_0571
XCC: XCC1703(ispF)
XCB: XC_2528(ispF)
XCV: XCV1755(ispF)
XAC: XAC1722(ispF)
XOO: XOO2960(ispF)
XOM: XOO_2811(ispF)
SML: Smlt1718(ispF)
SMT: Smal_1455
VCH: VC0529(ispF)
VCO: VC0395_A0057(ispF)
VVU: VV1_1583(ispF)
VVY: VV2814(ispF)
VPA: VP2558(ispF)
VFI: VF2072(ispF)
VHA: VIBHAR_03522
PPR: PBPRA3076(ispF)
PAE: PA3627(ispF)
PAU: PA14_17420(ispF)
PAP: PSPA7_1512(ispF)
PPU: PP_1618(ispF)
PPF: Pput_4159(ispF)
PPG: PputGB1_1172
PPW: PputW619_4057
PST: PSPTO_1560(ispF)
PSB: Psyr_1369(ispF)
PSP: PSPPH_3814(ispF)
PFL: PFL_1202(ispF)
PFO: PflO1_1127(ispF)
PEN: PSEEN4194(ispF)
PMY: Pmen_3026(ispF)
PSA: PST_1566(ispF)
CJA: CJA_2222(ispF)
PAR: Psyc_1243(ispF)
PCR: Pcryo_1149
PRW: PsycPRwf_0962
ACI: ACIAD1996(ispF)
ACB: A1S_1982
ABM: ABSDF1672(ispF)
ABY: ABAYE1569
ABC: ACICU_02105
SON: SO_3437(ispF)
SDN: Sden_1199
SFR: Sfri_1055
SAZ: Sama_1039
SBL: Sbal_3124
SBM: Shew185_3133
SBN: Sbal195_3276
SLO: Shew_1208
SPC: Sputcn32_2754
SSE: Ssed_1293
SPL: Spea_1188
SHE: Shewmr4_1118
SHM: Shewmr7_1189
SHN: Shewana3_1119
SHW: Sputw3181_1258
SHL: Shal_1225
SWD: Swoo_3347
ILO: IL0751(ispF)
CPS: CPS_1073(ispF)
PHA: PSHAa0685(ispF)
PAT: Patl_3858
SDE: Sde_1248
MAQ: Maqu_0924
AMC: MADE_03722
PIN: Ping_0673
MCA: MCA2518(ispF)
FTU: FTT1128(ispF)
FTF: FTF1128(ispF)
FTW: FTW_1161(ispF)
FTL: FTL_0833
FTH: FTH_0823(ispF)
FTA: FTA_0882(ispF)
FTN: FTN_1110(ispF)
FTM: FTM_1296(ispF)
FPH: Fphi_1496
NOC: Noc_0855
AEH: Mlg_1836
HHA: Hhal_1434
HCH: HCH_01870(ispF)
CSA: Csal_2637
ABO: ABO_1167(ispF)
MMW: Mmwyl1_1302

AHA: AHA_0824(ispF)
ASA: ASA_3472(ispF)
BCI: BCI_0210(ispF)
RMA: Rmag_0756(ispF)
VOK: COSY_0698(ispF)
NME: NMB1512(ispF)
NMA: NMA1712(ispF)
NMC: NMC1441(ispF)
NMN: NMCC_1417
NGO: NGO0971(ispF)
NGK: NGK_0825
CVI: CV_1259(ispF)
RSO: RSc1644(RS04019)
REU: Reut_A1362
REH: H16_A1457
RME: Rmet_1953
BMA: BMA1489(ispF)
BMV: BMASAVP1_A1986(ispF)
BML: BMA10229_A3320(ispF)
BMN: BMA10247_1258(ispF)
BXE: Bxe_A2311
BVI: Bcep1808_1869
BUR: Bcep18194_A5253
BCN: Bcen_6137
BCH: Bcen2424_1942
BCM: Bcenmc03_1966
BAM: Bamb_1930
BAC: BamMC406_1857
BMU: Bmul_1329
BMJ: BMULJ_01917(ispF)
BPS: BPSL2098(ispF)
BPM: BURPS1710b_2511(ispF)
BPL: BURPS1106A_2400(ispF)
BPD: BURPS668_2357(ispF)
BTE: BTH_I2090(ispF)
BPH: Bphy_0999
PNU: Pnuc_0931
PNE: Pnec_0910
BPE: BP0866(ispF)
BPA: BPP3365(ispF)
BBR: BB3816(ispF)
BPT: Bpet1696(ispF)
BAV: BAV1059(ispF)
RFR: Rfer_1332
POL: Bpro_2715
PNA: Pnap_2548
AAV: Aave_1582
AJS: Ajs_3155
VEI: Veis_4361
DAC: Daci_2850
MPT: Mpe_A1571
HAR: HEAR1911(ispF)
MMS: mma_1410
LCH: Lcho_2293
NEU: NE1402
NET: Neut_1300
NMU: Nmul_A2126
EBA: ebA6542(ispF)
AZO: azo1683(ispF)
DAR: Daro_1974(ispF)
TBD: Tbd_1004
MFA: Mfla_1117
HPY: HP1020(ispDF)
HPJ: jhp0404(ispDF)
HPA: HPAG1_0427(ispDF)
HPS: HPSH_02215(ispDF)
HHE: HH1582(ispDF)
HAC: Hac_1124(ispDF)
WSU: WS1940(ispDF)
TDN: Suden_1487(ispDF)
CJE: Cj1607(ispDF)
CJR: CJE1779(ispDF)
CJJ: CJJ81176_1594(ispDF)
CJU: C8J_1508
CJD: JJD26997_1961
CFF: CFF8240_0409(ispDF)
CCV: CCV52592_0202
CHA: CHAB381_0932
CCO: CCC13826_1467
ABU: Abu_0126(ispDF)
NIS: NIS_0595
SUN: SUN_0522
GSU: GSU3367(ispF)
GME: Gmet_0059
GUR: Gura_4164
GLO: Glov_3480
PCA: Pcar_0102(ispF)
PPD: Ppro_0012

DVU: DVU1454(ispD)
DVL: Dvul_1625
DDE: Dde_1726
LIP: LI0446
DPS: DP0257
DOL: Dole_1666
ADE: Adeh_1272
AFW: Anae109_2497
SAT: SYN_01400
SFU: Sfum_1636
WOL: WD1143
WBM: Wbm0409
WPI: WP0969
AMA: AM1356(ispF)
APH: APH_1276(ispF)
ERU: Erum1020(ispF)
ERW: ERWE_CDS_00990(ispF)
ERG: ERGA_CDS_00950(ispF)
ECN: Ecaj_0102
ECH: ECH_0156(ispF)
NSE: NSE_0134(ispF)
MLO: mll0395(ispDF)
MES: Meso_1621(ispDF)
PLA: Plav_3132
SME: SMc01040(ispDF)
SMD: Smed_1087(ispDF)
ATU: Atu1443(ispF)
ATC: AGR_C_2659
RET: RHE_CH01945(ispDF)
REC: RHECIAT_CH0002043(ispDF)
RLE: RL2254(ispDF)
BME: BMEI0863(ispDF)
BMF: BAB1_1143(ispDF)
BMB: BruAb1_1126(ispDF)
BMC: BAbS19_I10610
BMS: BR1120(ispDF)
BMT: BSUIS_A1169(ispF)
BOV: BOV_1078(ispDF)
BCS: BCAN_A1139(ispF)
OAN: Oant_2069
BJA: bll4485
BRA: BRADO3869(ispDF)
BBT: BBta_4067(ispDF)
RPA: RPA2590(ispD)
RPB: RPB_2885
RPC: RPC_2575
RPD: RPD_2587
RPE: RPE_2755
RPT: Rpal_2860
NWI: Nwi_1442
NHA: Nham_1834
BHE: BH05820
BQU: BQ04980(ispDF)
BBK: BARBAKC583_0540(ispDF)
BTR: Btr_0870
XAU: Xaut_4402
AZC: AZC_3089
MEX: Mext_2817
MRD: Mrad2831_2171
MET: M446_5927
BID: Bind_1516
CCR: CC_1738(ispDF)
CAK: Caul_2603
SIL: SPO2090(ispDF)
SIT: TM1040_1364
RSP: RSP_6071(ispF)
RSH: Rsph17029_1460
RSQ: Rsph17025_1484
RDE: RD1_2767(ispF)
PDE: Pden_3667
DSH: Dshi_1577
MMR: Mmar10_1439
ZMO: ZMO1128(ispDF)
NAR: Saro_1925(ispDF)
SAL: Sala_1278
SWI: Swit_0244(ispDF)
ELI: ELI_06290(ispDF)
GOX: GOX1669
GBE: GbCGDNIH1_1019
ACR: Acry_2031
GDI: GDI2269
RRU: Rru_A1674
MAG: amb2363
MGM: Mmc1_2673
ABA: Acid345_0187
SUS: Acid_1861
SWO: Swol_2360
CSC: Csac_1587

BSU: BSU00910(ispF)
BHA: BH0108(ispF)
BAN: BA0085(ispF)
BAR: GBAA0085(ispF)
BAA: BA_0675(ygbB)
BAT: BAS0086(ispF)
BCE: BC0107(ispF)
BCA: BCE_0086(ispF)
BCZ: BCZK0082(ispF)
BCY: Bcer98_0081
BTK: BT9727_0083(ispF)
BTL: BALH_0086(ispF)
BWE: BcerKBAB4_0081
BLI: BL03266(ispF)
BLD: BLi00109(ispF)
BCL: ABC0126(ispF)
BAY: RBAM_001160(yacN)
BPU: BPUM_0076
GKA: GK0082(ispF)
GTN: GTNG_0082(ispF)
LSP: Bsph_4645
ESI: Exig_0072
LMO: lmo0236(ispF)
LMF: LMOf2365_0248(ispF)
LIN: lin0268(ispF)
EFA: EF0042(ispF)
CAC: CAC0434
CPE: CPE2316(ispF)
CPF: CPF_2616(ispF)
CPR: CPR_2302(ispF)
CTC: CTC00232
CNO: NT01CX_0736(ispF)
CTH: Cthe_2946(ispF)
CDF: CD0048(ispF)
CBO: CBO0066(ispF)
CBA: CLB_0102(ispF)
CBH: CLC_0114(ispF)
CBL: CLK_3243(ispF)
CBK: CLL_A0353(ispF)
CBB: CLD_0719(ispF)
CBF: CLI_0123(ispF)
CBE: Cbei_0297
CKL: CKL_3774(ispF)
CPY: Cphy_3326
AMT: Amet_4505
AOE: Clos_0464
CHY: CHY_2341(ispF)
DSY: DSY0444
DRM: Dred_0188
PTH: PTH_0290(ispF)
HMO: HM1_1354(ispD)
FMA: FMG_1229
TTE: TTE2320(ispF)
TEX: Teth514_0841
TPD: Teth39_0348
MTA: Moth_2486
MPE: MYPE10270
MGA: MGA_0657
MTU: Rv3581c(ispF)
MTC: MT3687(ispF)
MRA: MRA_3620(ispF)
MTF: TBFG_13614(ispF)
MBO: Mb3612c(ispF)
MBB: BCG_3646c(ispF)
MLE: ML0322(ispF)
MPA: MAP0477(ispF)
MAV: MAV_0572(ispF)
MSM: MSMEG_6075(ispF)
MUL: MUL_4157(ispF)
MVA: Mvan_5339(ispF)
MGI: Mflv_1445(ispF)
MAB: MAB_0570
MMC: Mmcs_4738(ispF)
MKM: Mkms_4824(ispF)
MJL: Mjls_5124(ispF)
MMI: MMAR_5081(ispF)
CGL: NCgl2569(ispF)
CGB: cg2944(ispF)
CGT: cgR_2563(ispF)
CEF: CE2520(ispF)
CDI: DIP1972(ispF)
CJK: jk0309(ispF)
CUR: cu1674
NFA: nfa4370(ispF)
RHA: RHA1_ro04461(ispF)
SCO: SCO4234(ispF)
SMA: SAV3968(ispF)
SGR: SGR_4013

TWH: TWT348(ispDF)
TWS: TW422
LXX: Lxx18250(ispF)
CMI: CMM_2489(ispDF)
ART: Arth_0728
AAU: AAur_0899(ispF)
RSA: RSal33209_0410
KRH: KRH_18700(ispF)
PAC: PPA0354(ispF)
NCA: Noca_4024(ispF)
TFU: Tfu_2906(ispF)
FRA: Francci3_4253(ispF)
FRE: Franean1_0364
FAL: FRAAL6523(ispF)
ACE: Acel_0081
KRA: Krad_0900
SEN: SACE_0440(ispF)
STP: Strop_4260
SAQ: Sare_4690
BLO: BL0997(ispF)
BAD: BAD_0669(ispF)
RXY: Rxyl_2175
FNU: FN1788
RBA: RB3451(ispF)
OTE: Oter_2439
MIN: Minf_0771(ispF)
AMU: Amuc_1243
CTR: CT434(ispF)
CTA: CTA_0474(ispF)
CTB: CTL0693
CTL: CTLon_0689(ispF)
CMU: TC0718(ispF)
CPN: CPn0547(ispF)
CPA: CP0205(ispF)
CPJ: CPj0547(ispF)
CPT: CpB0568(ispF)
CCA: CCA00195(ispF)
CAB: CAB191(ispF)
CFE: CF0812(ispF)
PCU: pc0227(ispF)
TPA: TP0512
TPP: TPASS_0512
TDE: TDE2292(ispF)
LIL: LA3591(ygbB)
LIC: LIC10610(ispF)
LBJ: LBJ_0323(ispF)
LBL: LBL_2753(ispF)
LBI: LEPBI_I0322(ispF)
LBF: LBF_0313(ispF)
SYN: slr1542
SYW: SYNW1610
SYC: syc0380_d(ispF)
SYF: Synpcc7942_1170(ispF)
SYE: Syncc9902_1508
SYG: sync_0781(ispF)
SYR: SynRCC307_1730(ispF)
SYX: SynWH7803_1723(ispF)
SYP: SYNPCC7002_A1166(ispF)
CYA: CYA_0267(ispF)
CYB: CYB_0783(ispF)
TEL: tlr2035
MAR: MAE_31930
CYT: cce_0476(ispF)
GVI: glr3547
ANA: alr3883
NPU: Npun_F5826
AVA: Ava_1811(ispF)
PMA: Pro1354(trmD)
PMM: PMM1280
PMT: PMT0356
PMN: PMN2A_0847
PMI: PMT9312_1374
PMB: A9601_14791(trmD)
PMC: P9515_14411(trmD)
PMF: P9303_19461(trmD)
PMG: P9301_14651(trmD)
PMH: P9215_15051
PMJ: P9211_13261
PME: NATL1_17001(trmD)
TER: Tery_4716(ispF)
AMR: AM1_2915(ispF)
BTH: BT_3884
BFR: BF4006
BFS: BF3780(ispF)
BVU: BVU_1639
PGI: PG0028(ispF)
PGN: PGN_0024
PDI: BDI_2574

SRU: SRU_1651(ispF)
CHU: CHU_3180(ispF)
CTE: CT1601(ispF)
CPC: Cpar_1528
CCH: Cag_1782
CPH: Cpha266_0591
CPB: Cphamn1_0610
PVI: Cvib_1388
PLT: Plut_1598
PPH: Ppha_0719
CTS: Ctha_0565
PAA: Paes_0552
DET: DET0060(ispF)
DEH: cbdb_A75(ispF)
DEB: DehaBAV1_0054
EMI: Emin_1165
DRA: DR_0230
DGE: Dgeo_0073
TTH: TTC1438
TTJ: TTHA1790
AAE: aq_957
HYA: HY04AAS1_1161
SUL: SYO3AOP1_1107
TMA: TM0647
TPT: Tpet_0283
TLE: Tlet_0532
TRQ: TRQ2_0281
TME: Tmel_0239
FNO: Fnod_1503
PMO: Pmob_1172

Exemplary 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase nucleic acids and polypeptides

ATH: AT5G60600(GcpE)
OSA: 4329911(Os02g0603800)
PPP: PHYPADRAFT_130936(HDS3)
PHYPADRAFT_55802
OLU: OSTLU_12863(HDS)
CRE: CHLREDRAFT_55268(HDS1)
CME: CML284C
PFA: PF10_0221
PFH: PFHG_04116
PYO: PY01664
TAN: TA14455
TPV: TP02_0667
ECO: b2515(ispG)
ECJ: JW2499(ispG)
ECD: ECDH10B_2681(ispG)
ECE: Z3778(ispG)
ECS: ECs3377(ispG)
ECC: c3037(ispG)
ECI: UTI89_C2836(ispG)
ECP: ECP_2520
ECV: APECO1_4009(ispG)
ECW: EcE24377A_2799(ispG)
ECX: EcHS_A2666(ispG)
ECM: EcSMS35_2667(ispG)
ECL: EcolC_1162
STY: STY2768(ispG)
STT: t0333(ispG)
SPT: SPA0344(ispG)
SPQ: SPAB_00417
SEC: SC2520(ispG)
SEH: SeHA_C2781(ispG)
SEE: SNSL254_A2718(ispG)
SEW: SeSA_A2762(ispG)
SES: SARI_00355
STM: STM2523(ispG)
YPE: YPO2879(ispG)
YPK: y1353(ispG)
YPM: YP_2745(ispG)
YPA: YPA_2319(ispG)
YPN: YPN_1259
YPP: YPDSF_2224(ispG)
YPG: YpAngola_A0418(ispG)
YPS: YPTB2841(ispG)
YPI: YpsIP31758_1186(ispG)
YPY: YPK_1293
YPB: YPTS_2950
YEN: YE1073(ispG)
SFL: SF2561(ispG)
SFX: S2733(ispG)
SFV: SFV_2562(ispG)
SSN: SSON_2597(ispG)
SBO: SBO_2539(ispG)
SBC: SbBS512_E2890(ispG)
SDY: SDY_2711(ispG)
ECA: ECA3220(ispG)
ETA: ETA_10280(ispG)
PLU: plu1376(ispG)
BUC: BU287(gcpE)
BAS: BUsg276(gcpE)
WBR: WGLp573(gcpE)
SGL: SG1760(ispG)
ENT: Ent638_3009(ispG)
ESA: ESA_00745
KPN: KPN_02845(ispG)
CKO: CKO_00270
SPE: Spro_3609
BPN: BPEN_551(ispG)
HIN: HI0368(ispG)
HIT: NTHI0488(ispG)
HIP: CGSHiEE_01170
HIQ: CGSHiGG_04650(ispG)
HDU: HD1037(ispG)
HSO: HS_0404(ispG)
HSM: HSM_0729
PMU: PM2010(ispG)
MSU: MS1919(ispG)
APL: APL_1176(ispG)
APJ: APJL_1198(ispG)

APA: APP7_1235
ASU: Asuc_2027
XFA: XF2575(ispG)
XFT: PD1956(ispG)
XFM: Xfasm12_2147
XFN: XfasM23_2062
XCC: XCC1781(ispG)
XCB: XC_2455(ispG)
XCV: XCV1829(ispG)
XAC: XAC1799(ispG)
XOO: XOO2229(ispG)
XOM: XOO_2095(ispG)
SML: Smlt1786
SMT: Smal_1524
VCH: VC0759(ispG)
VCO: VC0395_A0288(ispG)
VVU: VV1_0427(ispG)
VVY: VV0766(ispG)
VPA: VP0608(ispG)
VFI: VF0629(ispG)
VHA: VIBHAR_01067
PPR: PBPRA0763(ispG)
PAE: PA3803(ispG)
PAU: PA14_14880(ispG)
PAP: PSPA7_1311(ispG)
PPU: PP_0853(ispG)
PPF: Pput_0883(ispG)
PPG: PputGB1_0896
PPW: PputW619_4325
PST: PSPTO_1434(ispG)
PSB: Psyr_1248(ispG)
PSP: PSPPH_1320(ispG)
PFL: PFL_4954(ispG)
PFO: PflO1_4601(ispG)
PEN: PSEEN1021(ispG)
PMY: Pmen_3500
PSA: PST_3031(ispG)
CJA: CJA_1481(ispG)
PAR: Psyc_0682(gcpE)
PCR: Pcryo_0652
PRW: PsycPRwf_1902
ACI: ACIAD0561(ispG)
ACB: A1S_0502
ABM: ABSDF3001(ispG)
ABY: ABAYE3263
ABC: ACICU_00511
SON: SO_3312(ispG)
SDN: Sden_1256(ispG)
SFR: Sfri_1116(ispG)
SAZ: Sama_2365(ispG)
SBL: Sbal_2990
SBM: Shew185_3005
SBN: Sbal195_3148
SLO: Shew_1290
SPC: Sputcn32_2652(ispG)
SSE: Ssed_1432
SPL: Spea_1305
SHE: Shewmr4_1228(ispG)
SHM: Shewmr7_1299(ispG)
SHN: Shewana3_1229(ispG)
SHW: Sputw3181_1355
SHL: Shal_1367
SWD: Swoo_1544
ILO: IL2034(ispG)
CPS: CPS_4252(ispG)
PHA: PSHAb0138(ispG)
PAT: Patl_3126
SDE: Sde_1434(ispG)
MAQ: Maqu_1127(ispG)
AMC: MADE_02981
PIN: Ping_1168
MCA: MCA2483(ispG)
FTU: FTT0607(ispG)
FTF: FTF0607(ispG)
FTW: FTW_1121(ispG)
FTL: FTL_0875(ispG)
FTH: FTH_0861(ispG)
FTA: FTA_0926(ispG)
FTN: FTN_1076(ispG)
FTM: FTM_0682(ispG)
FPH: Fphi_0034
NOC: Noc_1749
AEH: Mlg_1461(ispG)
HHA: Hhal_0132(ispG)
HCH: HCH_04456(ispG)
CSA: Csal_2854(ispG)
ABO: ABO_1860(ispG)
MMW: Mmwyl1_1356

AHA: AHA_1759(ispG)
ASA: ASA_2599(ispG)
BCI: BCI_0008(ispG)
RMA: Rmag_0384
VOK: COSY_0358(ispG)
NME: NMB1310(ispG)
NMA: NMA1524(ispG)
NMC: NMC1247(ispG)
NMN: NMCC_1223
NGO: NGO0594(ispG)
NGK: NGK_1324
CVI: CV_3538(ispG)
RSO: RSc1215(ispG)
REU: Reut_A2086(ispG)
REH: H16_A2364(ispG)
RME: Rmet_2106(ispG)
BMA: BMA1345(ispG)
BMV: BMASAVP1_A1835(ispG)
BML: BMA10229_A0062(ispG)
BMN: BMA10247_1107(ispG)
BXE: Bxe_A1594(ispG)
BVI: Bcep1808_1739
BUR: Bcep18194_A5113(ispG)
BCN: Bcen_6267(ispG)
BCH: Bcen2424_1812
BCM: Bcenmc03_1836
BAM: Bamb_1750(ispG)
BAC: BamMC406_1723
BMU: Bmul_1463
BMJ: BMULJ_01780(gcpE)
BPS: BPSL1513(ispG)
BPM: BURPS1710b_2355(ispG)
BPL: BURPS1106A_2228(ispG)
BPD: BURPS668_2190(ispG)
BTE: BTH_I2234(ispG)
BPH: Bphy_1420
PNU: Pnuc_1291(ispG)
PNE: Pnec_0664
BPE: BP2199(ispG)
BPA: BPP2855(ispG)
BBR: BB3176(ispG)
BPT: Bpet2019(ispG)
BAV: BAV2344(gcpE)
RFR: Rfer_2307
POL: Bpro_2608(ispG)
PNA: Pnap_1872(ispG)
AAV: Aave_1424(ispG)
AJS: Ajs_1170(ispG)
VEI: Veis_0080(ispG)
DAC: Daci_5019
MPT: Mpe_A1996(ispG)
HAR: HEAR1264(ispG)
MMS: mma_2127
LCH: Lcho_2868
NEU: NE0148 NE0149
NET: Neut_2168(ispG)
NMU: Nmul_A2377
EBA: ebA1261(ispG)
AZO: azo0927(ispG)
DAR: Daro_2985(ispG)
TBD: Tbd_0594(ispG)
MFA: Mfla_1620(ispG)
HPY: HP0625(ispG)
HPJ: jhp0569(ispG)
HPA: HPAG1_0608(ispG)
HPS: HPSH_03735(ispG)
HHE: HH0807(ispG)
HAC: Hac_0735(ispG)
WSU: WS1302(ispG)
TDN: Suden_0376(ispG)
CJE: Cj0686(ispG)
CJR: CJE0785(ispG)
CJJ: CJJ81176_0709(ispG)
CJU: C8J_0654(ispG)
CJD: JJD26997_1321(ispG)
CFF: CFF8240_0983(ispG)
CCV: CCV52592_0322(ispG)
CHA: CHAB381_0996(ispG)
CCO: CCC13826_0680(ispG)
ABU: Abu_0656(ispG)
NIS: NIS_0337(ispG)
SUN: SUN_2134(ispG)
GSU: GSU1459(ispG)
GME: Gmet_1353
GUR: Gura_2799
GLO: Glov_1907
PCA: Pcar_2368(ispG)
PPD: Ppro_1751

DVU: DVU1344(ispG)
DVL: Dvul_1724
DDE: Dde_2207
LIP: LI0024(gcpE)
DPS: DP1163
DOL: Dole_2059
ADE: Adeh_3949
AFW: Anae109_0476
SAT: SYN_00906
SFU: Sfum_2112
WOL: WD0116(ispG)
WBM: Wbm0782(ispG)
WPI: WP0196(ispG)
AMA: AM741(ispG)
APH: APH_0442(ispG)
ERU: Erum4730(ispG)
ERW: ERWE_CDS_04950(ispG)
ERG: ERGA_CDS_04850(ispG)
ECN: Ecaj_0471(ispG)
ECH: ECH_0559(ispG)
NSE: NSE_0799(ispG)
PUB: SAR11_0517(ispG)
MLO: mll3792(ispG)
MES: Meso_3337(ispG)
PLA: Plav_1746
SME: SMc03888(ispG)
SMD: Smed_3133(ispG)
ATU: Atu2723(gcpE)
ATC: AGR_C_4936
RET: RHE_CH04009(ispG)
REC: RHECIAT_CH0004297(gcpE)
RLE: RL4630(ispG)
BME: BMEI0269(ispG)
BMF: BAB1_1788(ispG)
BMB: BruAb1_1761(ispG)
BMC: BAbS19_I16710
BMS: BR1778(ispG)
BMT: BSUIS_B1254(ispG)
BOV: BOV_1713(ispG)
BCS: BCAN_A1816(ispG)
OAN: Oant_1123
BJA: blr0936(ispG)
BRA: BRADO0546(ispG)
BBT: BBta_7633(ispG)
RPA: RPA0519(ispG)
RPB: RPB_0522(ispG)
RPC: RPC_0491(ispG)
RPD: RPD_0317(ispG)
RPE: RPE_0183(ispG)
RPT: Rpal_0520
NWI: Nwi_0494(ispG)
NHA: Nham_0620(ispG)
BHE: BH15270(ispG)
BQU: BQ12180(ispG)
BBK: BARBAKC583_0119(ispG)
BTR: Btr_2457(gcpE)
XAU: Xaut_1889
AZC: AZC_4581
MEX: Mext_1597
MRD: Mrad2831_3931
MET: M446_5049
BID: Bind_0434
CCR: CC_0851
CAK: Caul_0957
SIL: SPO2594(ispG)
SIT: TM1040_0862(ispG)
RSP: RSP_2982(ispG)
RSH: Rsph17029_1628
RSQ: Rsph17025_1861
JAN: Jann_1935(ispG)
RDE: RD1_2825(ispG)
PDE: Pden_1820(ispG)
DSH: Dshi_1184
MMR: Mmar10_2256(ispG)
HNE: HNE_0621(ispG)
ZMO: ZMO0180(ispG)
NAR: Saro_0417
SAL: Sala_1848(ispG)
SWI: Swit_2126
ELI: ELI_10365(ispG)
GOX: GOX0034(ispG)
GBE: GbCGDNIH1_0604(ispG)
ACR: Acry_1012(ispG)
GDI: GDI1913(ispG)
RRU: Rru_A0747(ispG)
MAG: amb1616(ispG)
MGM: Mmc1_3591
ABA: Acid345_1423(ispG)

SUS: Acid_1193
SWO: Swol_0891
CSC: Csac_2351
BSU: BSU25070(ispG)
BHA: BH1401(ispG)
BAN: BA4502(ispG)
BAR: GBAA4502(ispG)
BAA: BA_4950
BAT: BAS4180(ispG)
BCE: BC4276(ispG)
BCA: BCE_4358(ispG)
BCZ: BCZK4028(ispG)
BCY: Bcer98_3006
BTK: BT9727_4018(ispG)
BTL: BALH_3871(ispG)
BWE: BcerKBAB4_4131
BLI: BL03725(ispG)
BLD: BLi02683(ispG)
BCL: ABC1708(ispG)
BAY: RBAM_023380
BPU: BPUM_2235
GKA: GK2466(ispG)
GTN: GTNG_2403(ispG)
LSP: Bsph_3646
ESI: Exig_0854
LMO: lmo1441(ispG)
LMF: LMOf2365_1460(ispG)
STH: STH1501
CAC: CAC1797(gcpE)
CPE: CPE1692(ispG)
CPF: CPF_1946(ispG)
CPR: CPR_1664(ispG)
CTC: CTC01270(gcpE)
CNO: NT01CX_2141(ispG)
CTH: Cthe_0997
CDF: CD2128(ispG)
CBO: CBO2424(ispG)
CBA: CLB_2288(gcpE)
CBH: CLC_2271(gcpE)
CBL: CLK_1800(gcpE)
CBK: CLL_A1267(ispG)
CBB: CLD_2216(gcpE)
CBF: CLI_2480(gcpE)
CBE: Cbei_1197(ispG)
CKL: CKL_1425(ispG)
CPY: Cphy_2620
AMT: Amet_2680
AOE: Clos_1521
CHY: CHY_1776(ispG)
DSY: DSY2537
DRM: Dred_1968
PTH: PTH_1262(gcpE)
DAU: Daud_0617
HMO: HM1_2266(ispG)
FMA: FMG_0730
TTE: TTE1400(ispG)
TEX: Teth514_1652
TPD: Teth39_1216
MTA: Moth_1043
MPE: MYPE9400
MGA: MGA_1156
MTU: Rv2868c(ispG)
MTC: MT2936(ispG)
MRA: MRA_2893(ispG)
MTF: TBFG_12884(ispG)
MBO: Mb2893c(ispG)
MBB: BCG_2890c(ispG)
MLE: ML1581(ispG)
MPA: MAP2938c(ispG)
MAV: MAV_3725(ispG)
MSM: MSMEG_2580(ispG)
MUL: MUL_2087(ispG)
MVA: Mvan_2262
MGI: Mflv_4081(ispG)
MAB: MAB_3169c
MMC: Mmcs_2044(ispG)
MKM: Mkms_2090(ispG)
MJL: Mjls_2027(ispG)
MMI: MMAR_0275(gcpE_2)
MMAR_1838(ispG)
CGL: NCgl1938(ispG)
CGB: cg2206(ispG)
CGT: cgR_1842(ispG)
CEF: CE1903(ispG)
CDI: DIP1498(ispG)
CJK: jk1165(ispG)
CUR: cu0833
NFA: nfa41180(ispG)

RHA: RHA1_ro06590(ispG)
SCO: SCO5696(ispG) SCO6767(ispG)
SMA: SAV1647(ispG) SAV2561(ispG)
SGR: SGR_1821(gcpE)
TWH: TWT186(ispG)
TWS: TW586(ispG)
LXX: Lxx12200(ispG)
CMI: CMM_2156(ispG)
ART: Arth_1404(ispG)
AAU: AAur_1546(ispG)
RSA: RSal33209_0641
KRH: KRH_16120(ispG)
PAC: PPA1506(ispG)
NCA: Noca_3202
TFU: Tfu_0749(ispG)
FRA: Francci3_3573(ispG)
FRE: Franean1_1170
FAL: FRAAL5772(ispG)
ACE: Acel_1522
KRA: Krad_1429
SEN: SACE_5992(ispG)
STP: Strop_1352(ispG)
SAQ: Sare_1304
BLO: BL0098(ispG)
BLJ: BLD_0116(gcpE)
BAD: BAD_1157(ispG)
RXY: Rxyl_1406
FNU: FN0478
RBA: RB2118(gcpE)
OTE: Oter_4634
MIN: Minf_1968(gcpE)
AMU: Amuc_1388
CTR: CT057(gcpE)
CTA: CTA_0061(gcpE)
CTB: CTL0313(gcpE)
CTL: CTLon_0308(gcpE)
CMU: TC0327(gcpE)
CPN: CPn0373(gcpE)
CPA: CP0383
CPJ: CPj0373(gcpE)
CPT: CpB0385(aarC)
CCA: CCA00423(gcpE)
CAB: CAB409
CFE: CF0584(gcpE)
PCU: pc0740(gcpE)
TPA: TP0446
TPP: TPASS_0446(gcpE)
TDE: TDE1265(ispG)
LIL: LA3160(gcpE)
LIC: LIC10955(gcpE)
LBJ: LBJ_0737(gcpE)
LBL: LBL_2341(gcpE)
LBI: LEPBI_I1285(ispG)
LBF: LBF_1231(gcpE)
SYN: slr2136(gcpE)
SYW: SYNW1174(ispG)
SYC: syc0817_d(ispG)
SYF: Synpcc7942_0713(ispG)
SYD: Syncc9605_1298(ispG)
SYE: Syncc9902_1179(ispG)
SYG: sync_1674(ispG)
SYR: SynRCC307_1462(ispG)
SYX: SynWH7803_1475(ispG)
SYP: SYNPCC7002_A0743(ispG)
CYA: CYA_2387(ispG)
CYB: CYB_0121(ispG)
TEL: tlr0996
MAR: MAE_28180
CYT: cce_2312(ispG)
GVI: gll3622
ANA: all2501
NPU: Npun_F5054
AVA: Ava_0433(ispG)
PMA: Pro1015(ispG)
PMM: PMM0676(ispG)
PMT: PMT0777(ispG)
PMN: PMN2A_0109(ispG)
PMI: PMT9312_0676(ispG)
PMB: A9601_07311(ispG)
PMC: P9515_07491(ispG)
PMF: P9303_14341(ispG)
PMG: P9301_07291(ispG)
PMH: P9215_07611(gcpE)
PMJ: P9211_07901(gcpE)
PME: NATL1_07341(ispG)
TER: Tery_4522(ispG)
AMR: AM1_0149(ispG)
BTH: BT_2517

BFR: BF4365
BFS: BF4164
BVU: BVU_1415
PGI: PG0952(ispG)
PGN: PGN_0998
PDI: BDI_3173
SRU: SRU_0682(ispG)
CHU: CHU_2192(ispG)
CTE: CT0147(gcpE)
CPC: Cpar_1891
CCH: Cag_0349
CPH: Cpha266_0225
CPB: Cphamn1_0311
PVI: Cvib_1613
PLT: Plut_1970
PPH: Ppha_2687
CTS: Ctha_1524
PAA: Paes_0280
DET: DET0369(ispG)
DEH: cbdb_A311(ispG)
DEB: DehaBAV1_0351
EMI: Emin_0687
DRA: DR_0386(ispG)
DGE: Dgeo_0704(ispG)
TTH: TTC1677(ispG)
TTJ: TTHA0305(ispG)
AAE: aq_1540(gcpE)
HYA: HY04AAS1_1229
SUL: SYO3AOP1_0412
TMA: TM0891
TPT: Tpet_0036
TLE: Tlet_0656
TRQ: TRQ2_0036
TME: Tmel_0263
FNO: Fnod_0952
PMO: Pmob_1941

Exemplary 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase nucleic acids and polypeptides

ATH: AT4G34350(CLB6)
OSA: 4334003(Os03g0731900)
PPP: PHYPADRAFT_194018
PHYPADRAFT_206243
OLU: OSTLU_32979(IDS)
CRE: CHLREDRAFT_59822(IDS1)
CME: CMJ152C
PFA: MAL1P1.35
PFD: PFDG_01560
PFH: PFHG_00328
PYO: PY01243
TAN: TA17670
TPV: TP03_0674
ECO: b0029(ispH)
ECJ: JW0027(ispH)
ECD: ECDH10B_0030(ispH)
ECE: Z0034(ispH)
ECS: ECs0032(ispH)
ECC: c0033(ispH)
ECI: UTI89_C0031(ispH)
ECP: ECP_0027
ECV: APECO1_1954(ispH)
ECW: EcE24377A_0029(ispH)
ECX: EcHS_A0031(ispH)
ECM: EcSMS35_0027(ispH)
ECL: EcolC_3626
STY: STY0058(ispH)
STT: t0051(ispH)
SPT: SPA0050(ispH)
SPQ: SPAB_00059
SEC: SC0043(ispH)
SEH: SeHA_C0053(ispH)
SEE: SNSL254_A0053(ispH)
SEW: SeSA_A0054(ispH)
SES: SARI_02945
STM: STM0049(ispH)
YPE: YPO0477(ispH)
YPK: y3697(ispH)
YPM: YP_3702(ispH)
YPA: YPA_4071(ispH)
YPN: YPN_0350
YPP: YPDSF_3154(ispH)
YPG: YpAngola_A0787(ispH)
YPS: YPTB0620(ispH)
YPI: YpsIP31758_3457(ispH)
YPY: YPK_3585
YPB: YPTS_0644
YEN: YE0619(ispH)
SFL: SF0026(ispH)
SFX: S0028(ispH)
SFV: SFV_0023(ispH)
SSN: SSON_0034(ispH)
SBO: SBO_0028(ispH)
SBC: SbBS512_E0033(ispH)
SDY: SDY_0051(ispH)
ECA: ECA3873(ispH)
ETA: ETA_07150(ispH)
PLU: plu0594(ispH)
BUC: BU147(lytB)
BAS: BUsg140(lytB)
WBR: WGLp292(lytB)
SGL: SG0417(ispH)
ENT: Ent638_0587(ispH)
ESA: ESA_03309
KPN: KPN_00024(ispH)
CKO: CKO_03363
SPE: Spro_0701
BPN: BPEN_124(ispH)
HIN: HI1007(ispH)
HIT: NTHI1182(ispH)
HIP: CGSHiEE_06935(ispH)
HIQ: CGSHiGG_08635(ispH)
HDU: HD0064(ispH)
HSO: HS_0184(ispH)
HSM: HSM_0050
PMU: PM1664(ispH)
MSU: MS1749(ispH)
APL: APL_1520(ispH)

APJ: APJL_1546(ispH)
APA: APP7_1580
ASU: Asuc_1874
XFA: XF2416(ispH)
XFT: PD1435(ispH)
XFM: Xfasm12_1576
XFN: XfasM23_1519
XCC: XCC1157(ispH)
XCB: XC_3085(ispH)
XCV: XCV1292(ispH)
XAC: XAC1256(ispH)
XOO: XOO1628(ispH)
XOM: XOO_1514(ispH)
SML: Smlt1342(ispH)
SMT: Smal_1127
VCH: VC0685(ispH)
VCO: VC0395_A0217(ispH)
VVU: VV1_0504(ispH)
VVY: VV0690(ispH)
VPA: VP0537(ispH)
VFI: VF0470(ispH)
VHA: VIBHAR_00983
PPR: PBPRA0594(ispH)
PAE: PA4557(ispH)
PAU: PA14_60330(ispH)
PAP: PSPA7_3192(ispH2)
PSPA7_5197(ispH1)
PPU: PP_0606(ispH)
PPF: Pput_0647(ispH)
PPG: PputGB1_0652
PPW: PputW619_4556
PST: PSPTO_0809(ispH)
PSB: Psyr_0713(ispH)
PSP: PSPPH_0724(ispH)
PFL: PFL_5318(ispH)
PFO: Pfl01_4849(ispH)
PEN: PSEEN4689(ispH)
PMY: Pmen_0956
PSA: PST_0967(ispH)
CJA: CJA_3214(ispH)
PAR: Psyc_1722(lytB)
PCR: Pcryo_2002
PRW: PsycPRwf_0578
ACI: ACIAD3322(ispH)
ACB: A1S_3169
ABM: ABSDF0323(ispH)
ABY: ABAYE0313
ABC: ACICU_03371
SON: SO_3529(lytB)
SDN: Sden_2720
SFR: Sfri_2887
SAZ: Sama_0927
SBL: Sbal_1057
SBM: Shew185_1124
SBN: Sbal195_1159
SLO: Shew_1102
SPC: Sputcn32_1062
SSE: Ssed_1197
SPL: Spea_1086
SHE: Shewmr4_2954
SHM: Shewmr7_3036
SHN: Shewana3_3133
SHW: Sputw3181_3103
SHL: Shal_1134
SWD: Swoo_1294
ILO: IL1125(lytB)
CPS: CPS_1211(ispH)
PHA: PSHAa0921(ispH)
PAT: Patl_3175
SDE: Sde_2563
MAQ: Maqu_0865
AMC: MADE_03027
PIN: Ping_3268
MCA: MCA1815(ispH)
FTU: FTT0833(ispH)
FTF: FTF0833(ispH)
FTW: FTW_1353(ispH)
FTL: FTL_0327
FTH: FTH_0325
FTA: FTA_0348(ispH)
FTM: FTM_0425(lytB)
FPH: Fphi_0475
NOC: Noc_1744
AEH: Mlg_0854
HHA: Hhal_1834
HCH: HCH_05930(ispH)
CSA: Csal_0484
ABO: ABO_0462(lytB)

MMW: Mmwyl1_4227
AHA: AHA_0685(ispH)
ASA: ASA_0687(lytB)
BCI: BCI_0558(ispH)
RMA: Rmag_1023
VOK: COSY_0924(lytB)
NME: NMB1831(ispH)
NMA: NMA0624(ispH)
NMC: NMC0385(ispH)
NMN: NMCC_0391(lytB)
NGO: NGO0072(ispH)
NGK: NGK_0106
CVI: CV_3567(ispH)
RSO: RSc2442(ispH)
REU: Reut_A2730(ispH) Reut_B4898(ispH)
REH: H16_A3031(ispH) H16_B2169(ispH)
RME: Rmet_2868(ispH) Rmet_4169(ispH)
BMA: BMA2228(ispH) BMAA1962(ispH)
BMV: BMASAVP1_0980(ispH)
BMASAVP1_A2644(ispH)
BML: BMA10229_1267(ispH)
BMA10229_A1018(ispH)
BMN: BMA10247_2097(ispH)
BMA10247_A2242(ispH)
BXE: Bxe_A0820(ispH) Bxe_B0018(ispH)
BVI: Bcep1808_2577 Bcep1808_3716
BUR: Bcep18194_A5831(ispH)
Bcep18194_B0106(ispH)
BCN: Bcen_1888(ispH) Bcen_5308(ispH)
BCH: Bcen2424_2499 Bcen2424_5552
BCM: Bcenmc03_2524 Bcenmc03_4720
BAM: Bamb_2546(ispH) Bamb_4876(ispH)
BAC: BamMC406_2417 BamMC406_5423
BMU: Bmul_0795 Bmul_3253
BMJ: BMULJ_02464(lytB)
BMULJ_05272(lytB)
BPS: BPSL0919(ispH) BPSS2168(ispH)
BPM: BURPS1710b_1141(ispH)
BURPS1710b_A1285(ispH)
BPL: BURPS1106A_0986(ispH)
BURPS1106A_A2929(ispH)
BPD: BURPS668_0981(ispH)
BURPS668_A3054(ispH)
BTE: BTH_I0783(ispH-1) BTH_II2243(ispH-2)
BPH: Bphy_0587 Bphy_4130
PNU: Pnuc_1731
PNE: Pnec_1449
BPE: BP1237(ispH)
BPA: BPP1852(ispH)
BBR: BB3256(ispH)
BPT: Bpet3147(lytB)
BAV: BAV2403(ispH)
RFR: Rfer_3248
POL: Bpro_0951
PNA: Pnap_3337
AAV: Aave_3771
AJS: Ajs_3448
VEI: Veis_1652
DAC: Daci_1906
MPT: Mpe_A2693
HAR: HEAR2466(ispH)
MMS: mma_2549
LCH: Lcho_0693
NEU: NE0649(lytB)
NET: Neut_1903
NMU: Nmul_A0089
EBA: ebA4444(ispH)
AZO: azo1202(ispH)
DAR: Daro_3043
TBD: Tbd_1860
MFA: Mfla_2431
HPY: HP0400(ispH)
HPJ: jhp0981(ispH)
HPA: HPAG1_0992(ispH)
HPS: HPSH_05405(ispH)
HHE: HH0138(ispH)
HAC: Hac_0458(ispH)
WSU: WS1310(ispH)
TDN: Suden_0872(ispH)
CJE: Cj0894c(ispH)
CJR: CJE0973(ispH)
CJJ: CJJ81176_0903(ispH)
CJU: C8J_0831(lytB)
CJD: JJD26997_0919(ispH)
CFF: CFF8240_1251(ispH)
CCV: CCV52592_0515(ispH)

CHA: CHAB381_0483(ispH)
CCO: CCC13826_1566(ispH)
ABU: Abu_2050(ispH)
NIS: NIS_0662(ispH)
SUN: SUN_0548(ispH)
GSU: GSU2604(lytB)
GME: Gmet_0866
GUR: Gura_1466
GLO: Glov_2146
PCA: Pcar_1883(lytB)
PPD: Ppro_1349
DVU: DVU0055(ispH)
DVL: Dvul_2906
DDE: Dde_0390
LIP: LI0728(lytB)
DPS: DP2166
DOL: Dole_0383
ADE: Adeh_1519
AFW: Anae109_2302
SAT: SYN_02454
SFU: Sfum_1812
WOL: WD1274(ispH)
WBM: Wbm0046(ispH)
WPI: WP0811(lytB)
AMA: AM804(ispH)
APH: APH_0380(ispH)
ERU: Erum5180(ispH)
ERW: ERWE_CDS_05430(ispH)
ERG: ERGA_CDS_05330(ispH)
ECN: Ecaj_0526(ispH)
ECH: ECH_0502(ispH)
NSE: NSE_0438(ispH)
PUB: SAR11_0124(lytB)
MLO: mlr7502(ispH)
MES: Meso_0748(ispH)
PLA: Plav_0686
SME: SMc00016(ispH)
SMD: Smed_0527(ispH)
ATU: Atu0774(lytB)
ATC: AGR_C_1414(lytB)
RET: RHE_CH00961(ispH)
REC: RHECIAT_CH0001056(ispH)
RLE: RL1030(ispH)
BME: BMEI1459(ispH)
BMF: BAB1_0501(ispH)
BMB: BruAb1_0497(ispH)
BMC: BAbS19_I04640
BMS: BR0475(ispH)
BMT: BSUIS_A0502(ispH)
BOV: BOV_0480(ispH)
BCS: BCAN_A0482(ispH)
OAN: Oant_0589
BJA: bll3007(ispH) blr1314
BRA: BRADO2632(ispH)
BRADO6588(ispH1)
BBT: BBta_0948(ispH1) BBta_2972(ispH)
RPA: RPA3734(ispH) RPA4271(lytB2)
RPB: RPB_1340 RPB_1729(ispH)
RPC: RPC_1726(ispH) RPC_4078
RPD: RPD_3570(ispH) RPD_4030
RPE: RPE_1816(ispH) RPE_4130
RPT: Rpal_4255 Rpal_4751
NWI: Nwi_2266(ispH) Nwi_2689
NHA: Nham_2679(ispH) Nham_3745
BHE: BH04410(ispH)
BQU: BQ03600(ispH)
BBK: BARBAKC583_0406(ispH)
BTR: Btr_0655(lytB)
XAU: Xaut_2355
AZC: AZC_1468
MEX: Mext_2593
MRD: Mrad2831_4312
MET: M446_6025
BID: Bind_1904
CCR: CC_3361
CAK: Caul_4391
SIL: SPO3207(ispH)
SIT: TM1040_2569
RSP: RSP_1666(lytB)
RSH: Rsph17029_0299
RSQ: Rsph17025_2580
JAN: Jann_0507
RDE: RD1_1355(ispH)
PDE: Pden_3619
DSH: Dshi_0188
MMR: Mmar10_2215
HNE: HNE_2713(ispH)
ZMO: ZMO0875(ispH)

NAR: Saro_1087
SAL: Sala_1136
SWI: Swit_2692
ELI: ELI_01560
GOX: GOX0179
GBE: GbCGDNIH1_1875
ACR: Acry_1832
GDI: GDI3102(ispH)
RRU: Rru_A0059
MAG: amb0764
MGM: Mmc1_3428
ABA: Acid345_1739
SUS: Acid_1259
BSU: BSU25160(ispH)
BHA: BH1382(ispH)
BAN: BA4511(ispH)
BAR: GBAA4511(ispH)
BAA: BA_4959
BAT: BAS4190(ispH)
BCA: BCE_4368(ispH)
BCZ: BCZK4038(ispH)
BCY: Bcer98_3015
BTK: BT9727_4028(ispH)
BTL: BALH_3881(ispH)
BWE: BcerKBAB4_4140
BLI: BL03721(ispH)
BLD: BLi02695(ispH)
BCL: ABC1694(ispH)
BAY: RBAM_023470(yqfP)
BPU: BPUM_2249(yqfP)
GKA: GK2477(ispH)
GTN: GTNG_2414(ispH)
LSP: Bsph_3685
ESI: Exig_0836
LMO: lmo1451(ispH)
LMF: LMOf2365_1470(ispH)
STH: STH910(ispH)
CPE: CPE1085(lytB)
CPF: CPF_1341(ispH)
CPR: CPR_1152(ispH)
CTC: CTC01314
CNO: NT01CX_2096
CTH: Cthe_0714
CDF: CD1818(ispH)
AMT: Amet_2625
AOE: Clos_1562
DRM: Dred_1154
TTE: TTE1352(lytB)
TEX: Teth514_1606
TPD: Teth39_1169
MPE: MYPE1330
MTU: Rv1110(ispH) Rv3382c(lytB1)
MTC: MT1141(ispH) MT3490(lytB-2)
MRA: MRA_1121(ispH) MRA_3422(lytB1)
MTF: TBFG_11132(ispH) TBFG_13416
MBO: Mb1140(ispH) Mb3414c(lytB1)
MBB: BCG_1170(ispH) BCG_3451c(lytB1)
MLE: ML1938(ispH)
MPA: MAP2684c(ispH)
MAV: MAV_1230(ispH)
MSM: MSMEG_5224(ispH)
MUL: MUL_0168(ispH)
MVA: Mvan_4631
MGI: Mflv_2079(ispH)
MAB: MAB_1257
MMC: Mmcs_4105(ispH)
MKM: Mkms_4181(ispH)
MJL: Mjls_4336(ispH)
MMI: MMAR_0277(lytB2)
MMAR_4355(ispH)
CGL: NCgl0982(ispH)
CGB: cg1164(ispH)
CGT: cgR_1109(ispH)
CEF: CE1079(ispH)
CDI: DIP0943(ispH)
CJK: jk1449(ispH)
CUR: cu0618
NFA: nfa47950(ispH)
RHA: RHA1_ro05870(ispH)
SCO: SCO5058(ispH)
SMA: SAV3210(ispH)
SGR: SGR_2472
TWH: TWT642(ispH)
TWS: TW664(ispH)
LXX: Lxx16760(ispH)
CMI: CMM_2228(ispH)
ART: Arth_2833(ispH)
RSA: RSal33209_1156

KRH: KRH_07120(ispH)
PAC: PPA0572(ispH)
NCA: Noca_1075
TFU: Tfu_0471(ispH)
FRA: Francci3_0824 Francci3_3881(ispH)
FRE: Franean1_0845 Franean1_5712
FAL: FRAAL1433(ispH) FRAAL6150(ispH)
ACE: Acel_1858
KRA: Krad_1123
SEN: SACE_0939(ispH) SACE_4326(ispH)
STP: Strop_0879(ispH)
SAQ: Sare_0824
BLO: BL1361(ispH)
BLJ: BLD_0227(lytB)
BAD: BAD_1081(ispH)
RXY: Rxyl_2212
RBA: RB9288(lytB)
OTE: Oter_3652
MIN: Minf_2119(lytB)
AMU: Amuc_1646
CTR: CT859(ispH)
CTA: CTA_0937(ispH)
CTB: CTL0234
CTL: CTLon_0234(ispH)
CMU: TC0249(ispH)
CPN: CPn1017(ispH)
CPA: CP0836(ispH)
CPJ: CPj1017(ispH)
CPT: CpB1055(ispH)
CCA: CCA00744(ispH)
CAB: CAB711(ispH)
CFE: CF0272(ispH)
PCU: pc1078(ispH)
TPA: TP0547
TPP: TPASS_0547(lytB)
TDE: TDE1096(ispH)
LIL: LA2420(lytB)
LIC: LIC11529(lytB)
LBJ: LBJ_1807(lytB)
LBL: LBL_1476(lytB)
LBI: LEPBI_I1588(ispH)
LBF: LBF_1537(lytB)
SYN: slr0348
SYW: SYNW0252(lytB)
SYC: syc1431_d(lytB)
SYF: Synpcc7942_0073
SYD: Syncc9605_0246
SYE: Syncc9902_0275
SYG: sync_0292(ispH)
SYR: SynRCC307_2319(lytB)
SYX: SynWH7803_0296(lytB)
SYP: SYNPCC7002_A0517(ispH)
CYA: CYA_1148(ispH)
CYB: CYB_2643(ispH)
TEL: tlr1041
MAR: MAE_16190
CYT: cce_1108
GVI: glr3299
ANA: all0985
NPU: Npun_R3286
AVA: Ava_2949
PMA: Pro0296(lytB)
PMM: PMM0264(lytB)
PMT: PMT1854(lytB)
PMN: PMN2A_1630
PMI: PMT9312_0266
PMB: A9601_02861(lytB)
PMC: P9515_02971(lytB)
PMF: P9303_24821(lytB)
PMG: P9301_02871(lytB)
PMH: P9215_02881(lytB)
PMJ: P9211_02911(lytB)
PME: NATL1_03421(lytB)
TER: Tery_4479
AMR: AM1_4950(ispH)
BTH: BT_2061(ispH)
BFR: BF3748(ispH)
BFS: BF3536(ispH)
BVU: BVU_1936
PGI: PG0604(ispH)
PGN: PGN_0647
PDI: BDI_3740(ispH)
SRU: SRU_1880(ispH)
CHU: CHU_0087(ispH)
CTE: CT0283(ispH)
CPC: Cpar_1751
CCH: Cag_0579(ispH)
CPH: Cpha266_0414(ispH)

CPB: Cphamn1_0456
PVI: Cvib_1518(ispH)
PLT: Plut_1736(ispH)
PPH: Ppha_0448
CTS: Ctha_0114
PAA: Paes_0419
DET: DET1344(ispH)
DEH: cbdb_A1294(ispH)
DEB: DehaBAV1_1155
EMI: Emin_0409
DRA: DR_2164
DGE: Dgeo_1010
TTH: TTC1983(lytB)
TTJ: TTHA0015
AAE: aq_1739(lytB)
HYA: HY04AAS1_1048
SUL: SYO3AOP1_1148
TMA: TM1444
TPT: Tpet_1350
TLE: Tlet_1650
TRQ: TRQ2_1336
PMO: Pmob_1619

Exemplary isopentenyl-diphosphate delta-isomerase nucleic acids and polypeptides

HSA: 3422(IDI1) 91734(IDI2)
PTR: 450262(IDI2) 450263(IDI1)
MCC: 710052(LOC710052)
721730(LOC721730)
MMU: 319554(Idi1)
RNO: 89784(Idi1)
BTA: 514293(IDI1)
MDO: 100021550(LOC100021550)
100021613(LOC100021613)
100021638(LOC100021638)
OAA: 100080658(LOC100080658)
GGA: 420459(IDI1)
XLA: 494671(LOC494671)
XTR: 496783(idi2)
SPU: 586184(LOC586184)
NVE:
NEMVE_v1g121175(NEMVEDRAFT_v1g1
21175)
DME: Dmel_CG5919(CG5919)
DPO: Dpse_GA19228
AGA: AgaP_AGAP001704
AAG: AaeL_AAEL006144
TCA: 660176(LOC660176)
CEL: K06H7.9(idi-1)
CBR: CBG22969
BMY: Bm1_16940
ATH: AT3G02780(IDI2/IPIAT1/IPP2)
AT5G16440(IPP1)
OSA: 4338791(Os05g0413400)
4343523(Os07g0546000)
PPP: PHYPADRAFT_56143
OLU: OSTLU_13493
CRE: CHLREDRAFT_24471(IDI1)
CME: CMB062C
SCE: YPL117C(IDI1)
AGO: AGOS_ADL268C
KLA: KLLA0F00924g
DHA: DEHA0G20009g
PIC: PICST_68990(IDI1)
VPO: Kpol_479p9
CGR: CAGL0J06952g
YLI: YALI0F04015g
SPO: SPBC106.15(idi1)
NCR: NCU07719
PAN: PODANSg7228
MGR: MGG_07125
FGR: FG09722.1
ANI: AN0579.2
AFM: AFUA_6G11160
AOR: AO090023000500
ANG: An08g07570
CNE: CNA02550
CNB: CNBA2380
LBC: LACBIDRAFT_291469
UMA: UM04838.1
MGL: MGL_1929
ECU: ECU02_0230
MBR: MONBRDRAFT_34433
GLA: GL50803_6335
DDI: DDB_0191342(ipi)
TET: TTHERM_00237280
TTHERM_00438860
PTM: GSPATT00007643001
GSPATT00011951001
TBR: Tb09.211.0700
TCR: 408799.19 510431.10
LMA: LmjF35.5330
EHI: EHI_194410
TVA: TVAG_116230 TVAG_495540
ECO: b2889(idi)
ECJ: JW2857(idi)
ECD: ECDH10B_3063(idi)
ECE: Z4227
ECS: ECs3761
ECC: c3467
ECI: UTI89_C3274
ECP: ECP_2882
ECV: APECO1_3638
ECW: EcE24377A_3215(idi)
ECX: EcHS_A3048(idi)
ECM: EcSMS35_3022(idi)
ECL: EcolC_0820

STY: STY3195
STT: t2957
SPT: SPA2907(idi)
SPQ: SPAB_03786
SEC: SC2979(idi)
SEH: SeHA_C3270(idi)
SEE: SNSL254_A3273(idi)
SEW: SeSA_A3207(idi)
SES: SARI_04611
STM: STM3039(idi)
SFL: SF2875
SFX: S3074
SFV: SFV_2937
SSN: SSON_3042 SSON_3489(yhfK)
SBO: SBO_3103
SBC: SbBS512_E3308(idi)
SDY: SDY_3193
ECA: ECA2789
ETA: ETA_22390(idi)
PLU: plu3987
ENT: Ent638_3307
ESA: ESA_00346
KPN: KPN_03317(idi)
CKO: CKO_04250
SPE: Spro_2201
VPA: VPA0278
VFI: VF0403
VHA: VIBHAR_04924
PPR: PBPRA0469
PEN: PSEEN4850
PSA: PST_3876
CBU: CBU_0607(mvaD)
CBS: COXBURSA331_A0720(mvaD)
CBD: CBUD_0619(mvaD)
LPN: lpg2051
LPF: lpl2029
LPP: lpp2034
LPC: LPC_1537(fni)
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)
SCL: sce1761(idi)
RPR: RP452
RTY: RT0439(idi)
RCO: RC0744
RFE: RF_0785(fni)
RBE: RBE_0731(fni)
RBO: A1I_04760
RAK: A1C_04195
RCM: A1E_02555
RRI: A1G_04195
RRJ: RrIowa_0882
RMS: RMA_0766(fni)
MLO: mlr6371
MES: Meso_4299
RET: RHE_PD00245(ypd00046)
REC: RHECIAT_PB0000285
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
SWO: Swol_1341
BSU: BSU22870(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
BWE: BcerKBAB4_1422
BLI: BL02217(fni)
BLD: BLi02426

BAY: RBAM_021020(fni)
BPU: BPUM_2020(fni)
OIH: OB0537
SAU: SA2136(fni)
SAV: SAV2346(fni)
SAW: SAHV_2330(fni)
SAM: MW2267(fni)
SAR: SAR2431(fni)
SAS: SAS2237
SAC: SACOL2341(fni)
SAB: SAB2225c(fni)
SAA: SAUSA300_2292(fni)
SAX: USA300HOU_2327
SAO: SAOUHSC_02623
SAJ: SaurJH9_2370
SAH: SaurJH1_2416
SAE: NWMN_2247(idi)
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
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SPF: SpyM51123(fni)
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SPW: SPCG_0379(fni)
SPX: SPG_0349
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SAN: gbs1393
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STL: stu0562(idi)
STE: STER_0601
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SSU: SSU05_0292
SSV: SSU98_0288
SGO: SGO_0242
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LSA: LSA0905(idi)
LSL: LSL_0682
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CGB: cg2531(idi)
CGT: cgR_2177
CEF: CE2207
CDI: DIP1730(idi)
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KRH: KRH_03040(idi)
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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
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Exemplary isoprene synthase nucleic acids and polypeptides

Genbank Accession Nos.
AY341431
AY316691
AY279379
AJ457070
AY182241

CLAIMS

What is claimed is:

1. Cells comprising (i) a heterologous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide or (ii) a duplicate copy of an endogenous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide.

2. The cells of claim 1, wherein the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide.

3. The cells of claim 1 or 2, wherein the cells in culture produce greater than about 400 nmole/g_{wcm}/hr of isoprene.

4. The cells of claim 1 or 2, wherein more than about 0.02 molar percent of the carbon that the cells consume from a cell culture medium is converted into isoprene.

5. A method of producing isoprene, the method comprising

(a) culturing cells comprising (i) a heterologous nucleic acid encoding a heterologous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide or (ii) a duplicate copy of an endogenous nucleic acid encoding an iron-sulfur cluster-interacting redox polypeptide, a DXP pathway polypeptide, a MVA pathway polypeptide, and an isoprene synthase polypeptide under suitable culture conditions for the production of isoprene, and

(b) producing isoprene.

6. The method of claim 5, wherein the cells further comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding an IDI (isopentenyl-diphosphate delta-isomerase) polypeptide.

7. The method of claim 5 or 6, wherein the cells in culture produce greater than about 400 nmole/g_{wcm}/hr of isoprene.

8. The method of claim 5 or 6, wherein more than about 0.02 molar percent of the carbon that the cells consume from a cell culture medium is converted into isoprene.

Figure 1

1-
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Figure 2

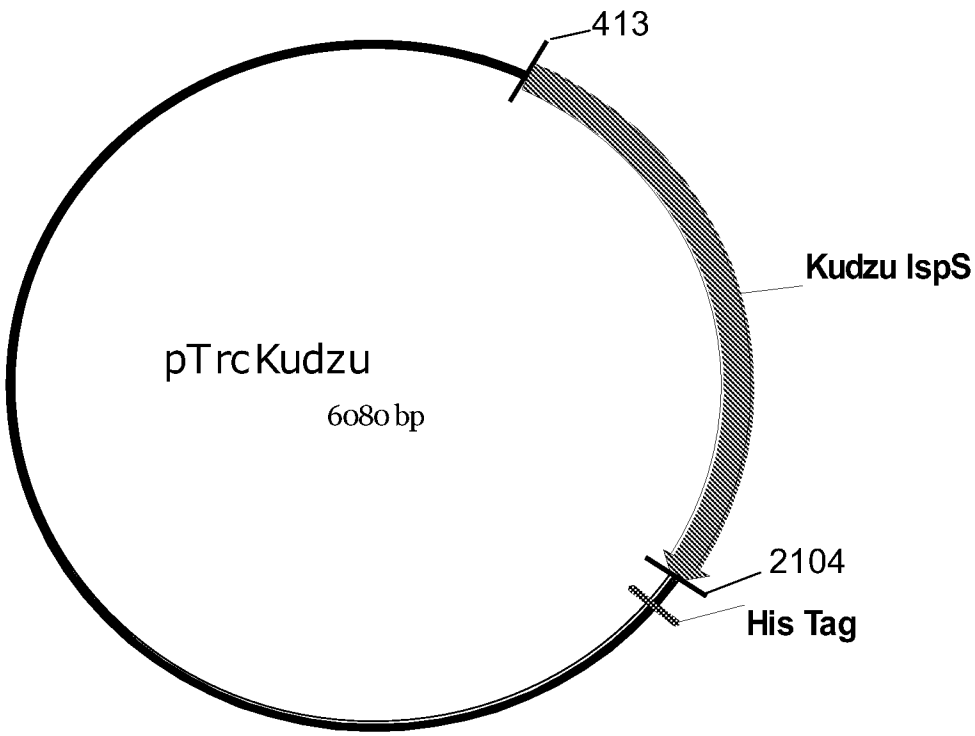

Figure 3A

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Figure 3B

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Figure 3C

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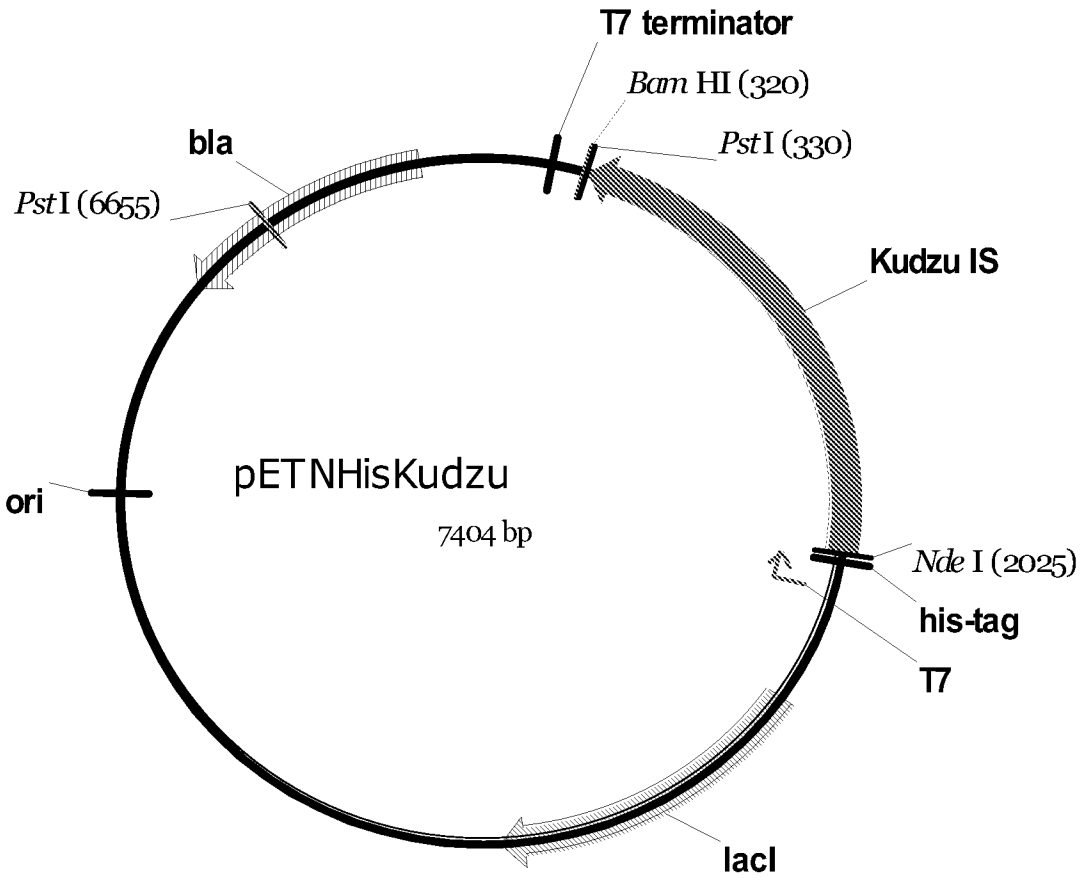
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Figure 5A

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Figure 5B

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Figure 5C

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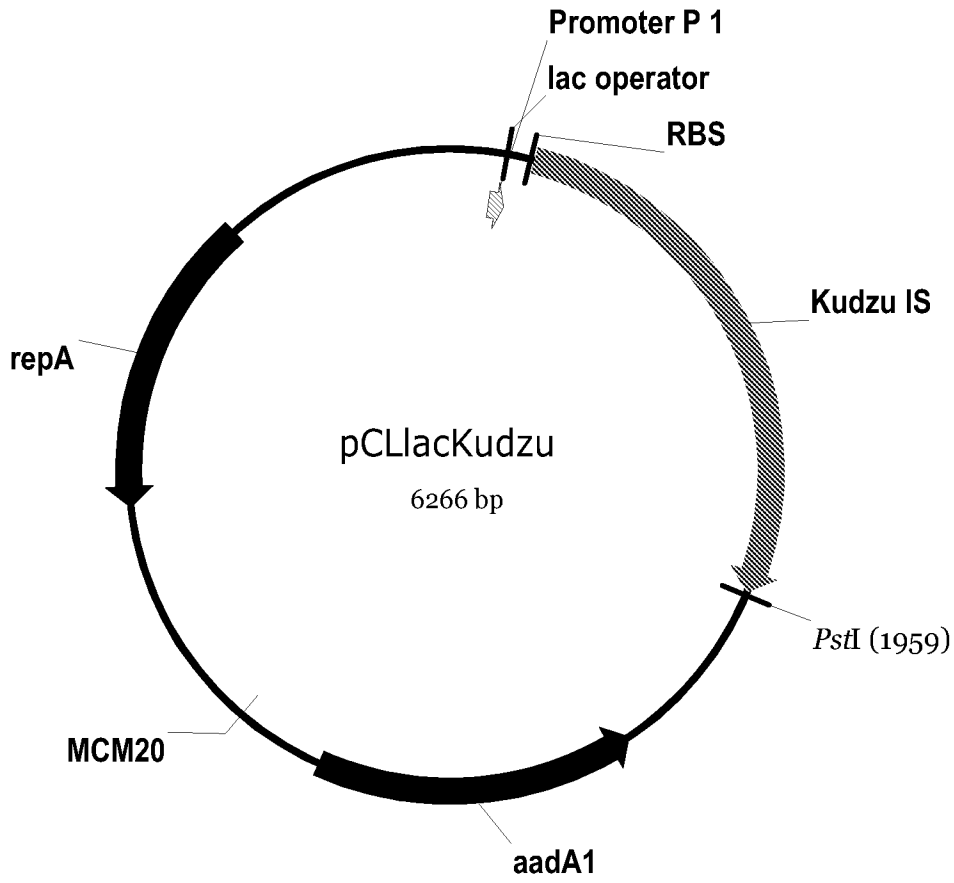
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Figure 7A

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Figure 7C

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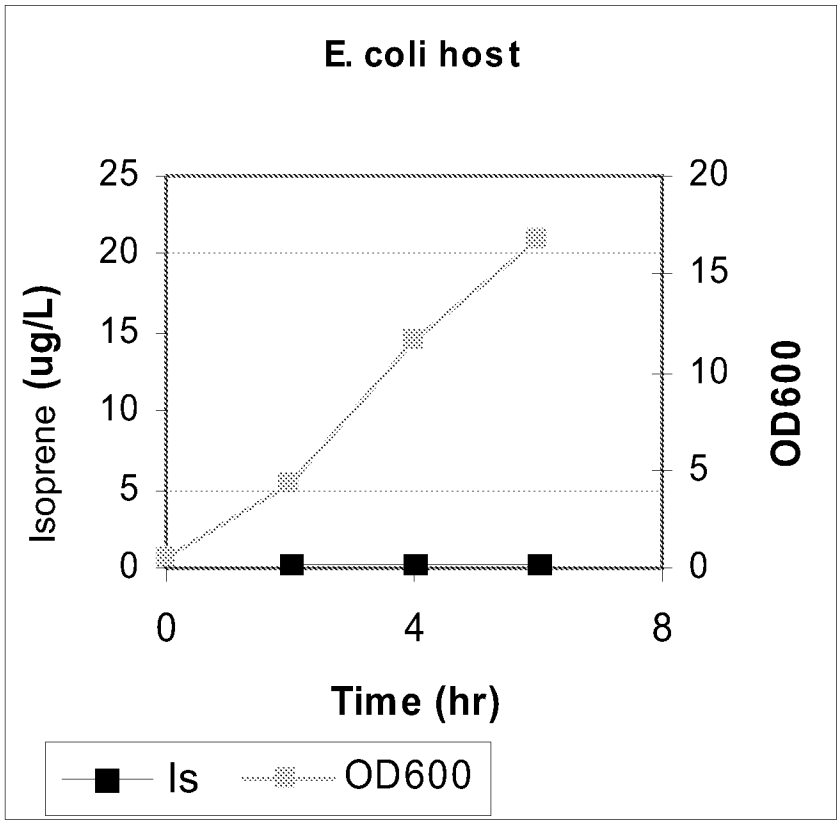
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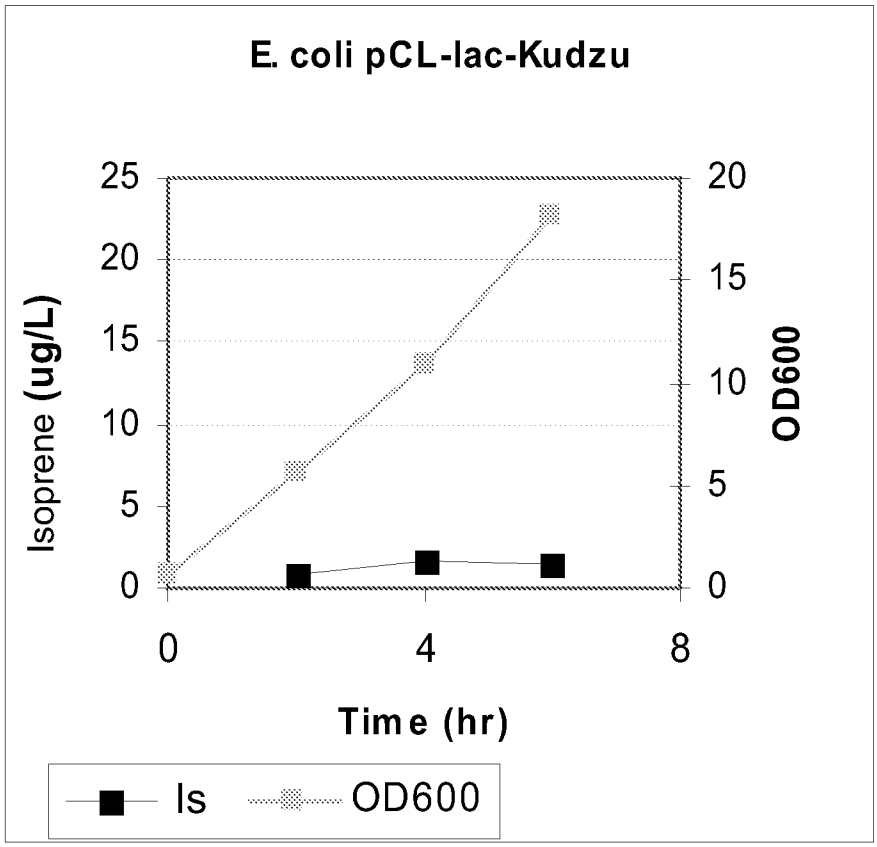
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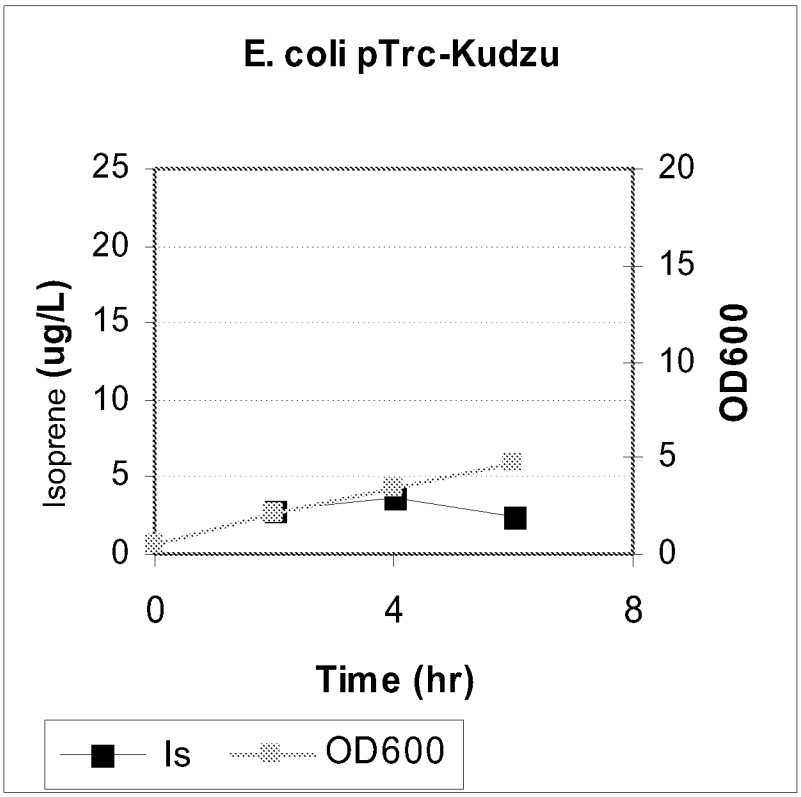
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OD600

Figure 8D

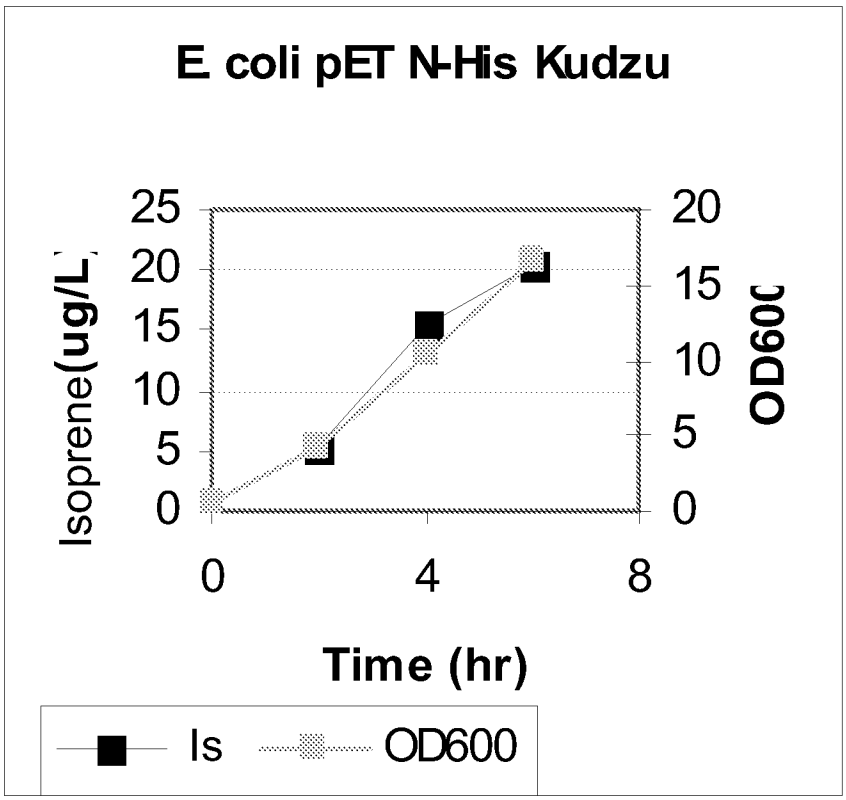
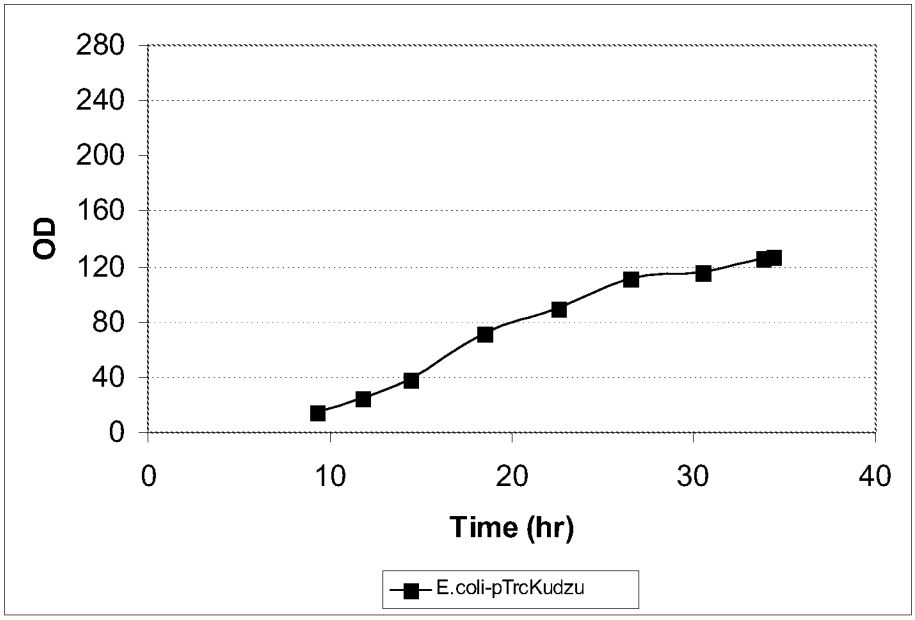

E. coli pET N-His Kudzu
Isoprene(ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 9

A.


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

B.

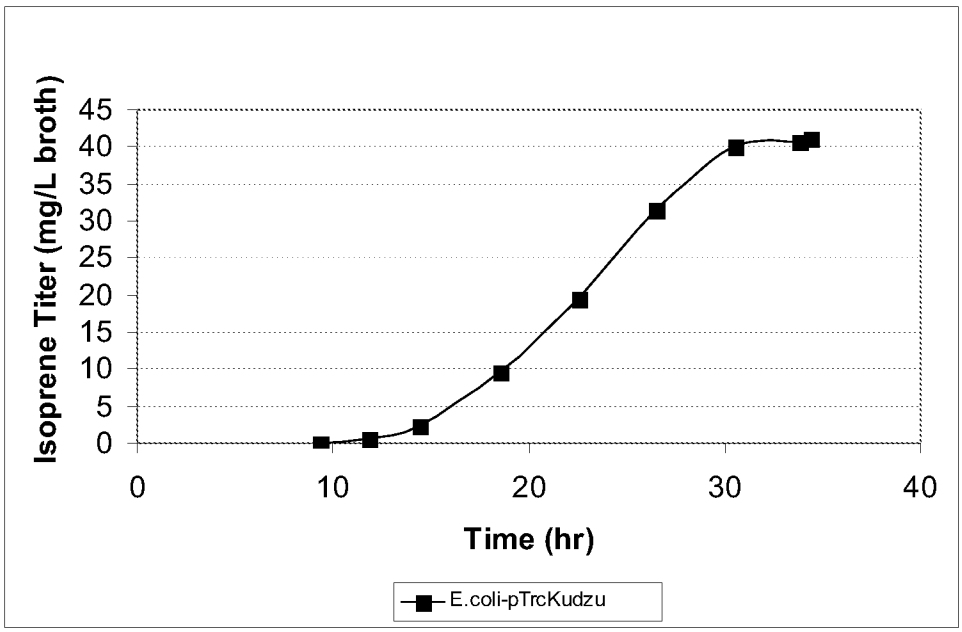

Isoprene Titer (mg/L broth)
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
Time (hr)
E.coli-pTrcKudzu

Figure 10A

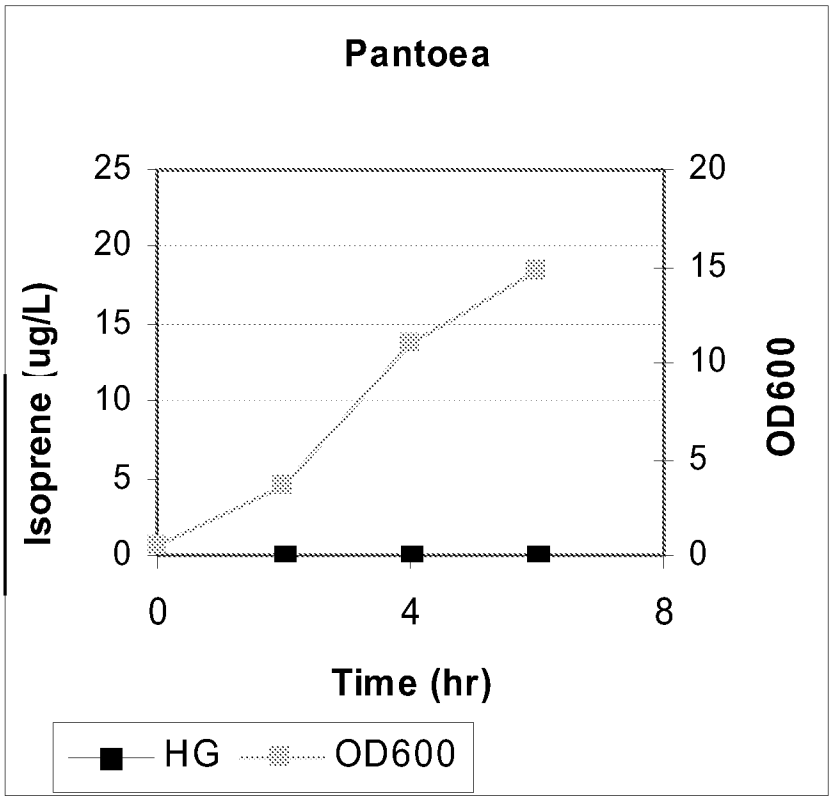

Pantoea
Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
HG
OD600

Figure 10B

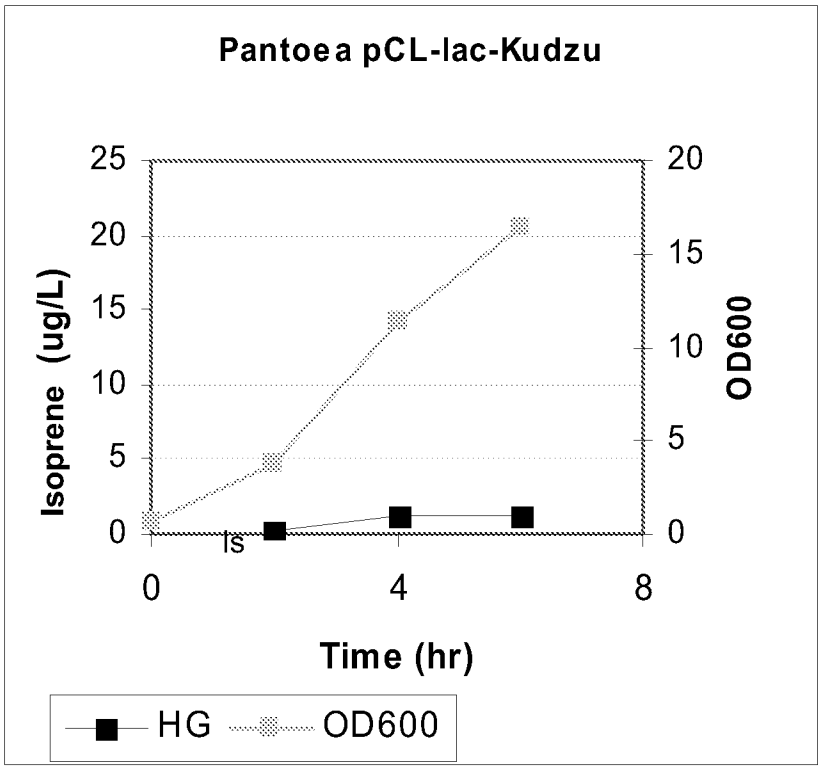

Pantoea pCL-lac-Kudzu
Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
ls
0
4
8
Time (hr)
HG
OD600

Figure 10C

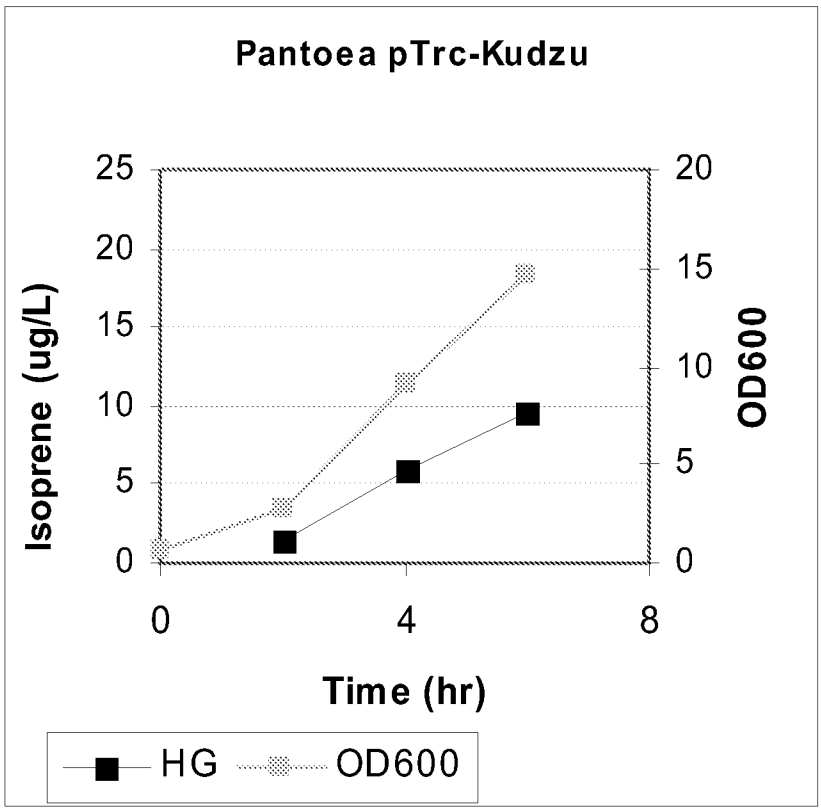

Pantoea pTrc-Kudzu
Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
HG
OD600

Figure 11

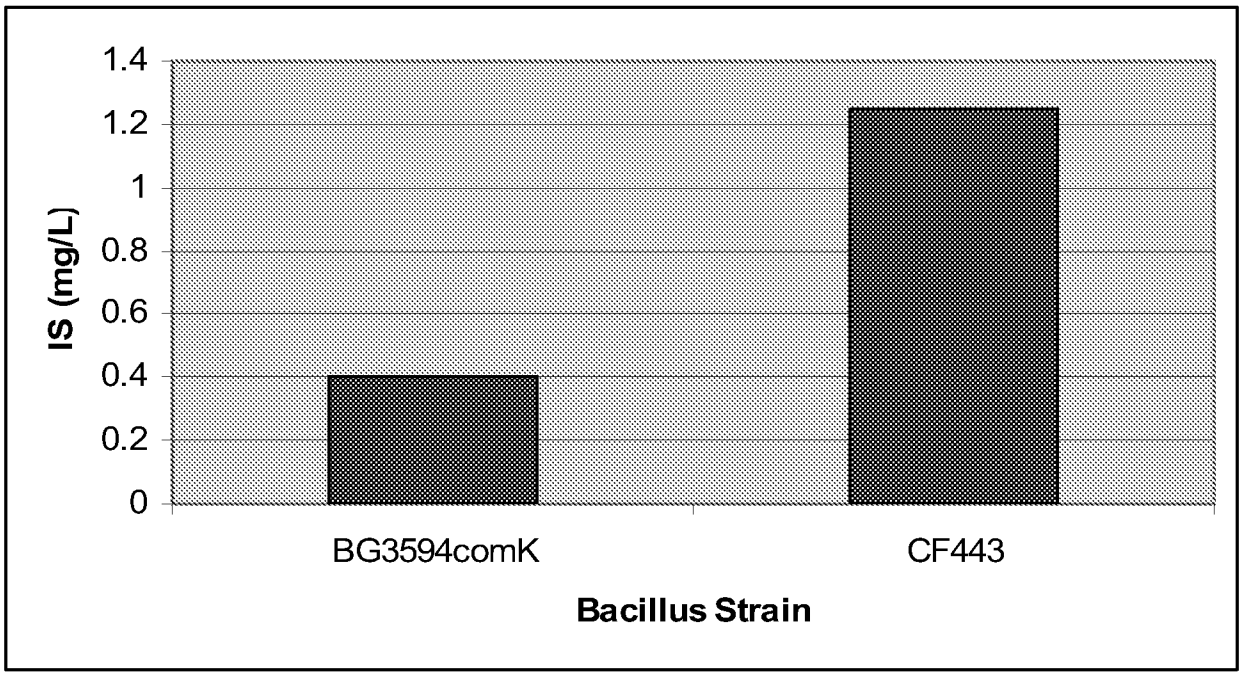


Figure 12A

1-
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Figure 12B

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Figure 12C

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(SEQ ID NO:56)

Figure 13

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GACGTCTTCGAGAGATTTAAGGACAAGGAGGGAGGATTTAGCGGCGAGCTGAAGGGAGACGTTC
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Figure 14

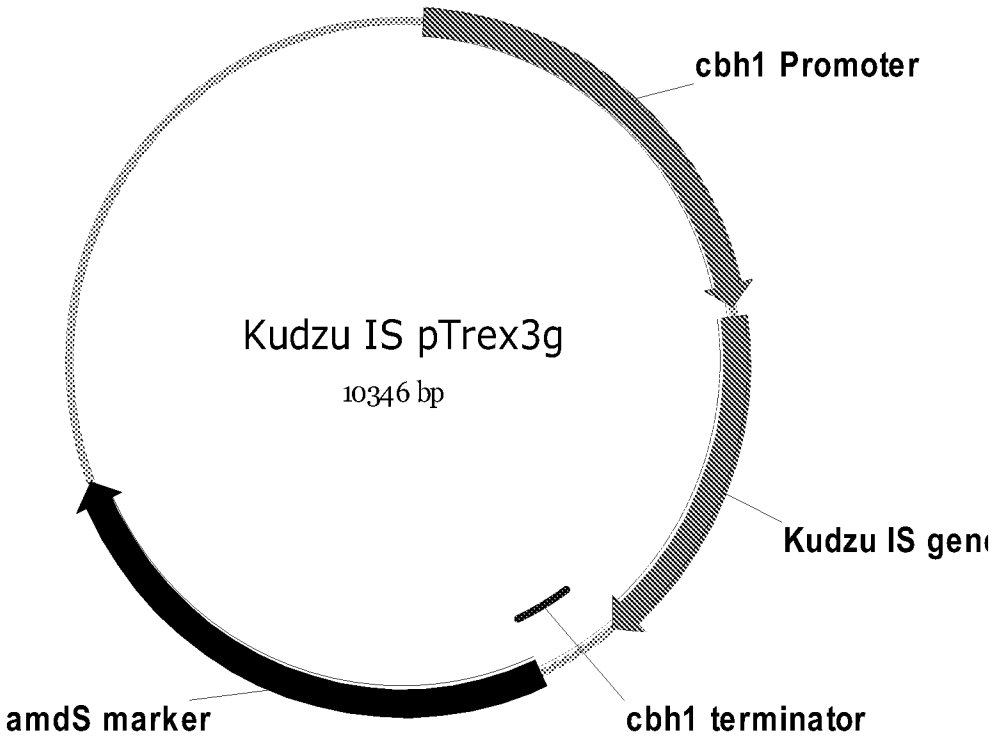
cbh1 Promoter
Kudzu IS pTrex3g
10346 bp
Kudzu IS gen
amdS marker
cbh1 terminator

Figure 15A

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  61 AAAGAAAGCA ATTGAAAACA AAACAAAACA ATTTTCATTC CTTCTCTTAT CATTCCTTTT
 121 CTTTTCTTTT CTCTCATTCA ACGCACTCCA TCGTATCCGT ATTCCTCTTA TTTTTTCTCT
 181 TTCTCTATAT CCATTTCTTT CTCTCTAGGT GTGTCCTCTC TCTCTCTTCA ATTTCTCTAC
 241 TCCGCATTCC AACGCATCCT TCCCCCAACC TCCCATTTCC TCCTTACGGC CCGATAGCGA
 301 TCGTCTTTCC CTCGCTATCA CTCGCTACCG GCCCCTCCTC TGCACCGTAA CCTCCTACGT
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 421 TGGCTTCCGG ATTATCTTCT TGTCCATAAG GTGATCCATG CTTCCTGAAG ATTCCCGAAA
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 721 CTATCACGAA TTCACATACA TTATGAAGAT CACCGCTGTC ATTGCCCTTT TATTCTCACT
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 841 TGCTGATTTC CTTCGTGTTT ACCAAAGTTG GAACACTTTT GCTAATCCTG ATAGACCCAA
 901 CCTTAAGAAG AGAAATGATA CACCTGCAAG TGGATATCAA GTTGAAAAAG TCGTAATTTT
 961 GTCACGTCAC GGTGTTAGGG CCCCTACAAA AATGACTCAA ACCATGCGTG ATGTCACTCC
1021 TAATACATGG CCAGAATGGC CCGTTAAATT AGGATATATT ACACCAAGAG GTGAACACTT
1081 GATATCACTT ATGGGCGGTT TTTACCGTCA AAAATTCCAG CAACAAGGAA TCCTTTCTCA
1141 GGGCTCCTGT CCTACTCCTA ACTCCATATA TGTCTGGGCT GACGTCGATC AGCGTACTTT
1201 AAAAACTGGT GAAGCATTCC TTGCTGGTTT GGCACCACAA TGTGGCTTGA CAATTCATCA
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1381 TTTGAATCAA CATTACATCC CCTTTTTAGC TTTAATGAAT ACAACATTAA ATTTTAGTAC
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1981 TCAAACACTA GAACAACTTC GATCACAGAC TCCCCTTTCT CTAAATCAGC CTGCCGGATC
2041 TGTTCAACTT AAAATTCCAG GTTGCAATGA TCAAACAGCC GAGGGTTACT GTCCTCTTTC
2101 CACTTTTACA AGAGTTGTTT CCCAATCTGT TGAACCTGGA TGCCAACTTC AATAATGAGG
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2221 GTTCTTTTCT TTTGTTCTTT ATGTCGTTTT TCGATGGTAC GGCCGTTGTC AATCTCAGTT
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2461 TCCTCCTCGT TCATCTTGAG GTTTGACGTG TCTGTTTAGA ATTTTGAGCT GTTGCAGCAT
2521 CTTATTTTTT GTTTTGCGAA AACGAAGCGC TTTACTCTCT TCATCAGTTG GACGATTGTA
2581 CCTTTGAAAA CCAACTACTT TTGCATGTTT TGTATAGAAA TCAATGATAT TAGAATCCCA
2641 TCCTTTAATT TCTTTCAAAG TAGTTGAGCT ATAGTTAAGT GTAAGGGCCC TACTGCGAAA
2701 GCATTTGCCA AGGATGTTTT CATTAATCAA GAACGAAAGT TAGGGGATCG AAGACGATCA
2761 GATACCGTCG TAGTCTTAAC CATAAACTAT GCCGACTAGG GATCGGGCAA TGTTTCATTT
2821 ATCGACTTGC TCGGCACCTT ACGAGAAATC AAAGTCTTTG GGTTCCGGGG GGAGTATGGT
2881 CGCAAGGCTG AAACTTAAAG GAATTGACGG AAGGGCACCA CAATGGAGTG GAGCCTGCGG
2941 CTTAATTTGA CTCAACACGG GGAAACTCAC CAGGTCCAGA CATAGTAAGG ATTGACAGAT
3001 TGAGAGCTCT TTCTTGATTC TATGGGTGGT GGTGCATGGC CGTTCTTAGT TGGTGGAGTG
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3121 CATTTTGGCT GATCATTAGC TTCTTAGAGG GACTATTGGC ATAAAGCCAA TGGAAGTTTG
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3361 ATTCGATGTT GCAGATTTTA CAAGTTTTTA AAATGTATTT CATTATTACT TTTTATATGC
3421 CTAATAAAAA AGCCATAGTT TAATCTATAG ATAACTTTTT TTCCAGTGCA CTAACGGACG
```

Figure 15B

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3481 TTACATTCCC ATACAAAACT GCGTAGTTAA AGCTAAGGAA AAGTTAATAT CATGTTAATT
3541 AAATACGCTA TTTACAATAA GACATTGAAC TCATTTATAT CGTTGAATAT GAATAACCAA
3601 TTTCAGCGAA TTTTTAACAA ACATCGTTCA CCTCGTTTAA GGATATCTTG TGTATGGGGT
3661 GTTGACTTGC TTTATCGAAT AATTACCGTA CCTGTAATTG GCTTGCTGGA TATAGCGGTA
3721 GTCTAATATC TAGCAAAAAT CTTTTGGGTG AAAAGGCTTG CAATTTCACG ACACCGAACT
3781 ATTTGTCATT TTTTAATAAG GAAGTTTTCC ATAAATTCCT GTAATTCTCG GTTGATCTAA
3841 TTGAAAAGAG TAGTTTTGCA TCACGATGAG GAGGGCTTTT GTAGAAAGAA ATACGAACGA
3901 AACGAAAATC AGCGTTGCCA TCGCTTTGGA CAAAGCTCCC TTACCTGAAG AGTCGAATTT
3961 TATTGATGAA CTTATAACTT CCAAGCATGC AAACCAAAAG GGAGAACAAG TAATCCAAGT
4021 AGACACGGGA ATTGGATTCT TGGATCACAT GTATCATGCA CTGGCTAAAC ATGCAGGCTG
4081 GAGCTTACGA CTTTACTCAA GAGGTGATTT AATCATCGAT GATCATCACA CTGCAGAAGA
4141 TACTGCTATT GCACTTGGTA TTGCATTCAA GCAGGCTATG GGTAACTTTG CCGGCGTTAA
4201 AAGATTTGGA CATGCTTATT GTCCACTTGA CGAAGCTCTT TCTAGAAGCG TAGTTGACTT
4261 GTCGGGACGG CCCTATGCTG TTATCGATTT GGGATTAAAG CGTGAAAAGG TTGGGGAATT
4321 GTCCTGTGAA ATGATCCCTC ACTTACTATA TTCCTTTTCG GTAGCAGCTG GAATTACTTT
4381 GCATGTTACC TGCTTATATG GTAGTAATGA CCATCATCGT GCTGAAAGCG CTTTTAAATC
4441 TCTGGCTGTT GCCATGCGCG CGGCTACTAG TCTTACTGGA AGTTCTGAAG TCCCAAGCAC
4501 GAAGGGAGTG TTGTAAAGAT GAATTGGATT ATGTCAGGAA AAGAACGACA ATTTTGCATC
4561 CAAATTGTCT AAATTTTAGA GTTGCTTGAA AACAATAGAA CCTTACTTGC TTTATAATTA
4621 CGTTAATTAG AAGCGTTATC TCGTGAAGGA ATATAGTACG TAGCCGTATA AATTGAATTG
4681 AATGTTCAGC TTATAGAATA GAGACACTTT GCTGTTCAAT GCGTCGTCAC TTACCATACT
4741 CACTTTATTA TACGACTTTA AGTATAAACT CCGCGGTTAT GGTAAAATTA ATGATGCACA
4801 AACGTCCGAT TCCATATGGG TACACTACAA TTAAATACTT TTAAGCTGAT CCCCCACACA
4861 CCATAGCTTC AAAATGTTTC TACTCCTTTT TTACTCTTCC AGATTTTCTC GGACTCCGCG
4921 CATCGCCGTA CCACTTCAAA ACACCCAAGC ACAGCATACT AAATTTTCCC TCTTTCTTCC
4981 TCTAGGGTGT CGTTAATTAC CCGTACTAAA GGTTTGGAAA AGAAAAAAGA GACCGCCTCG
5041 TTTCTTTTTC TTCGTCGAAA AAGGCAATAA AAATTTTTAT CACGTTTCTT TTTCTTGAAA
5101 TTTTTTTTTT TAGTTTTTTT CTCTTTCAGT GACCTCCATT GATATTTAAG TTAATAAACG
5161 GTCTTCAATT TCTCAAGTTT CAGTTTCATT TTTCTTGTTC TATTACAACT TTTTTTACTT
5221 CTTGTTCATT AGAAAGAAAG CATAGCAATC TAATCTAAGG GCGGTGTTGA CAATTAATCA
5281 TCGGCATAGT ATATCGGCAT AGTATAATAC GACAAGGTGA GGAACTAAAC CATGGCCAAG
5341 TTGACCAGTG CCGTTCCGGT GCTCACCGCG CGCGACGTCG CCGGAGCGGT CGAGTTCTGG
5401 ACCGACCGGC TCGGGTTCTC CCGGGACTTC GTGGAGGACG ACTTCGCCGG TGTGGTCCGG
5461 GACGACGTGA CCCTGTTCAT CAGCGCGGTC CAGGACCAGG TGGTGCCGGA CAACACCCTG
5521 GCCTGGGTGT GGGTGCGCGG CCTGGACGAG CTGTACGCCG AGTGGTCGGA GGTCGTGTCC
5581 ACGAACTTCC GGGACGCCTC CGGGCCGGCC ATGACCGAGA TCGGCGAGCA GCCGTGGGGG
5641 CGGGAGTTCG CCCTGCGCGA CCCGGCCGGC AACTGCGTGC ACTTCGTGGC CGAGGAGCAG
5701 GACTGACACG TCCGACGGCG GCCCACGGGT CCCAGGCCTC GGAGATCCGT CCCCCTTTTC
5761 CTTTGTCGAT ATCATGTAAT TAGTTATGTC ACGCTTACAT TCACGCCCTC CCCCCACATC
5821 CGCTCTAACC GAAAAGGAAG GAGTTAGACA ACCTGAAGTC TAGGTCCCTA TTTATTTTTT
5881 TATAGTTATG TTAGTATTAA GAACGTTATT TATATTTCAA ATTTTTCTTT TTTTTCTGTA
5941 CAGACGCGAG CTTCCCAGTA AATGTGCCAT CTCGTAGGCA GAAAACGGTT CCCCCGTAGG
6001 GTCTCTCTCT TGGCCTCCTT TCTAGGTCGG GCTGATTGCT CTTGAAGCTC TCTAGGGGGG
6061 CTCACACCAT AGGCAGATAA CGTTCCCCAC CGGCTCGCCT CGTAAGCGCA CAAGGACTGC
6121 TCCCAAAGAT CCTAGGCGGG ATTTTGCCGA TTTCGGCCTA AAGGAACCGG AACACGTAGA
6181 AAGCCAGTCC GCAGAAACGG TGCTGACCCC GGATGAATGT CAGCTACTGG GCTATCTGGA
6241 CAAGGGAAAA CGCAAGCGCA AAGAGAAAGC AGGTAGCTTG CAGTGGGCTT ACATGGCGAT
6301 AGCTAGACTG GGCGGTTTTA TGGACAGCAA GCGAACCGGA ATTGCCAGCT GGGGCGCCCT
6361 CTGGTAAGGT TGGGAAGCCC TGCAAAGTAA ACTGGATGGC TTTCTTGCCG CCAAGGATCT
6421 GATGGCGCAG GGGATCAAGA TCTGATCAAG AGACAGGATG AGGATCGTTT CGCATGATTG
6481 AACAAGATGG ATTGCACGCA GGTTCTCCGG CCGCTTGGGT GGAGAGGCTA TTCGGCTATG
6541 ACTGGGCACA ACAGACAATC GGCTGCTCTG ATGCCGCCGT GTTCCGGCTG TCAGCGCAGG
6601 GGCGCCCGGT TCTTTTTGTC AAGACCGACC TGTCCGGTGC CCTGAATGAA CTGCAGGACG
6661 AGGCAGCGCG GCTATCGTGG CTGGCCACGA CGGGCGTTCC TTGCGCAGCT GTGCTCGACG
6721 TTGTCACTGA AGCGGGAAGG GACTGGCTGC TATTGGGCGA AGTGCCGGGG CAGGATCTCC
6781 TGTCATCTCG CCTTGCTCCT GCCGAGAAAG TATCCATCAT GGCTGATGCA ATGCGGCGGC
6841 TGCATACGCT TGATCCGGCT ACCTGCCCAT TCGACCACCA AGCGAAACAT CGCATCGAGC
6901 GAGCACGTAC TCGGATGGAA GCCGGTCTTG TCGATCAGGA TGATCTGGAC GAAGAGCATC
6961 AGGGGCTCGC GCCAGCCGAA CTGTTCGCCA GGCTCAAGGC GCGCATGCCC GACGGCGAGG
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Figure 15C

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7021 ATCTCGTCGT GATCCATGGC GATGCCTGCT TGCCGAATAT CATGGTGGAA AATGGCCGCT
7081 TTTCTGGATT CAACGACTGT GGCCGGCTGG GTGTGGCGGA CCGCTATCAG GACATAGCGT
7141 TGGATACCCG TGATATTGCT GAAGAGCTTG GCGGCGAATG GGCTGACCGC TTCCTCGTGC
7201 TTTACGGTAT CGCCGCTCCC GATTCGCAGC GCATCGCCTT CTATCGCCTT CTTGACGAGT
7261 TCTTCTGAAT TGAAAAAGGT ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAAACT
7321 TCATTTTTAA TTTAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA TGACCAAAAT
7381 CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC GTAGAAAAGA TCAAAGGATC
7441 TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG CAAACAAAAA AACCACCGCT
7501 ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT CTTTTTCCGA AGGTAACTGG
7561 CTTCAGCAGA GCGCAGATAC CAAATACTGT CCTTCTAGTG TAGCCGTAGT TAGGCCACCA
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
8041 CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT TTTGCTCACA TGTTCTTTCC
8101 TGCGTTATCC CCTGATTCTG TGGATAACCG TATTACCGCC TTTGAGTGAG CTGATACCGC
8161 TCGCCGCAGC CGAACGACCG AGCGCAGCGA G
```

(SEQ ID NO:11)

Figure 16

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   1 GAATTCAAAA CAAAATGTGT GCAACCTCCT CCCAGTTTAC TCAGATTACC GAGCATAATT
  61 CTCGACGATC TGCTAACTAC CAGCCGAACC TTTGGAACTT TGAGTTTCTC CAGTCTCTCG
 121 AAAATGACCT GAAGGTGGAA AAGCTCGAGG AGAAGGCGAC CAAACTCGAG GAGGAGGTGC
 181 GATGTATGAT CAACAGAGTT GACACCCAAC CCCTGTCTTT GCTGGAGCTG ATCGACGATG
 241 TGCAGCGGTT GGGTTTGACT TATAAATTCG AGAAGGACAT TATCAAGGCA CTGGAGAACA
 301 TTGTGCTCCT CGACGAGAAC AAGAAGAACA AGTCTGATCT TCACGCTACC GCTCTCTCTT
 361 TCCGACTTCT TCGACAACAC GGCTTCGAGG TGTCGCAGGA CGTCTTCGAG AGATTTAAGG
 421 ACAAGGAGGG AGGATTTAGC GGCGAGCTGA AGGGAGACGT TCAGGGTCTT CTCTCCTTGT
 481 ACGAGGCGTC CTACCTGGGA TTCGAGGGAG AGAACCTCCT GGAGGAAGCT CGTACATTTT
 541 CCATCACTCA CCTTAAGAAT AACCTTAAGG AGGGAATTAA CACCAAGGTG GCCGAGCAGG
 601 TTTCTCACGC CCTGGAGCTC CCCTACCACC AACGGCTCCA TAGACTGGAG GCTCGTTGGT
 661 TCCTGGACAA ATATGAGCCA AAGGAGCCTC ATCATCAGTT GCTGTTGGAG TTGGCCAAGC
 721 TGGACTTCAA TATGGTTCAG ACGCTGCACC AAAAGGAGTT GCAGGACCTG TCTCGATGGT
 781 GGACCGAGAT GGGATTGGCC TCGAAGCTGG ATTTTGTCCG TGACCGACTT ATGGAGGTCT
 841 ATTTTTGGGC CCTTGGAATG GCGCCTGACC CCCAGTTCGG AGAGTGCCGG AAGGCGGTGA
 901 CGAAGATGTT CGGTCTTGTG ACTATCATCG ACGACGTCTA CGATGTCTAC GGCACACTCG
 961 ACGAGTTGCA GCTGTTCACT GACGCCGTCG AGCGATGGGA TGTGAACGCC ATTAATACTC
1021 TCCCTGACTA TATGAAGCTG TGCTTCCTGG CTCTGTACAA CACTGTCAAC GATACCTCGT
1081 ACTCTATCCT CAAGGAGAAG GGACACAACA ATCTCTCCTA CTTGACCAAA TCCTGGCGAG
1141 AACTGTGCAA GGCTTTTCTG CAGGAGGCTA AATGGTCCAA TAACAAGATC ATTCCTGCTT
1201 TTTCTAAATA CCTGGAAAAT GCCTCGGTGT CGAGCTCTGG CGTCGCCCTT CTGGCCCCTT
1261 CCTACTTCTC CGTCTGCCAG CAGCAGGAGG ATATTTCCGA TCATGCTCTT AGATCGCTGA
1321 CCGATTTTCA CGGCCTCGTG CGATCTTCCT GCGTGATTTT TCGGTTGTGT AATGACCTTG
1381 CGACCTCTGC TGCTGAGCTG GAACGAGGCG AGACTACAAA TTCCATTATT TCTTACATGC
1441 ACGAAAACGA TGGAACATCT GAAGAACAGG CTAGAGAGGA ACTGCGAAAG TTGATCGACG
1501 CCGAGTGGAA GAAGATGAAC AGAGAGCGGG TGTCCGACTC TACCCTGCTT CCCAAGGCCT
1561 TCATGGAGAT CGCCGTGAAC ATGGCTCGAG TTTCCCATTG TACTTACCAG TACGGTGACG
1621 GCCTGGGTCG TCCGGACTAC GCTACAGAGA ACCGAATCAA GCTGCTGCTC ATCGACCCCT
1681 TCCCTATCAA CCAATTGATG TACGTGTAAT AGTCTAGAGG ATCC
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(SEQ ID NO:12)

Figure 17

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   1 GAATTCAACA AAAATGTGCT CTGTTTCCAC TGAGAACGTG TCCTTTACTG AGACTGAGAC
  61 TGAAGCACGT AGAAGCGCCA ACTACGAACC CAACTCCTGG GATTATGACT TTCTGCTGTC
 121 TTCTGACACC GACGAGTCGA TCGAGGTTTA TAAGGATAAG GCCAAGAAAC TTGAGGCCGA
 181 GGTCAGACGA GAGATTAACA ACGAGAAGGC CGAGTTCCTG ACCCTTCTTG AGCTGATCGA
 241 CAACGTTCAA CGACTTGGTC TTGGTTACCG TTTCGAATCC GATATCCGAC GTGCATTGGA
 301 TCGATTTGTC TCGTCCGGAG GTTTCGATGG TGTGACTAAG ACGTCGCTGC ACGCCACAGC
 361 TCTTTCCTTC AGACTGTTGC GGCAGCATGG ATTTGAGGTT TCCCAGGAAG CCTTTTCTGG
 421 TTTCAAGGAT CAGAACGGAA ACTTTTTGGA GAATCTCAAG GAGGACACCA AGGCCATCCT
 481 GTCGTTGTAT GAGGCCTCGT TCCTGGCTCT TGAGGGCGAG AATATTCTGG ATGAGGCTCG
 541 GGTTTTCGCT ATTTCGCACC TGAAGGAGTT GTCGGAGGAA AAGATCGGAA AGGAACTGGC
 601 CGAGCAGGTC AACCATGCAC TTGAACTTCC CCTGCATCGA CGTACCCAGC GACTGGAGGC
 661 CGTGTGGAGC ATCGAGGCGT ACAGAAAAAA GGAGGATGCT AATCAGGTTC TGCTCGAACT
 721 CGCTATCCTC GACTATAACA TGATTCAGAG CGTGTACCAG CGTGACTTGC GAGAGACAAG
 781 CCGGTGGTGG CGACGGGTGG GACTGGCCAC GAAGCTCCAC TTTGCTAAAG ATCGATTGAT
 841 TGAGTCGTTC TACTGGGCAG TGGGTGTGGC CTTTGAGCCT CAGTACTCCG ACTGCCGAAA
 901 CTCCGTTGCA AAGATGTTTT CTTTTGTCAC TATCATCGAC GACATCTACG ATGTTTACGG
 961 CACTCTCGAT GAACTCGAAC TCTTCACGGA CGCTGTCGAG CGATGGGATG TGAATGCCAT
1021 TAATGATCTG CCAGATTATA TGAAGTTGTG TTTCTTGGCG CTCTACAACA CAATTAATGA
1081 AATTGCCTAC GACAACCTCA AGGACAAGGG AGAGAACATT CTGCCCTACC TTACTAAAGC
1141 CTGGGCCGAC CTGTGTAACG CCTTTTTGCA GGAAGCCAAG TGGCTCTATA ACAAATCTAC
1201 TCCTACATTT GATGACTACT TCGGCAACGC TTGGAAGTCT TCCAGCGGCC CTCTCCAGTT
1261 GATCTTCGCT TACTTTGCAG TGGTCCAGAA CATCAAGAAA GAGGAGATTG AGAACCTCCA
1321 GAAGTATCAC GACATCATCT CCCGACCTTC GCACATCTTT CGACTGTGCA ATGACCTTGC
1381 CTCCGCATCC GCTGAGATTG CCCGAGGAGA AACAGCCAAT TCTGTGTCGT GTTACATGCG
1441 TACAAAGGGC ATCTCCGAGG AGCTGGCTAC CGAGTCTGTG ATGAACCTGA TCGATGAAAC
1501 CTGTAAGAAG ATGAACAAAG AGAAACTGGG CGGTTCTCTG TTCGCCAAAC CATTTGTTGA
1561 AACCGCGATC AATCTGGCTC GTCAGTCTCA TTGTACTTAC CATAACGGTG ACGCGCACAC
1621 TTCGCCGGAC GAATTGACCC GTAAGCGTGT GCTTTCGGTG ATTACCGAGC CGATCCTGCC
1681 GTTCGAAAGA TAATAGGATC C
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(SEQ ID NO:13)

Figure 18A1

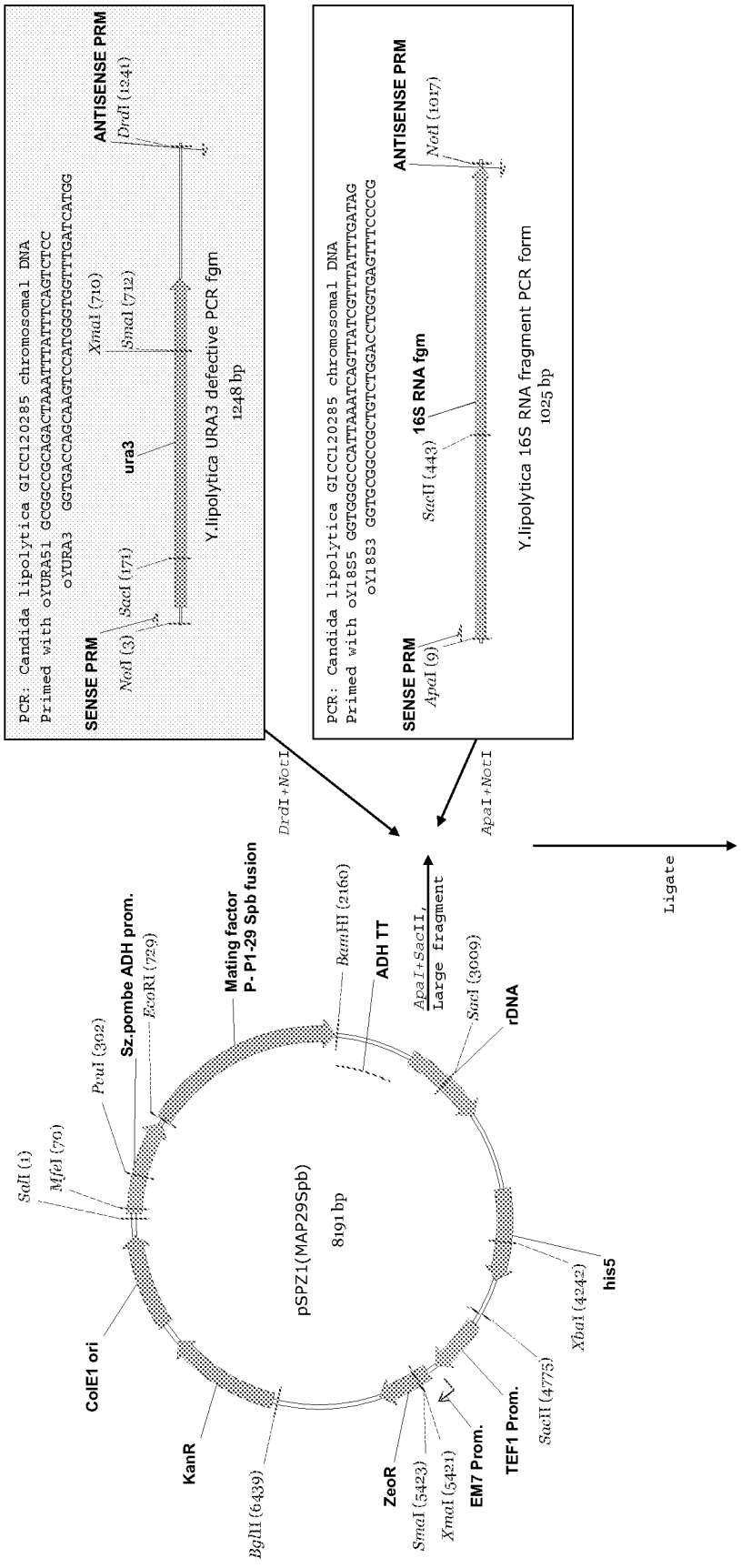
PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oYURA51 GCGGCCGCAGACTAAATTTATTTCAGTCTCC
oYURA3 GGTGACCAGCAAGTCCATGGGTGGTTTGATCATGG
SENSE PRM
NotI (3)
SacI (171)
ura3
XmaI (710)
SmaI (712)
ANTISENSE PRM
DrdI (1241)
Y.lipolytica URA3 defective PCR fgm
1248 bp
DrdI+NotI
PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oY18S5 GGTGGGCCCATTAAATCAGTTATCGTTTATTTGATAG
oY18S3 GGTGCGGCCGCTGTCTGGACCTGGTGAGTTTCCCCG
SENSE PRM
ApaI (9)
SacII (443)
16S RNA fgm
ANTISENSE PRM
NotI (1017)
Y.lipolytica 16S RNA fragment PCR form
1025 bp
ApaI+NotI
Ligate
pSPZ1(MAP29Spb)
8191 bp
ApaI+SacII,
Large fragment
SalI (1)
MfeI (70)
PmaI (392)
Sz.pombe ADH prom.
EcoRI (729)
Mating factor
P- P1-29 Spb fusion
BamHI (2160)
ADH TT
SacI (3009)
rDNA
his5
XbaI (4242)
SacII (4773)
TEF1 Prom.
EM7 Prom.
XmaI (5421)
SmaI (5423)
ZeoR
BglII (6439)
KanR
ColE1 ori

Figure 18A2

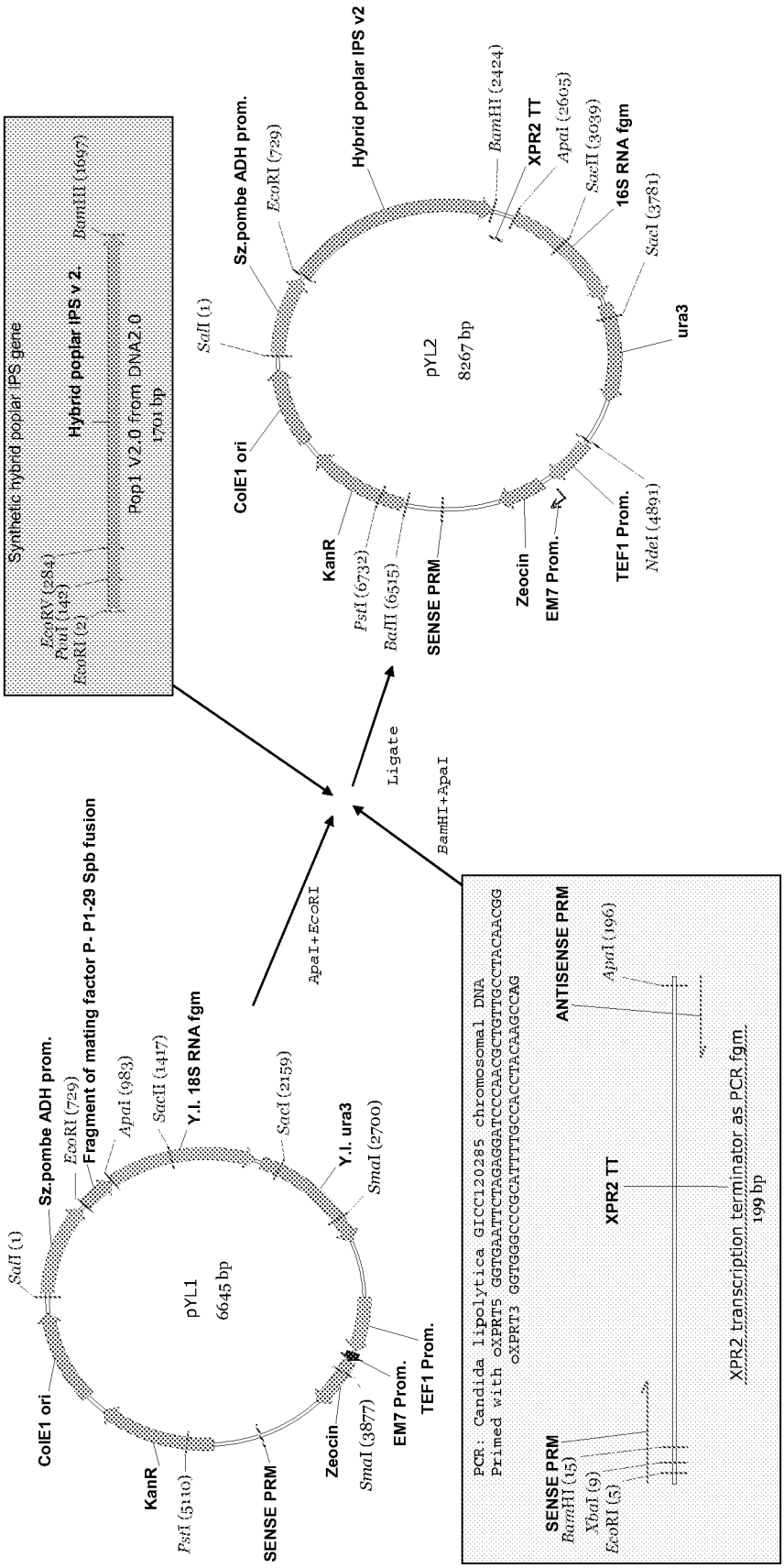
Synthetic hybrid poplar IPS gene
EcoRV (284)
PstI (146)
EcoRI (2)
Hybrid poplar IPS v 2.
BamHI (1697)
Pop1 V2.0 from DNA2.0
1701 bp
SalI (1)
Sz.pombe ADH prom.
EcoRI (729)
Hybrid poplar IPS v2
BamHI (2424)
XPR2 TT
ApaI (2605)
SacII (3039)
16S RNA fgm
SacI (3781)
ura3
ColE1 ori
pYL2
8267 bp
KanR
PstI (6732)
BclI (6515)
SENSE PRM
Zeocin
EM7 Prom.
TEF1 Prom.
NdeI (4891)
ApaI+EcoRI
Ligate
BamHI+ApaI
SalI (1)
Sz.pombe ADH prom.
EcoRI (729)
Fragment of mating factor P- P1-29 Spb fusion
ApaI (983)
SacII (1417)
Y.l. 18S RNA fgm
SacI (2159)
Y.l. ura3
SmaI (2700)
ColE1 ori
pYL1
6645 bp
KanR
PstI (5110)
SENSE PRM
Zeocin
SmaI (3877)
EM7 Prom.
TEF1 Prom.
PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oXPRT5 GGTGAATTCTAGAGGATCCCAACGCTGTTGCCTACAACGG
oXPRT3 GGTGGGCCCGCATTTTGCCACCTACAAGCCAG
SENSE PRM
BamHI (15)
XbaI (9)
EcoRI (5)
XPR2 TT
ANTISENSE PRM
ApaI (196)
XPR2 transcription terminator as PCR fgm
199 bp

Figure 18B

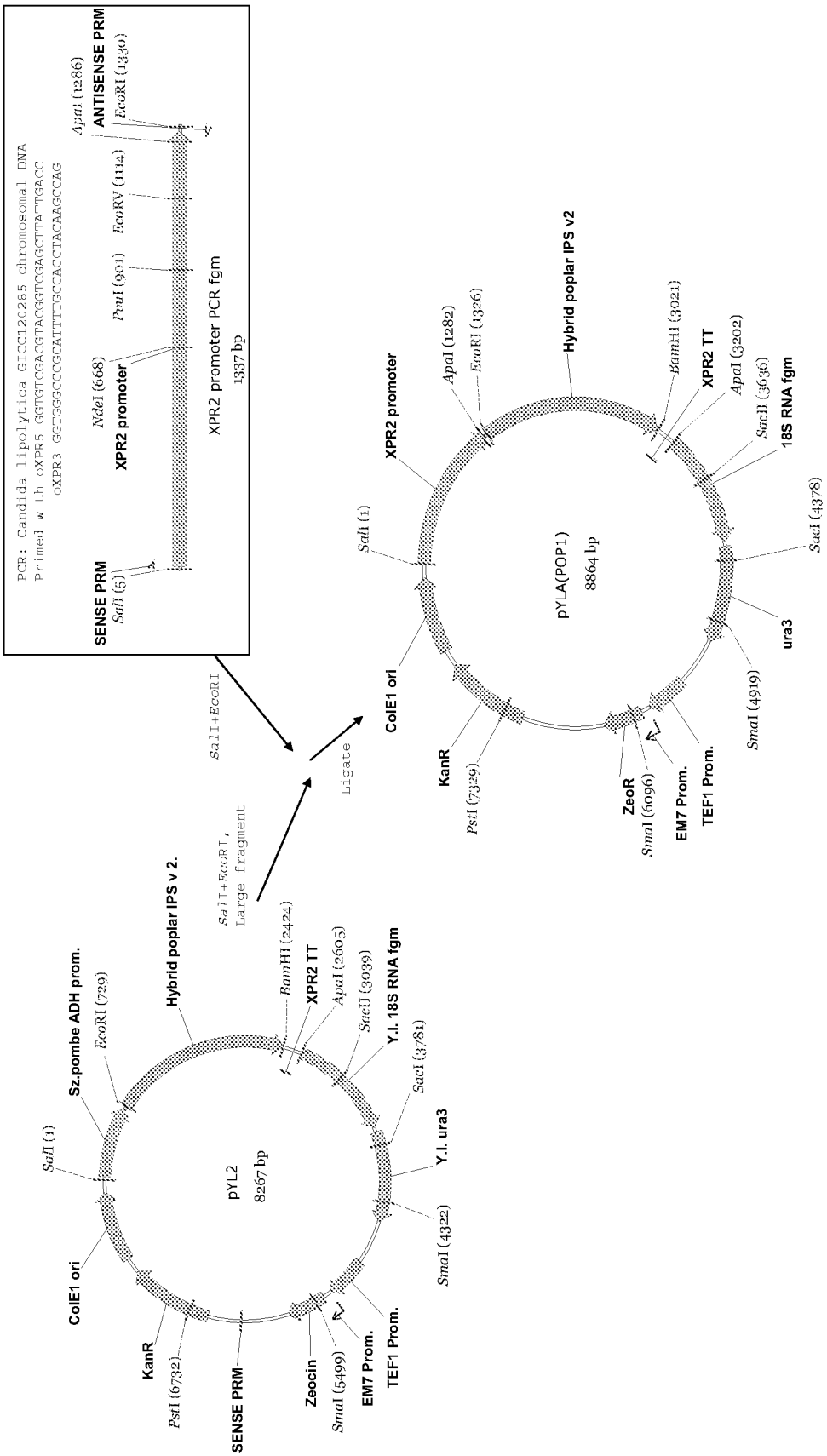
PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oXPR5 GGTGTCGACGTACGGTCGAGCTTATTGACC
oXPR3 GGTGGGCCCGCATTTTGCCACCTACAAGCCAG
SENSE PRM
SalI (5)
XPR2 promoter
NdeI (668)
PmaI (901)
EcoRV (1114)
ApaI (1286)
ANTISENSE PRM
EcoRI (1330)
XPR2 promoter PCR fgm
1337 bp
SalI+EcoRI
SalI+EcoRI, Large fragment
Ligate
pYL2
8267 bp
SalI (1)
Sz.pombe ADH prom.
EcoRI (729)
Hybrid poplar IPS v 2.
BamHI (2424)
XPR2 TT
ApaI (2605)
SacII (3039)
Y.l. 18S RNA fgm
SacI (3781)
Y.l. ura3
SmaI (4322)
TEF1 Prom.
EM7 Prom.
Zeocin
SmaI (5499)
SENSE PRM
PstI (6732)
KanR
ColE1 ori
pYLA(POP1)
8864 bp
SalI (1)
XPR2 promoter
ApaI (1282)
EcoRI (1326)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
SacI (4378)
ura3
SmaI (4919)
TEF1 Prom.
EM7 Prom.
ZeoR
SmaI (6096)
KanR
PstI (7329)
ColE1 ori

Figure 18C

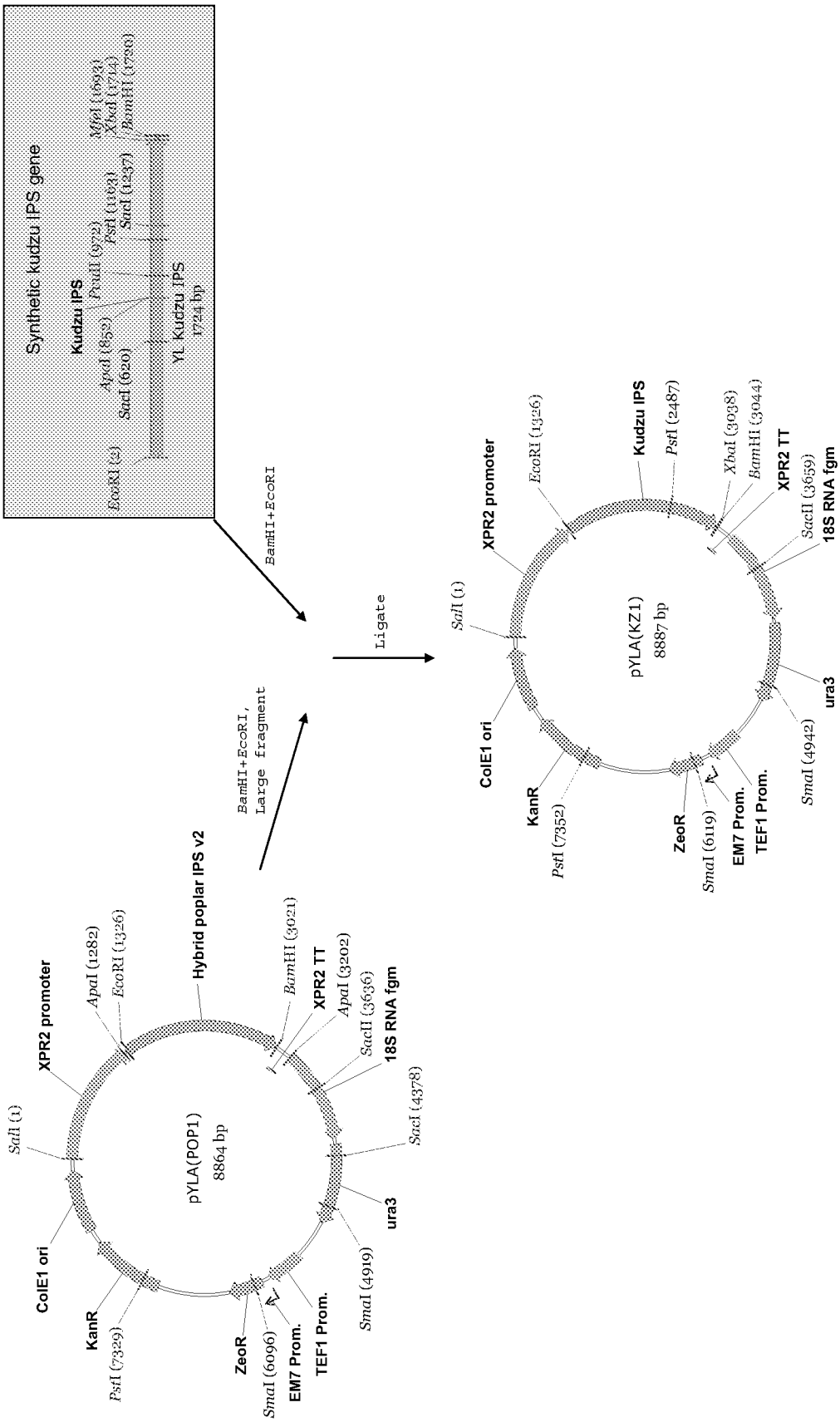
Synthetic kudzu IPS gene
Kudzu IPS
EcoRI (1)
ApaI (859)
SacI (620)
PvuII (972)
PstI (1163)
SacI (1237)
MfeI (1693)
XbaI (1714)
BamHI (1730)
YL Kudzu IPS
1724 bp
BamHI+EcoRI
BamHI+EcoRI,
Large fragment
Ligate
pYLA(POP1)
8864 bp
SalI (1)
XPR2 promoter
ApaI (1282)
EcoRI (1326)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
SacI (4378)
ura3
SmaI (4919)
TEF1 Prom.
EM7 Prom.
ZeoR
SmaI (6096)
PstI (7329)
KanR
ColE1 ori
pYLA(KZ1)
8887 bp
SalI (1)
XPR2 promoter
EcoRI (1326)
Kudzu IPS
PstI (2487)
XbaI (3038)
BamHI (3044)
XPR2 TT
SacII (3659)
18S RNA fgm
ura3
SmaI (4942)
TEF1 Prom.
EM7 Prom.
SmaI (6119)
ZeoR
PstI (7352)
KanR
ColE1 ori

Figure 18D

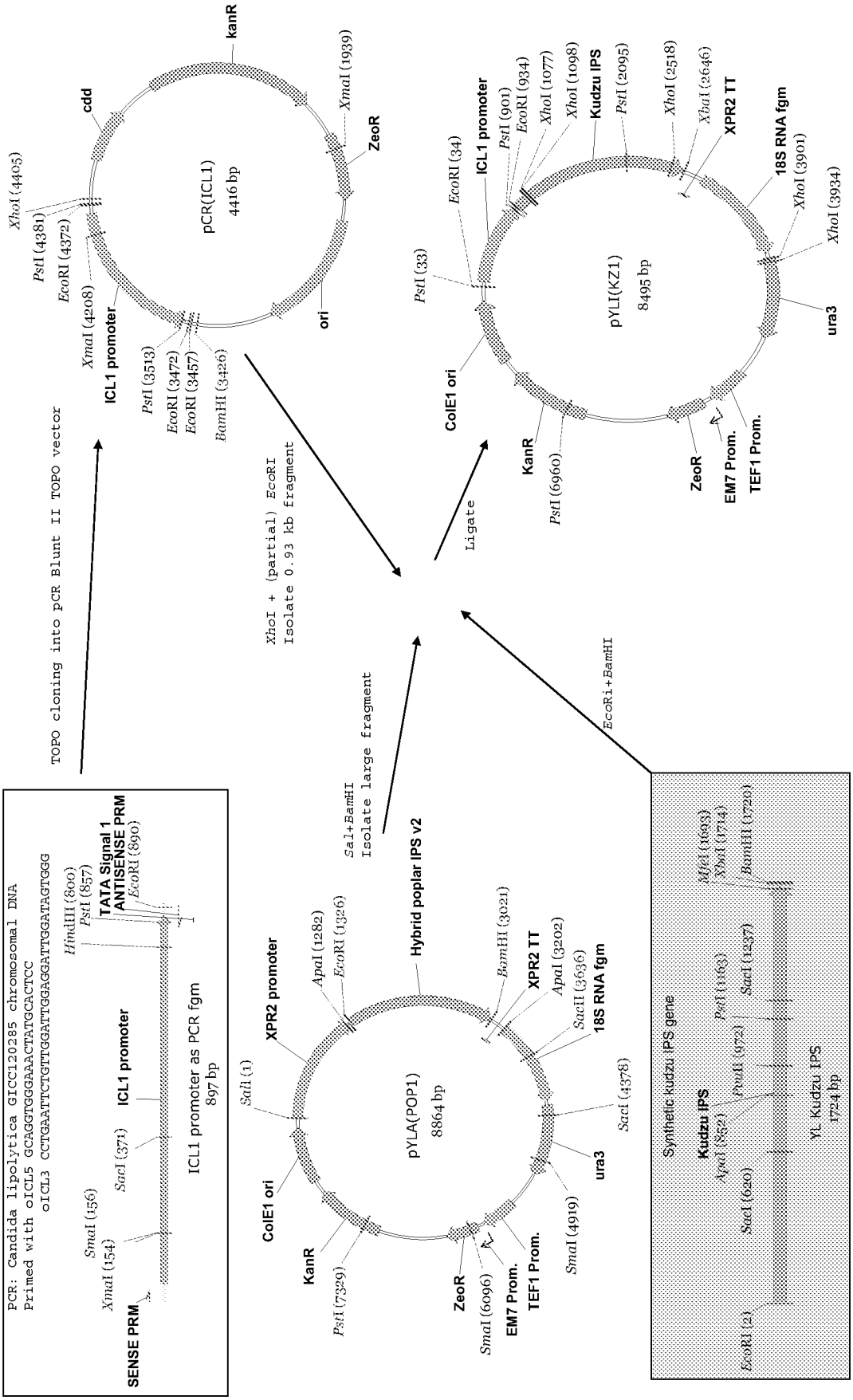
PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oICL5 GCAGGTGGGAAACTATGCACTCC
oICL3 CCTGAATTCTGTTGGATTGGAGGATTGGATAGTGGG
HindIII (80)
PstI (857)
TATA Signal 1
ANTISENSE PRM
EcoRI (890)
SmaI (156)
XmaI (154)
SacI (371)
ICL1 promoter
SENSE PRM
ICL1 promoter as PCR fgm
897 bp
TOPO cloning into pCR Blunt II TOPO vector
XhoI (4405)
PstI (4381)
EcoRI (4372)
cdd
XmaI (4208)
ICL1 promoter
PstI (3513)
EcoRI (3472)
EcoRI (3457)
BamHI (3426)
pCR(ICL1)
4416 bp
kanR
XmaI (1939)
ZeoR
ori
XhoI + (partial) EcoRI
Isolate 0.93 kb fragment
Sal+BamHI
Isolate large fragment
Hybrid poplar IPS v2
EcoRI+BamHI
Ligate
SalI (1)
XPR2 promoter
ApaI (1282)
EcoRI (1326)
ColE1 ori
KanR
PstI (7329)
pYLA(POP1)
8864 bp
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
SacI (4378)
ZeoR
SmaI (6096)
EM7 Prom.
TEF1 Prom.
SmaI (4919)
ura3
Synthetic kudzu IPS gene
MfeI (1893)
XhoI (1714)
Kudzu IPS
ApaI (822)
PstI (1163)
EcoRI (2)
SacI (620)
PmaII (972)
SacI (1237)
BamHI (1720)
YL kudzu IPS
1724 bp
PstI (33)
EcoRI (34)
ICL1 promoter
PstI (901)
EcoRI (934)
XhoI (1077)
XhoI (1098)
Kudzu IPS
pYLI(KZ1)
8495 bp
PstI (2095)
XhoI (2518)
XbaI (2646)
XPR2 TT
18S RNA fgm
XhoI (3901)
XhoI (3934)
ura3
ColE1 ori
KanR
PstI (6960)
ZeoR
EM7 Prom.
TEF1 Prom.

Figure 18E

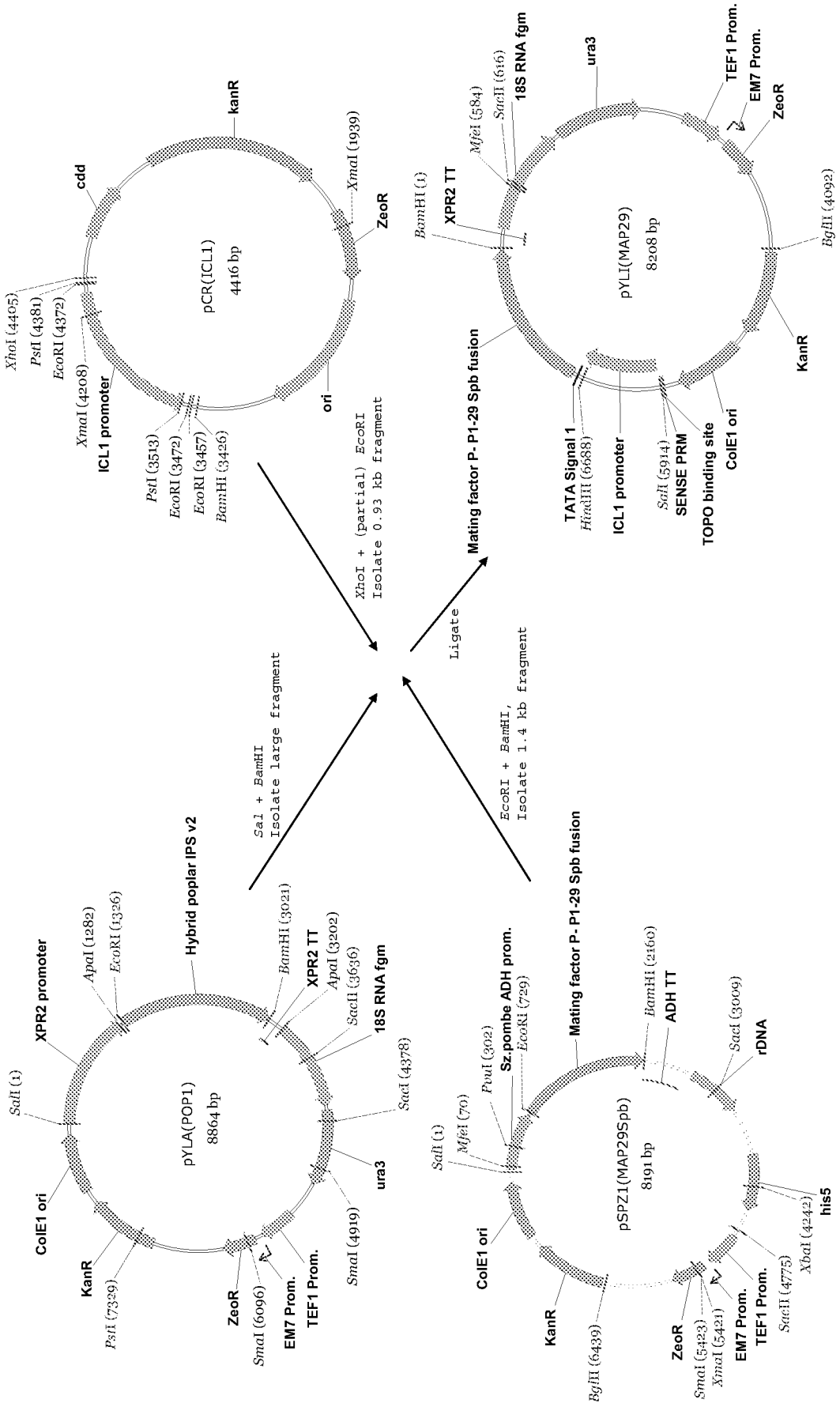
pCR(ICL1)
4416 bp
kanR
ZeoR
ori
cdd
ICL1 promoter
XmaI (1939)
XmaI (4208)
EcoRI (4372)
PstI (4381)
XhoI (4405)
PstI (3513)
EcoRI (3472)
EcoRI (3457)
BamHI (3426)
XhoI + (partial) EcoRI
Isolate 0.93 kb fragment
pYLI(MAP29)
8208 bp
BamHI (1)
XPR2 TT
MfeI (584)
SacII (616)
18S RNA fgm
ura3
TEF1 Prom.
EM7 Prom.
ZeoR
BglII (4092)
KanR
ColE1 ori
TOPO binding site
SENSE PRM
SalI (5914)
ICL1 promoter
TATA Signal 1
HindIII (6688)
Mating factor P- P1-29 Spb fusion
Ligate
pYLA(POP1)
8864 bp
SalI (1)
XPR2 promoter
ApaI (1282)
EcoRI (1326)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
SacI (4378)
ura3
SmaI (4919)
TEF1 Prom.
EM7 Prom.
ZeoR
SmaI (6096)
PstI (7329)
KanR
ColE1 ori
Sal + BamHI
Isolate large fragment
EcoRI + BamHI,
Isolate 1.4 kb fragment
pSPZ1(MAP29Spb)
8191 bp
SalI (1)
MfeI (70)
PvuI (302)
Sz.pombe ADH prom.
EcoRI (729)
Mating factor P- P1-29 Spb fusion
BamHI (2160)
ADH TT
SacI (3069)
rDNA
his5
XbaI (4242)
SacII (4775)
TEF1 Prom.
EM7 Prom.
ZeoR
SmaI (5423)
XmaI (5421)
BglII (6439)
KanR
ColE1 ori

Figure 18F

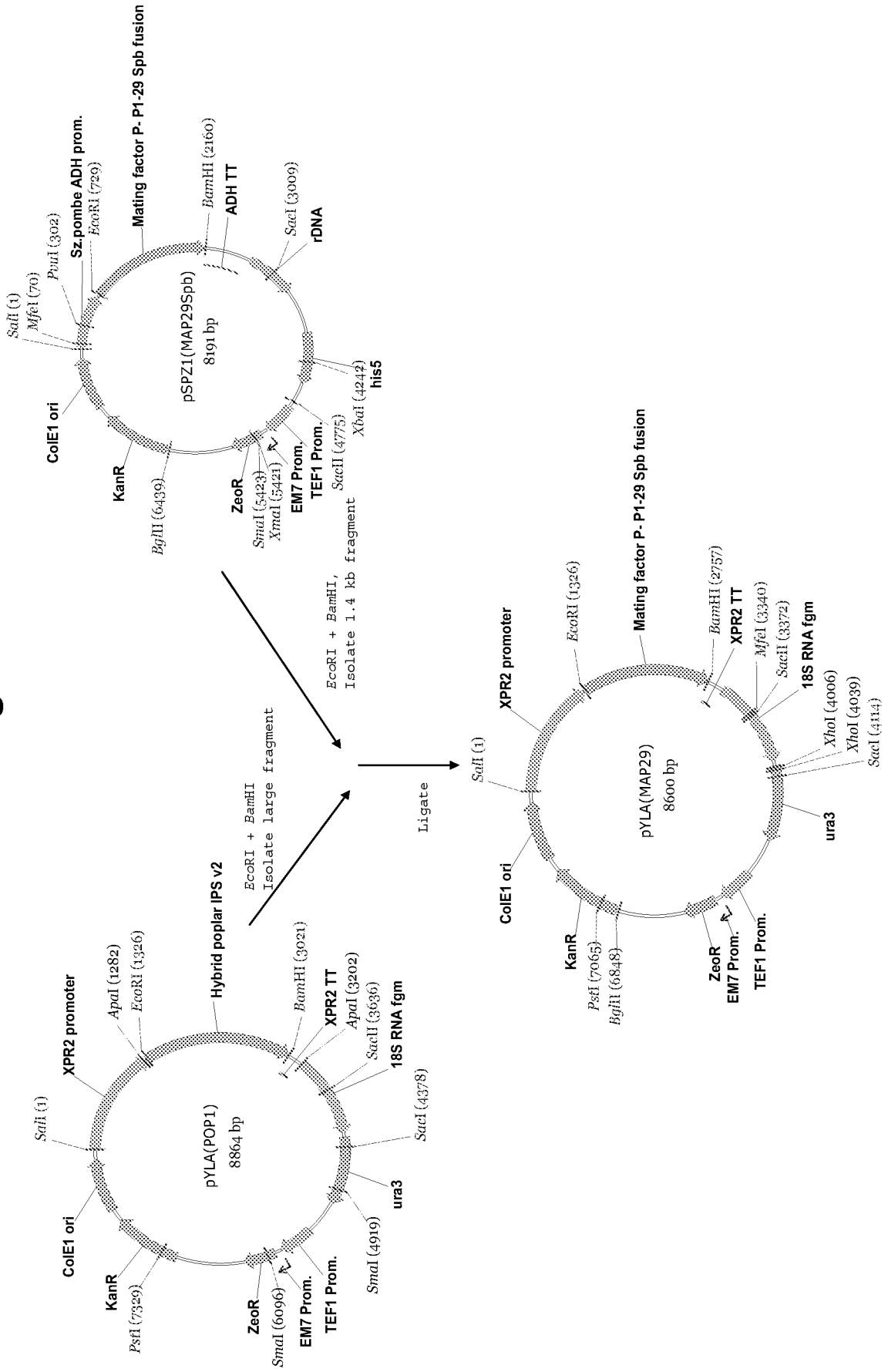
pSPZ1(MAP29Spb)
8191 bp
Mating factor P- P1-29 Spb fusion
Sz.pombe ADH prom.
ADH TT
rDNA
his5
ColE1 ori
KanR
ZeoR
EM7 Prom.
TEF1 Prom.
SalI (1)
MfeI (70)
PvuI (302)
EcoRI (729)
BamHI (2160)
SacI (3009)
XbaI (4242)
SacII (4775)
SmaI (5423)
XmaI (5421)
BglII (6439)
EcoRI + BamHI
Isolate large fragment
EcoRI + BamHI,
Isolate 1.4 kb fragment
Ligate
pYLA(POP1)
8864 bp
Hybrid poplar IPS v2
XPR2 promoter
ApaI (1282)
EcoRI (1326)
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
SacI (4378)
ura3
SmaI (4919)
SmaI (6096)
PstI (7329)
pYLA(MAP29)
8600 bp
EcoRI (1326)
Mating factor P- P1-29 Spb fusion
BamHI (2757)
XPR2 TT
MfeI (3340)
SacII (3372)
18S RNA fgm
XhoI (4006)
XhoI (4039)
SacI (4114)
PstI (7065)
BglII (6848)

Figure 19

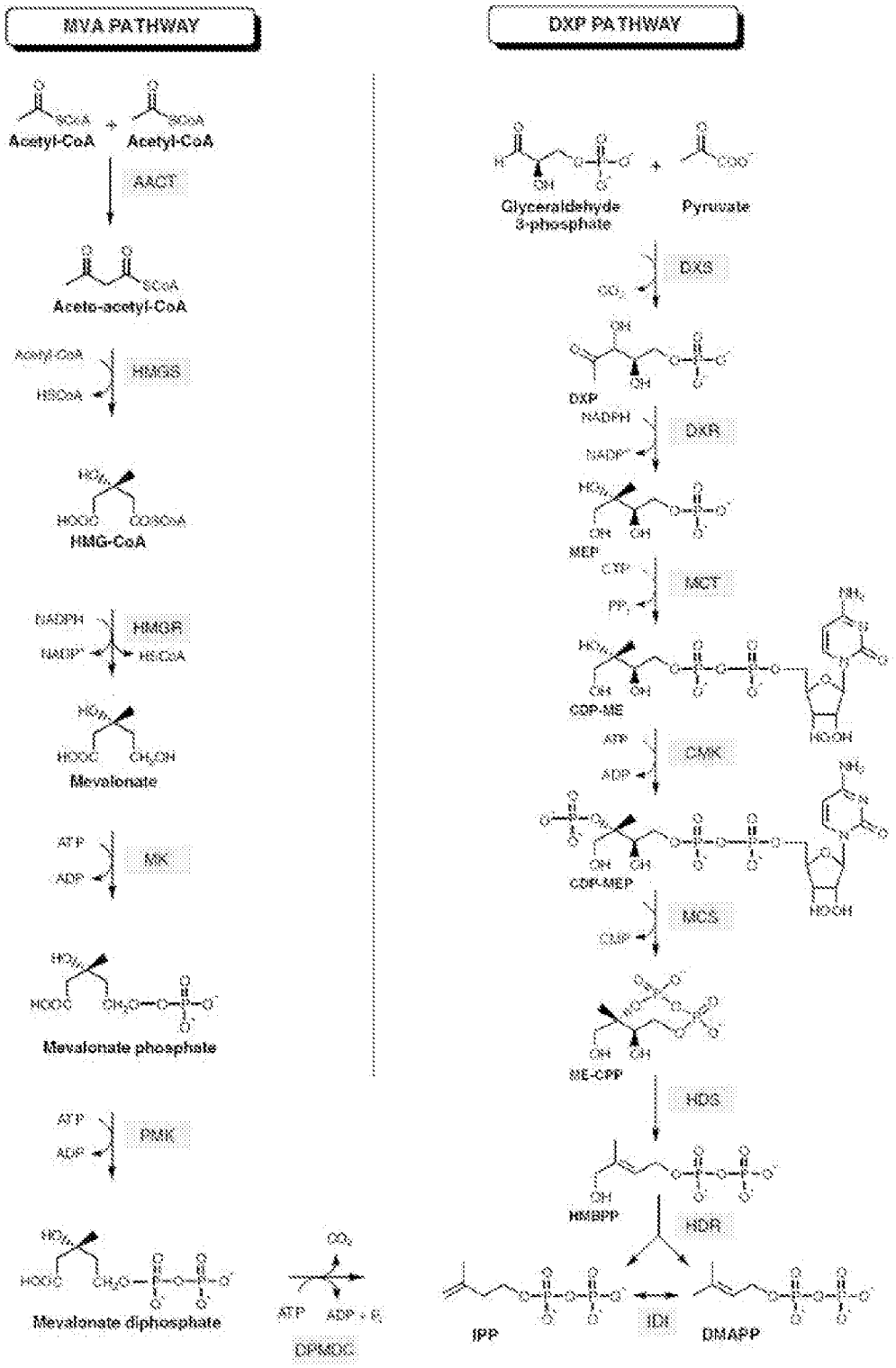
MVA PATHWAY
DXP PATHWAY
Acetyl-CoA
Acetyl-CoA
AACT
Aceto-acetyl-CoA
HMGS
HMG-CoA
NADPH
NADP+
HMGR
Mevalonate
ATP
ADP
MK
Mevalonate phosphate
ATP
ADP
PMK
Mevalonate diphosphate
ATP
ADP + Pi
DPMDC
Glyceraldehyde 3-phosphate
Pyruvate
DXS
DXP
NADPH
NADP+
DXR
MEP
CTP
PPi
MCT
CDP-ME
ATP
ADP
CMK
CDP-MEP
CMP
MCS
ME-cPP
HDS
HMBPP
HDR
IPP
IDI
DMAPP

Figure 20

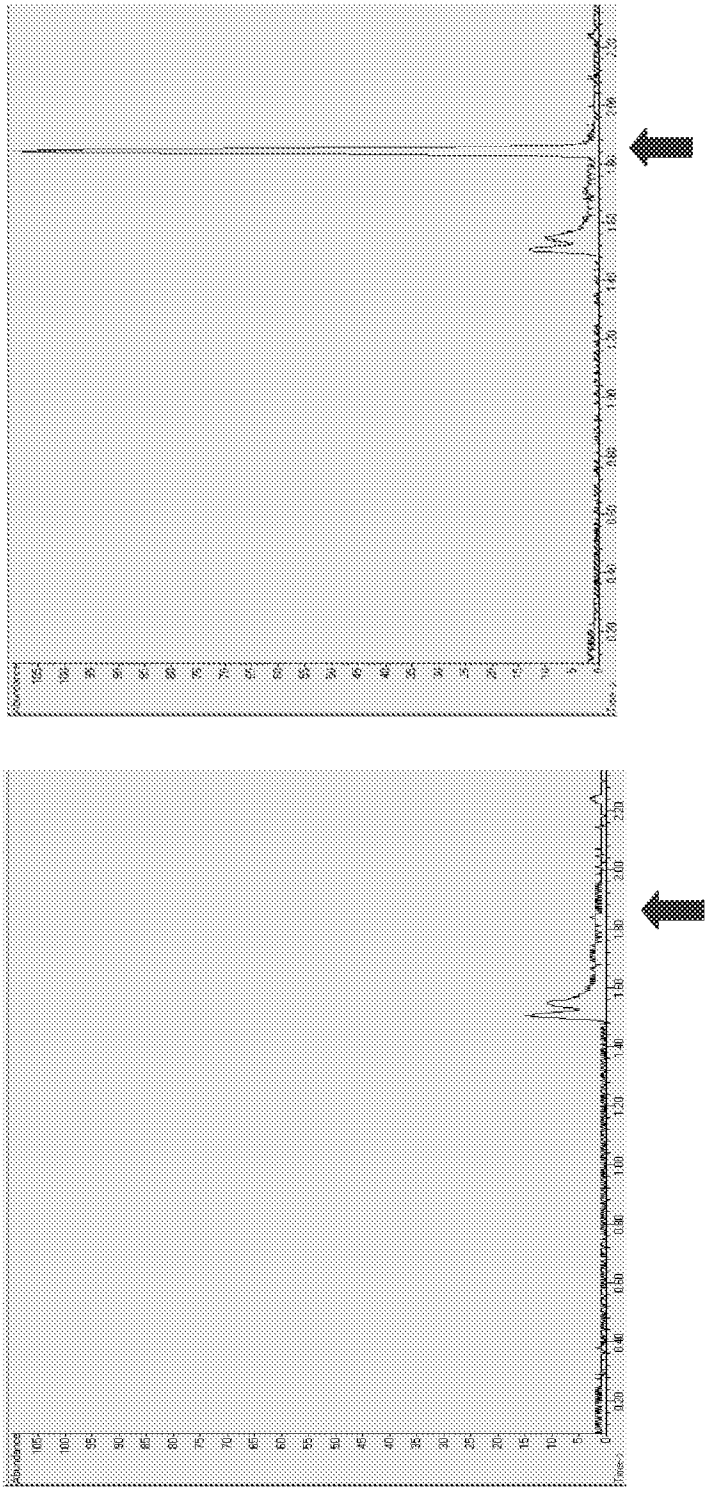
Y. lipolytica CLIB122:: pYLA(KZ1)
Y. lipolytica CLIB122:: pYLA(MAP29)

Figure 21

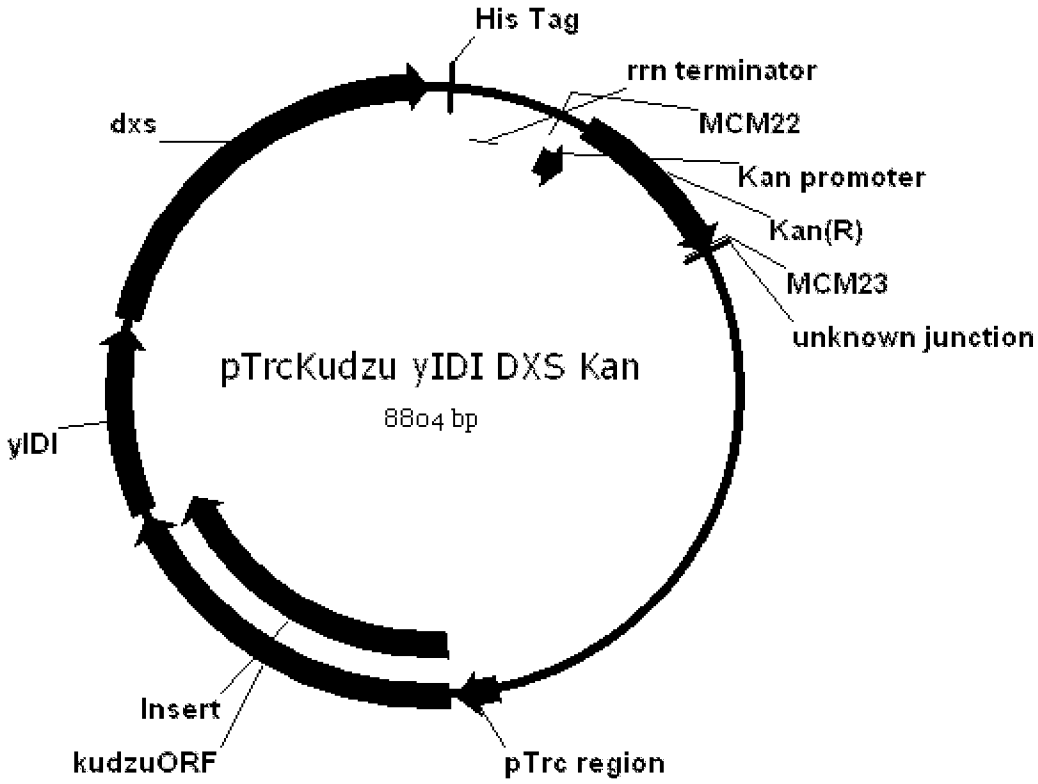
His Tag
rrn terminator
MCM22
Kan promoter
Kan(R)
MCM23
unknown junction
dxs
pTrcKudzu yIDI DXS Kan
8804 bp
yIDI
Insert
kudzuORF
pTrc region

Figure 22A

1-
gctggtaccatatgggaattcgaagctttctagaacaaaaactcatctcagaagaggatctgaa
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ccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgt
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Figure 22B

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attgtcgcggcgattaaatctcgcgccgatcaactgggtgccagcgtggtggtgtcgatggtag
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Figure 22C

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Figure 22D

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cgccagccatgaagcgctggtcaccgtagaagaaaacgccattatgggcggcgcaggcagcggc
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Figure 23A

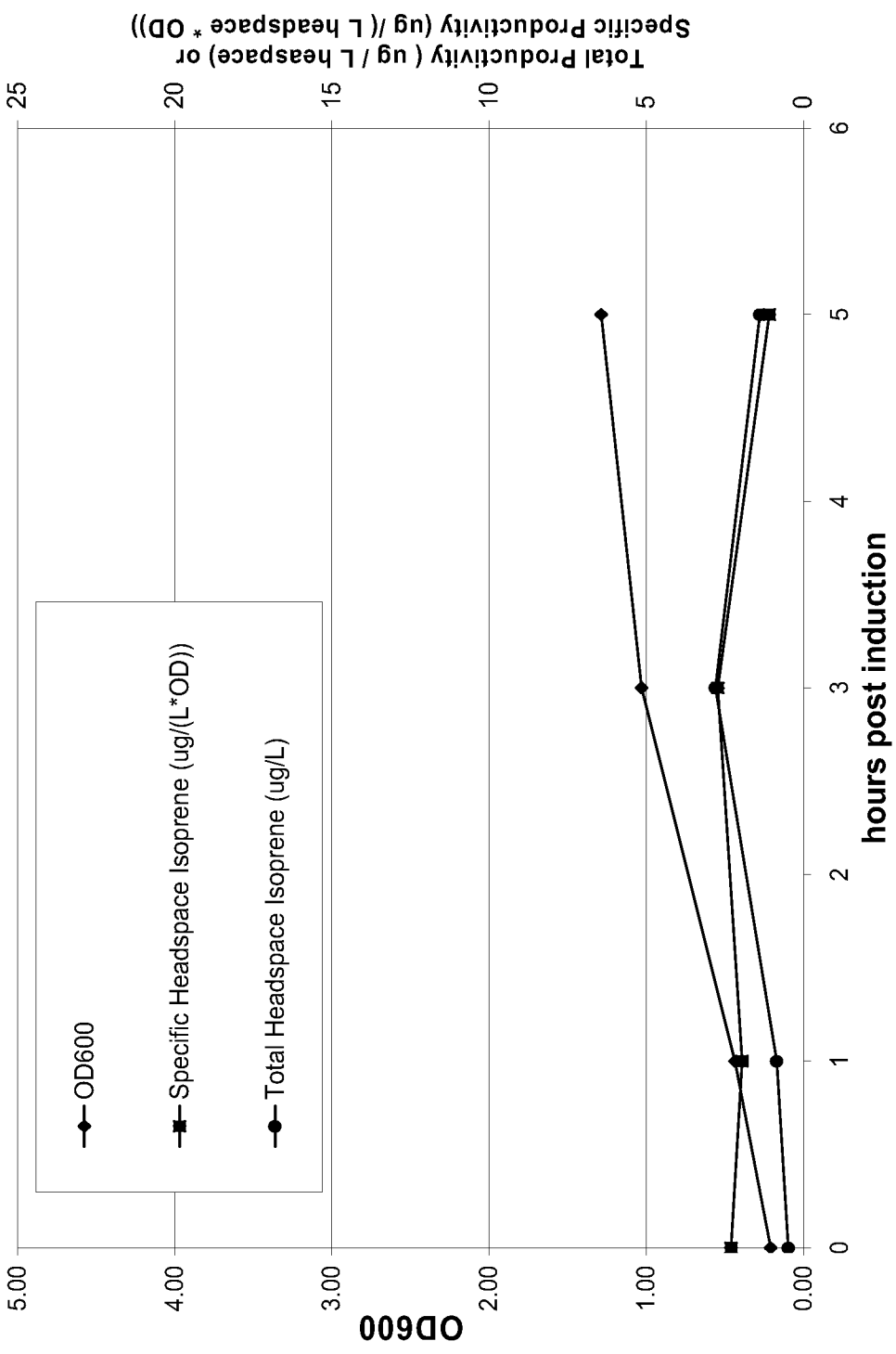

pTrcKudzu kan
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Total Headspace Isoprene (ug/L)
Specific Headspace Isoprene (ug/(L*OD))
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23B

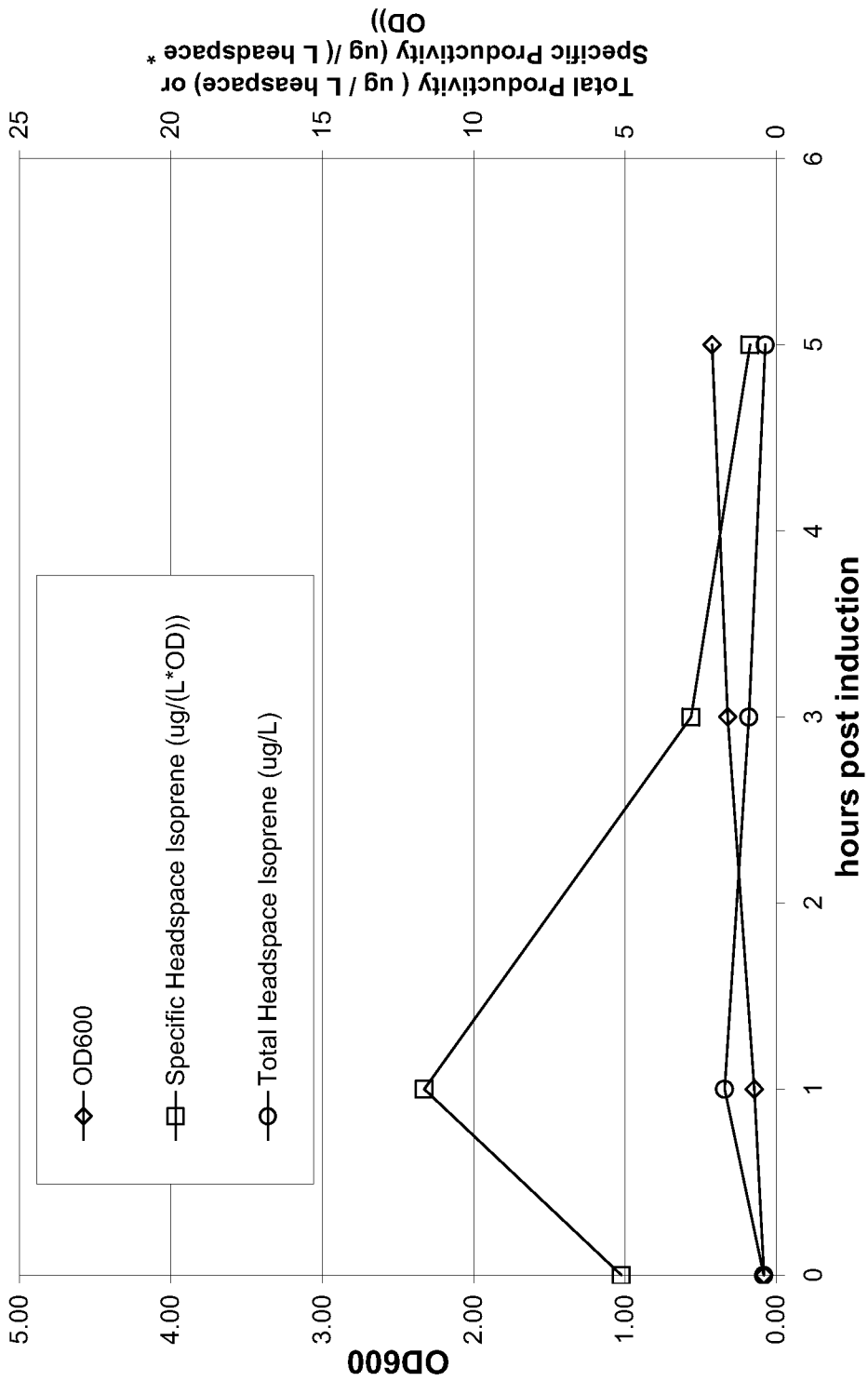

pTrcKudzu yIDI kan
OD600
Total Productivity (ug / L headspace) or Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23C

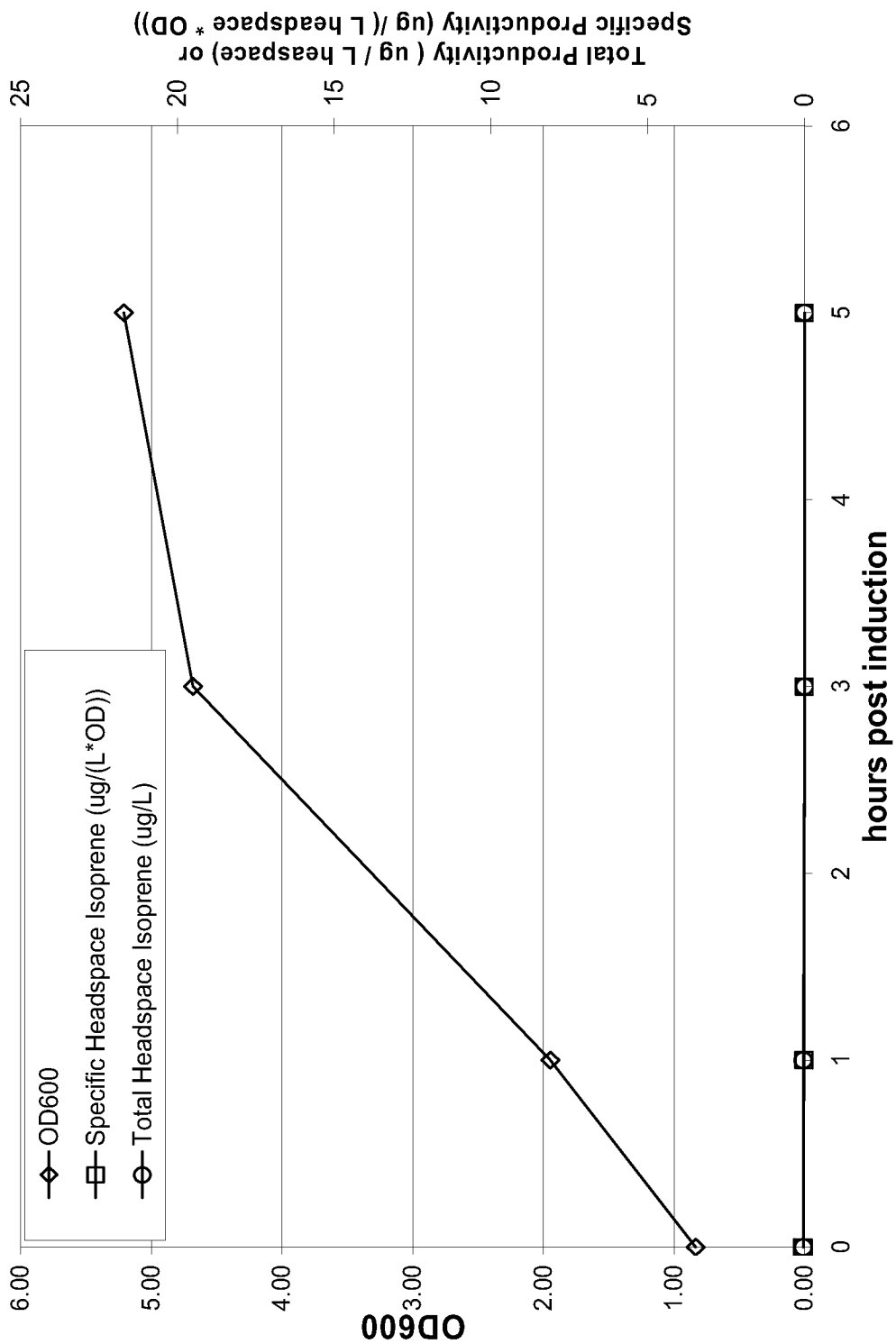

pTrcKudzu DXS kan
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
25
20
15
10
5
0
0
1
2
3
4
5
6

Figure 23D

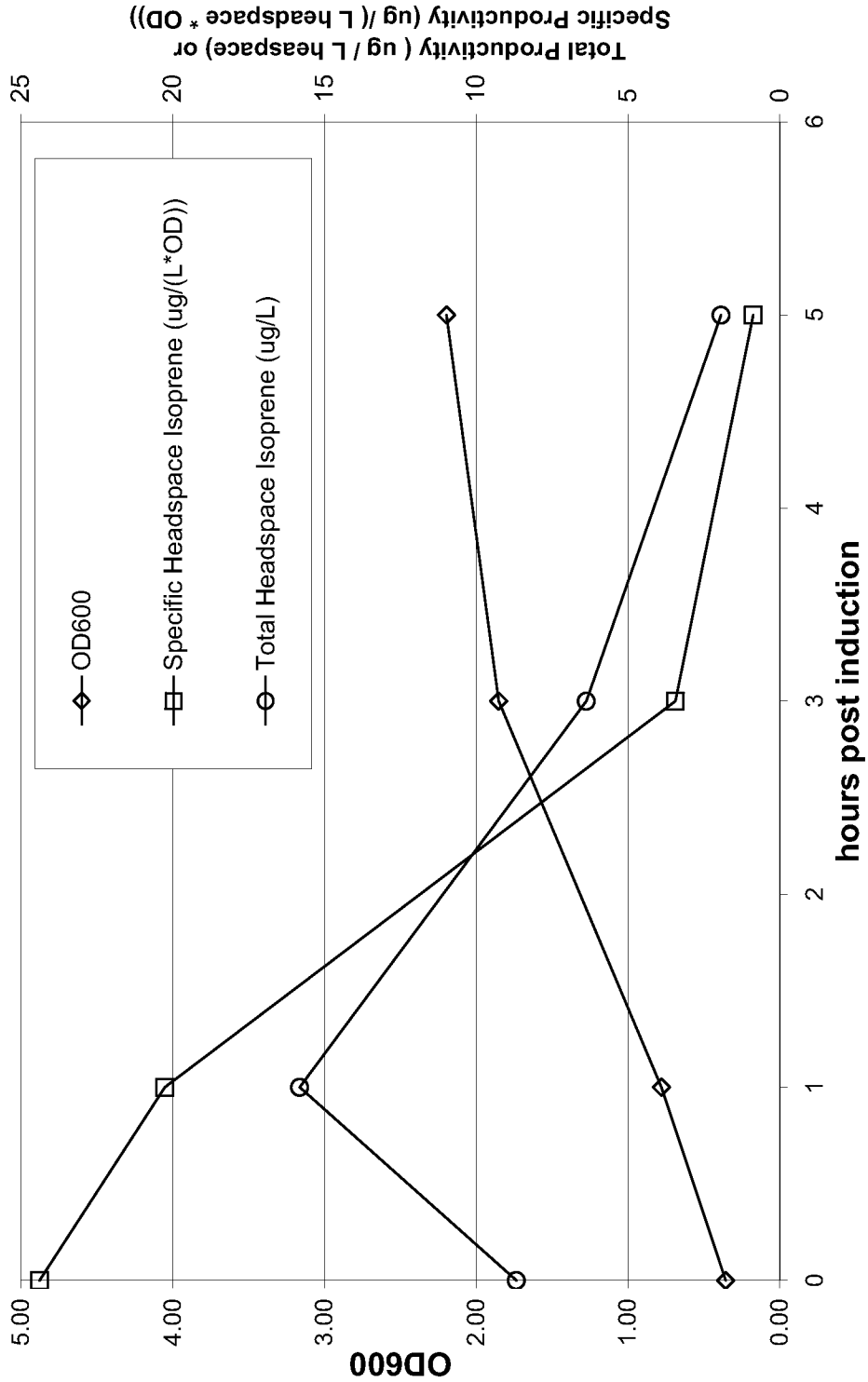
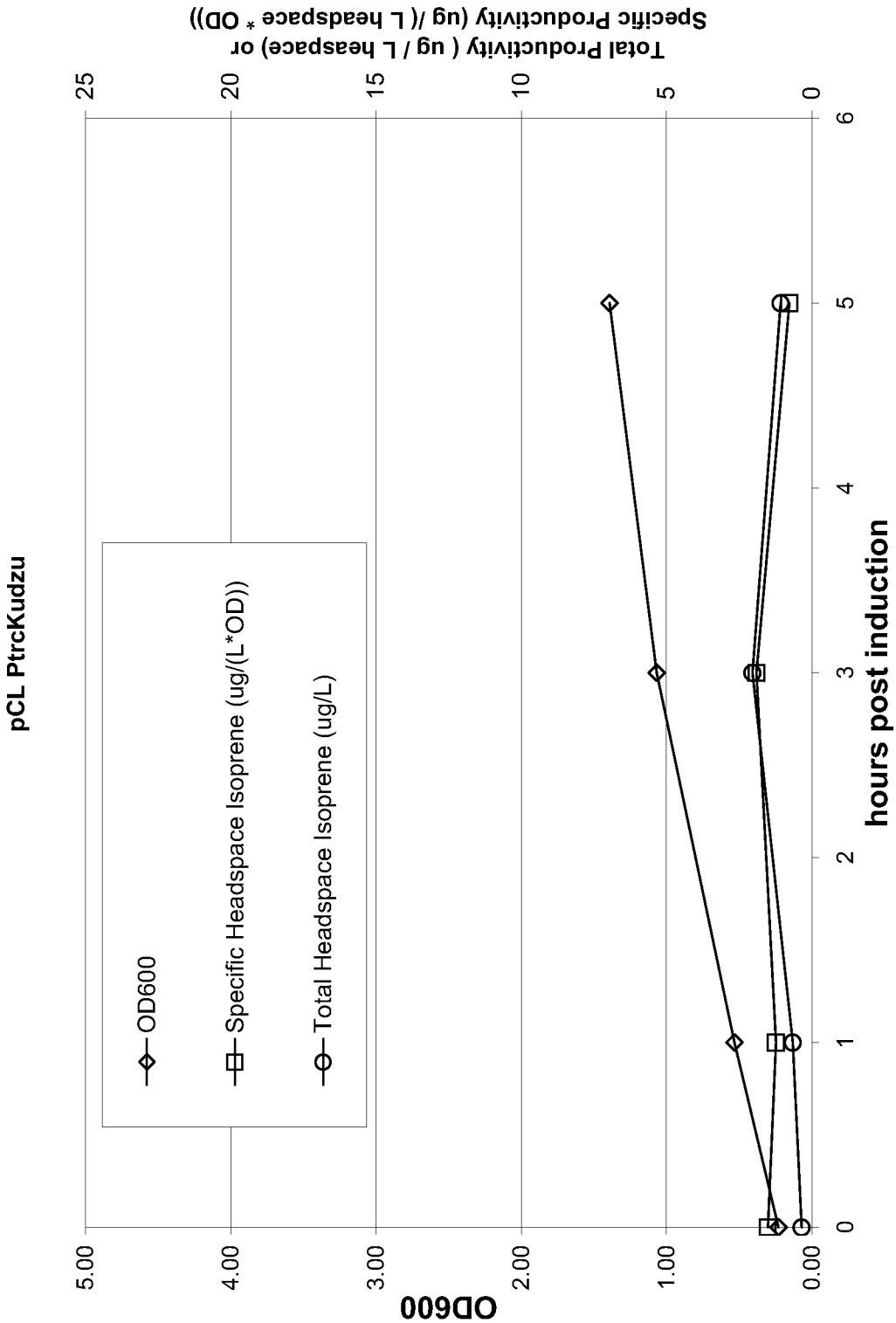

pTrcKudzu yIDI DXS kan
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23E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OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23F

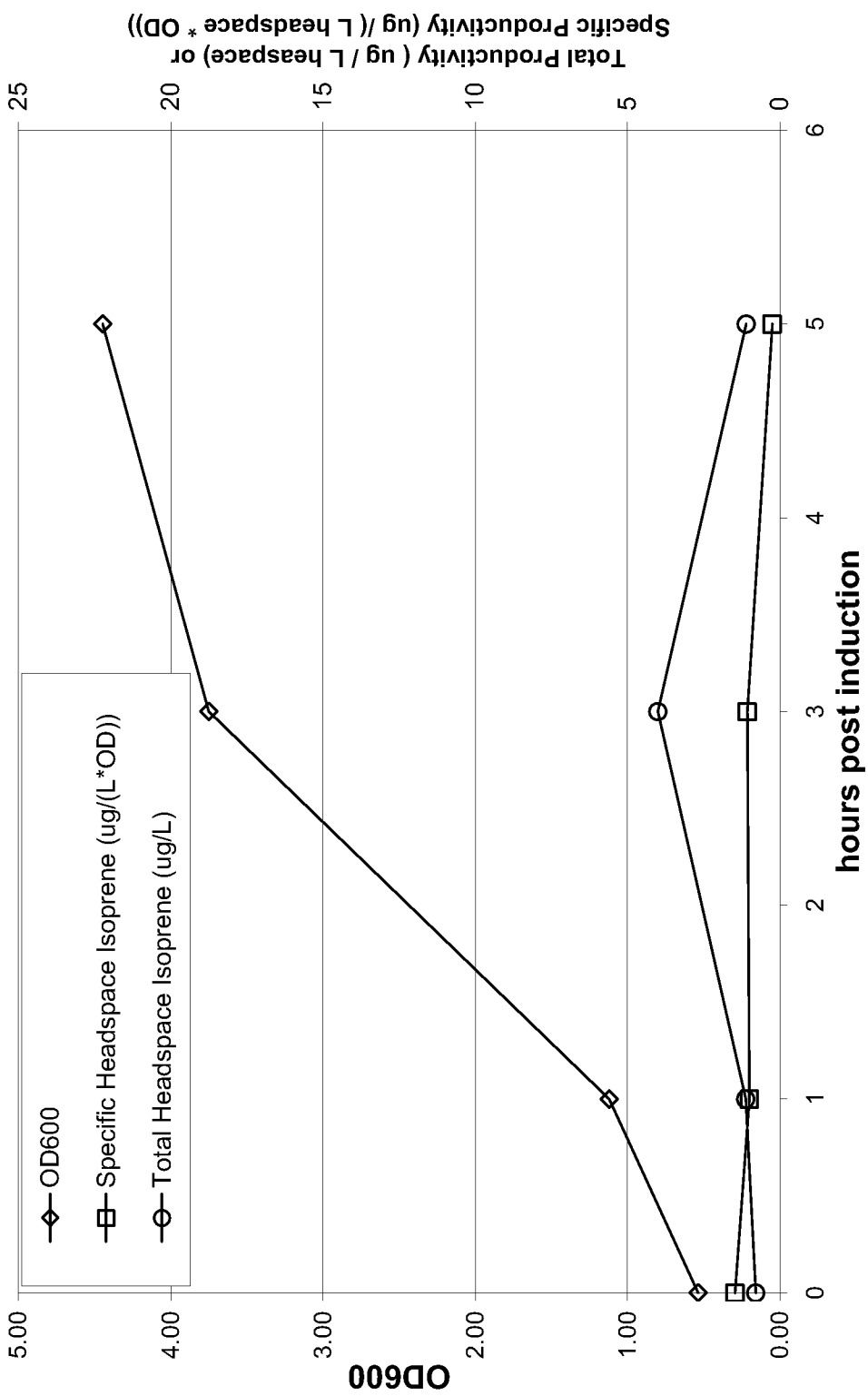
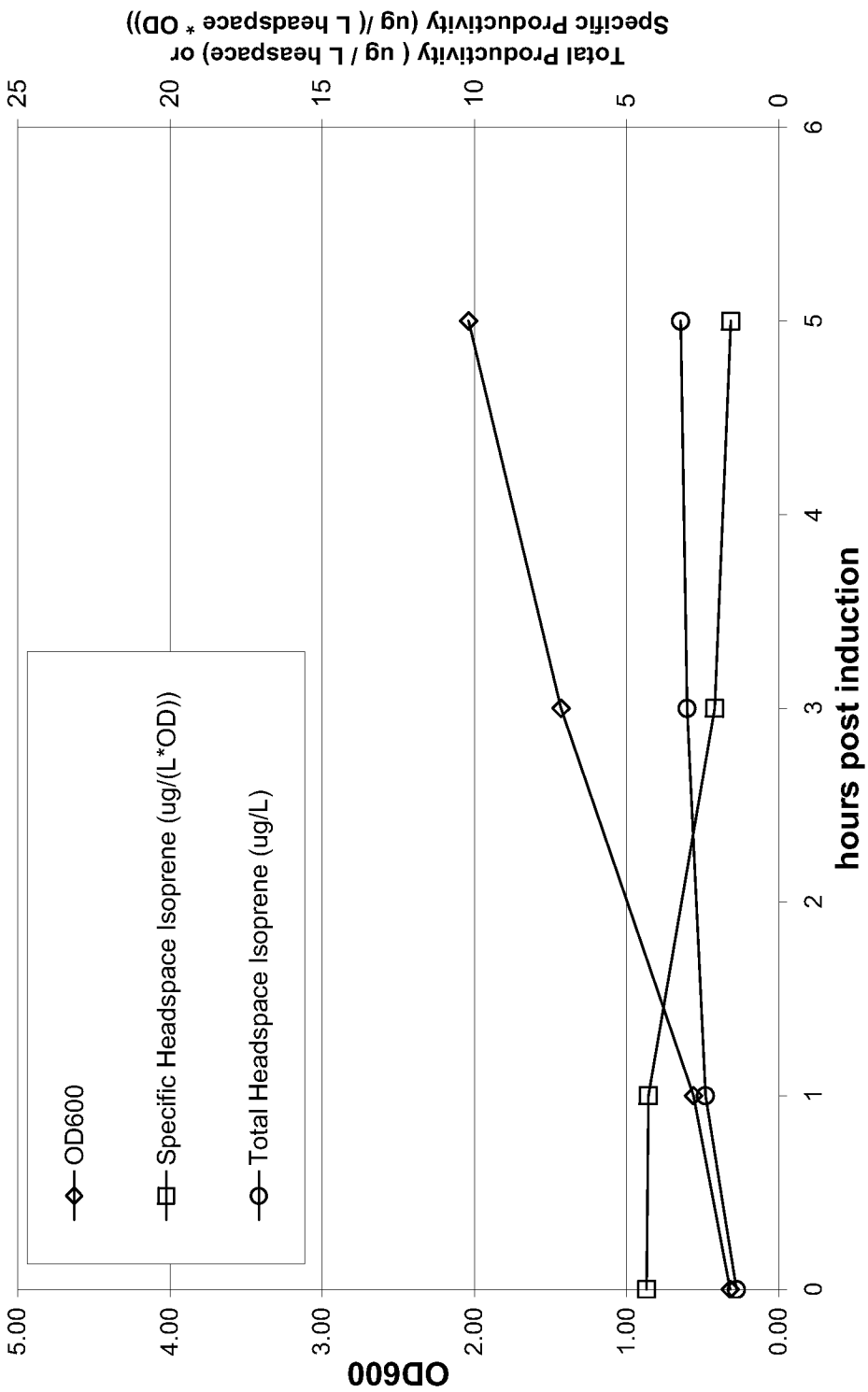

pCL PtrcKudzu ylDI
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23G


pCL PtrcKudzu DXS
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
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

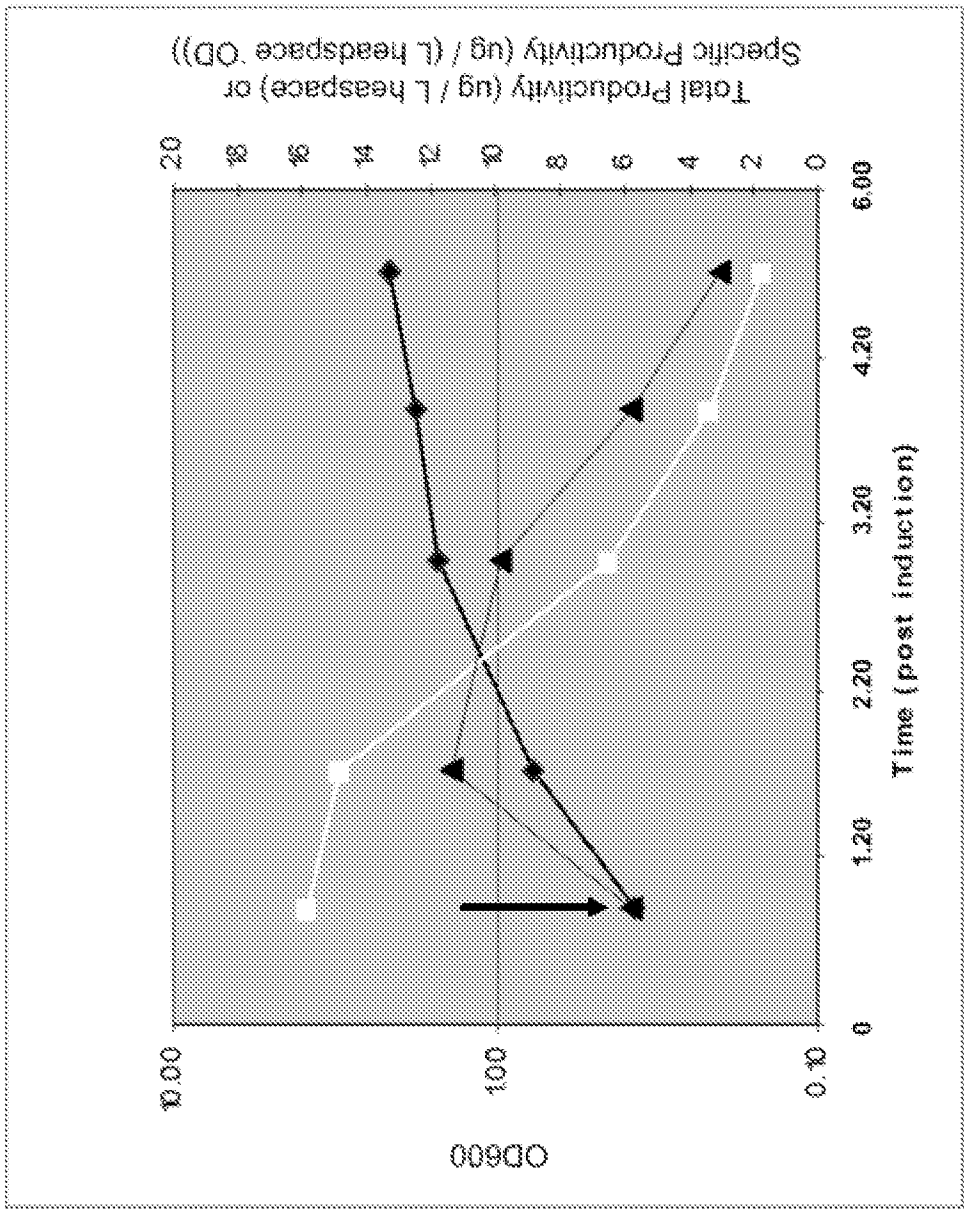
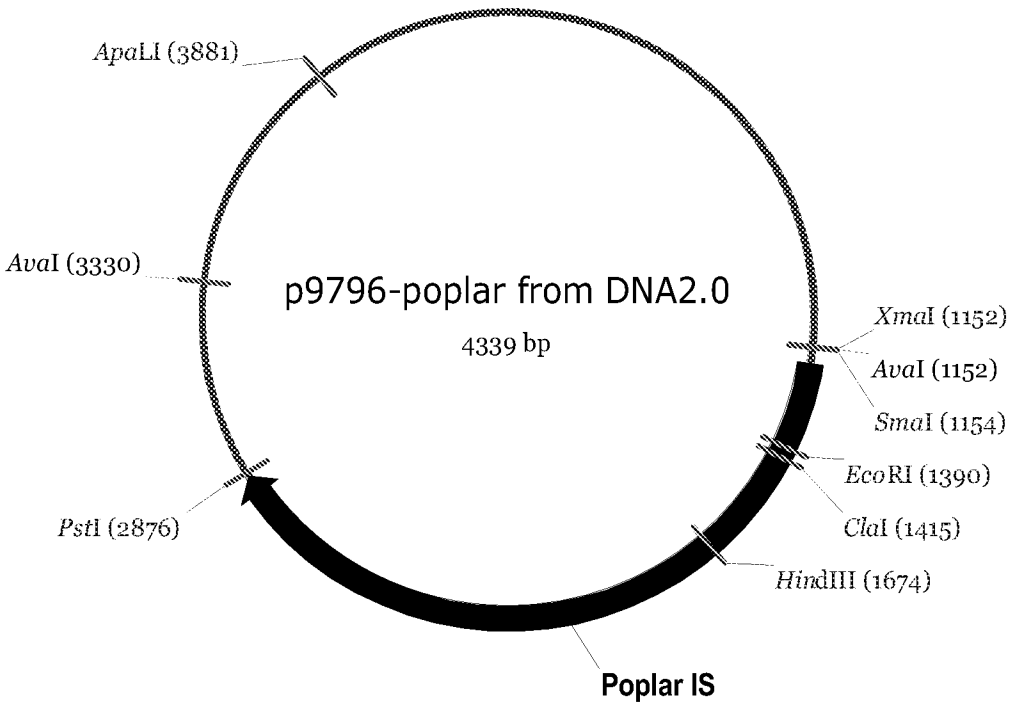

Total Productivity (ug / L headspace) or
Specific Productivity (ug / (L headspace *OD))
20
18
16
14
12
10
8
6
4
2
0
6.00
4.20
3.20
2.20
1.20
0
Time (post induction)
OD600
10.00
1.00
0.10

Figure 23H

Figure 24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AvaI (3330)
p9796-poplar from DNA2.0
4339 bp
XmaI (1152)
AvaI (1152)
SmaI (1154)
EcoRI (1390)
ClaI (1415)
HindIII (1674)
PstI (2876)
Poplar IS

Figure 25A

5'-
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Figure 25B

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Figure 26

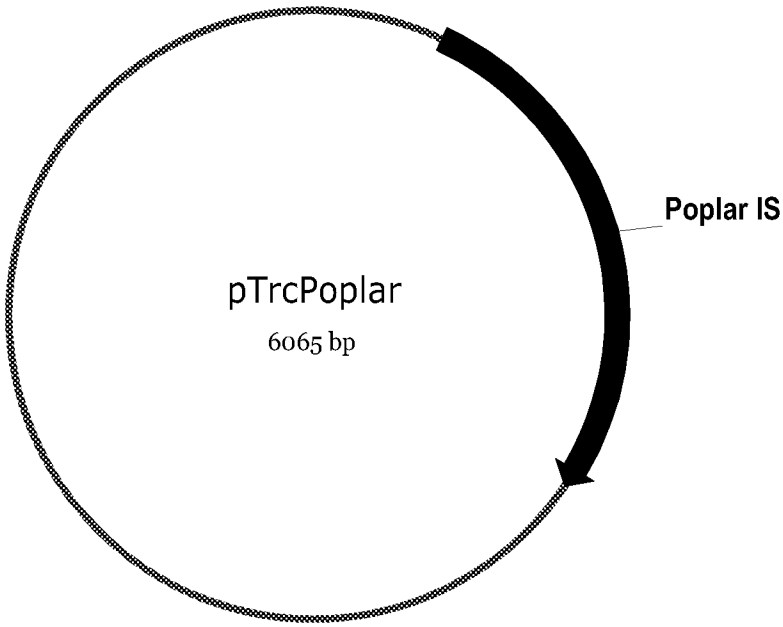

Figure 27A

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Figure 27B

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Figure 27C

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Figure 28

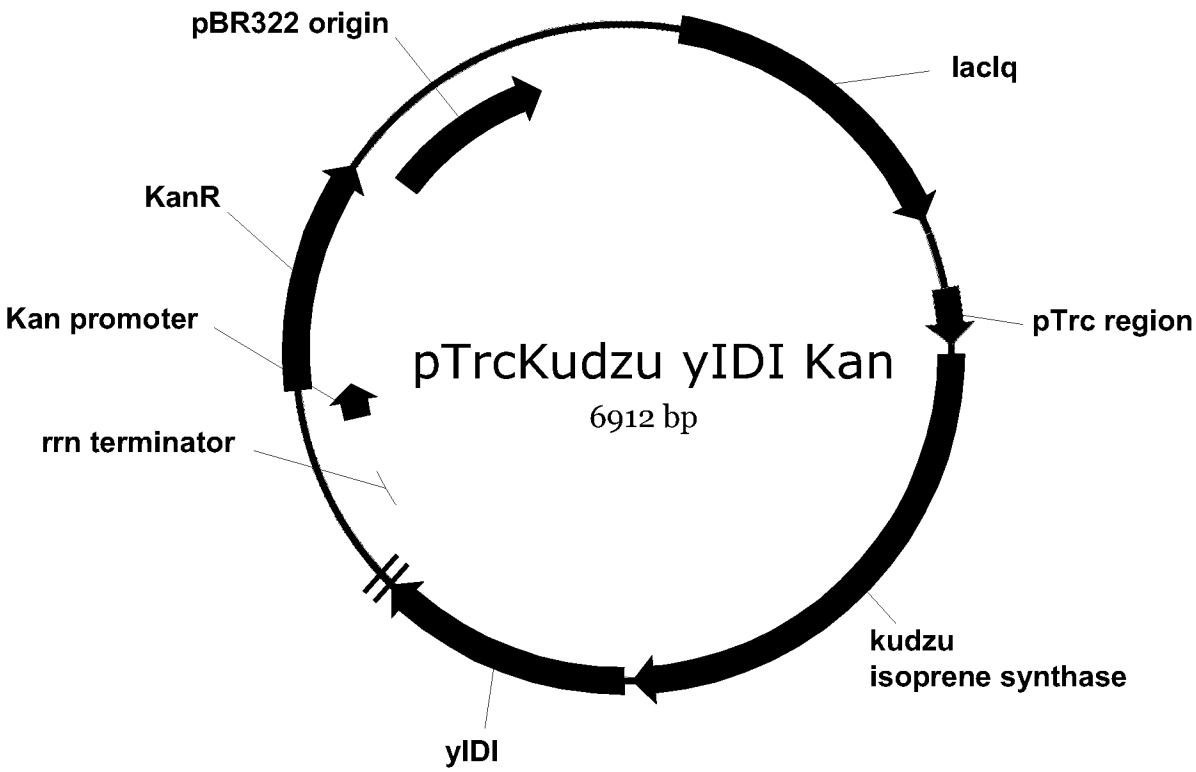
pBR322 origin
laclq
KanR
Kan promoter
pTrc region
pTrcKudzu yIDI Kan
6912 bp
rrn terminator
kudzu
isoprene synthase
yIDI

Figure 29A

5'-
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Figure 29B

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Figure 29C

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Figure 30

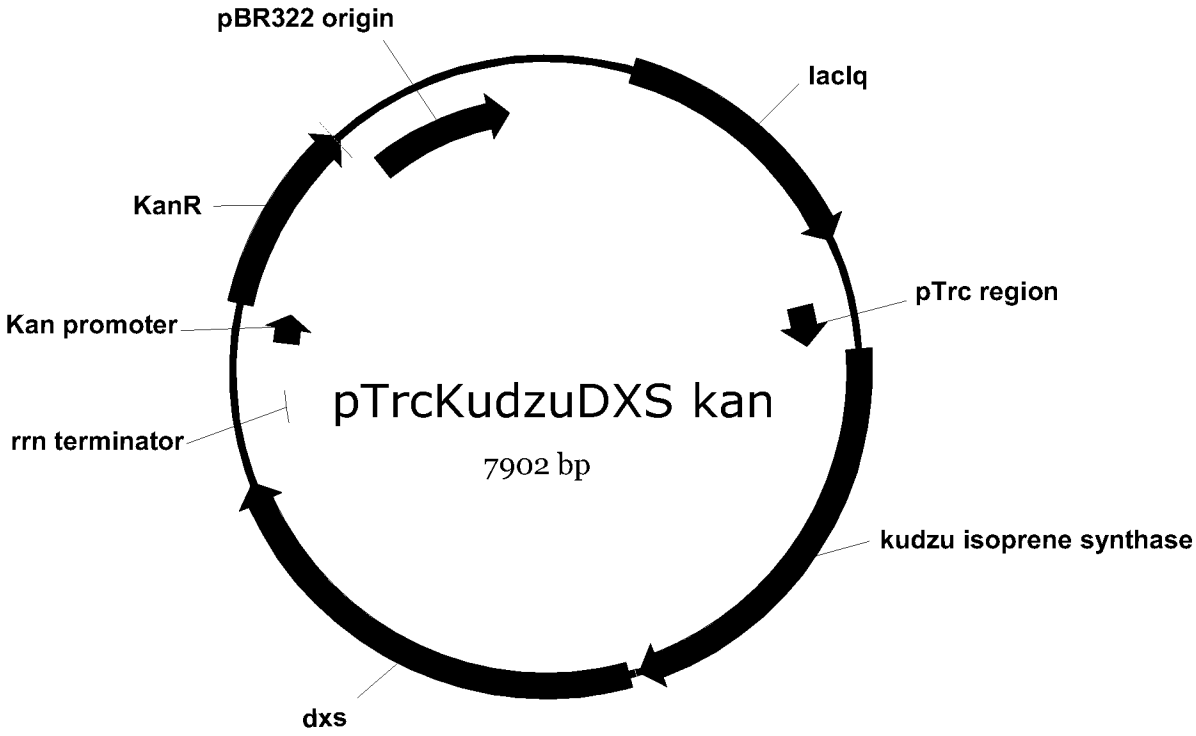
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Figure 31A

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Figure 31B

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Figure 31C

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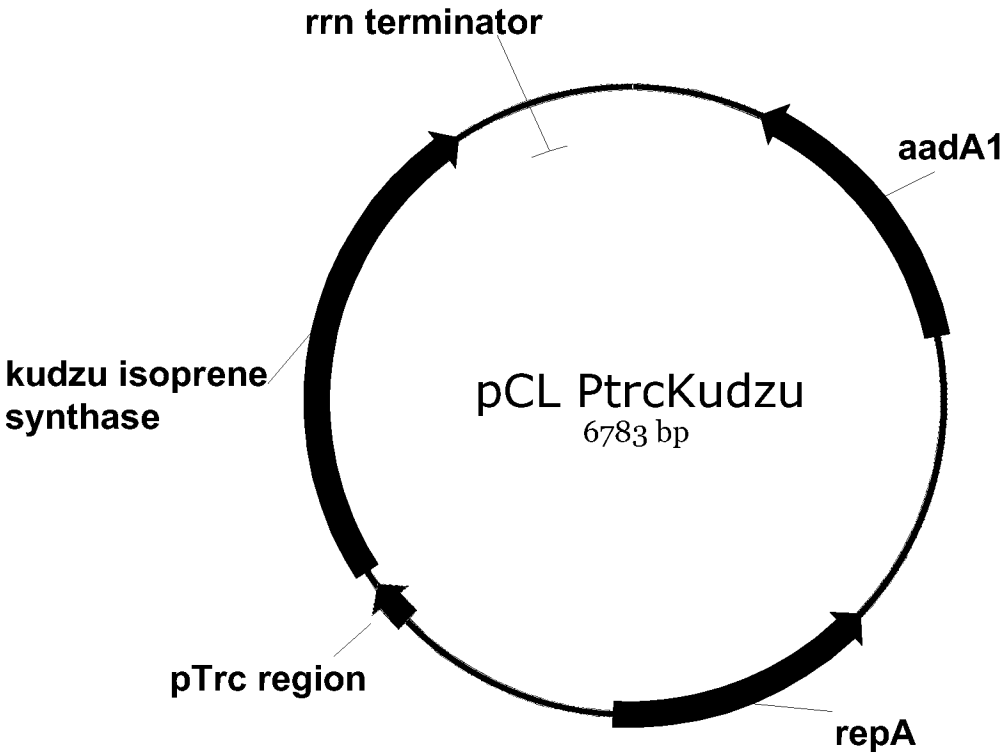
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Figure 33A

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Figure 33B

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Figure 33C

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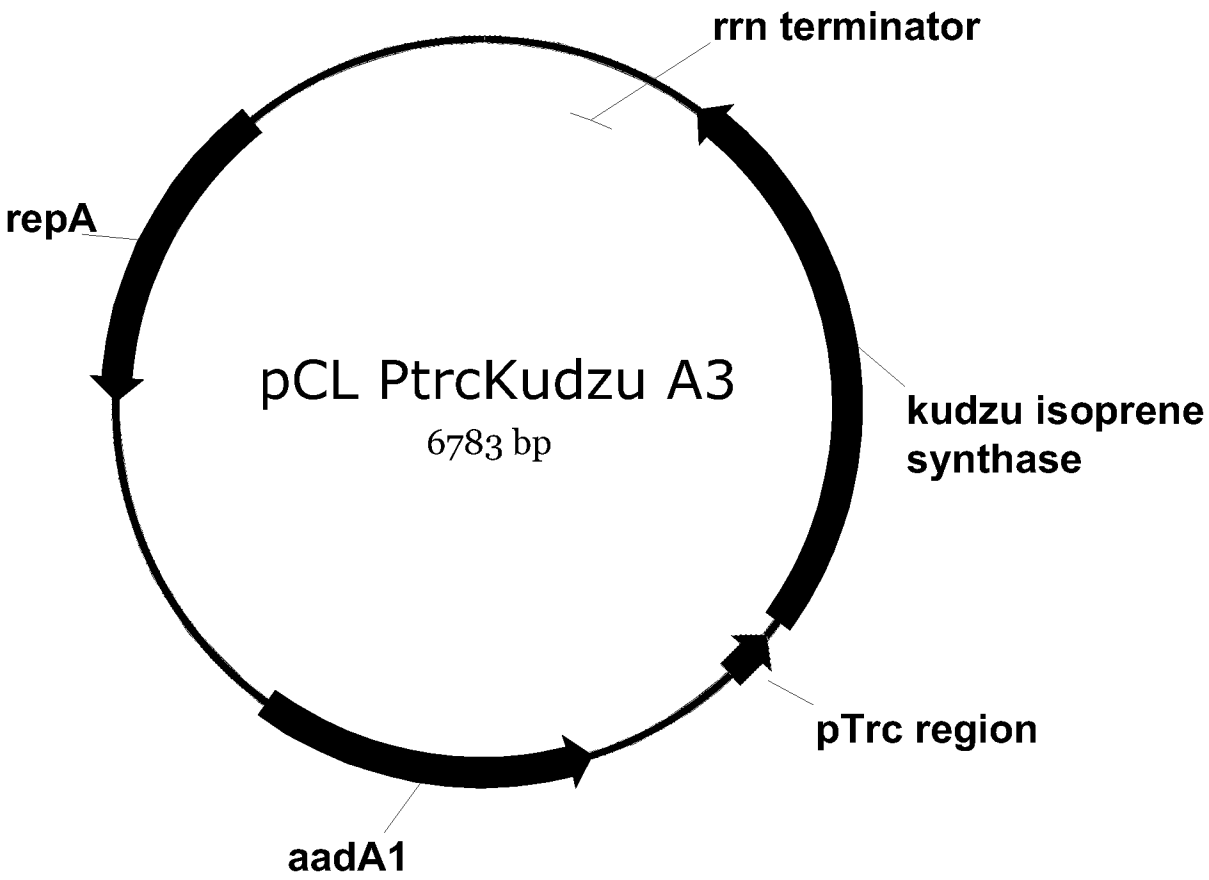
Figure 34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kudzu isoprene synthase
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Figure 35A

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Figure 35B

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Figure 35C

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Figure 36

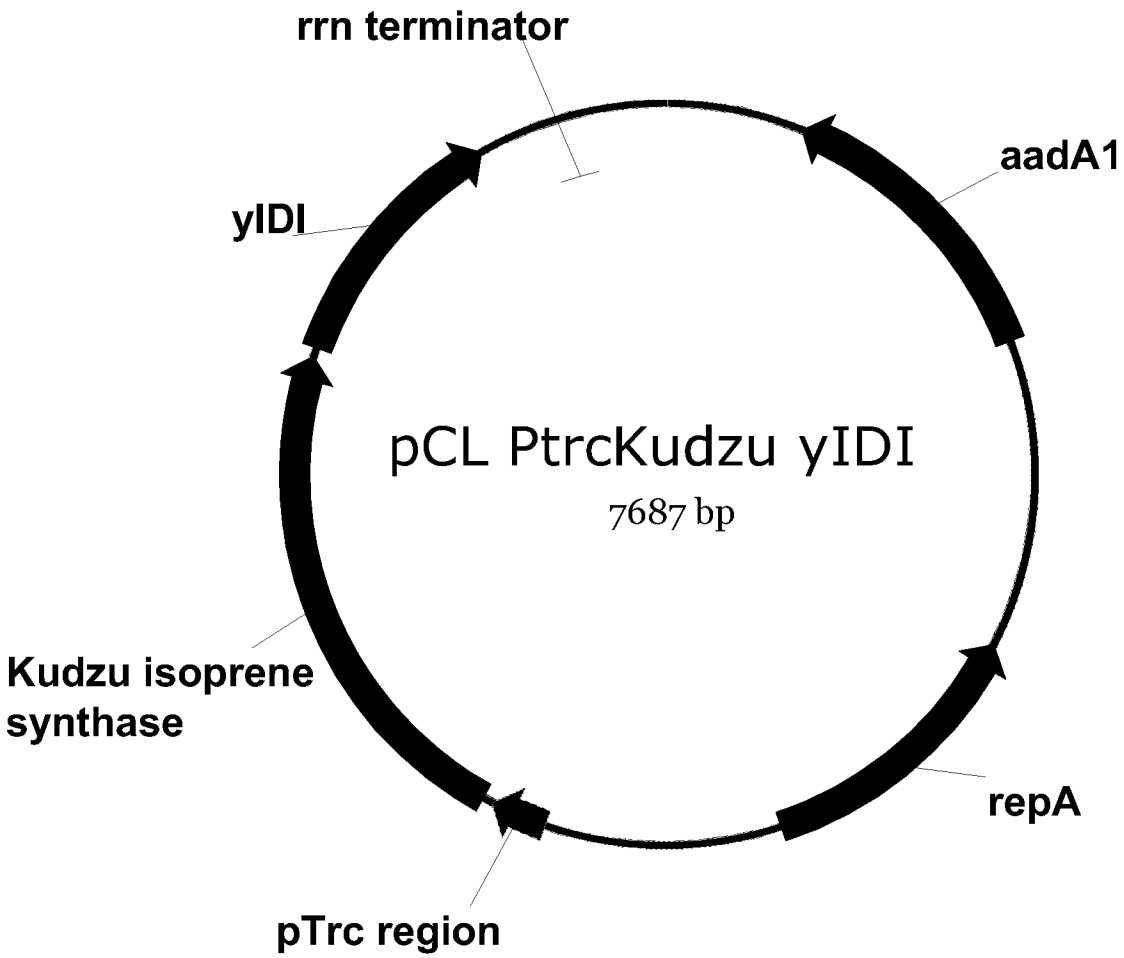
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Figure 37A

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Figure 37B

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Figure 37C

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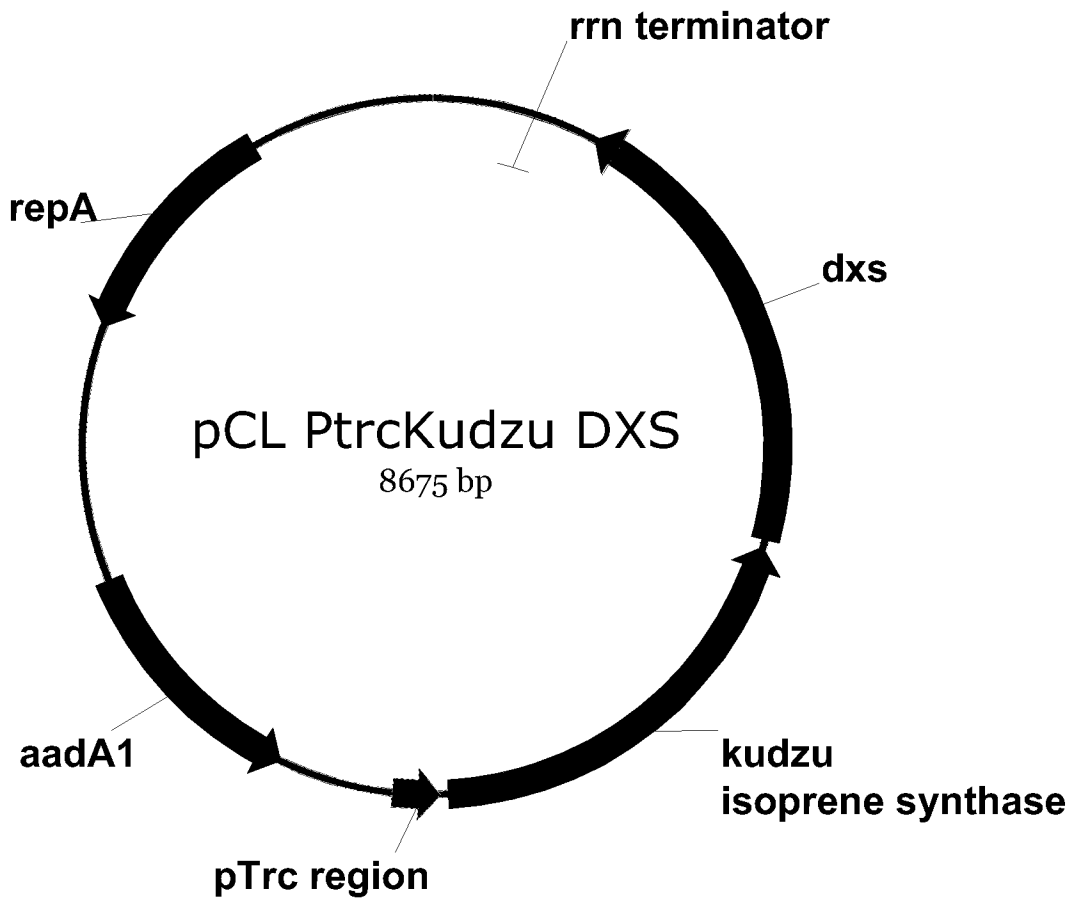
Figure 38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dxs
pCL PtrcKudzu DXS
8675 bp
aadA1
kudzu
isoprene synthase
pTrc region

Figure 39A

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Figure 39B

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Figure 39C

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tatattttatgaatttttttaactggaaaagataaggcaatatctcttcactaaaaactaattc
taatttttcgcttgagaacttggcatagtttgtccactggaaaatctcaaagcctttaaccaaa
ggattcctgatttccacagttctcgtcatcagctctctggttgctttagctaatacaccataag
catttcccctactgatgttcatcatctgagcgtattggttataagtgaacgataccgtccgttc
tttccttgtagggttttcaatcgtggggttgagtagtgccacacagcataaaattagcttggtt
tcatgctccgttaagtcatagcgactaatcgctagttcatttgctttgaaaacaactaattcag
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ataattactagtccttttccttgagttgtgggtatctgtaaattctgctagacctttgctgga
aaacttgtaaattctgctagaccctctgtaaattccgctagacctttgtgtgttttttttgttt
atattcaagtggttataatttatagaataaagaaagaataaaaaaagataaaaagaatagatcc
cagccctgtgtataactcactactttagtcagttccgcagtattacaaaaggatgtcgcaaacg
ctgtttgctcctctacaaaacagaccttaaaaccctaaaggcttaagtagcaccctcgcaagct
cgggcaaatcgctgaatattccttttgtctccgaccatcaggcacctgagtcgctgtctttttc

Figure 39D

Gtgacattcagttcgctgcgctcacggctctggcagtgaatgggggtaaatggcactacaggcg
ccttttatggattcatgcaaggaaactacccataatacaagaaaagcccgtcacgggcttctca
gggcgttttatggcgggtctgctatgtggtgctatctgacttttgctgttcagcagttcctgc
cctctgattttccagtctgaccacttcggattatcccgtgacaggtcattcagactggctaatg
cacccagtaaggcagcggtatcatcaacaggctta
(SEQ ID NO:28)

Figure 40A

A.

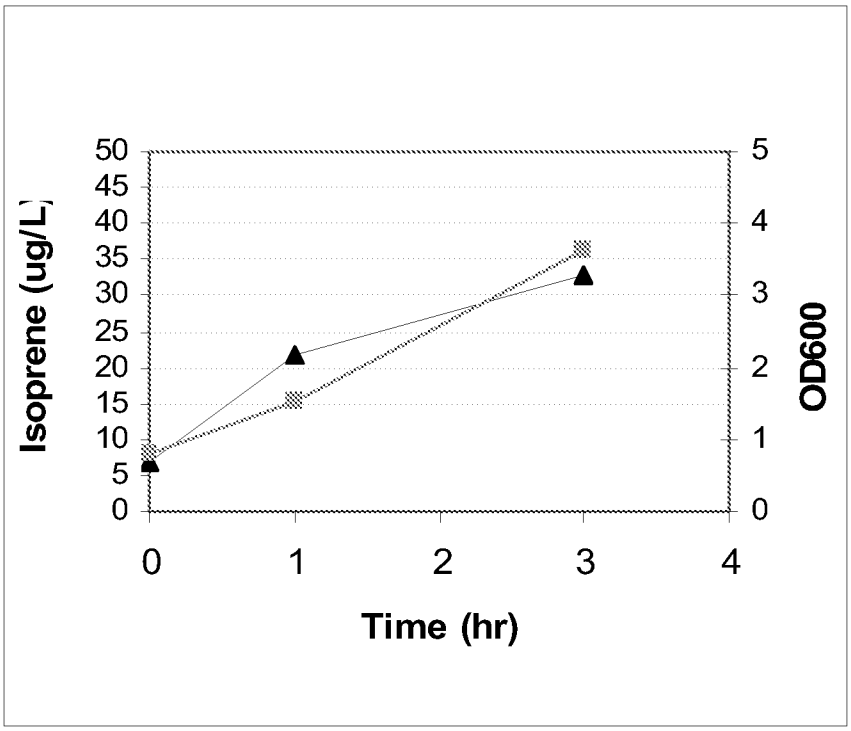

Isoprene (ug/L)
OD600
Time (hr)
0
5
10
15
20
25
30
35
40
45
50
0
1
2
3
4
0
1
2
3
4
5

Figure 40B

B.

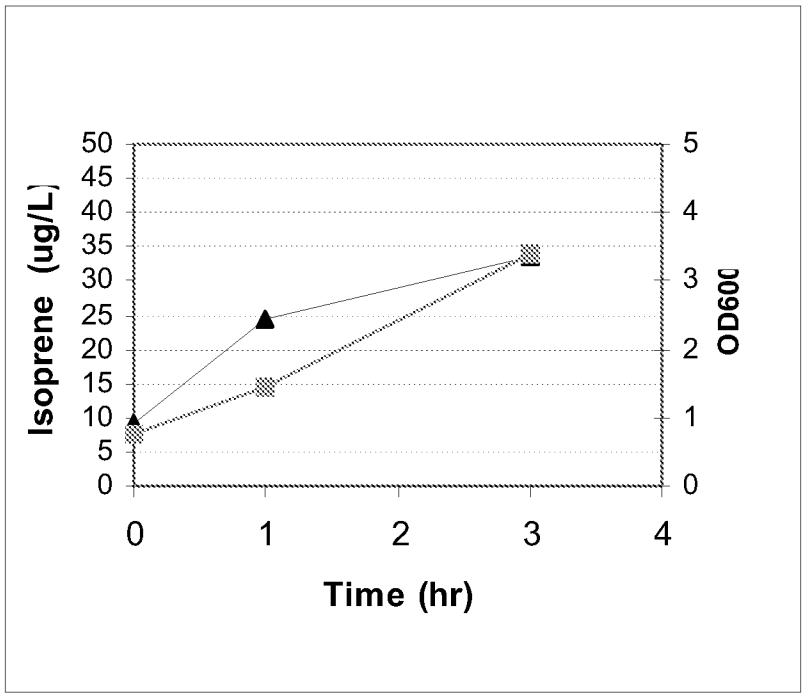

Isoprene (ug/L)
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
Time (hr)
5
4
3
2
1
0
OD600

Figure 40C

C.

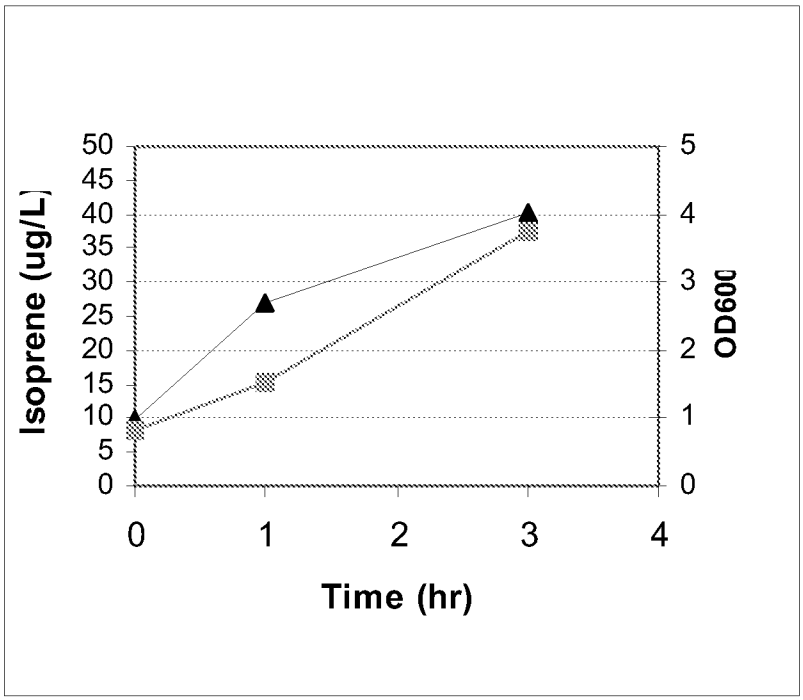

Isoprene (ug/L)
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
Time (hr)
5
4
3
2
1
0
OD600

Figure 40D

D.

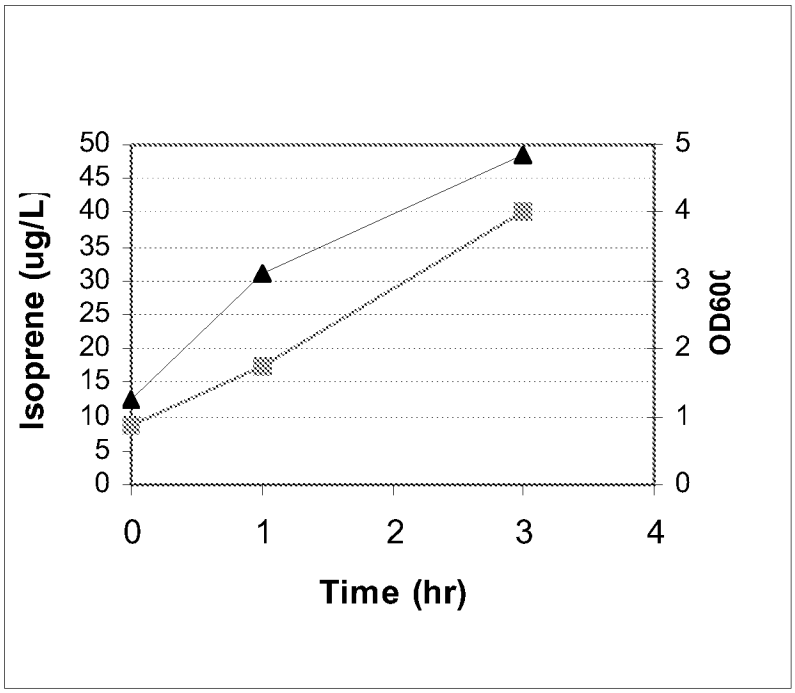

Isoprene (ug/L)
50
45
40
35
30
25
20
15
10
5
0
OD60C
5
4
3
2
1
0
0
1
2
3
4
Time (hr)

Figure 40E

E.

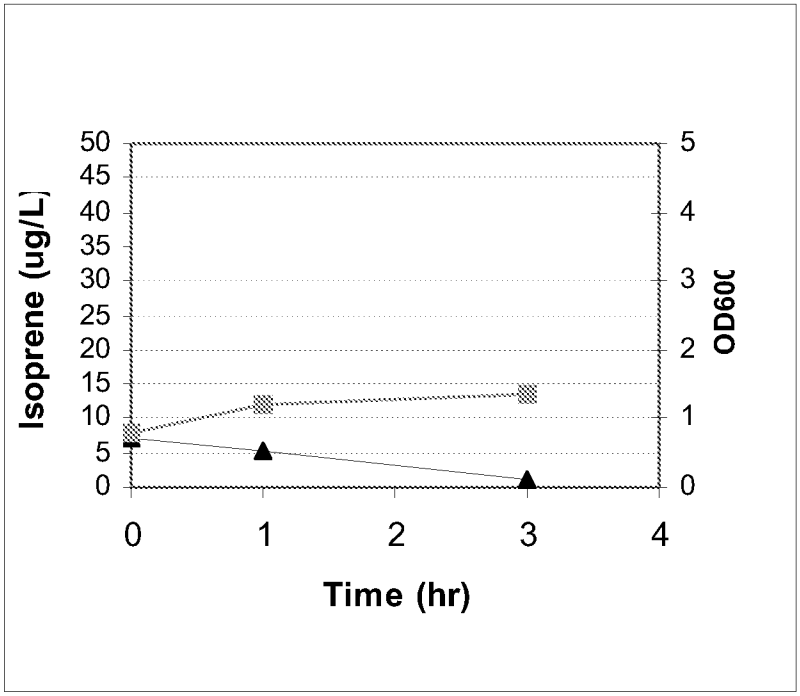

Isoprene (ug/L)
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
Time (hr)
5
4
3
2
1
0
OD60(

Figure 41A

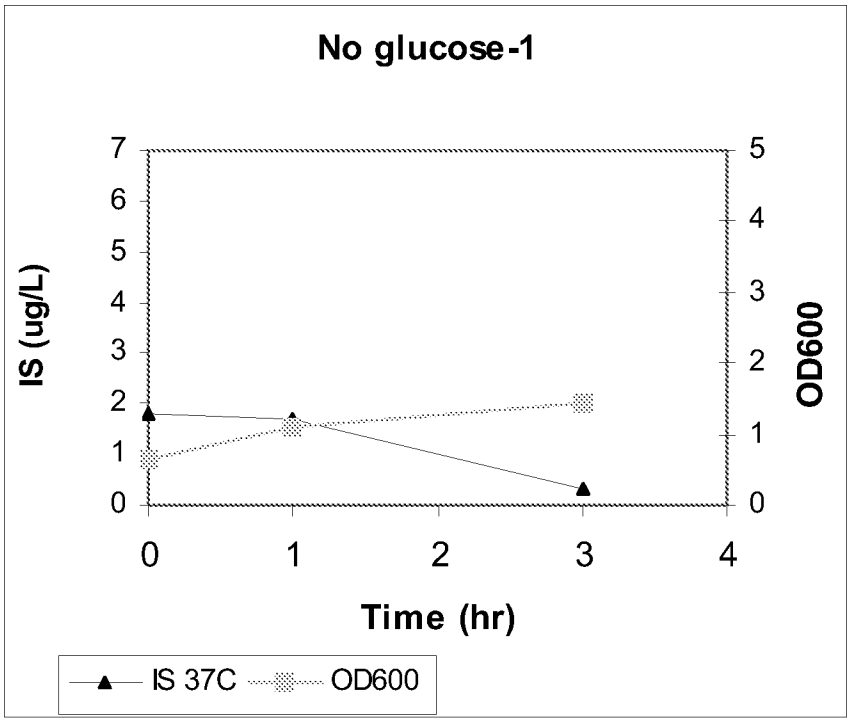


Figure 41B

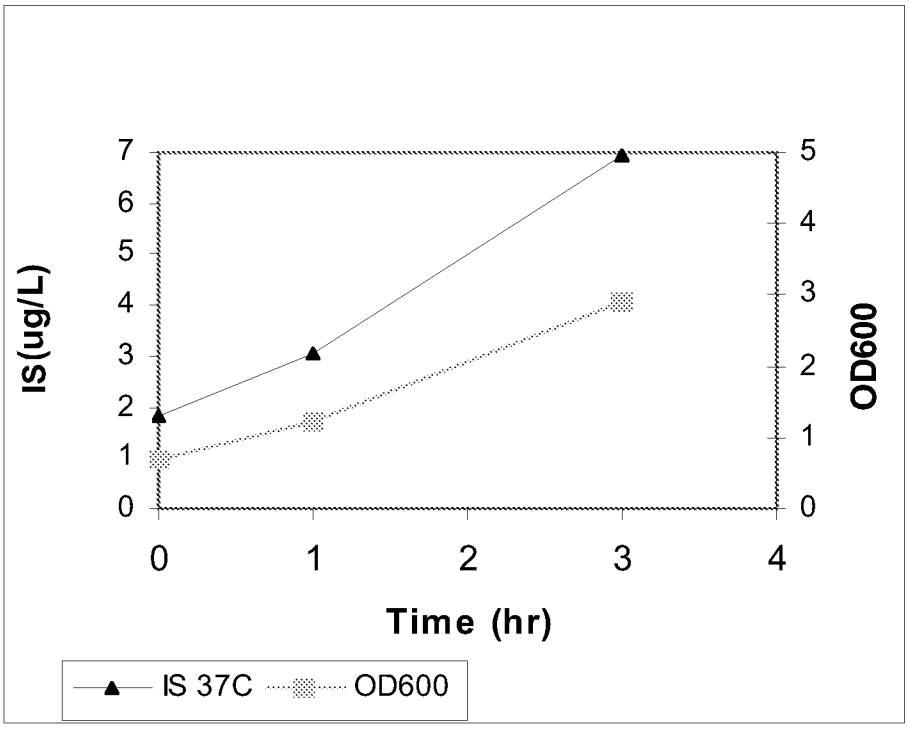


Figure 41C

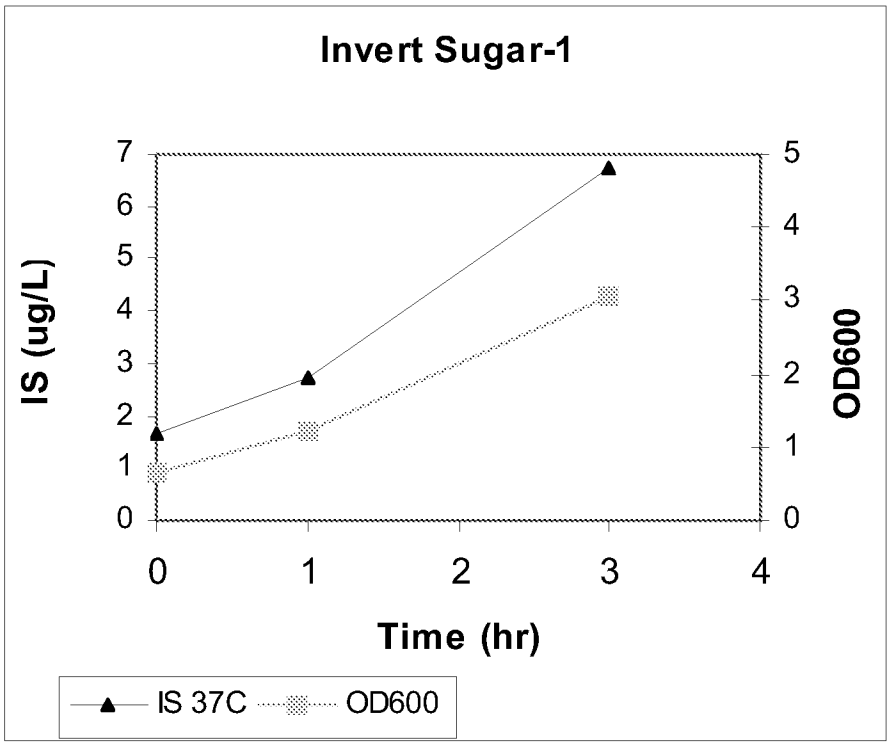


Figure 41D

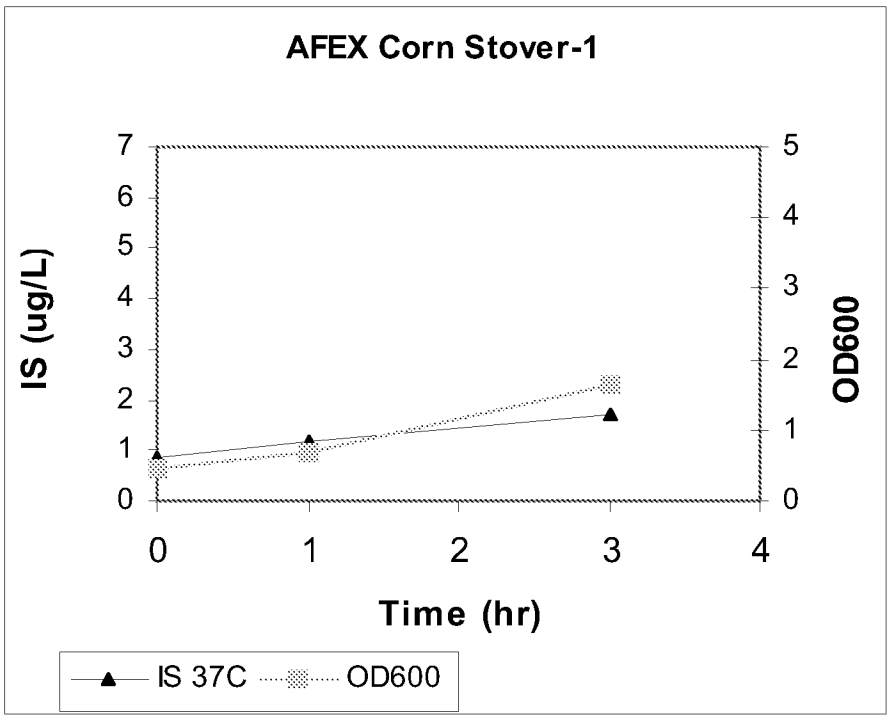


Figure 42A

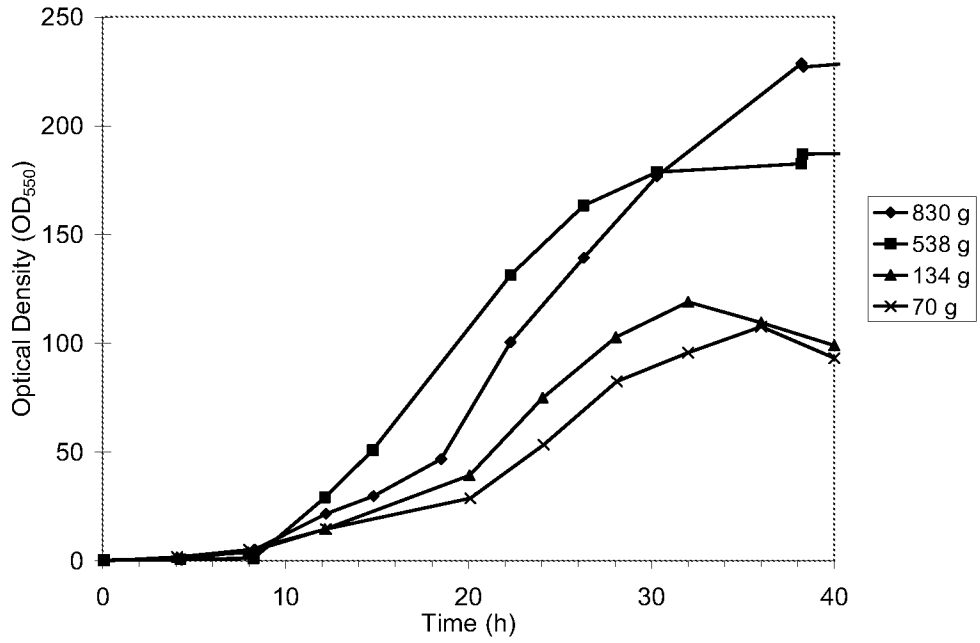


Figure 42B

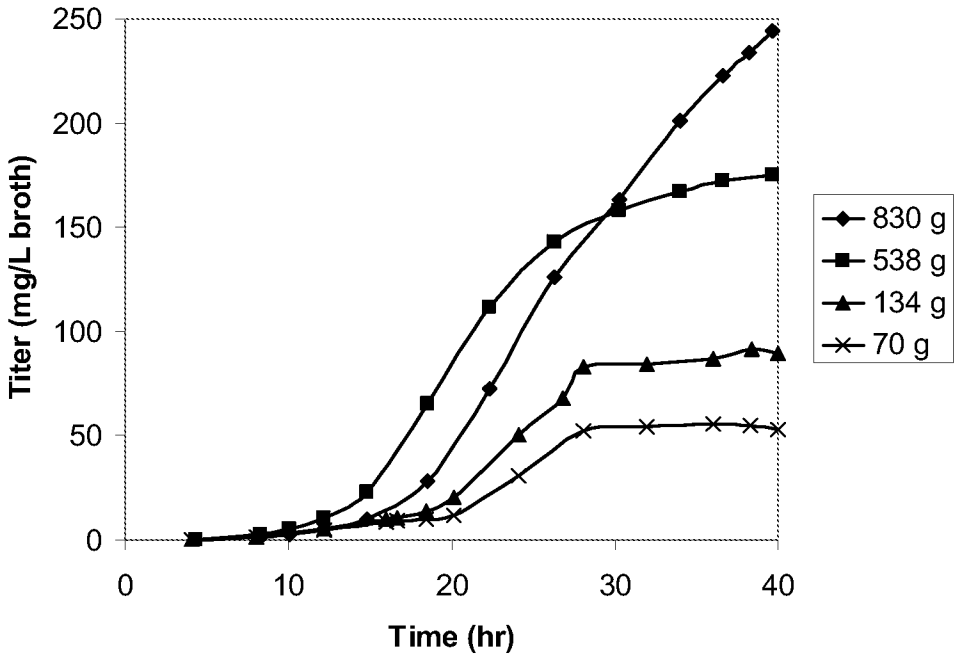


Figure 42C

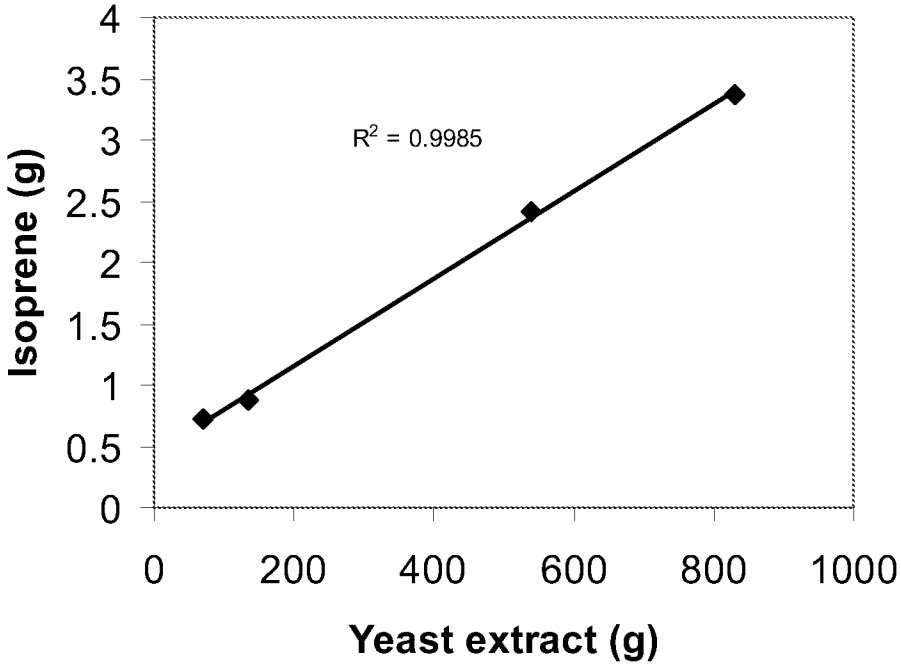

Isoprene (g)
Yeast extract (g)
R^2 = 0.9985
0
0.5
1
1.5
2
2.5
3
3.5
4
0
200
400
600
800
1000

Figure 43A

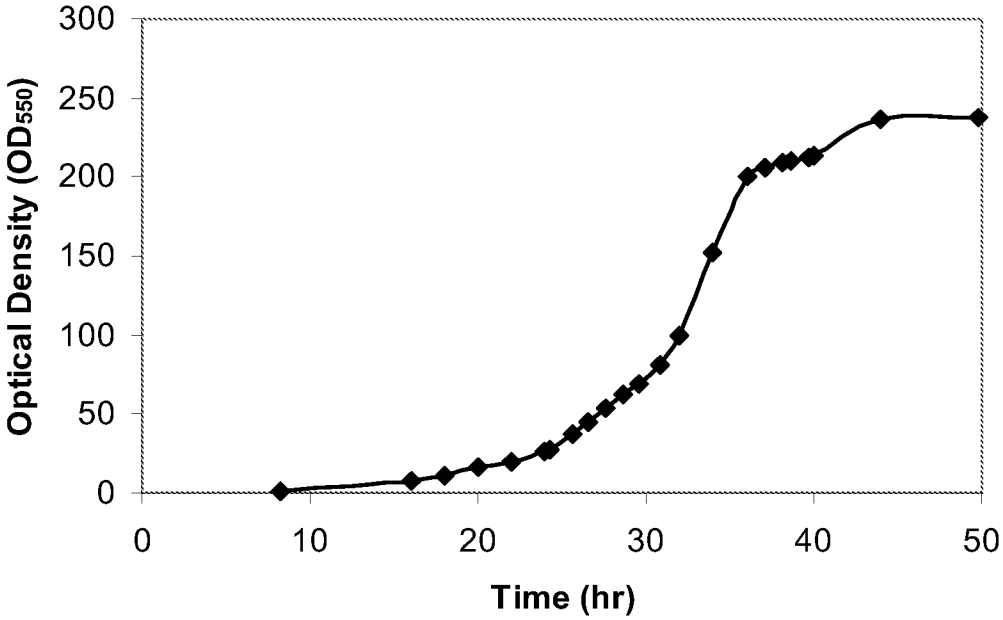


Figure 43B

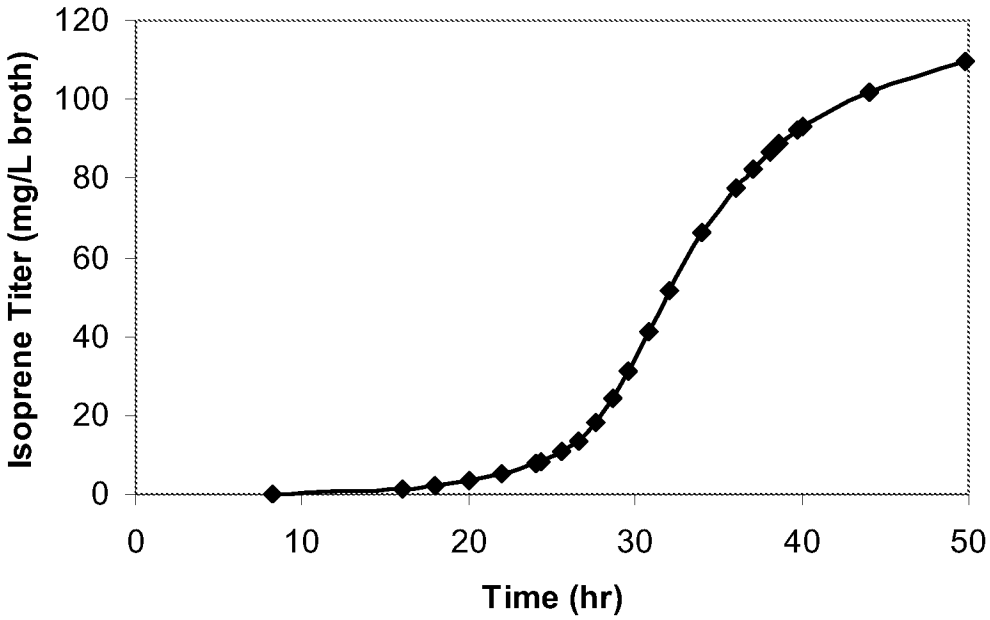


Figure 43C

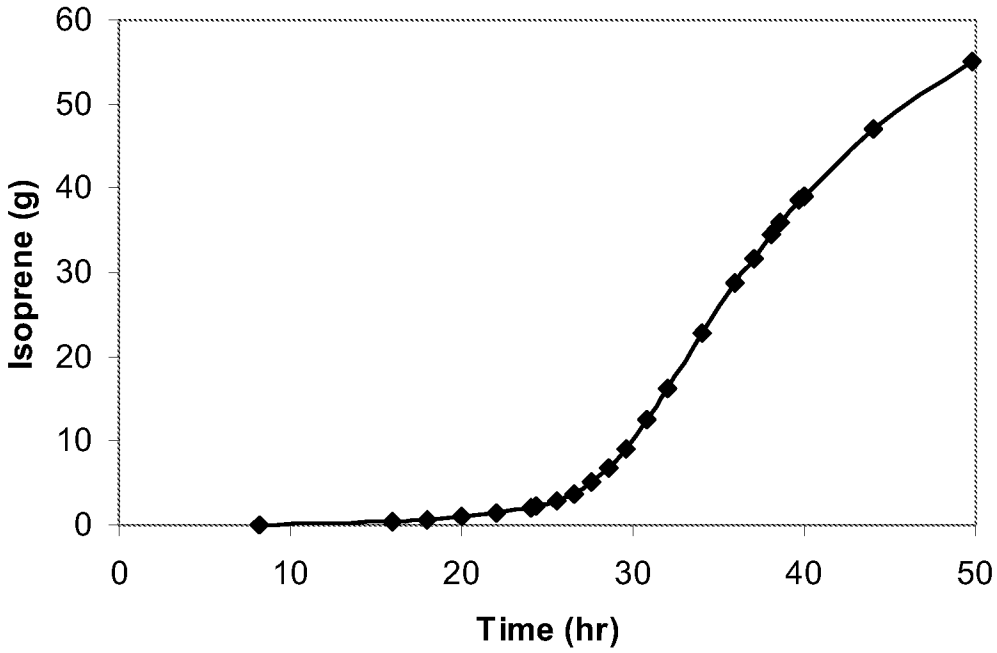


Figure 44

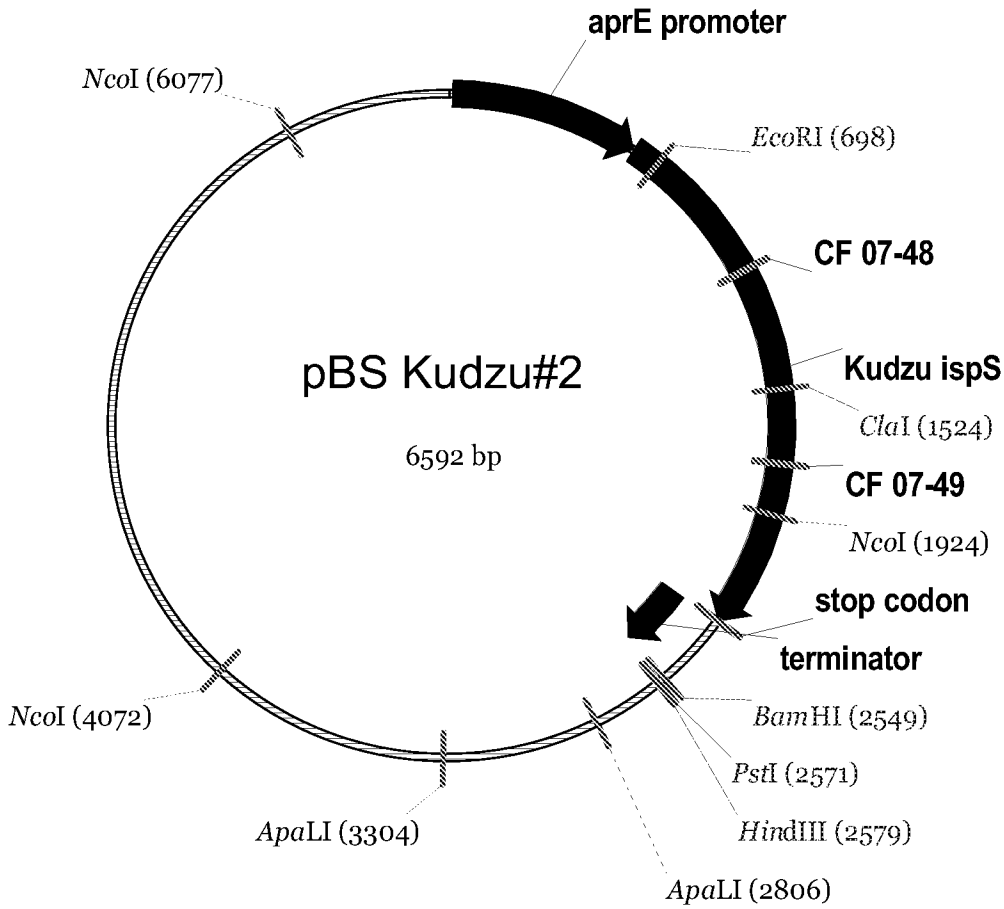

aprE promoter
NcoI (6077)
EcoRI (698)
CF 07-48
pBS Kudzu#2
6592 bp
Kudzu ispS
ClaI (1524)
CF 07-49
NcoI (1924)
stop codon
terminator
NcoI (4072)
BamHI (2549)
PstI (2571)
ApaLI (3304)
HindIII (2579)
ApaLI (2806)

Figure 45A

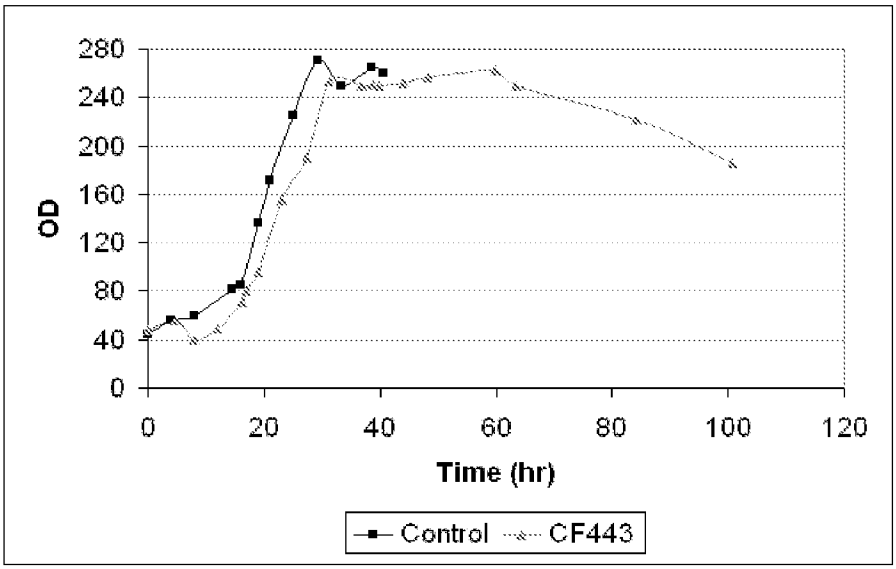
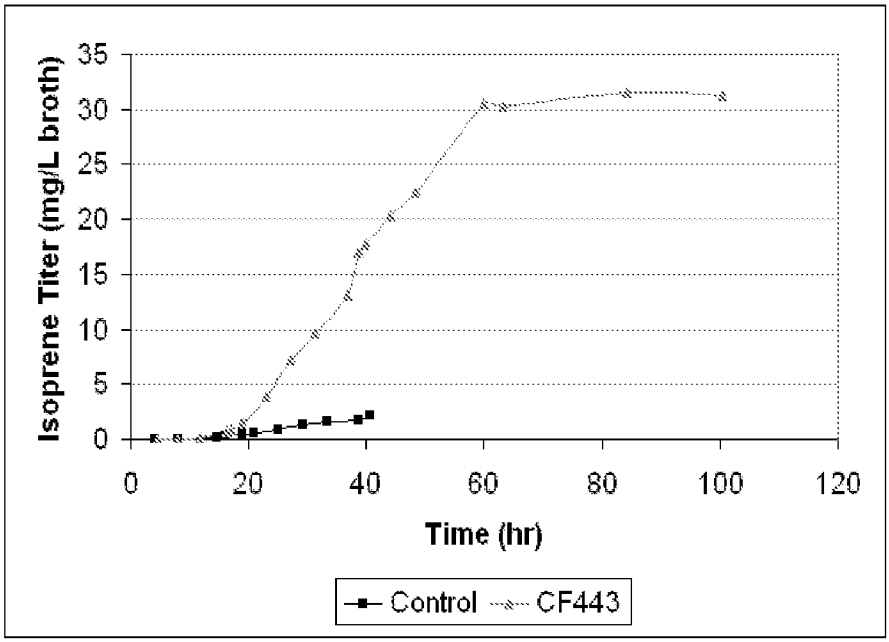
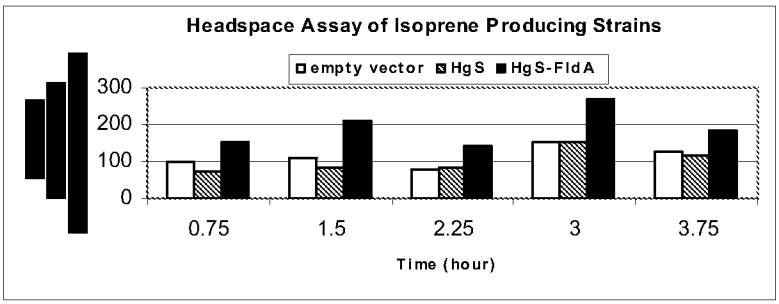


Figure 45B

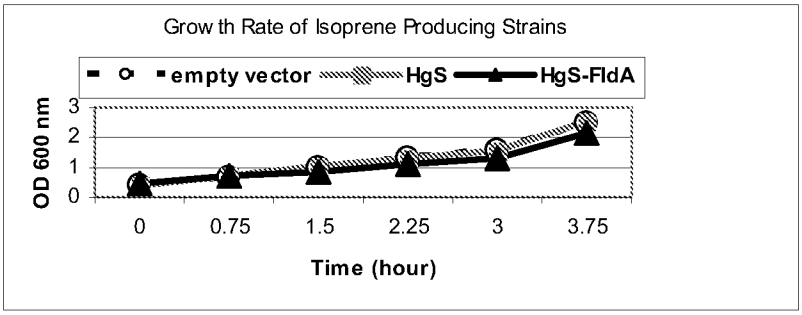


Figures 46A-46D

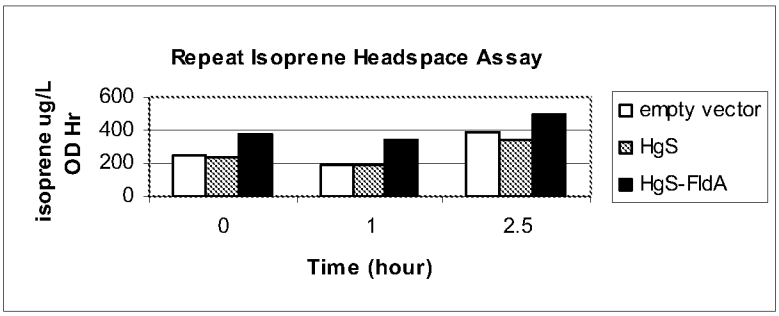
A



B



C



D

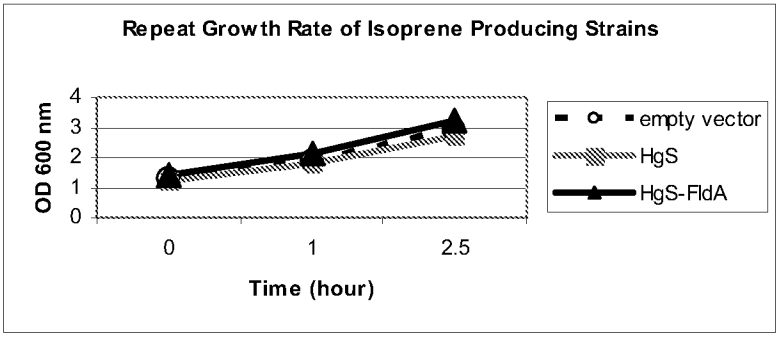


Figure 46E

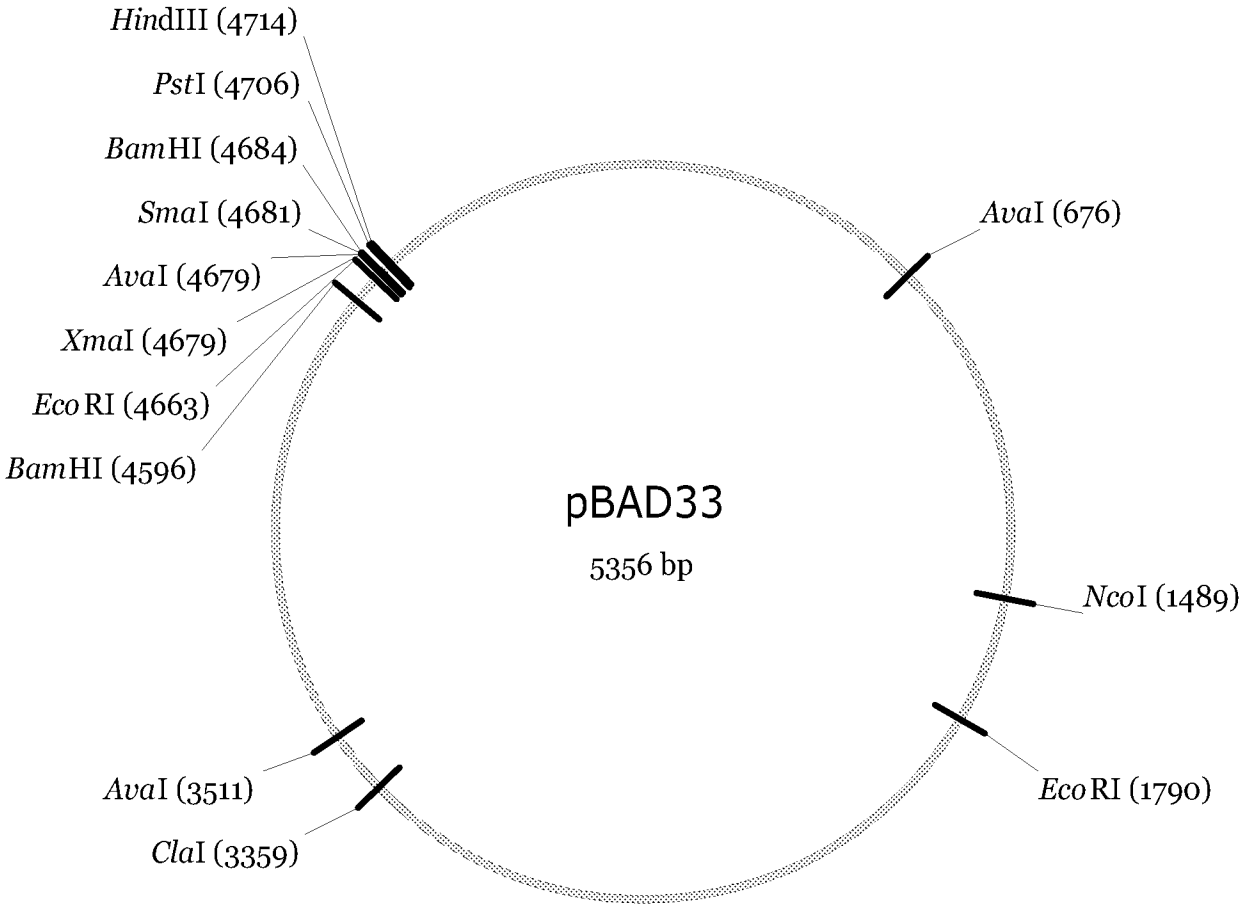

HindIII (4714)
PstI (4706)
BamHI (4684)
SmaI (4681)
AvaI (4679)
XmaI (4679)
EcoRI (4663)
BamHI (4596)
AvaI (676)
NcoI (1489)
EcoRI (1790)
AvaI (3511)
ClaI (3359)
pBAD33
5356 bp

Figure 46F

1-
tctataatcacggcagaaaagtccacattgattatttgcacggcgtcacactttgctatgccatagcatttttatccataagattagcggatcctac
ctgacgctttttatcgcaactctctactgtttctccatacccgtttttttgggctagcgaattcgagctcggtacccggggatcctctagagtcgac
ctgcaggcatgcaagcttggctgttttggcggatgagagaagattttcagcctgatacagattaaatcagaacgcagaagcggtctgataaa
acagaatttgcctggcggcagtagcgcggtggtcccacctgaccccatgccgaactcagaagtgaaacgccgtagcgccgatggtagtgt
ggggtctccccatgcgagagtagggaactgccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtt
tgtcggtgaacgctctcctgagtaggacaaatccgccgggagcggatttgaacgttgcgaagcaacggcccggagggtggcgggcagg
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ccctgccctgaaccgacgaccgggtcgaatttgctttcgaatttctgccattcatccgcttattatcacttattcaggcgtagcaccaggcgtttta
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gcgaagaagttgtccatattggccacgtttaaatcaaaactggtgaaactcacccagggattggctgagacgaaaaacatattctcaataaaac
cctttagggaaataggccaggttttcaccgtaacacgccacatcttgcgaatatatgtgtagaaactgccggaaatcgtcgtggtattcactcc
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acggaattccggatgagcattcatcaggcgggcaagaatgtgaataaaggccggataaaacttgtgcttatttttctttacggtctttaaaaag
gccgtaatatccagctgaacggtctggttataggtacattgagcaactgactgaaatgcctcaaaatgttctttacgatgccattgggatatatc
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caggcgtttccccctggcggctccctcgtgcgctctcctgttcctgcctttcggtttaccggtgtcattccgctgttatggccgcgtttgtctcatt
ccacgcctgacactcagttccgggtaggcagttcgctccaagctggactgtatgcacgaaccccccgttcagtccgaccgctgcgccttatc
cggtaactatcgtcttgagtccaacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagttagtcttg
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cagagaaccttcgaaaaaccgccctgcaaggcggtttttcgttttcagagcaagagattacgcgcagaccaaaacgatctcaagaagatca

Figure 46G

tcttattaatcagataaaatatttgctcatgagcccgaagtggcgagcccgatcttccccatcggtgatgtcggcgatataggcgccagcaac
cgcacctgtggcgccggtgatgccggccacgatgcgtccggcgtagaggatctgctcatgtttgacagcttatcatcgatgcataatgtgcc
tgtcaaatggacgaagcagggattctgcaaaccctatgctactccgtcaagccgtcaattgtctgattcgttaccaattatgacaacttgacgg
ctacatcattcacttttcttcacaaccggcacggaactcgctcgggctggccccggtgcatttttaaataccgcgagaaatagagttgatcg
tcaaaaccaacattgcgaccgacggtggcgataggcatccgggtggtgctcaaaagcagcttcgcctggctgatacgttggtcctcgcgcc
agcttaagacgctaatccctaactgctggcggaaaagatgtgacagacgcgacggcgacaagcaaacatgctgtgcgacgctggcgatat
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caggtcgctgaaatgcggctggtgcgcttcatccgggcgaaagaaccccgtattggcaaatattgacggccagttaagccattcatgccagt
aggcgcgcggacgaaagtaaacccactggtgataccattcgcgagcctccggatgacgaccgtagtgatgaatctctcctggcgggaaca
gcaaaatatcacccggtcggcaaacaaattctcgtccctgatttttcaccaccccctgaccgcgaatggtgagattgagaatataacctttcatt
cccagcggtcggtcgataaaaaaatcgagataaccgttggcctcaatcggcgttaaacccgccaccagatgggcattaaacgagtatcccg
gcagcaggggatcattttgcgcttcagccatacttttcatactcccgccattcagagaagaaaccaattgtccatattgcatcagacattgccgt
cactgcgtcttttactggctcttctcgctaaccaaaccggtaaccccgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaa
aaacgcgtaacaaaagtg (SEQ ID NO:51)

Figure 46H

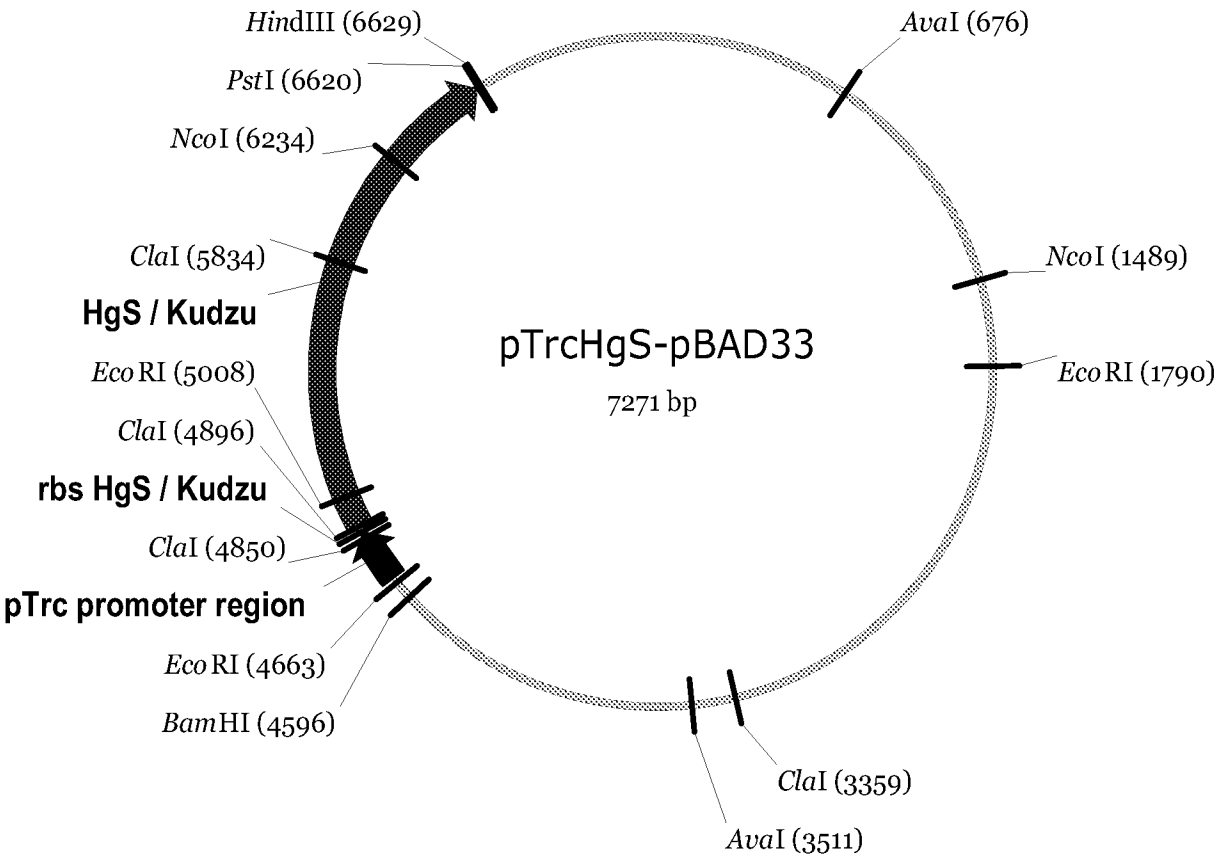
HindIII (6629)
PstI (6620)
NcoI (6234)
ClaI (5834)
HgS / Kudzu
EcoRI (5008)
ClaI (4896)
rbs HgS / Kudzu
ClaI (4850)
pTrc promoter region
EcoRI (4663)
BamHI (4596)
AvaI (676)
NcoI (1489)
EcoRI (1790)
pTrcHgS-pBAD33
7271 bp
ClaI (3359)
AvaI (3511)

Figure 46I

1-
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Figure 46J

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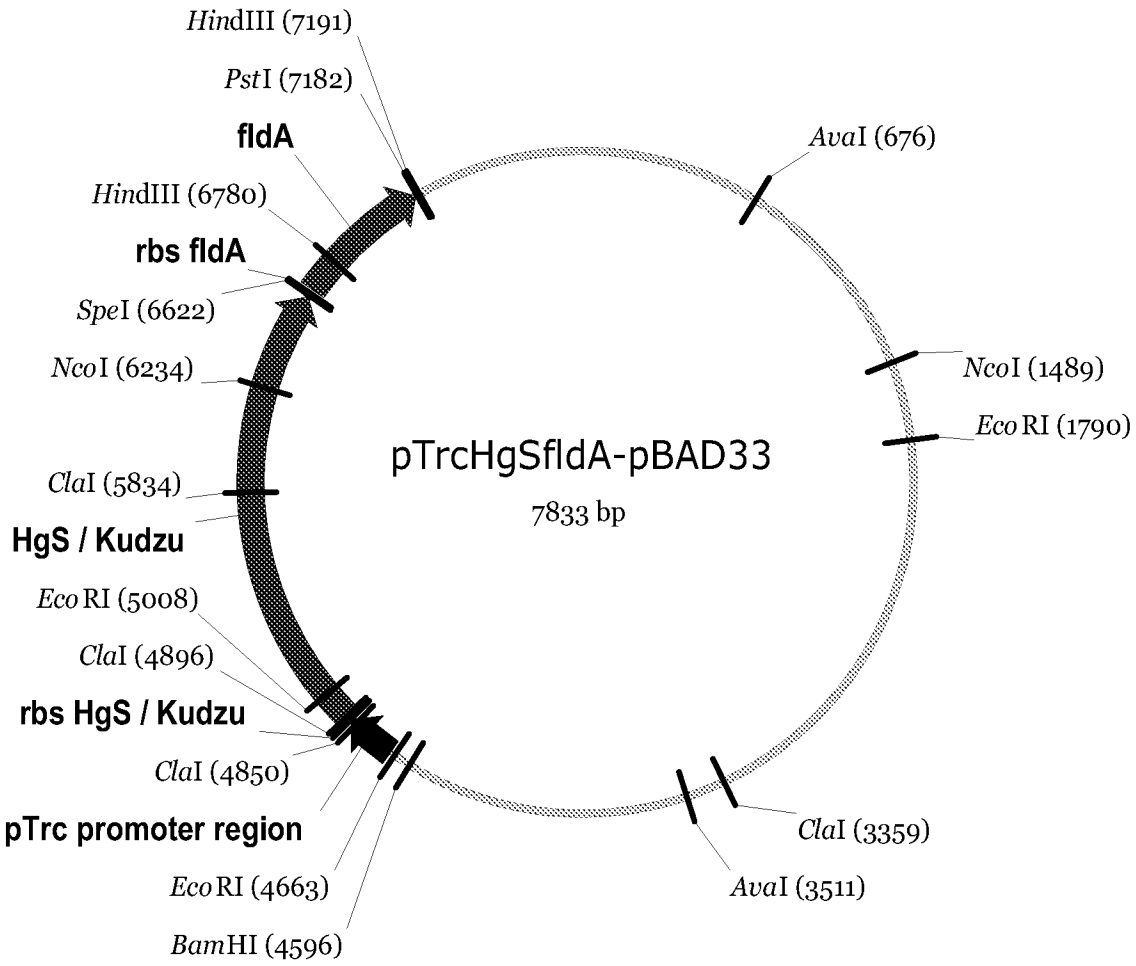
Figure 46K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PstI (7182)
fldA
HindIII (6780)
rbs fldA
SpeI (6622)
NcoI (6234)
ClaI (5834)
HgS / Kudzu
EcoRI (5008)
ClaI (4896)
rbs HgS / Kudzu
ClaI (4850)
pTrc promoter region
EcoRI (4663)
BamHI (4596)
AvaI (676)
NcoI (1489)
EcoRI (1790)
ClaI (3359)
AvaI (3511)
pTrcHgSfldA-pBAD33
7833 bp

Figure 46L

1-
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Figure 46M

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(SEQ ID NO:53)

Figure 47

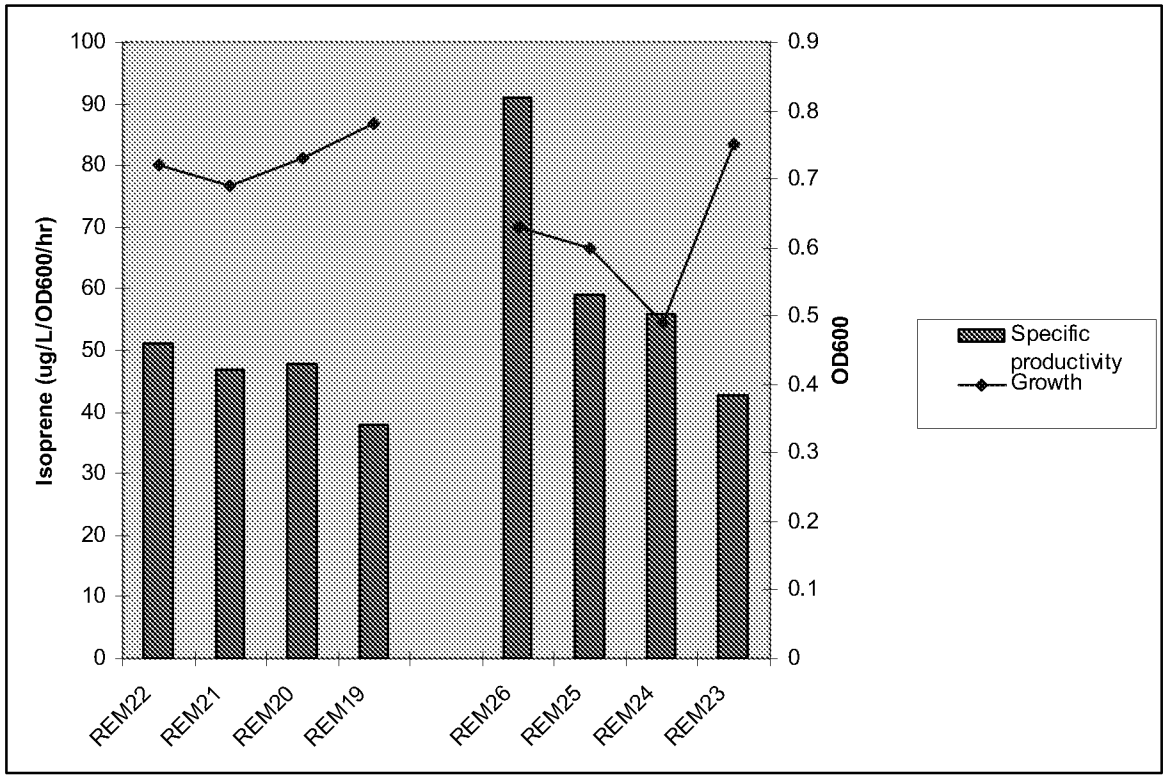

Isoprene (ug/L/OD600/hr)
OD600
100
90
80
70
60
50
40
30
20
10
0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
REM22
REM21
REM20
REM19
REM26
REM25
REM24
REM23
Specific productivity
Growth

Figure 48

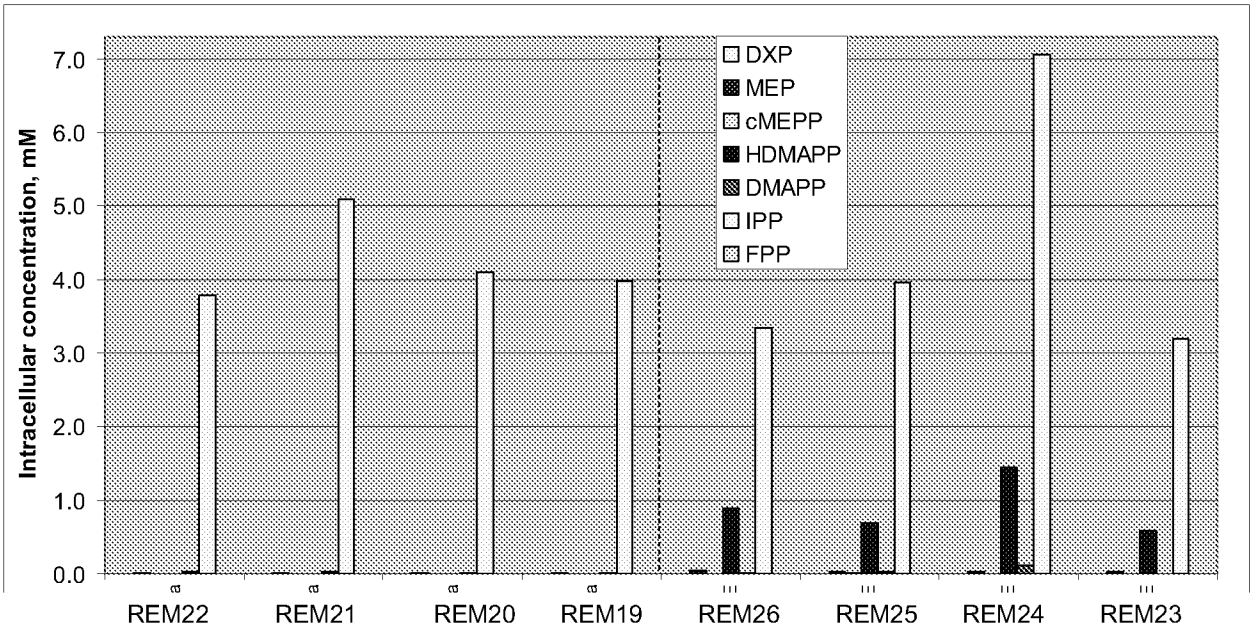

Intracellular concentration, mM
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
DXP
MEP
cMEPP
HDMAPP
DMAPP
IPP
FPP
a
a
a
a
III
III
III
III
REM22
REM21
REM20
REM19
REM26
REM25
REM24
REM23

Figure 49

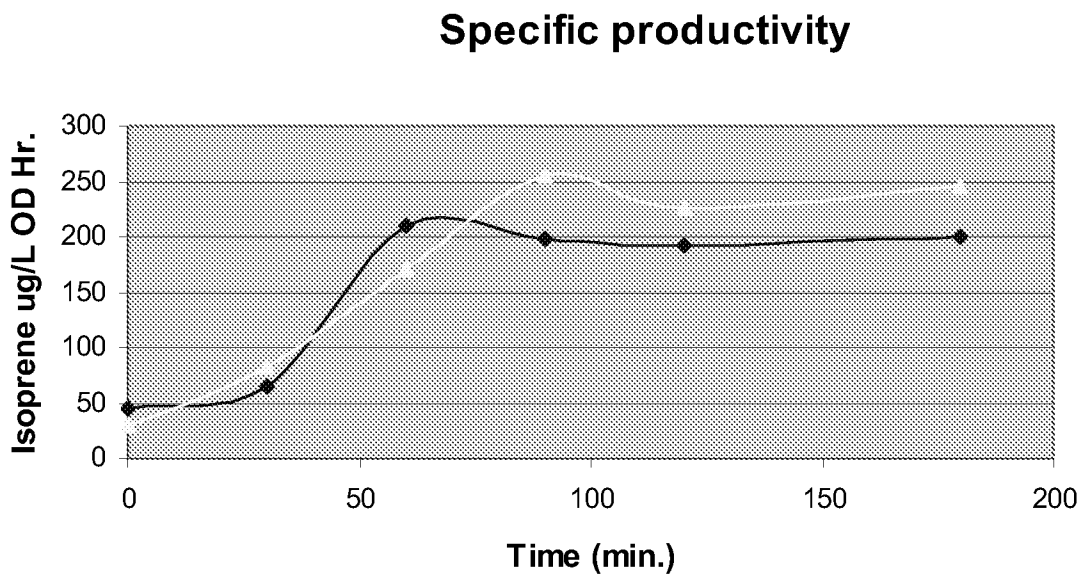
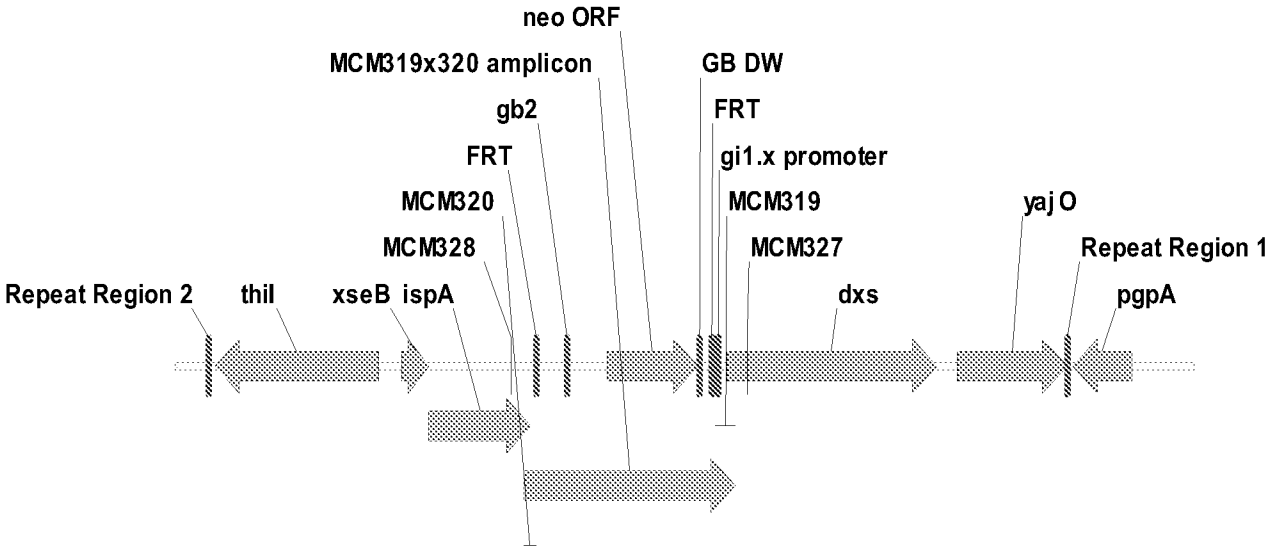

Specific productivity
Isoprene ug/L OD Hr.
300
250
200
150
100
50
0
0
50
100
150
200
Time (min.)

Figure 50



MCM617-625 - gi1.x integrated at dxs

9013 bp

Figure 51

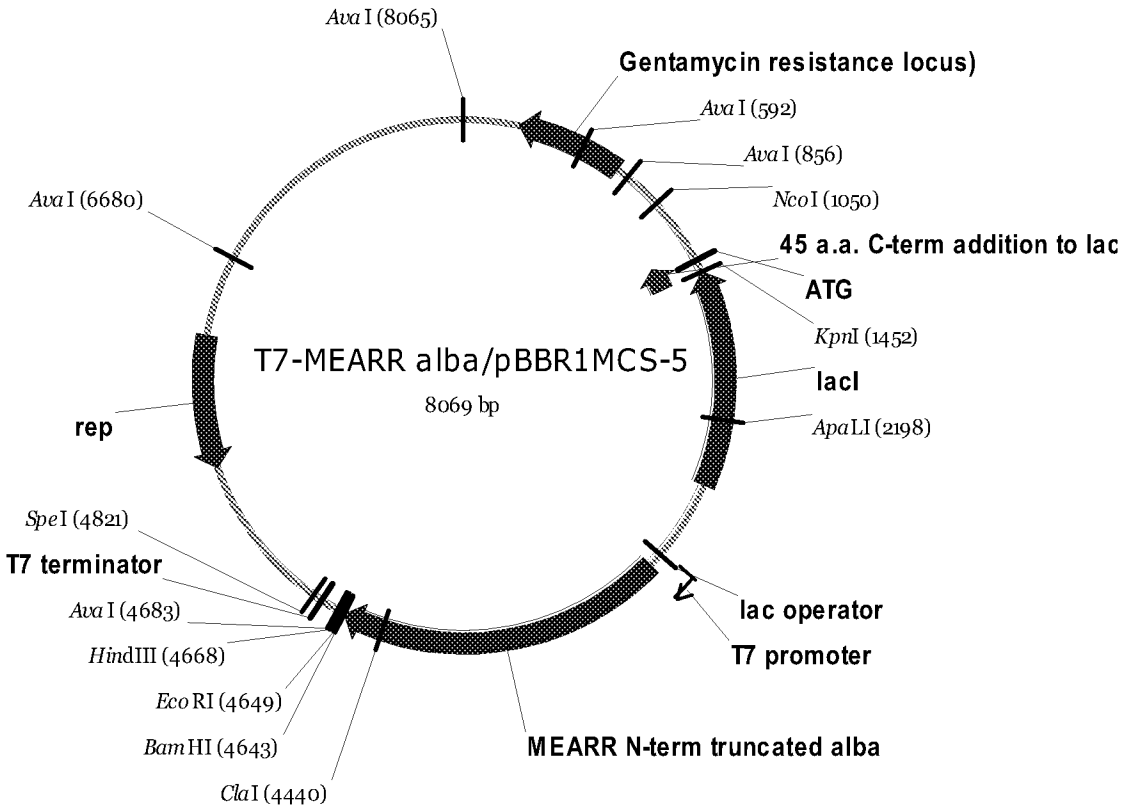

AvaI (8065)
Gentamycin resistance locus)
AvaI (592)
AvaI (856)
NcoI (1050)
45 a.a. C-term addition to lac
ATG
KpnI (1452)
lacI
ApaLI (2198)
AvaI (6680)
T7-MEARR alba/pBBR1MCS-5
8069 bp
rep
SpeI (4821)
T7 terminator
AvaI (4683)
HindIII (4668)
EcoRI (4649)
BamHI (4643)
ClaI (4440)
lac operator
T7 promoter
MEARR N-term truncated alba

Figure 52

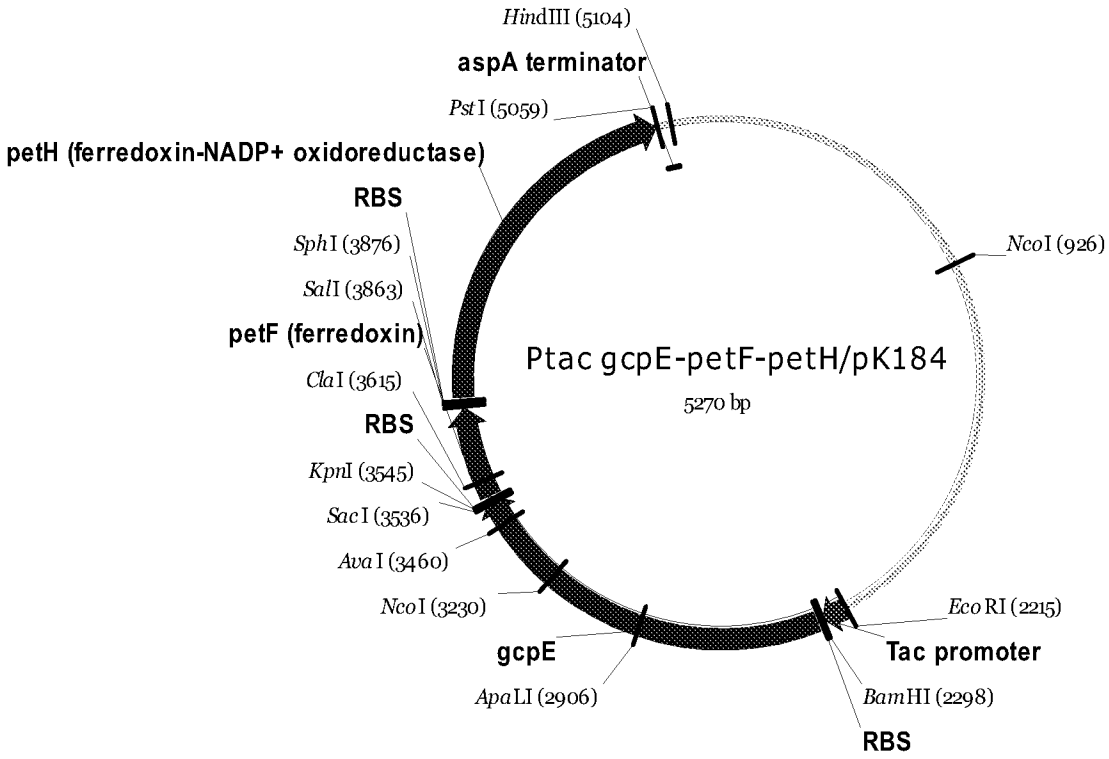

HindIII (5104)
aspA terminator
PstI (5059)
petH (ferredoxin-NADP+ oxidoreductase)
RBS
SphI (3876)
SalI (3863)
petF (ferredoxin)
ClaI (3615)
RBS
KpnI (3545)
SacI (3536)
AvaI (3460)
NcoI (3230)
gcpE
ApaLI (2906)
NcoI (926)
Ptac gcpE-petF-petH/pK184
5270 bp
EcoRI (2215)
Tac promoter
BamHI (2298)
RBS

Figure 53A

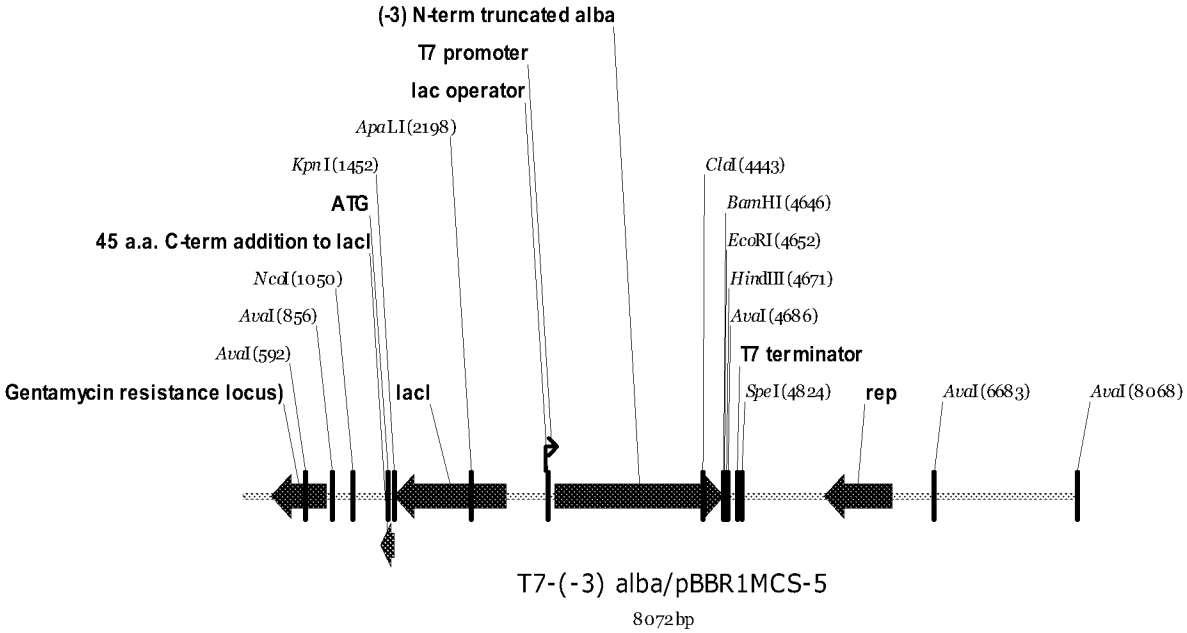


Figure 53B

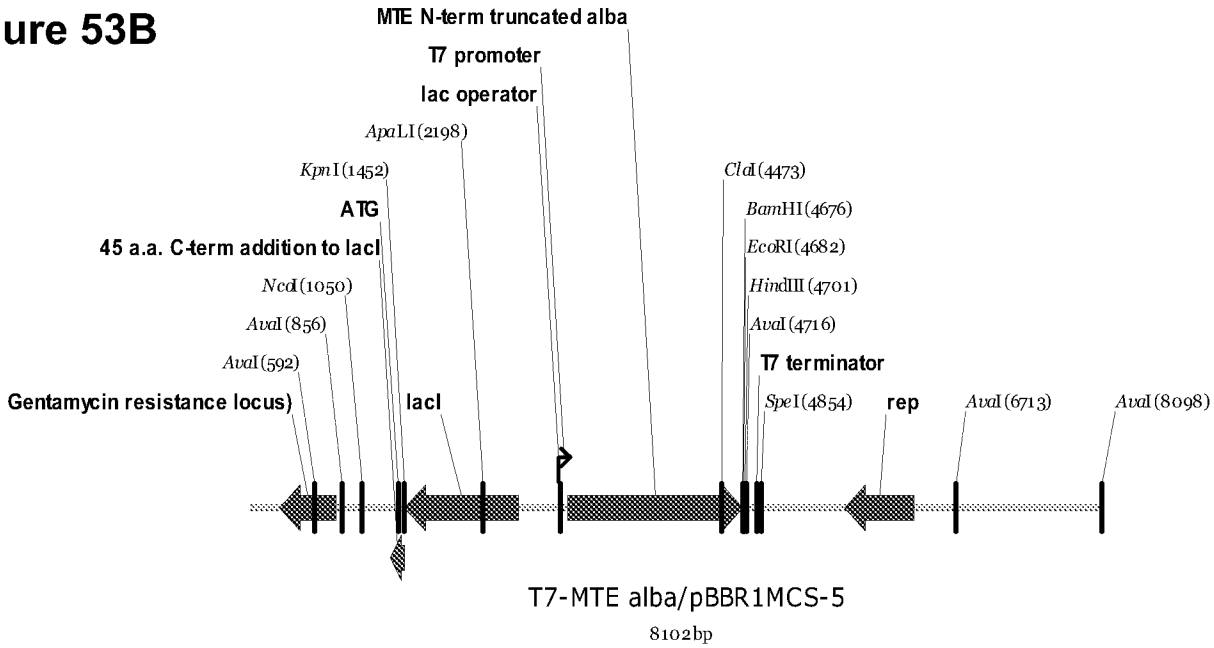


Figure 54

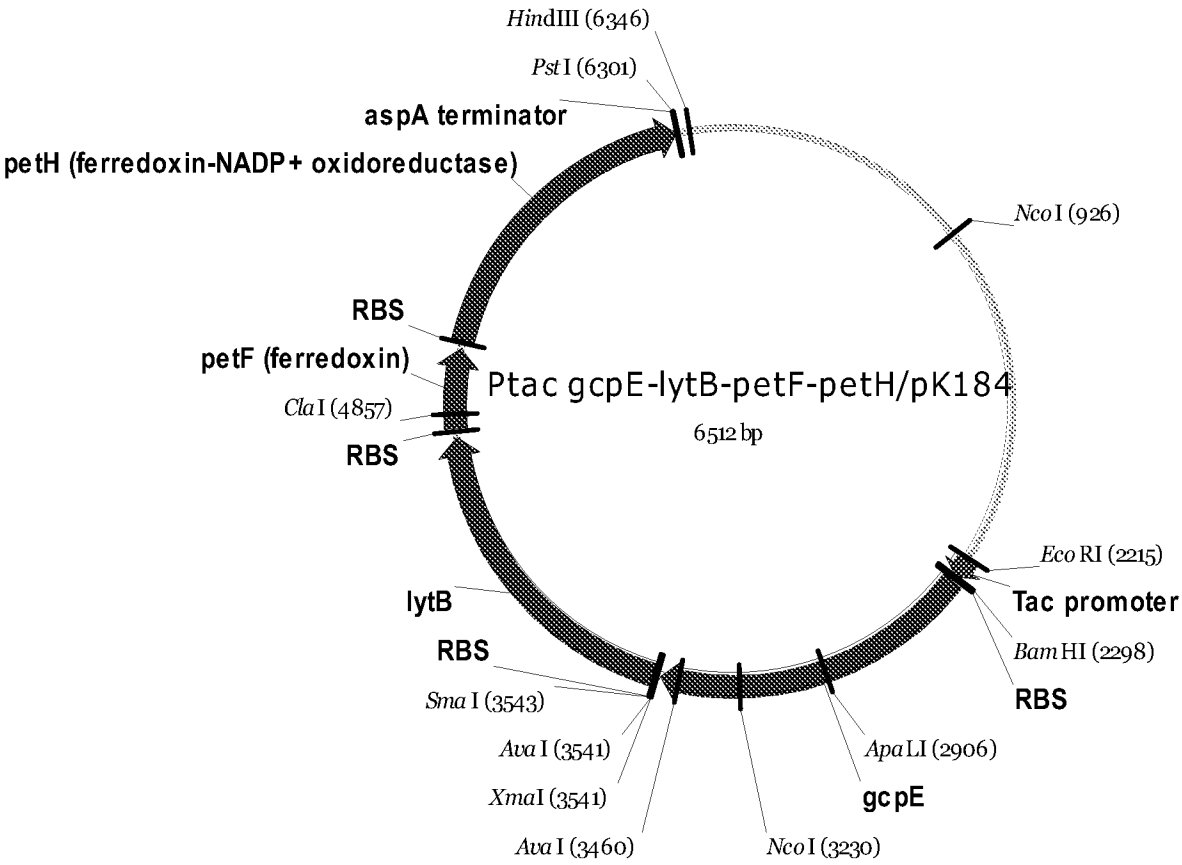

HindIII (6346)
PstI (6301)
aspA terminator
petH (ferredoxin-NADP+ oxidoreductase)
NcoI (926)
RBS
petF (ferredoxin)
ClaI (4857)
RBS
Ptac gcpE-lytB-petF-petH/pK184
6512 bp
EcoRI (2215)
Tac promoter
BamHI (2298)
RBS
lytB
RBS
SmaI (3543)
AvaI (3541)
XmaI (3541)
AvaI (3460)
NcoI (3230)
ApaLI (2906)
gcpE

Figure 55A

accttcgggagcgcctgaagcccgttctggacgccctggggccgttgaatcgggatatgcaggccaaggccgccgcgatcatcaaggcc
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Figure 55B

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Figure 55C

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Figure 56A

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Figure 56B

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Figure 57A

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Figure 57B

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Figure 57C

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Figure 58A

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Figure 58B

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Figure 58C

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Figure 59A

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Figure 59B

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Figure 60A

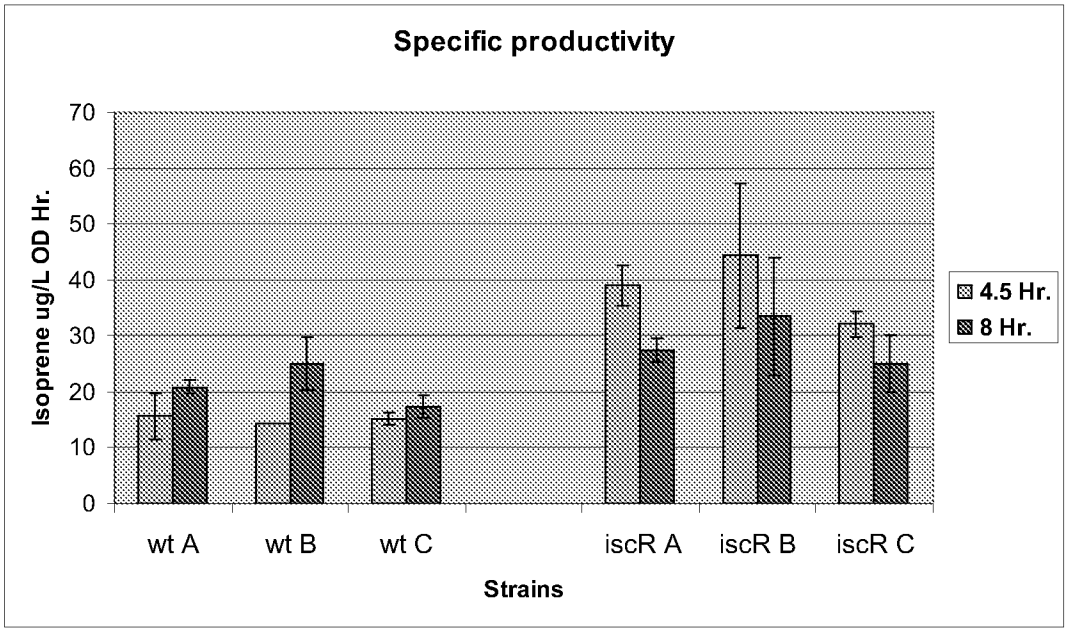


Figure 60B

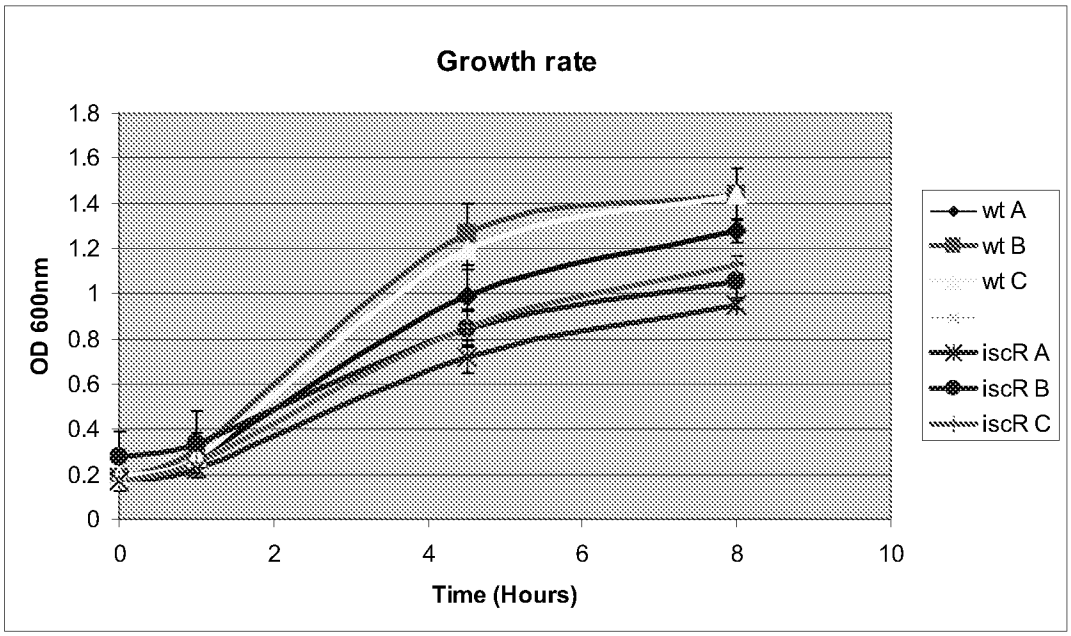
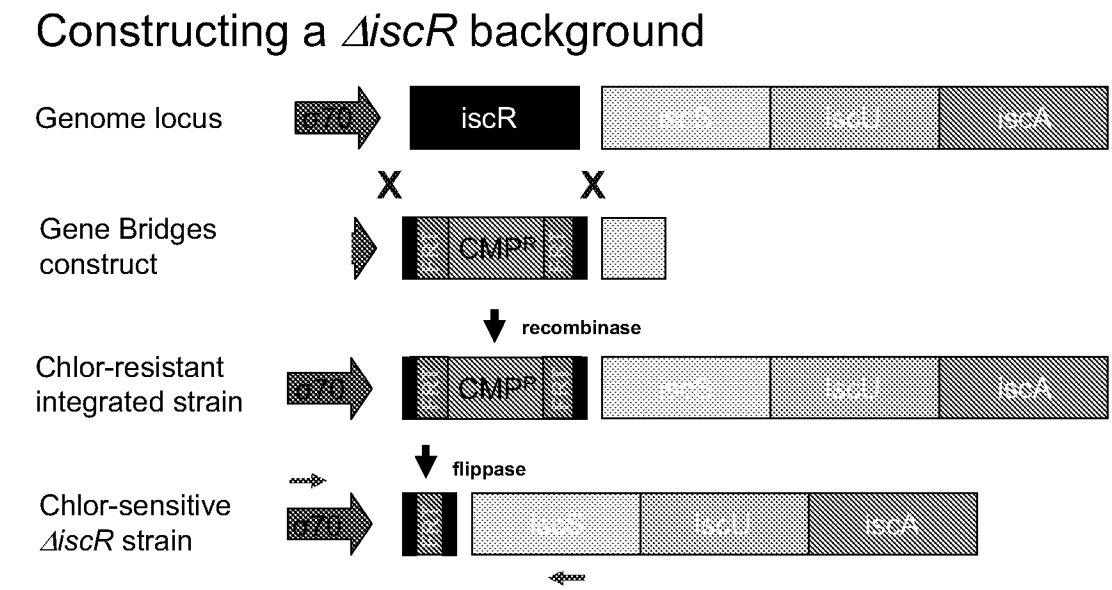


Figure 61



Diagnostic primers used to verify the removal of *iscR* from the BL21(DE3) genome

Figure 62

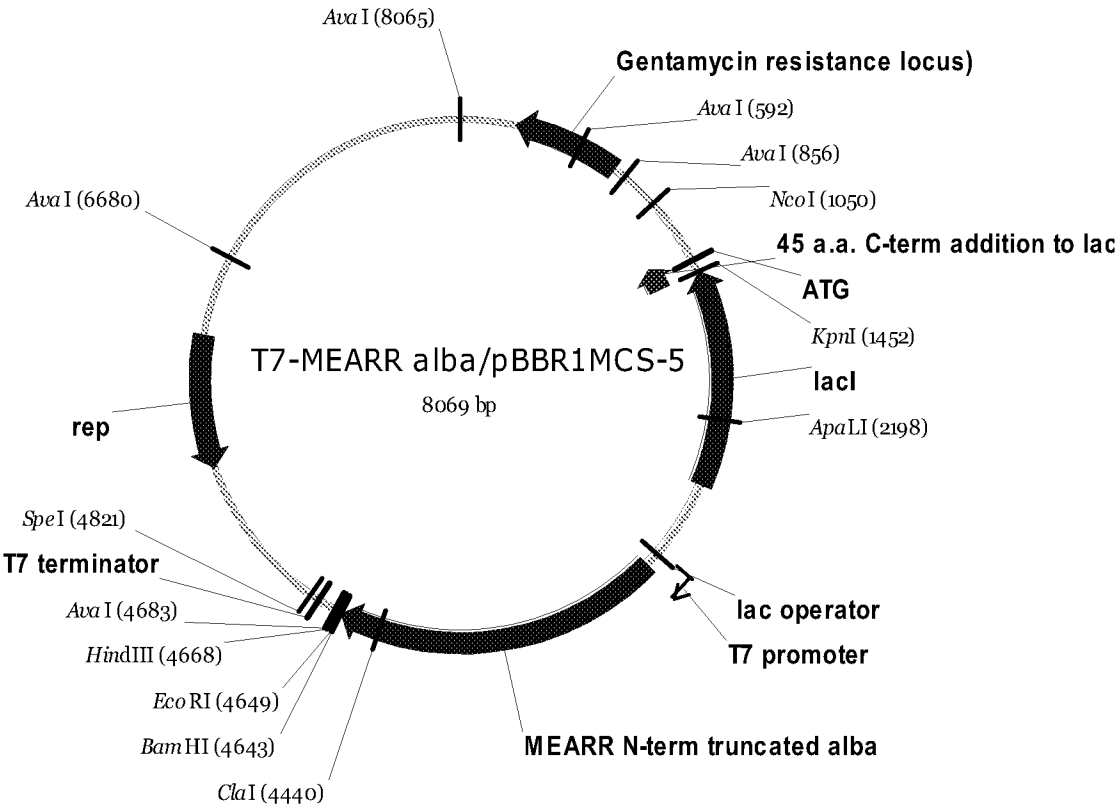
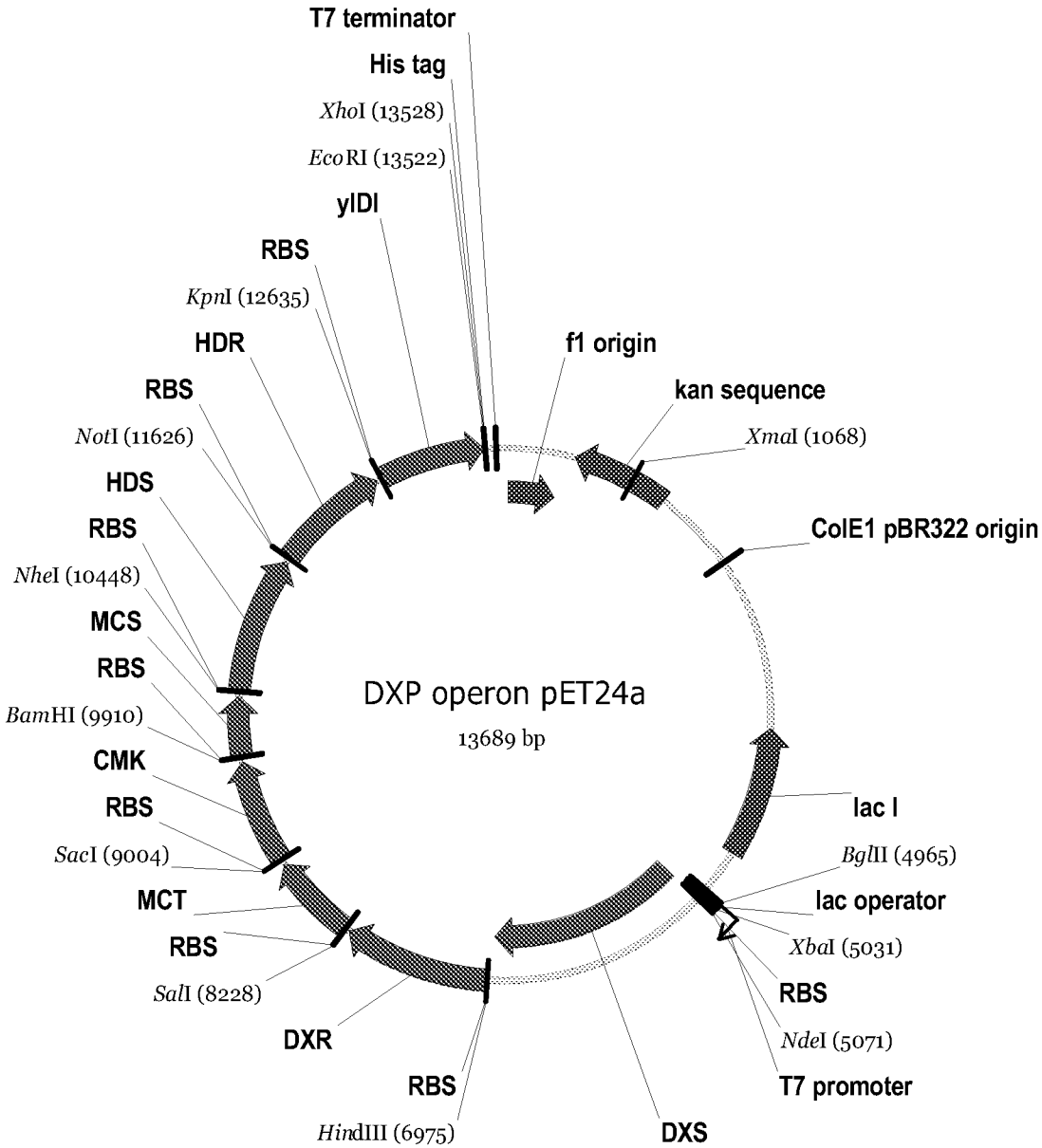

AvaI (8065)
Gentamycin resistance locus)
AvaI (592)
AvaI (856)
NcoI (1050)
45 a.a. C-term addition to lac
ATG
KpnI (1452)
lacI
ApaLI (2198)
AvaI (6680)
T7-MEARR alba/pBBR1MCS-5
8069 bp
rep
SpeI (4821)
T7 terminator
AvaI (4683)
HindIII (4668)
EcoRI (4649)
BamHI (4643)
ClaI (4440)
lac operator
T7 promoter
MEARR N-term truncated alba

Figure 63


T7 terminator
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XhoI (13528)
EcoRI (13522)
ylDI
RBS
KpnI (12635)
HDR
RBS
NotI (11626)
HDS
RBS
NheI (10448)
MCS
RBS
BamHI (9910)
CMK
RBS
SacI (9004)
MCT
RBS
SalI (8228)
DXR
RBS
HindIII (6975)
DXS
T7 promoter
RBS
NdeI (5071)
lac operator
BglII (4965)
lac I
ColE1 pBR322 origin
XmaI (1068)
kan sequence
f1 origin
DXP operon pET24a
13689 bp

Figure 64A

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Figure 64B

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Figure 64C

Gcgcgttgagacgccggaagccgtggccgaccggctgacaaaagcggttcggcaggggtatgagcctgccctacaggccgccgcag gagcgcgtgagatgcgcaagaaggccgatcaagcccaagagacggcccgag (SEQ ID NO:78)

Figure 65A

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Figure 65B

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Figure 65C

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Figure 65D

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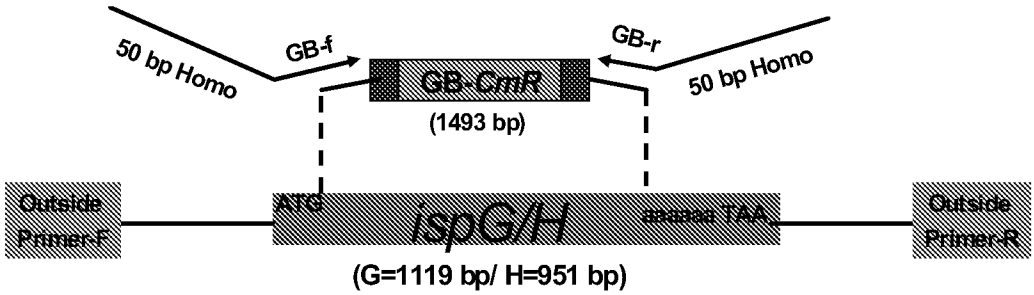
Figure 66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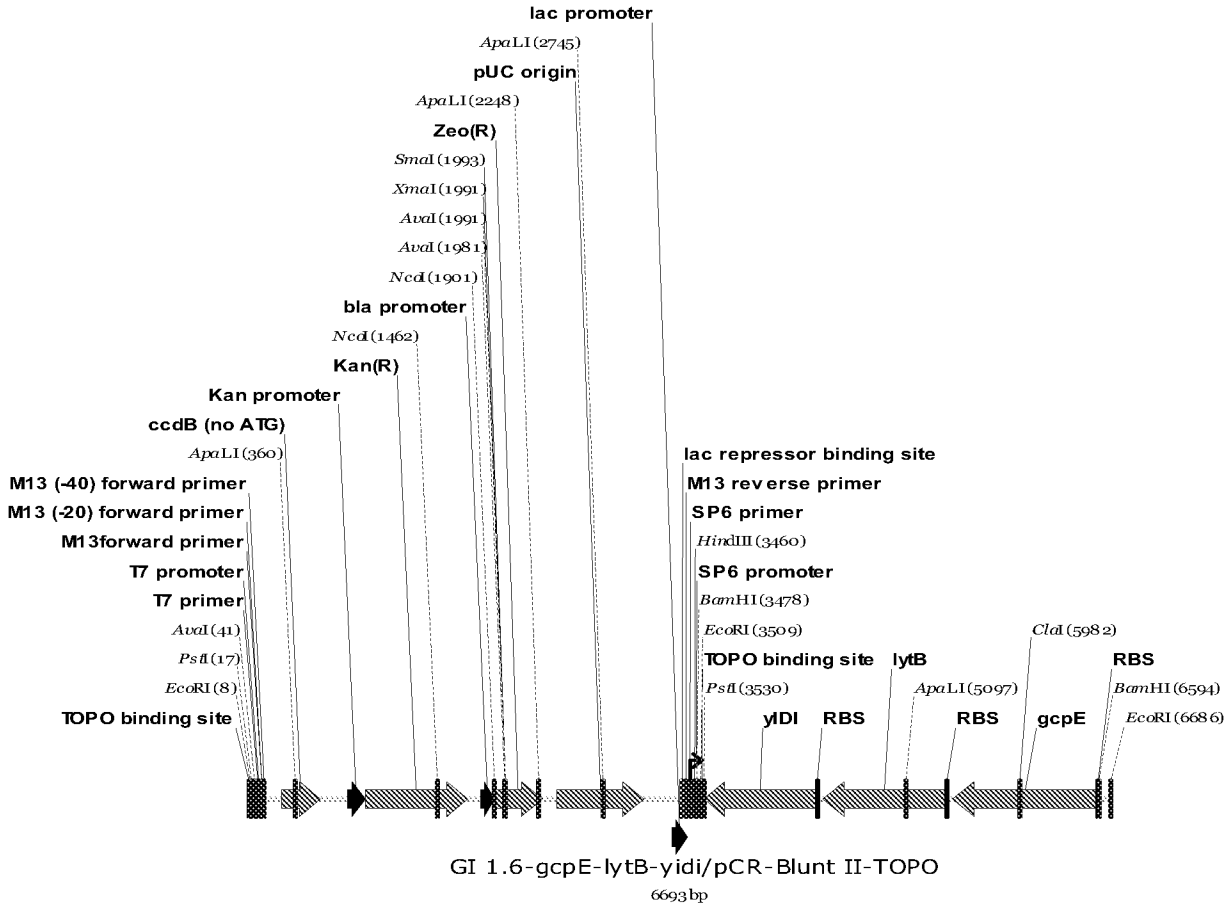
GB-r
50 bp Homo
GB-CmR
(1493 bp)
Outside Primer-F
ATG
ispG/H
aaaaa TAA
Outside Primer-R
(G=1119 bp/ H=951 bp)

Figure 67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pUC origin
ApaLI(2248)
Zeo(R)
SmaI(1993)
XmaI(1991)
AvaI(1991)
AvaI(1981)
NcoI(1901)
bla promoter
NcoI(1462)
Kan(R)
Kan promoter
ccdB (no ATG)
ApaLI(360)
M13 (-40) forward primer
M13 (-20) forward primer
M13forward primer
T7 promoter
T7 primer
AvaI(41)
PstI(17)
EcoRI(8)
TOPO binding site
lac repressor binding site
M13 reverse primer
SP6 primer
HindIII(3460)
SP6 promoter
BamHI(3478)
EcoRI(3509)
TOPO binding site
PstI(3530)
lytB
ApaLI(5097)
ClaI(5982)
RBS
BamHI(6594)
EcoRI(6686)
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RBS
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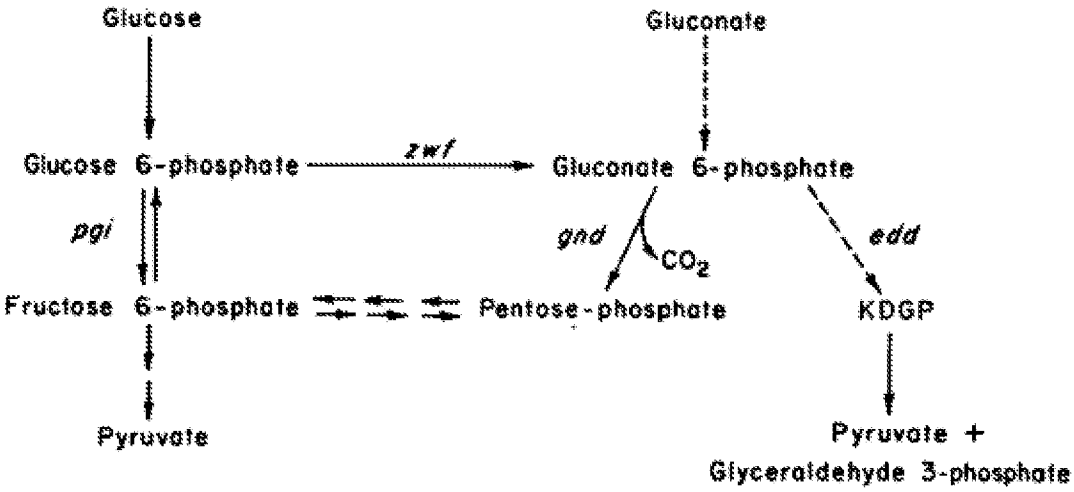
6693 bp

Figure 68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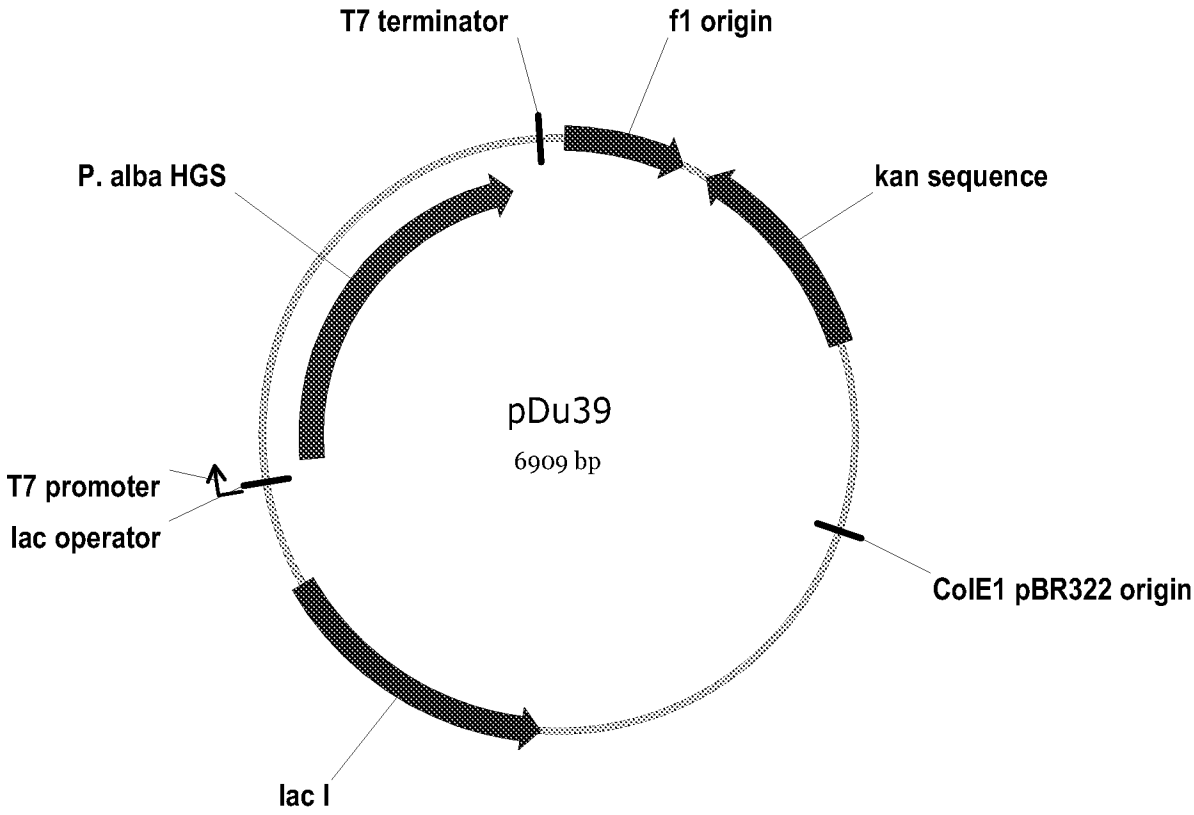
Gluconate
Glucose 6-phosphate
zwf
Gluconate 6-phosphate
pgi
gnd
CO2
edd
Fructose 6-phosphate
Pentose-phosphate
KDGP
Pyruvate
Pyruvate +
Glyceraldehyde 3-phosphate

Figure 69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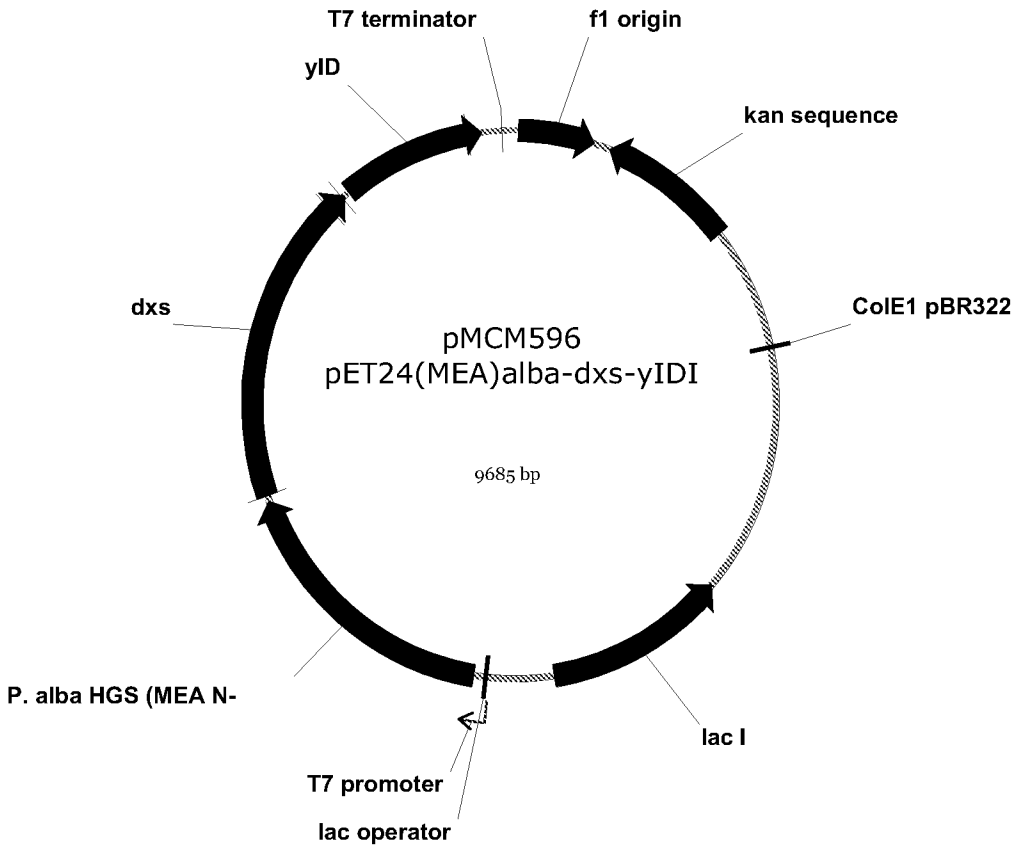
P. alba HGS
kan sequence
pDu39
6909 bp
T7 promoter
lac operator
ColE1 pBR322 origin
lac I

Figure 70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yID
kan sequence
dxs
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P. alba HGS (MEA N-
lac I
T7 promoter
lac operator

Figure 71A

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Figure 71B

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Figure 72B

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Figure 72C

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Figure 73A

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Figure 73B

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aaaccgcgatcaacctggcacgtcaatctcactgcacttatcataacggcgacgcgcatacctctccggatgagctgacccgcaaacgcgt
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Figure 73C

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Figure 74

Figure 75A

1-
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gctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcag
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ggctctcaagggcatcggtcgagatcccggtgcctaatgagtgagctaacttacattaattgcgttgcgctcactgcccgctttccagtcggg
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Figure 75B

aaatcctgtttgatggtggttaacggcgggatataacatgagctgtcttcggtatcgtcgtatcccactaccgagatatccgcaccaacgcgc
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atttgcatggtttgttgaaaaccggacatggcactccagtcgccttcccgttccgctatcggctgaatttgattgcgagtgagatatttatgccag
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taatgctattggtgccggtaccaagaaagtttgtcatttaatggaaaatattgaaaagggtttactacatcgtgcattctccgtctttattttcaatg
aacaaggtgaattacttttacaacaaagagccactgaaaaaataactttccctgatctttggactaacacatgctgctctcatccactatgtattg
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gctgtcaaacatgagaa (SEQ ID NO:113)

Figure 76

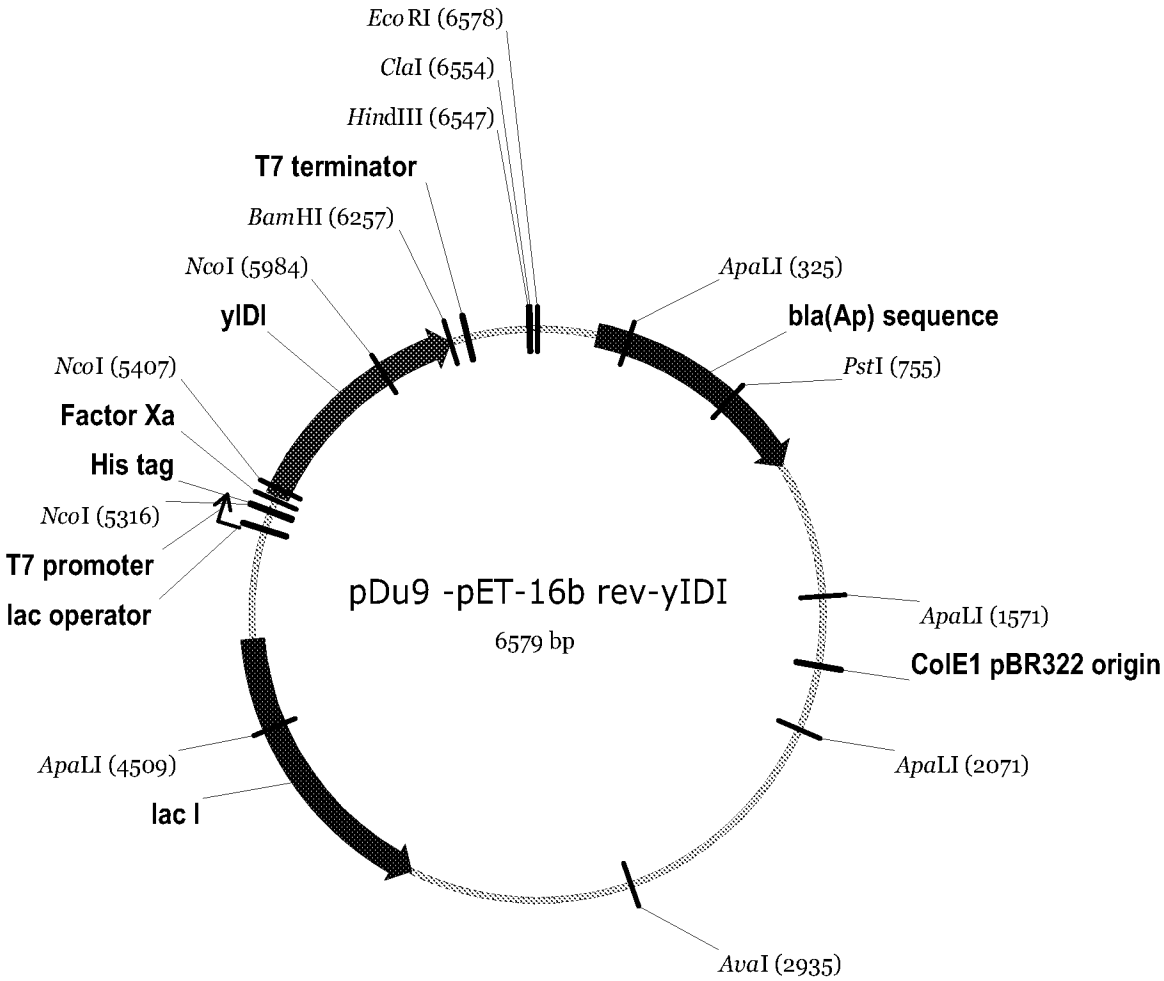
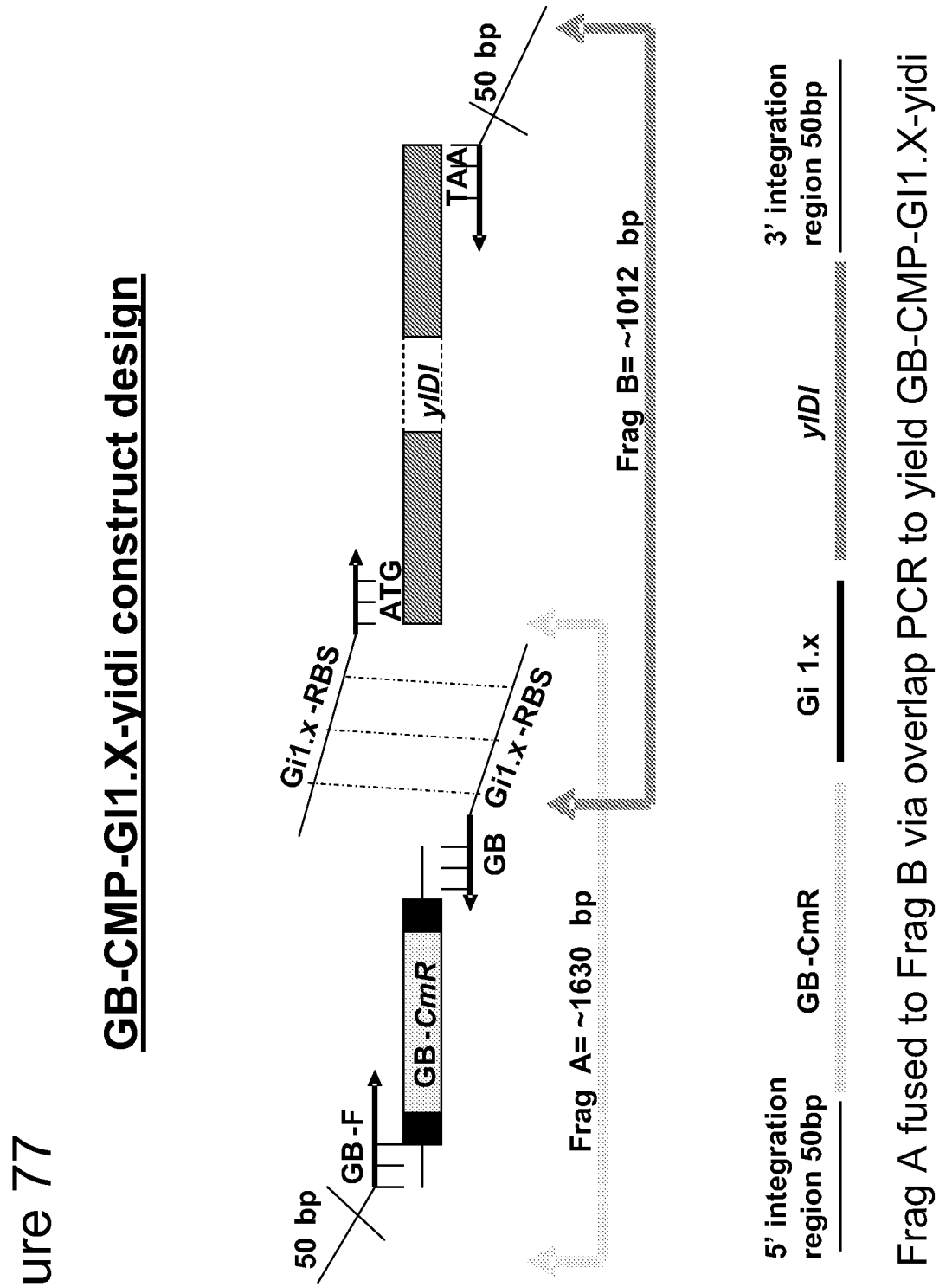

EcoRI (6578)
ClaI (6554)
HindIII (6547)
T7 terminator
BamHI (6257)
NcoI (5984)
yIDI
NcoI (5407)
Factor Xa
His tag
NcoI (5316)
T7 promoter
lac operator
ApaLI (4509)
lac I
AvaI (2935)
ApaLI (2071)
ColE1 pBR322 origin
ApaLI (1571)
PstI (755)
bla(Ap) sequence
ApaLI (325)
pDu9 -pET-16b rev-yIDI
6579 bp

Figure 77



Frag A fused to Frag B via overlap PCR to yield GB-CMP-GI1.X-yidi

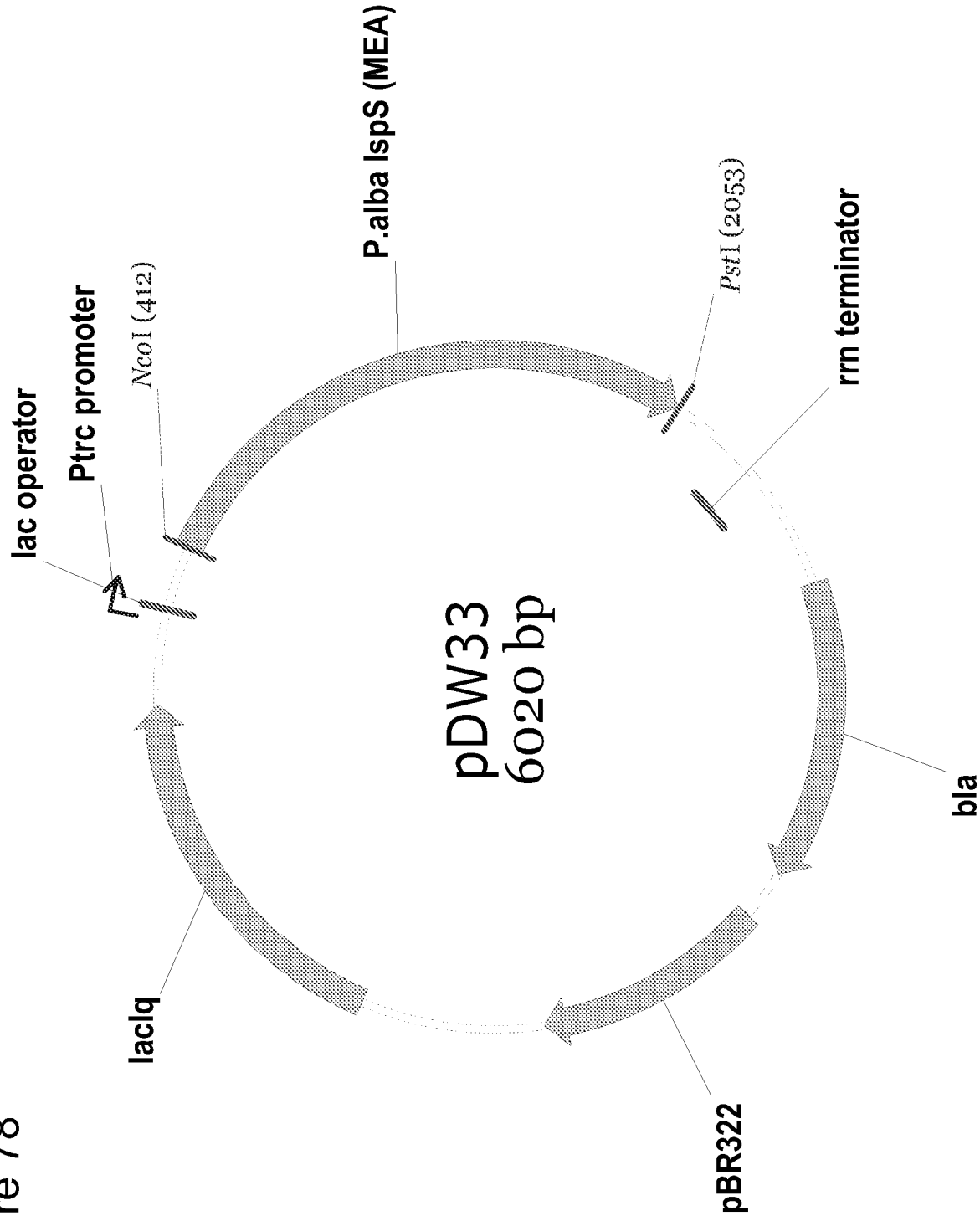

pDW33
6020 bp
P.alba IspS (MEA)
PstI (2053)
rrn terminator
bla
pBR322
laclq
NcoI (412)
Ptrc promoter
lac operator

Figure 78

Figure 79A

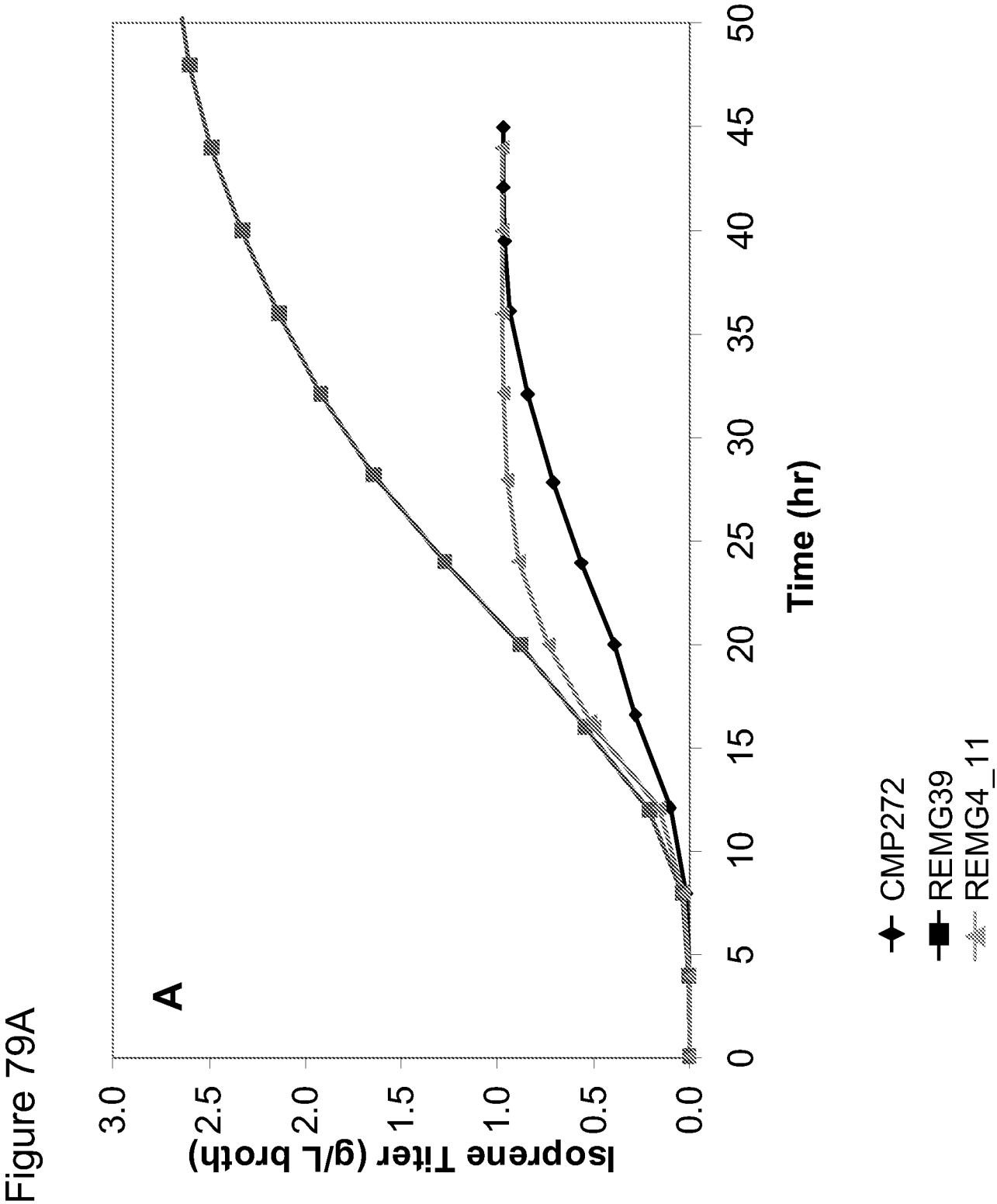

A
Isoprene Titer (g/L broth)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
40
45
50
Time (hr)
CMP272
REMG39
REMG4_11

Figure 79B

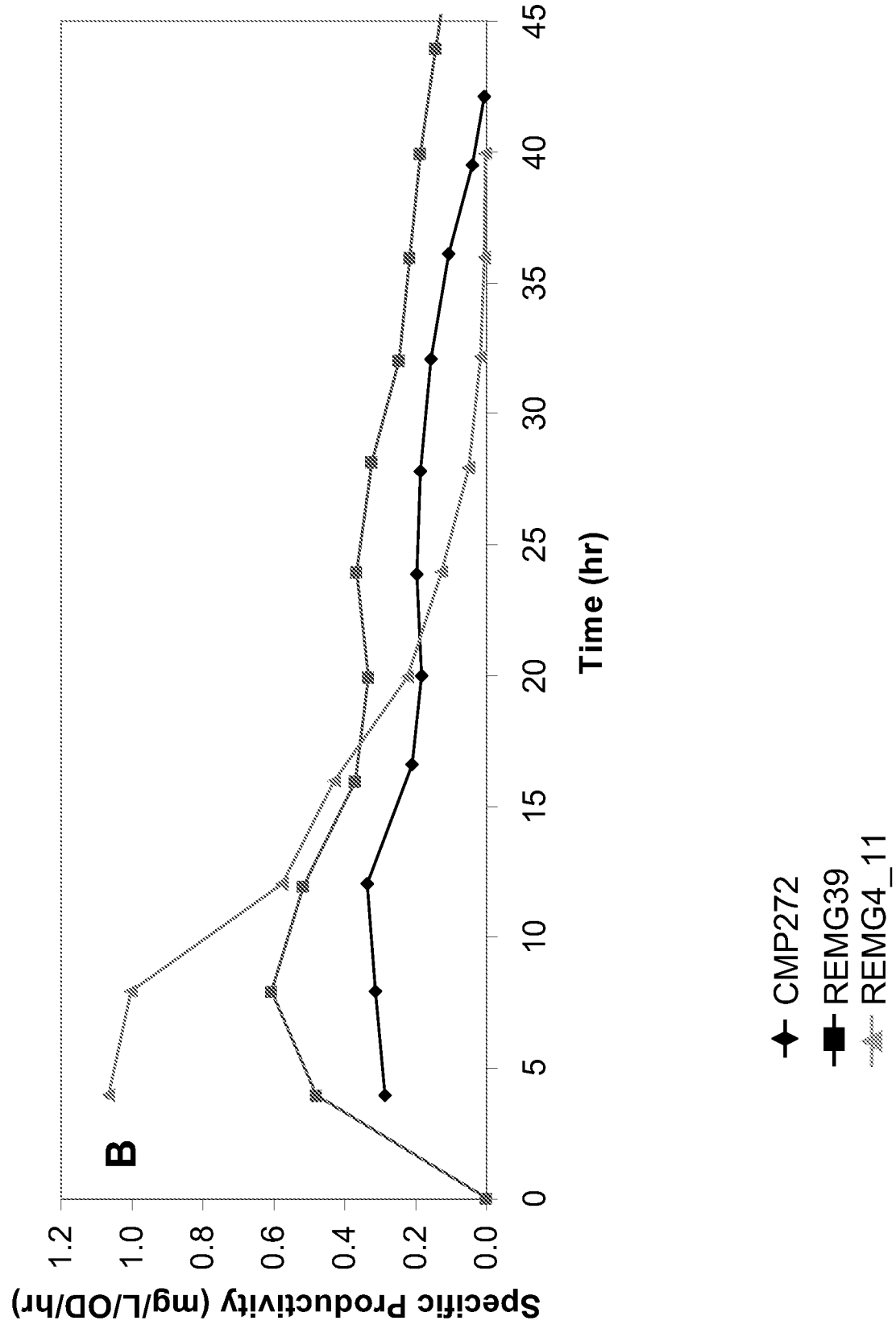

B
Specific Productivity (mg/L/OD/hr)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
45
Time (hr)
CMP272
REMG39
REMG4_11

Figure 79C

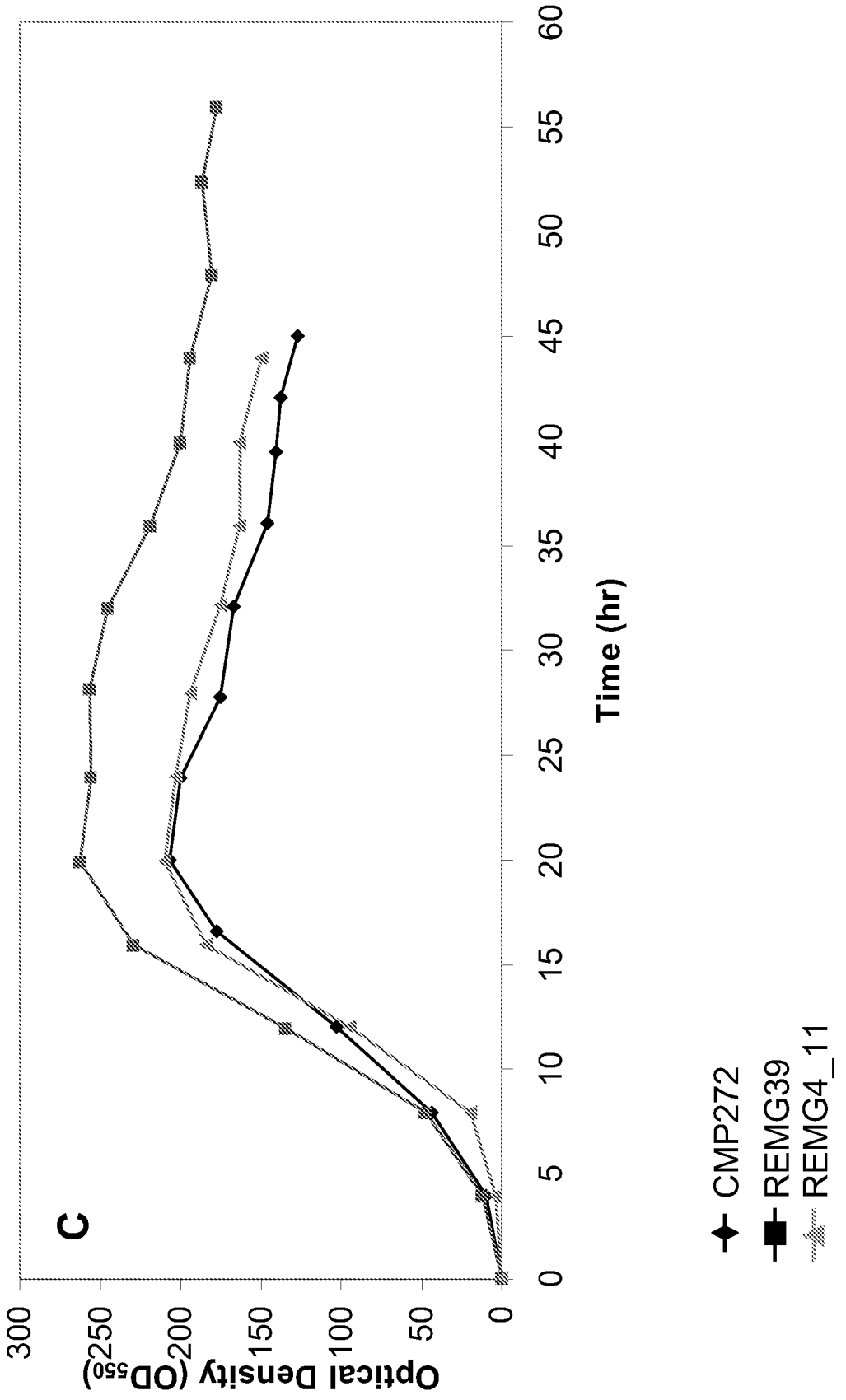

c
Optical Density (OD550)
Time (hr)
0
50
100
150
200
250
300
0
5
10
15
20
25
30
35
40
45
50
55
60
CMP272
REMG39
REMG4_11

Figure 79 D

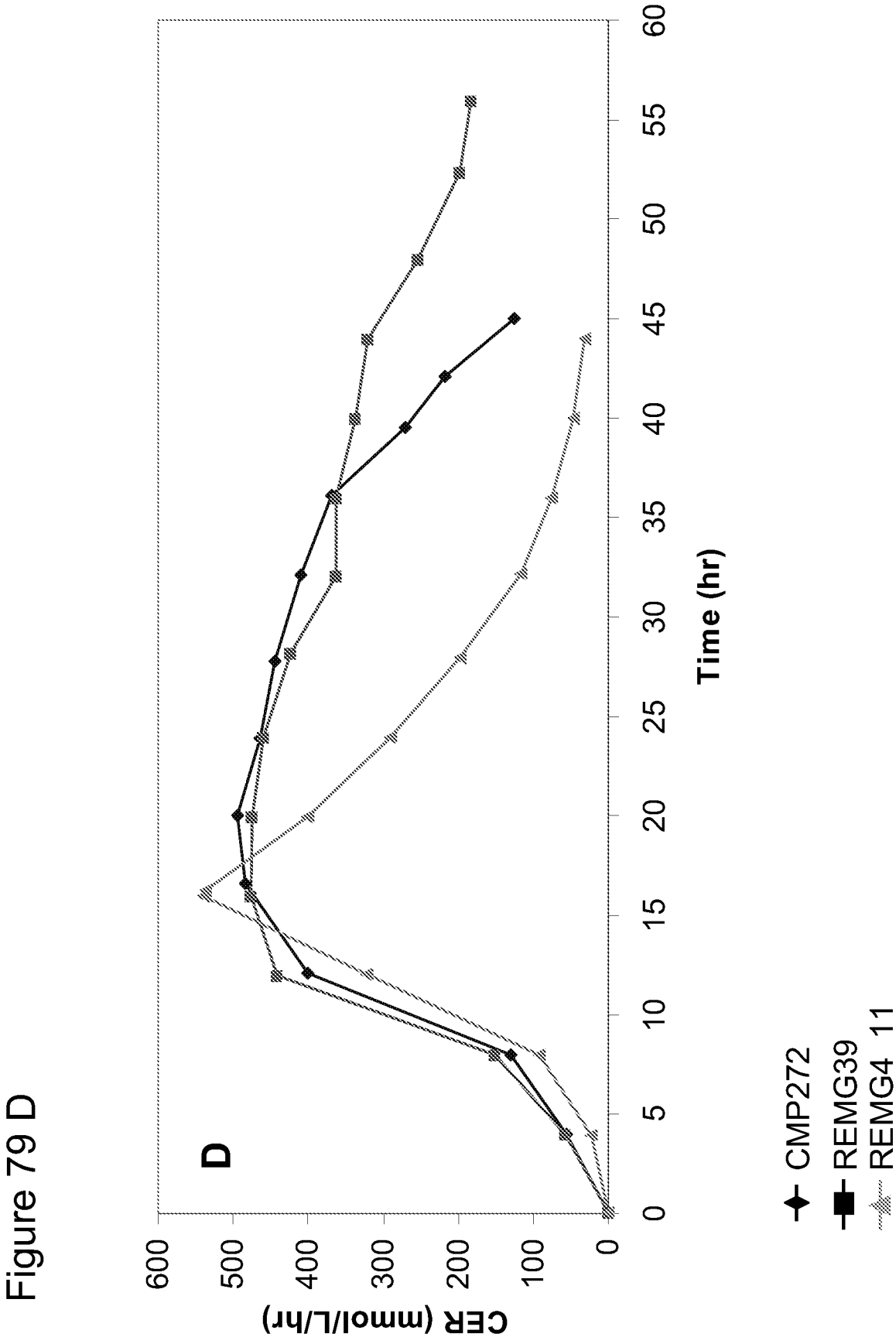

D
CER (mmol/L/hr)
0
100
200
300
400
500
600
Time (hr)
0
5
10
15
20
25
30
35
40
45
50
55
60
CMP272
REMG39
REMG4_11

Figure 79 E

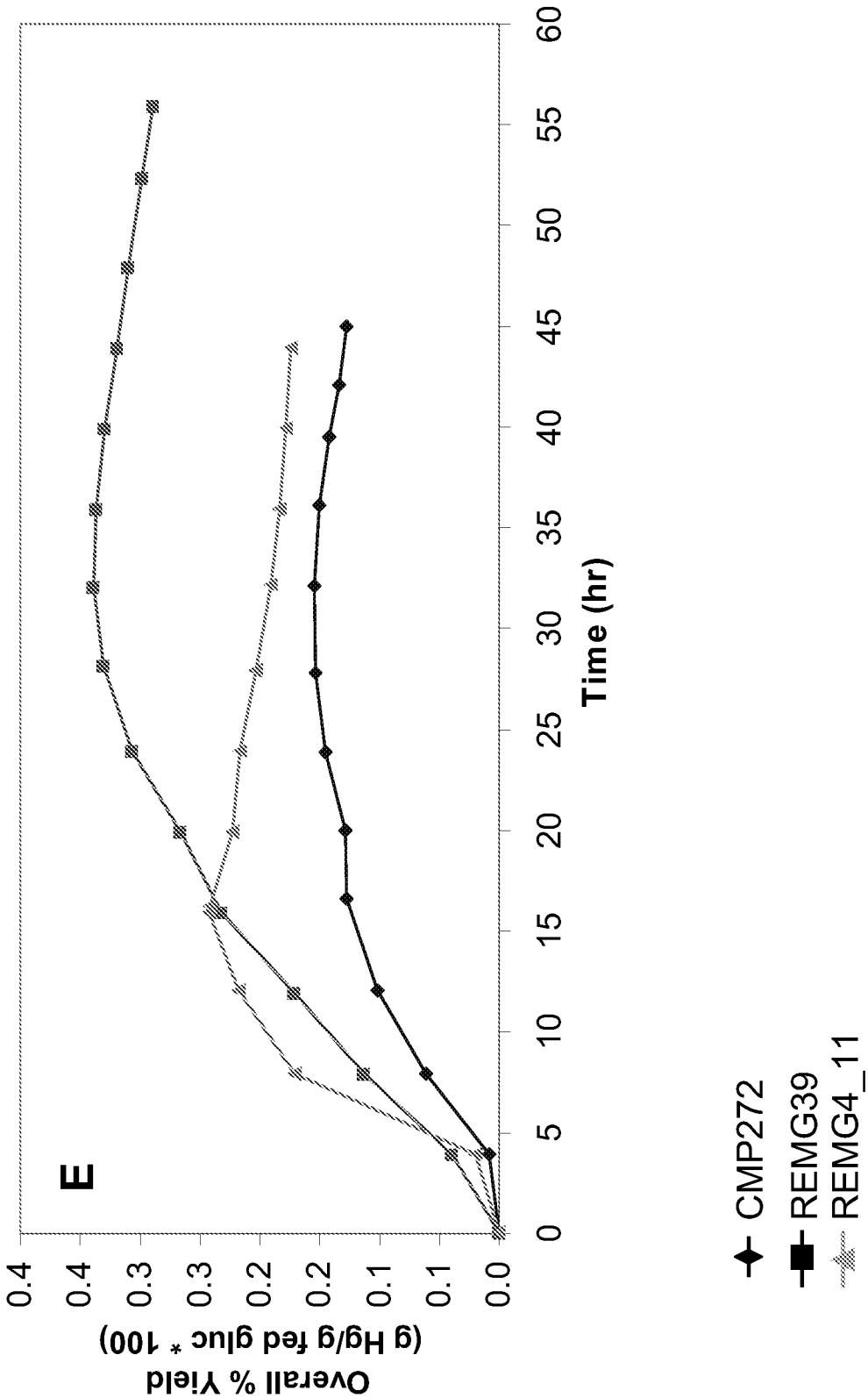

E
Overall % Yield
(g Hg/g fed gluc * 100)
0.0
0.1
0.1
0.2
0.2
0.3
0.3
0.4
0.4
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)
CMP272
REMG39
REMG4_11

Figure 80A

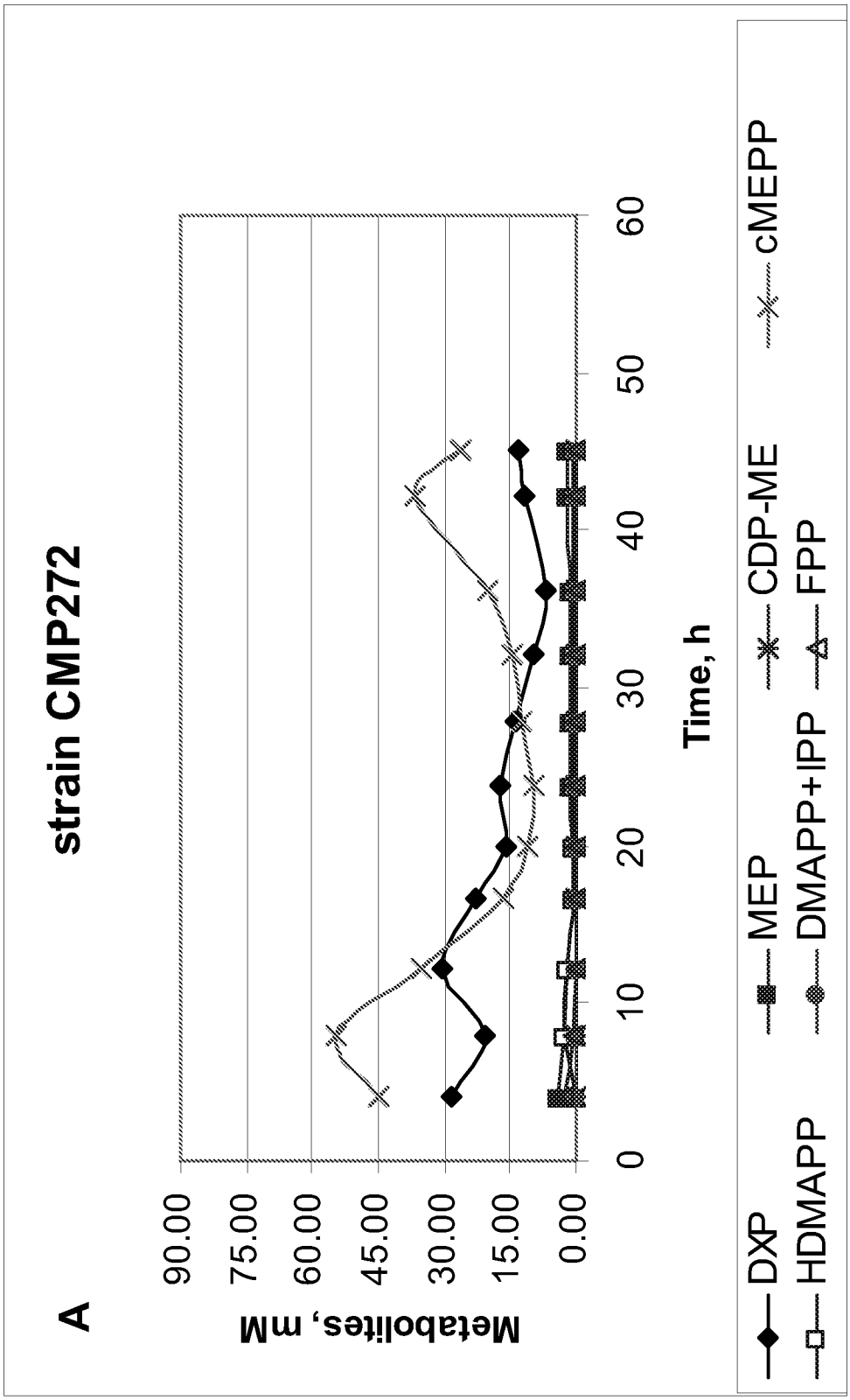

A
strain CMP272
Metabolites, mM
90.00
75.00
60.00
45.00
30.00
15.00
0.00
0
10
20
30
40
50
60
Time, h
DXP
MEP
CDP-ME
cMEPP
HDMAPP
DMAPP+IPP
FPP

Figure 80B

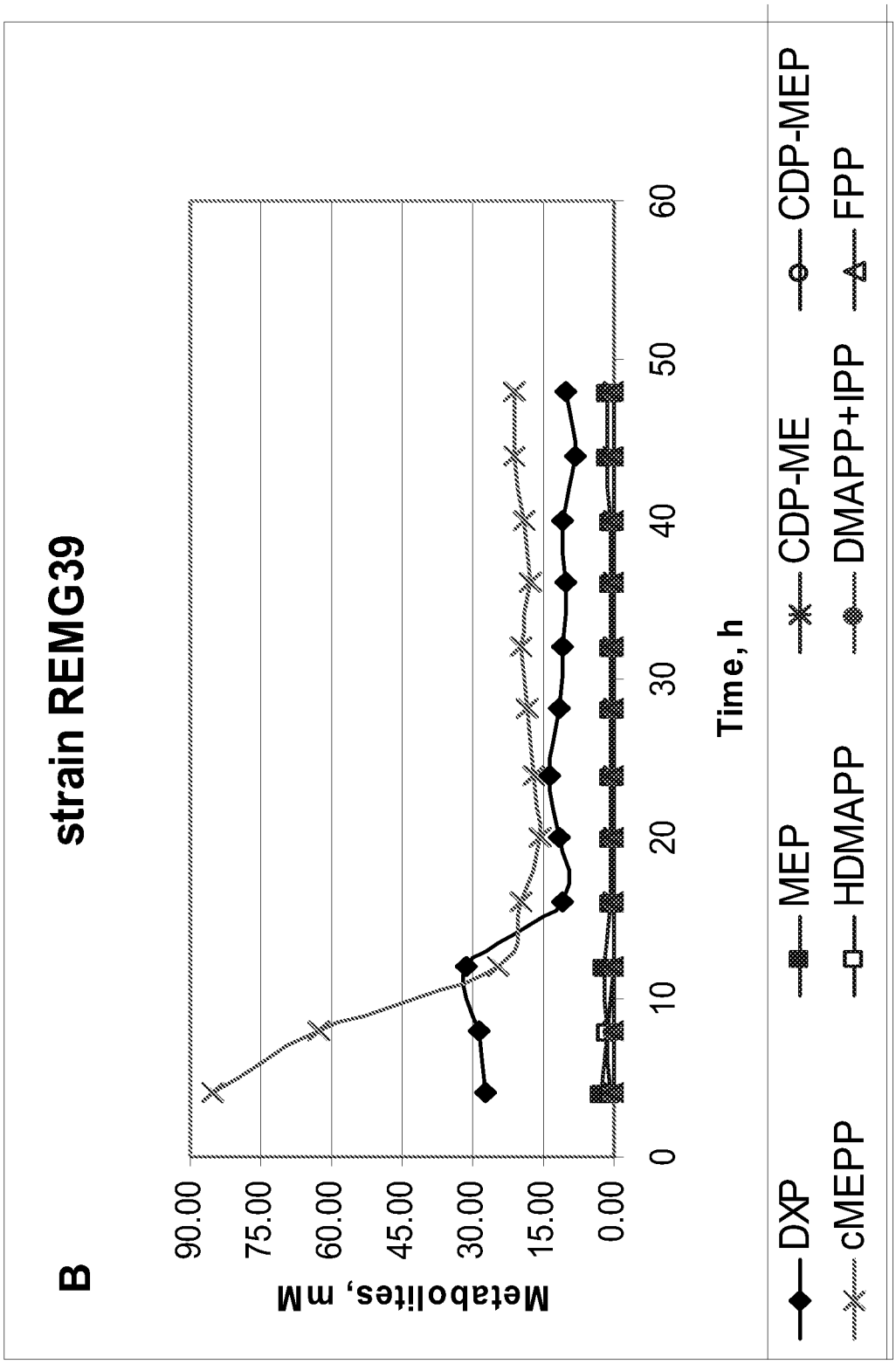

B
strain REMG39
Metabolites, mM
90.00
75.00
60.00
45.00
30.00
15.00
0.00
0
10
20
30
40
50
60
Time, h
DXP
MEP
CDP-ME
CDP-MEP
cMEPP
HDMAPP
DMAPP+IPP
FPP

Figure 80C

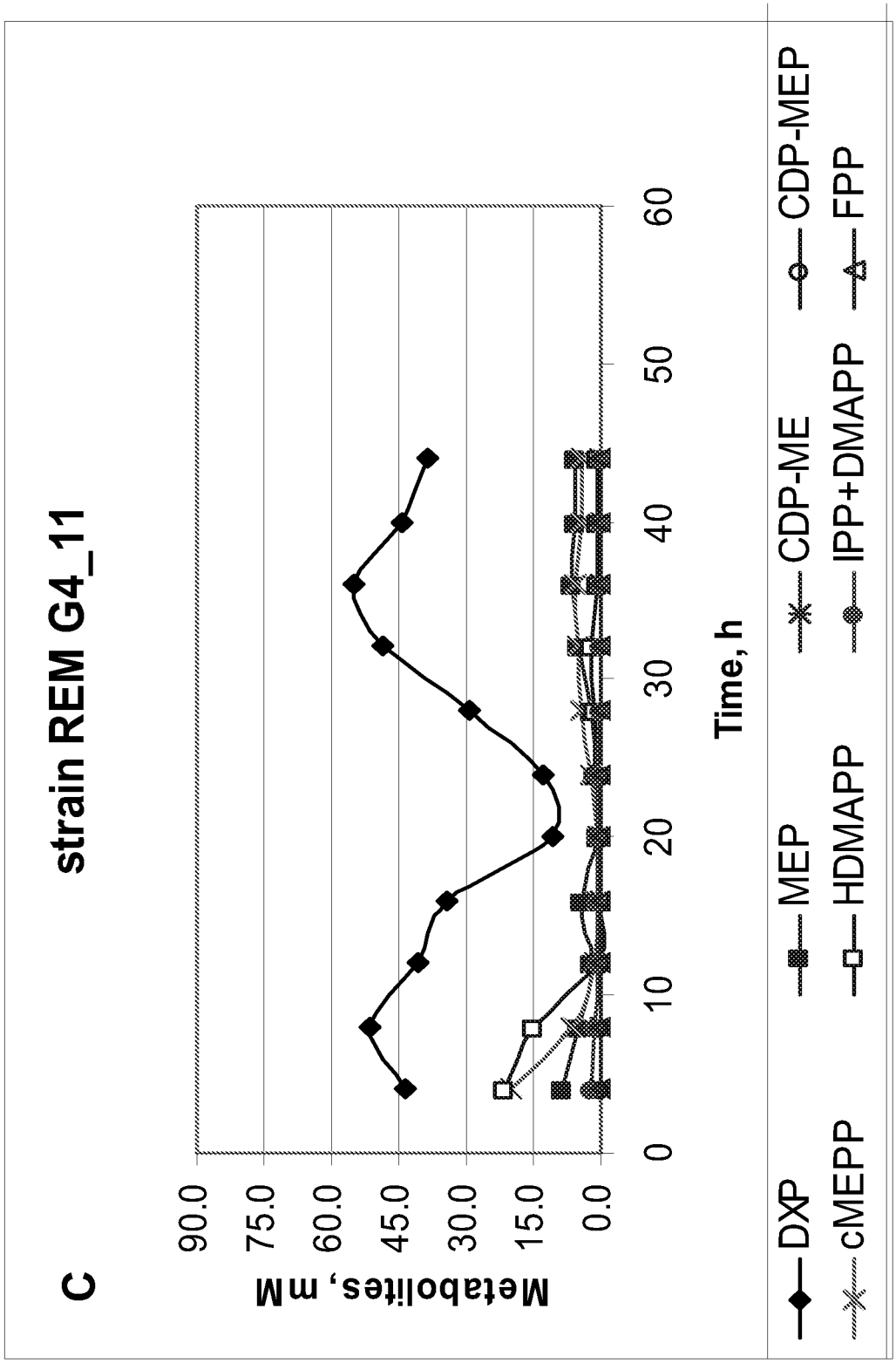
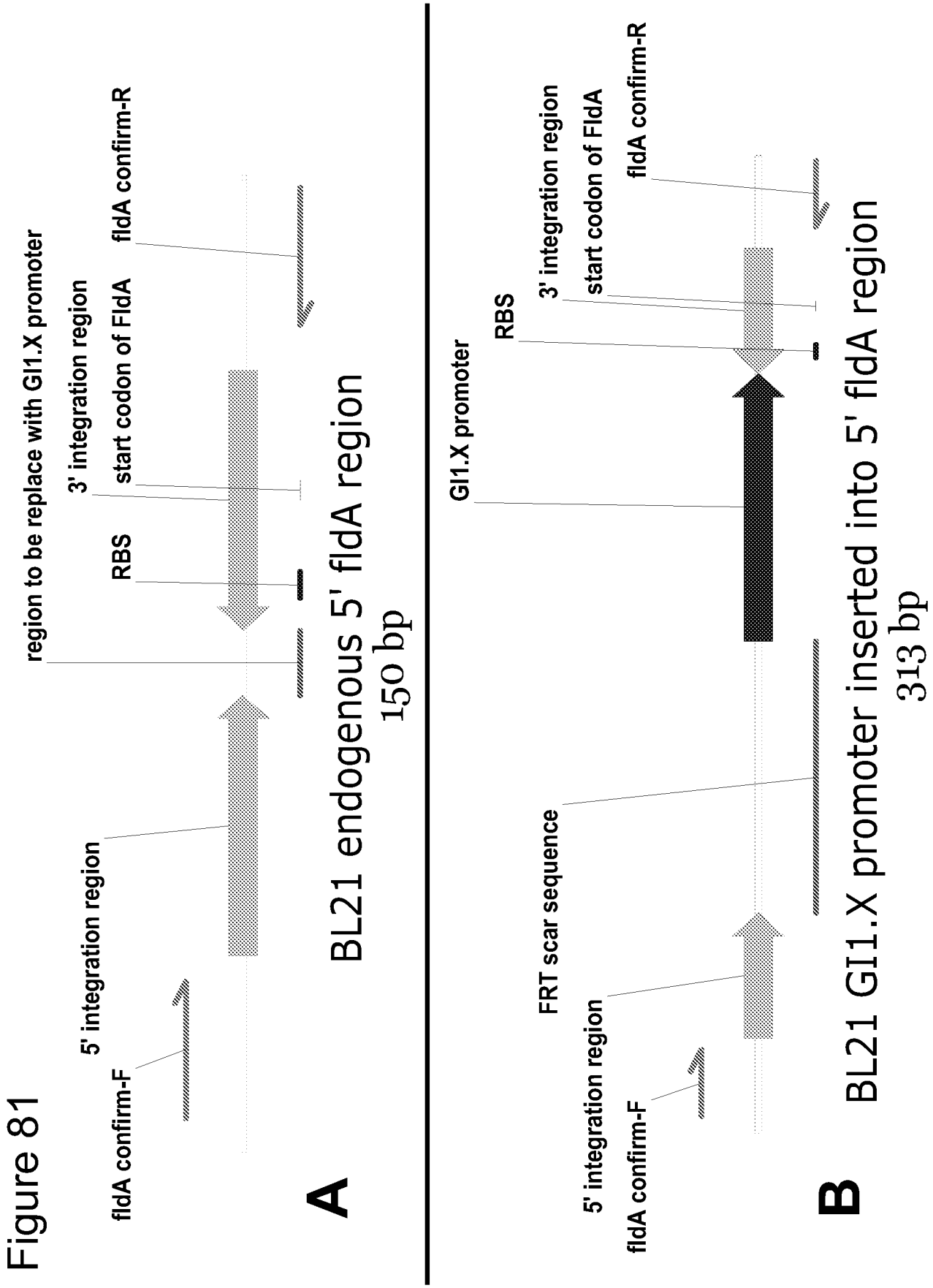

C
strain REM G4_11
Metabolites, mM
90.0
75.0
60.0
45.0
30.0
15.0
0.0
0
10
20
30
40
50
60
Time, h
DXP
MEP
CDP-ME
CDP-MEP
cMEPP
HDMAPP
IPP+DMAPP
FPP

Figure 81


region to be replace with GI1.X promoter
3' integration region
start codon of FldA
fldA confirm-R
RBS
5' integration region
fldA confirm-F
A
BL21 endogenous 5' fldA region
150 bp
GI1.X promoter
RBS
3' integration region
start codon of FldA
fldA confirm-R
FRT scar sequence
5' integration region
fldA confirm-F
B
BL21 GI1.X promoter inserted into 5' fldA region
313 bp

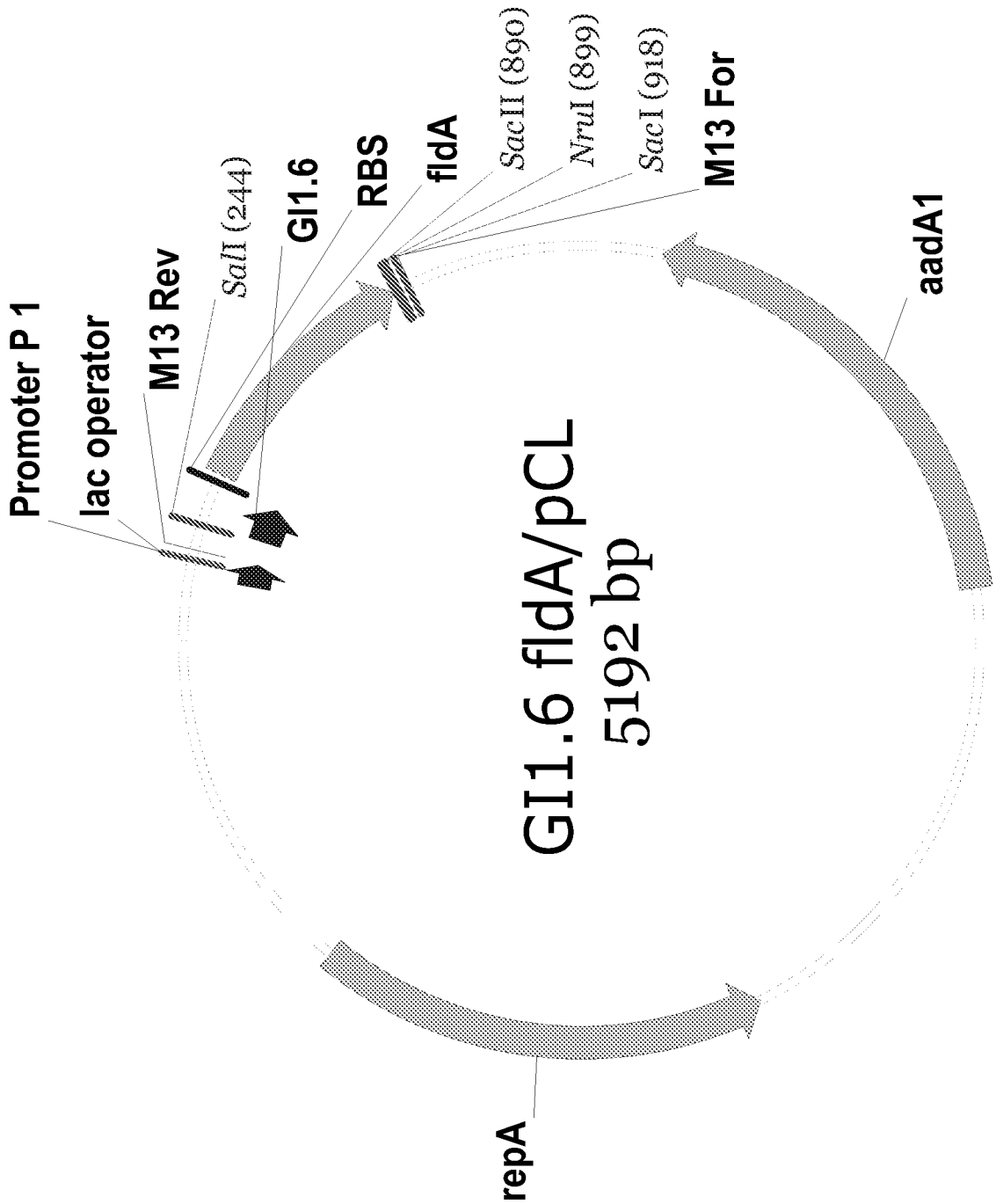

Promoter P 1
lac operator
M13 Rev
SalI (244)
GI1.6
RBS
fldA
SacII (890)
NruI (899)
SacI (918)
M13 For
aadA1
repA
GI1.6 fldA/pCL
5192 bp

Figure 82

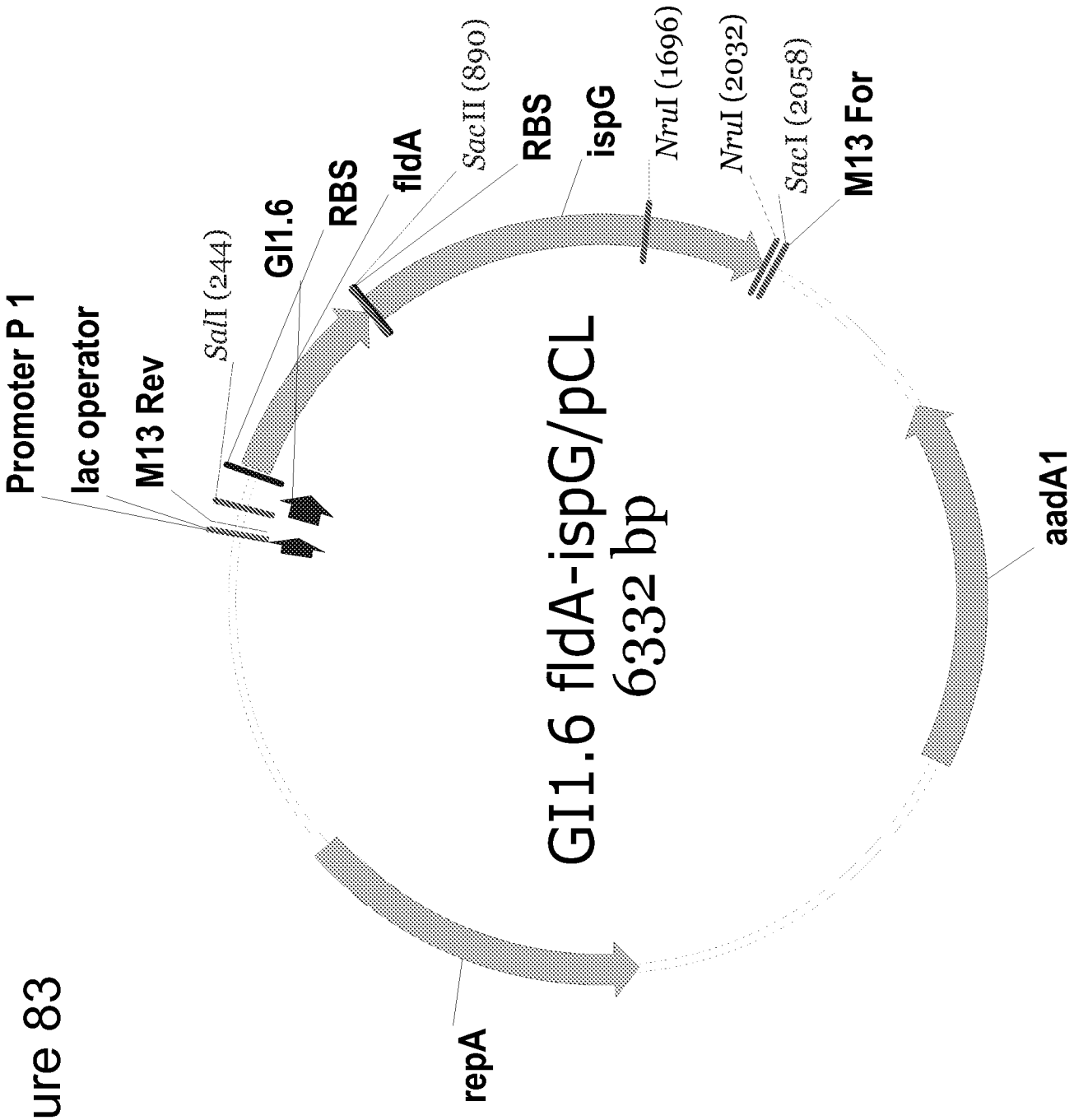

Promoter P 1
lac operator
M13 Rev
SalI (244)
GI1.6
RBS
fldA
SacII (890)
RBS
ispG
NruI (1696)
NruI (2032)
SacI (2058)
M13 For
aadA1
repA
GI1.6 fldA-ispG/pCL
6332 bp

Figure 83

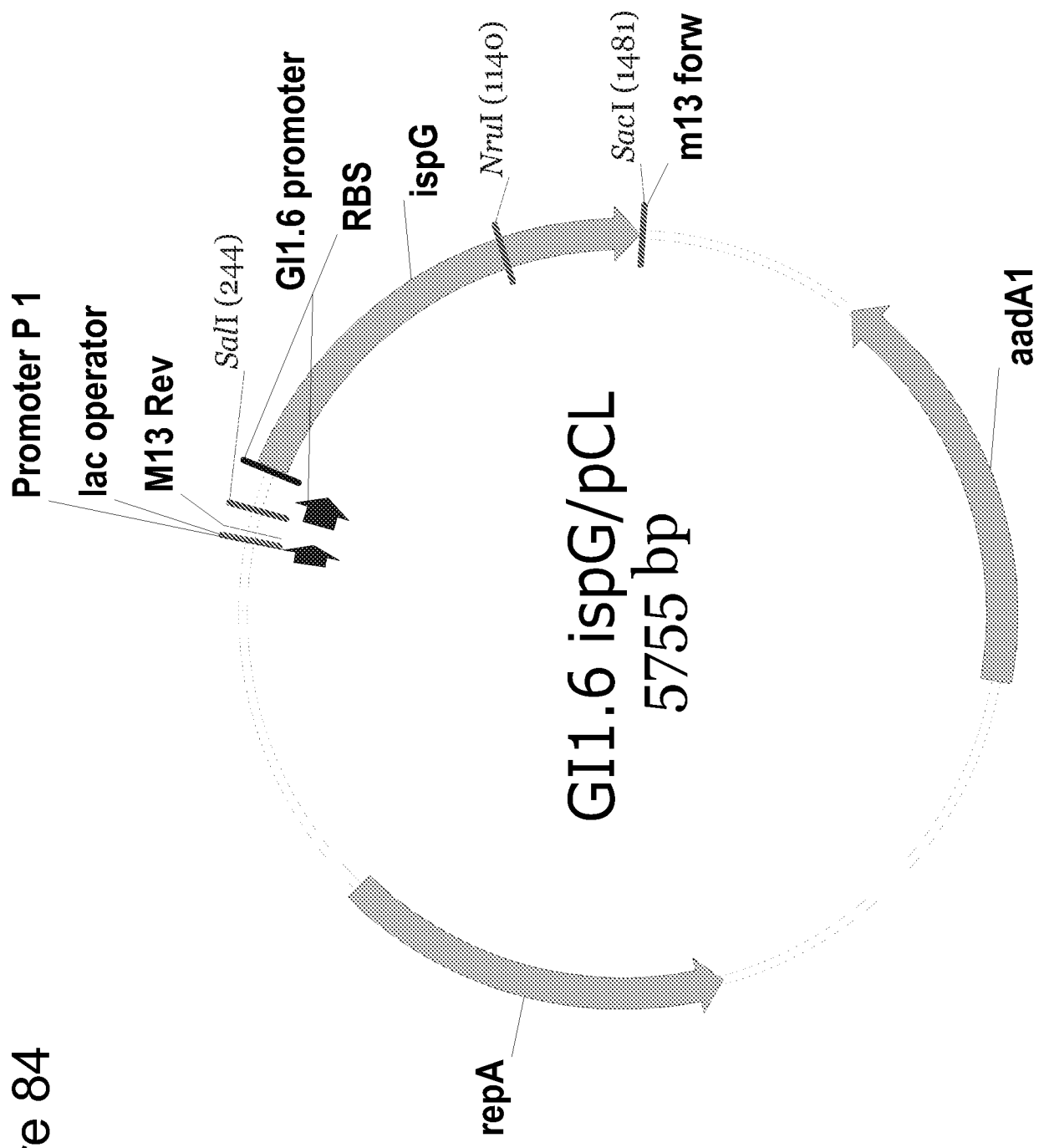

Promoter P 1
lac operator
M13 Rev
SalI (244)
GI1.6 promoter
RBS
ispG
NruI (1140)
SacI (1481)
m13 forw
aadA1
repA
GI1.6 ispG/pCL
5755 bp

Figure 84

Figure 85A

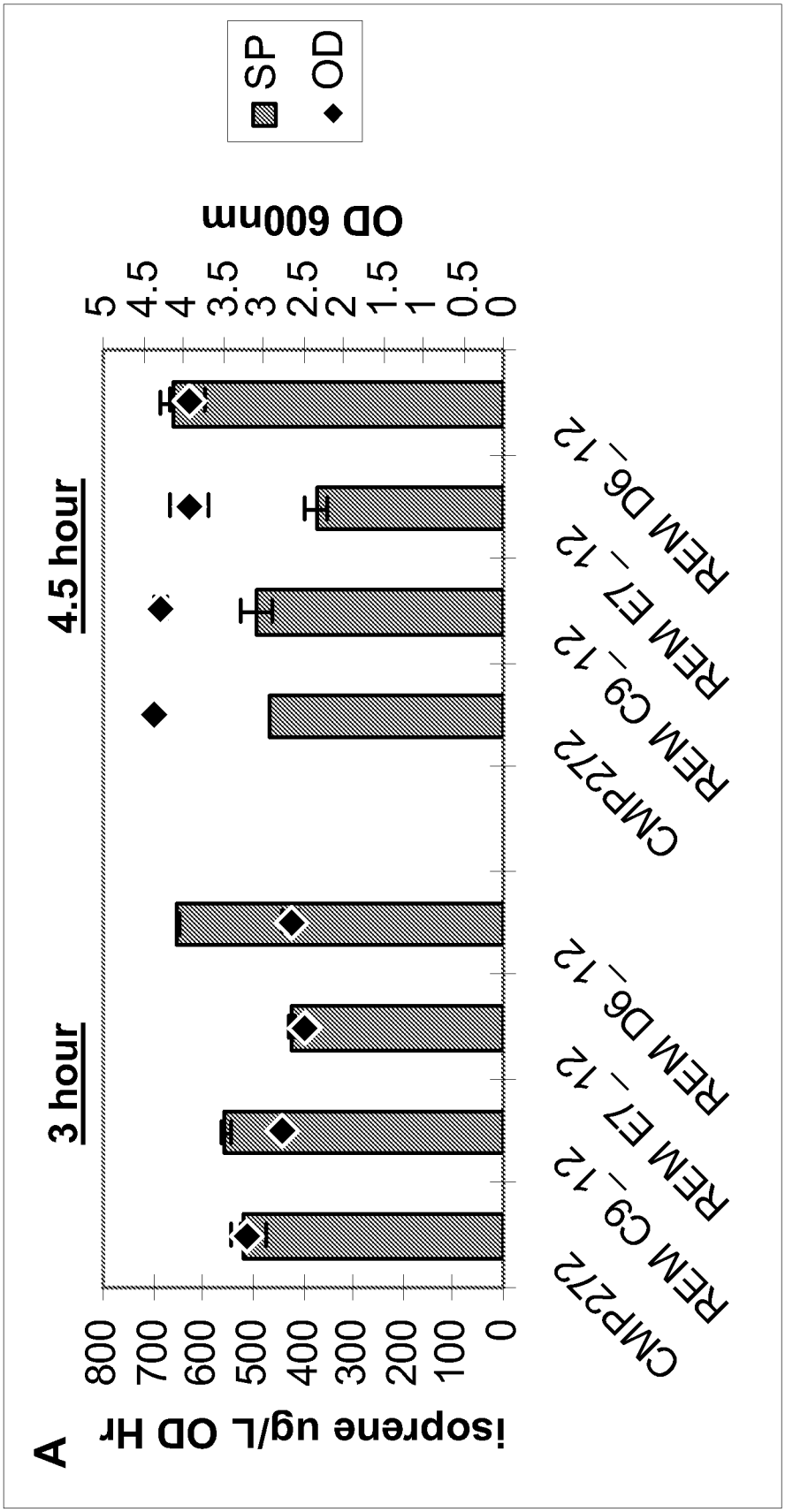

A
isoprene ug/L OD Hr
800
700
600
500
400
300
200
100
0
OD 600nm
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
SP
OD
3 hour
4.5 hour
CMP272
REM C9_12
REM E7_12
REM D6_12
CMP272
REM C9_12
REM E7_12
REM D6_12

Figure 85B

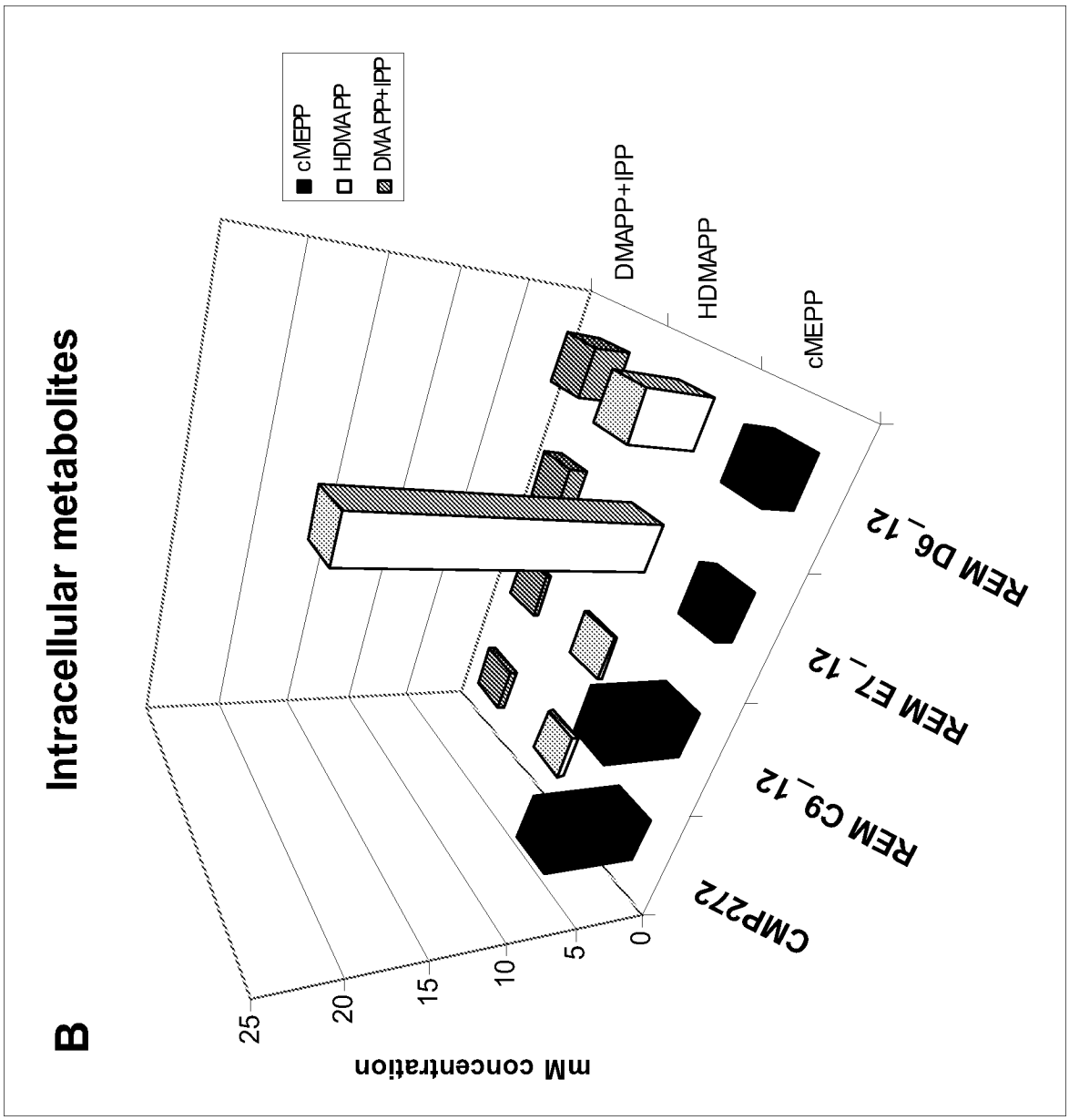

B
Intracellular metabolites
mM concentration
0
5
10
15
20
25
CMP272
REM C9_12
REM E7_12
REM D6_12
cMEPP
HDMAPP
DMAPP+IPP
cMEPP
HDMAPP
DMAPP+IPP

Figure 86A

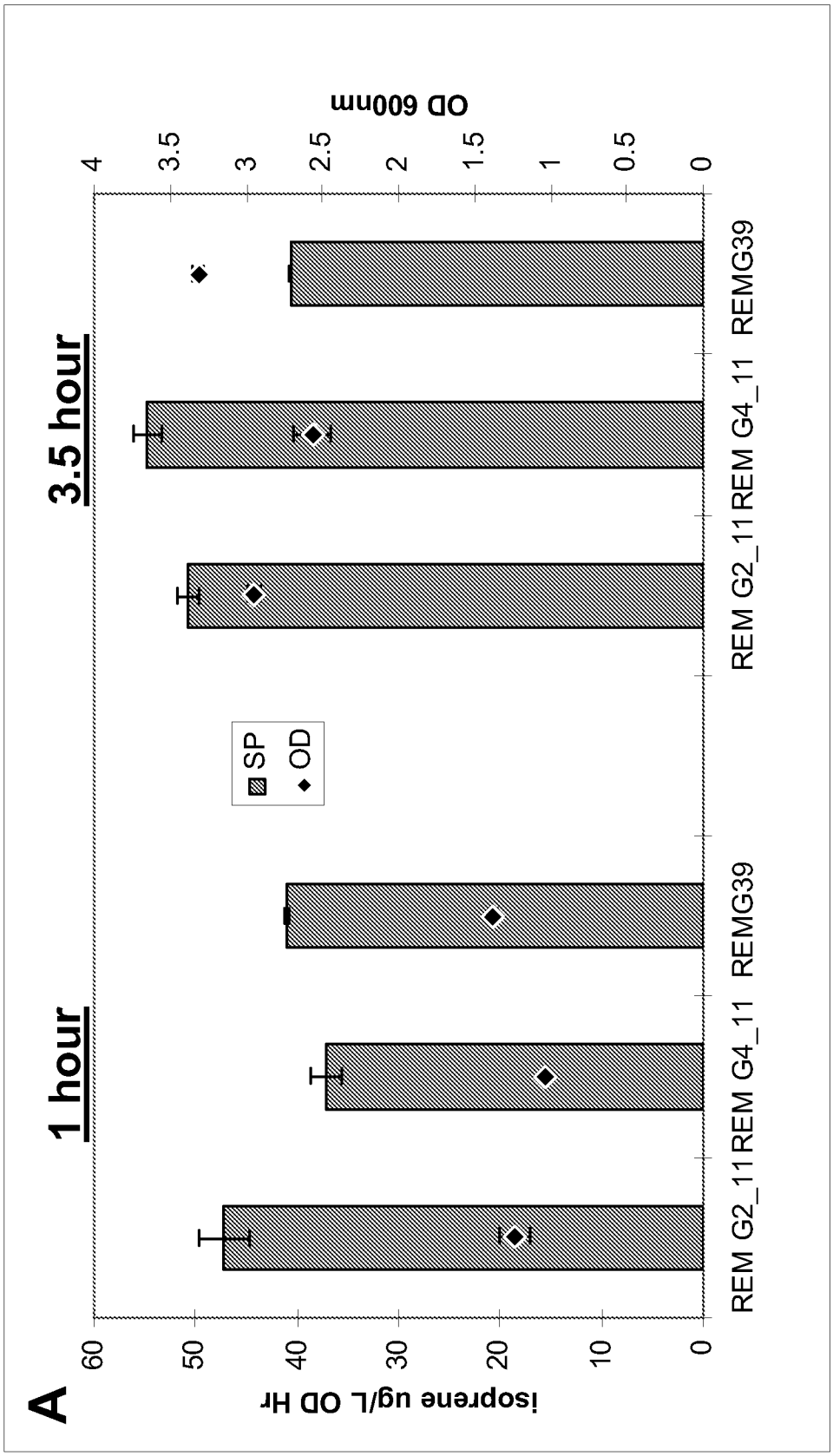

A
1 hour
3.5 hour
isoprene ug/L OD Hr
OD 600nm
SP
OD
0
10
20
30
40
50
60
0
0.5
1
1.5
2
2.5
3
3.5
4
REM G2_11
REM G4_11
REMG39
REM G2_11
REM G4_11
REMG39

Figure 86B

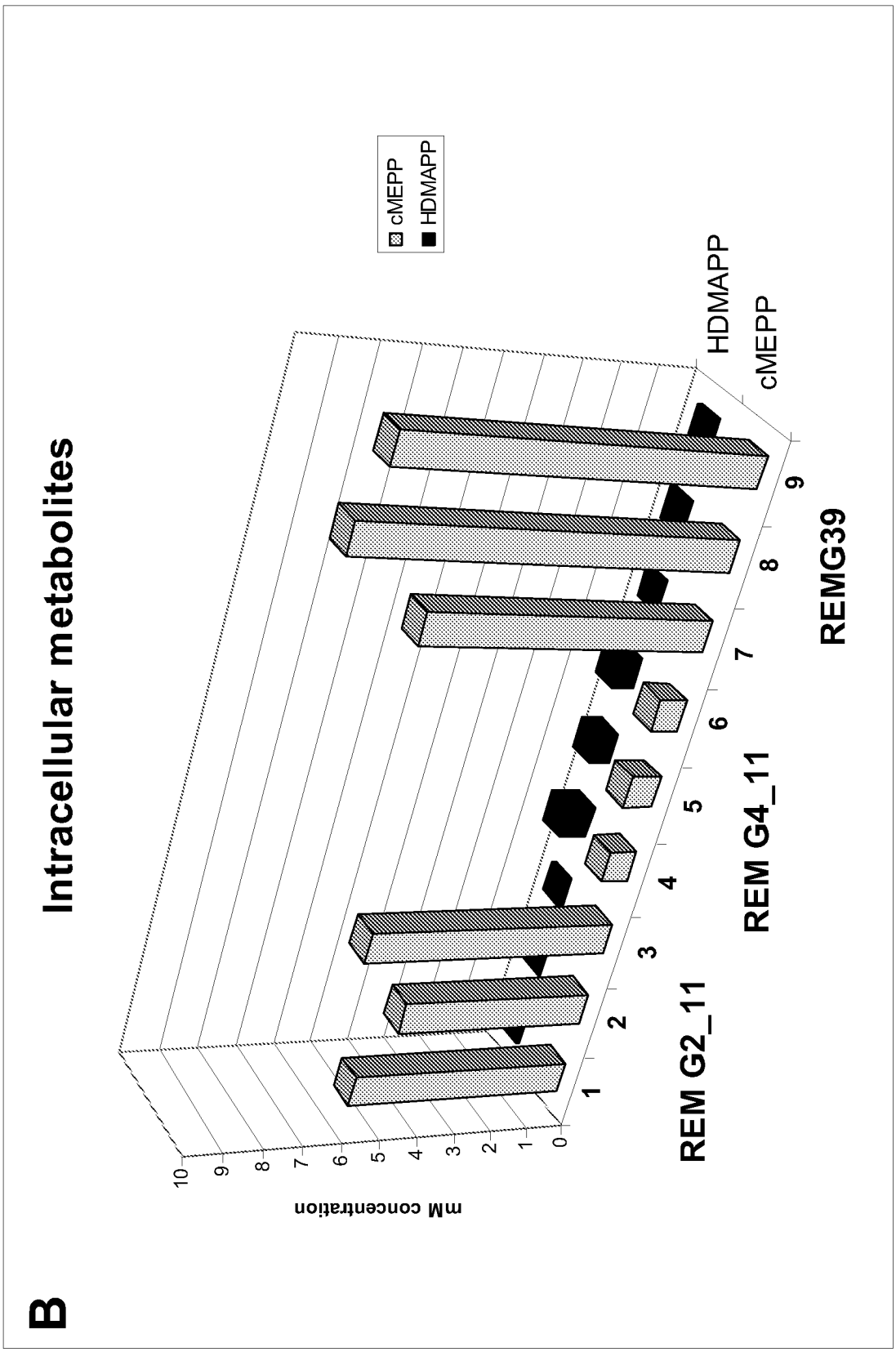

B
Intracellular metabolites
mM concentration
0
1
2
3
4
5
6
7
8
9
10
REM G2_11
REM G4_11
REMG39
cMEPP
HDMAPP
cMEPP
HDMAPP

Figure 87

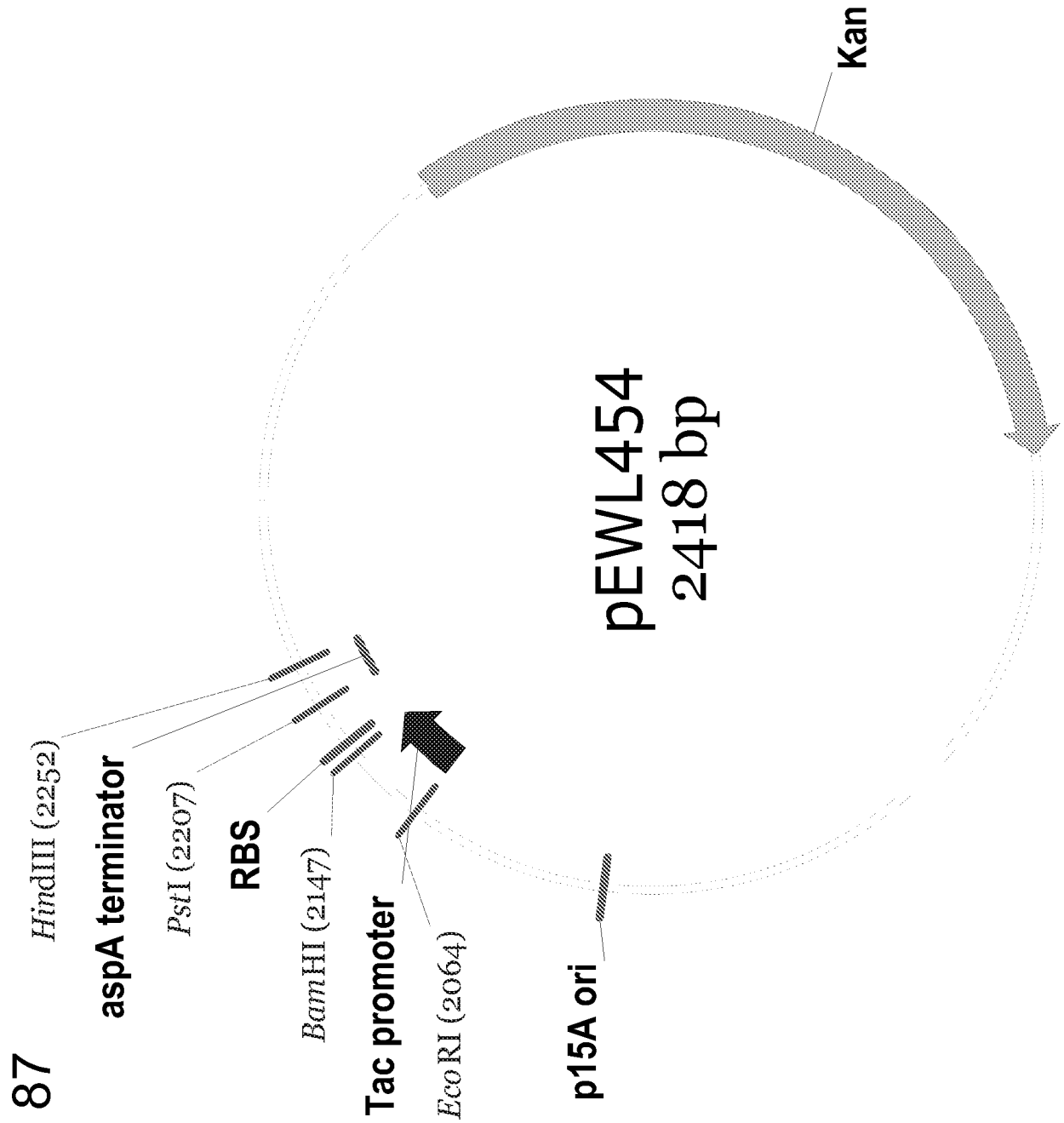

pEWL454
2418 bp
Kan
HindIII (2252)
aspA terminator
PstI (2207)
RBS
BamHI (2147)
Tac promoter
EcoRI (2064)
p15A ori

Figure 88

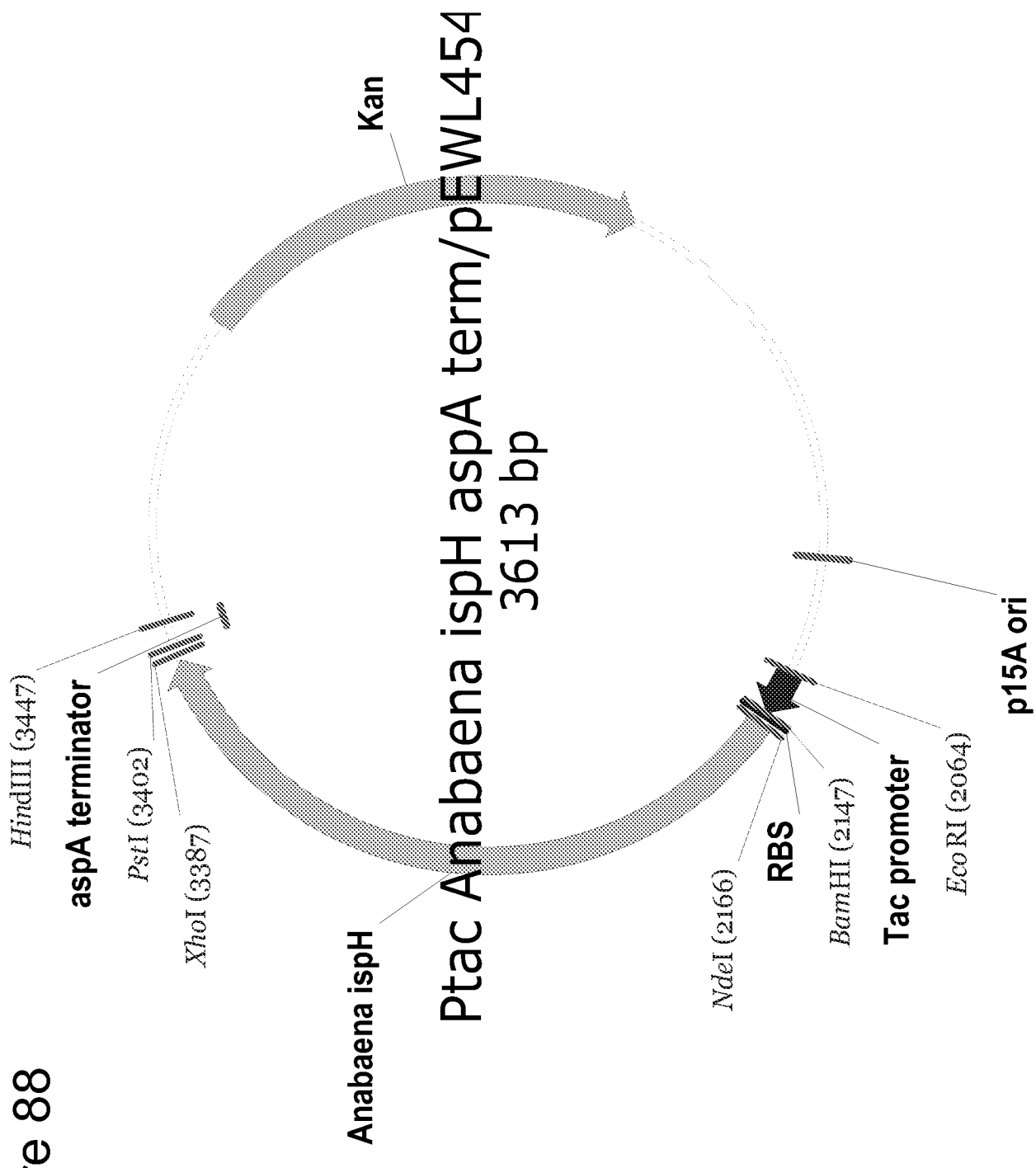

Ptac Anabaena ispH aspA term/pEWL454
3613 bp
Kan
p15A ori
Tac promoter
RBS
Anabaena ispH
aspA terminator
HindIII (3447)
PstI (3402)
XhoI (3387)
NdeI (2166)
BamHI (2147)
EcoRI (2064)

Figure 89

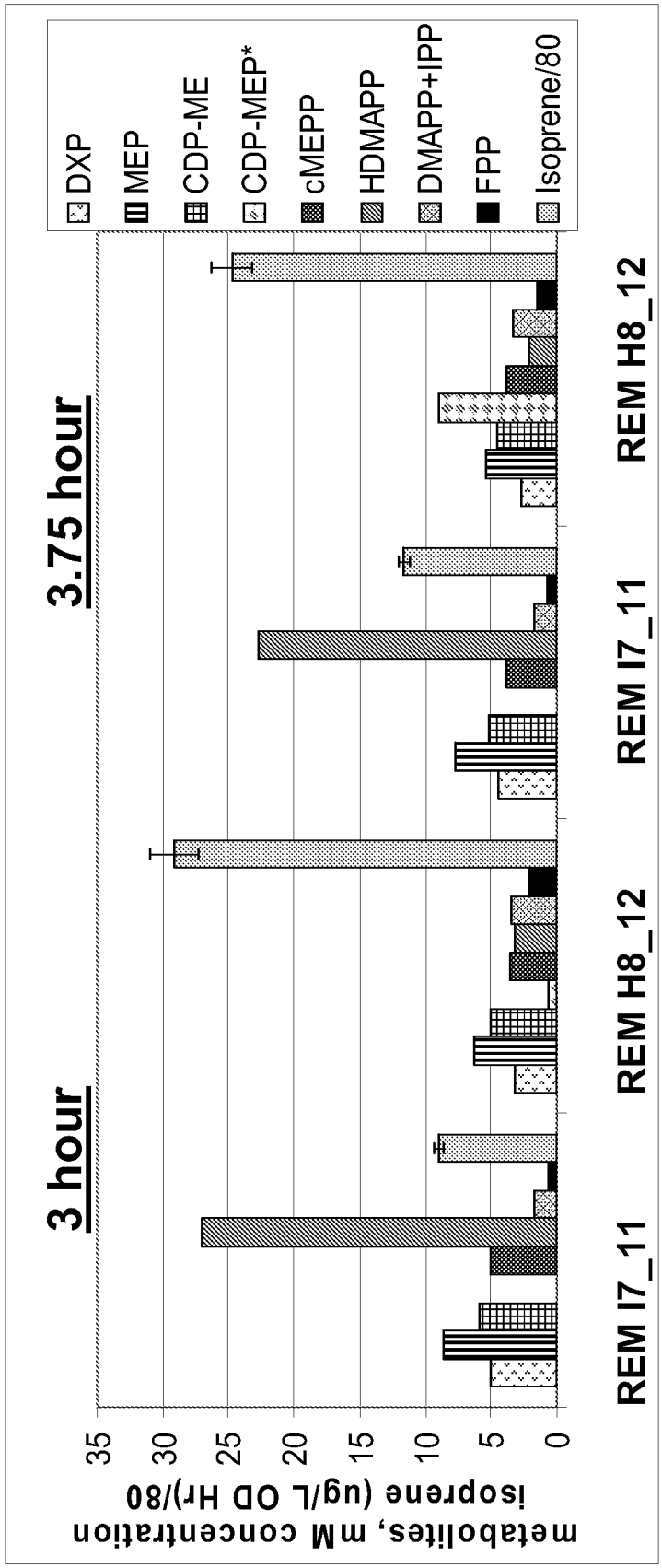

3 hour
3.75 hour
DXP
MEP
CDP-ME
CDP-MEP*
cMEPP
HDMAPP
DMAPP+IPP
FPP
Isoprene/80
metabolites, mM concentration
isoprene (ug/L OD Hr)/80
0
5
10
15
20
25
30
35
REM I7_11
REM H8_12
REM I7_11
REM H8_12

Figure 90A

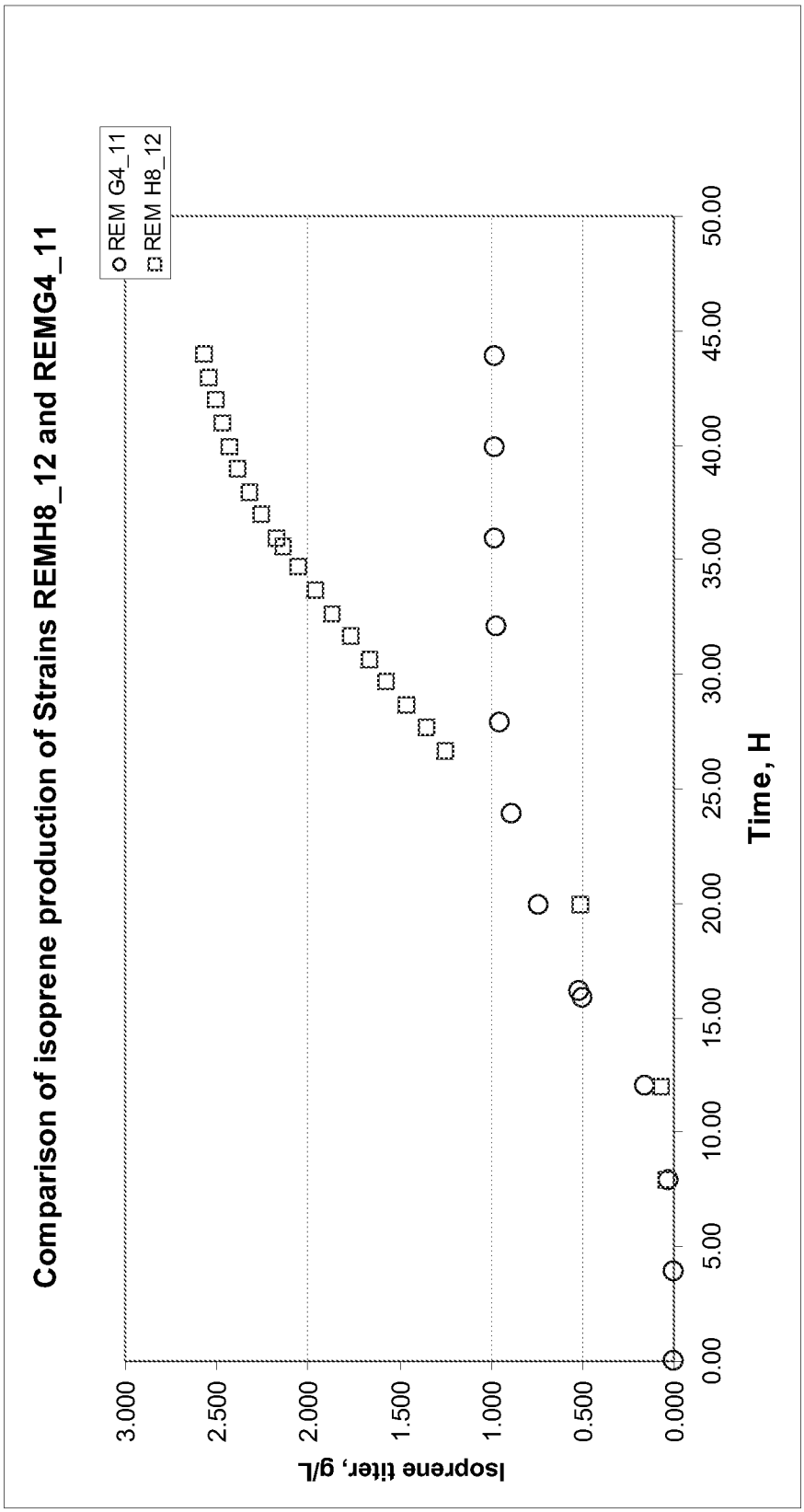

Comparison of isoprene production of Strains REMH8_12 and REMG4_11
REM G4_11
REM H8_12
Isoprene titer, g/L
Time, H
0.000
0.500
1.000
1.500
2.000
2.500
3.000
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00

Figure 90B

B

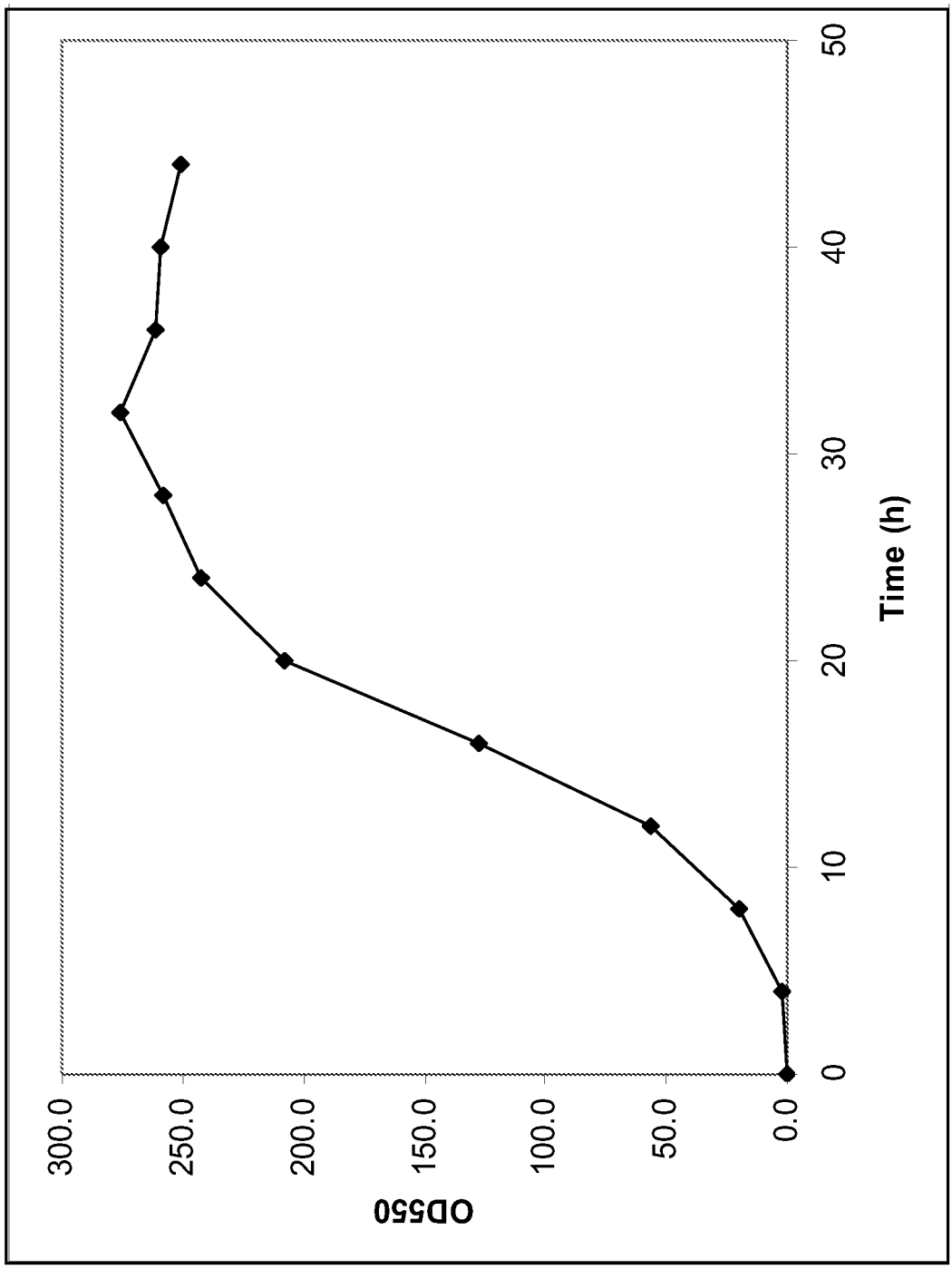
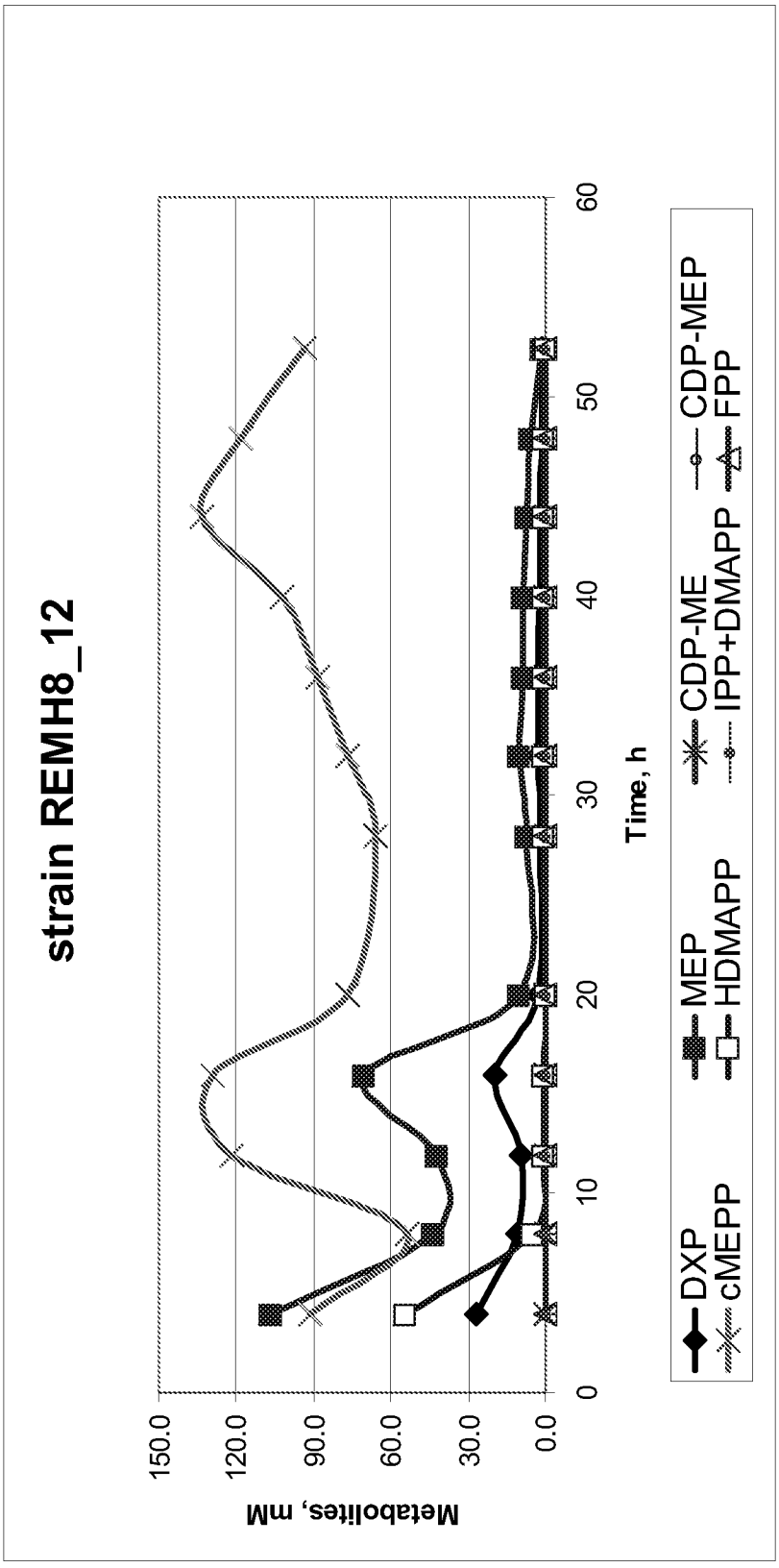


Figure 90C


strain REMH8_12
Metabolites, mM
150.0
120.0
90.0
60.0
30.0
0.0
0
10
20
30
40
50
60
Time, h
DXP
cMEPP
MEP
HDMAPP
CDP-ME
IPP+DMAPP
CDP-MEP
FPP

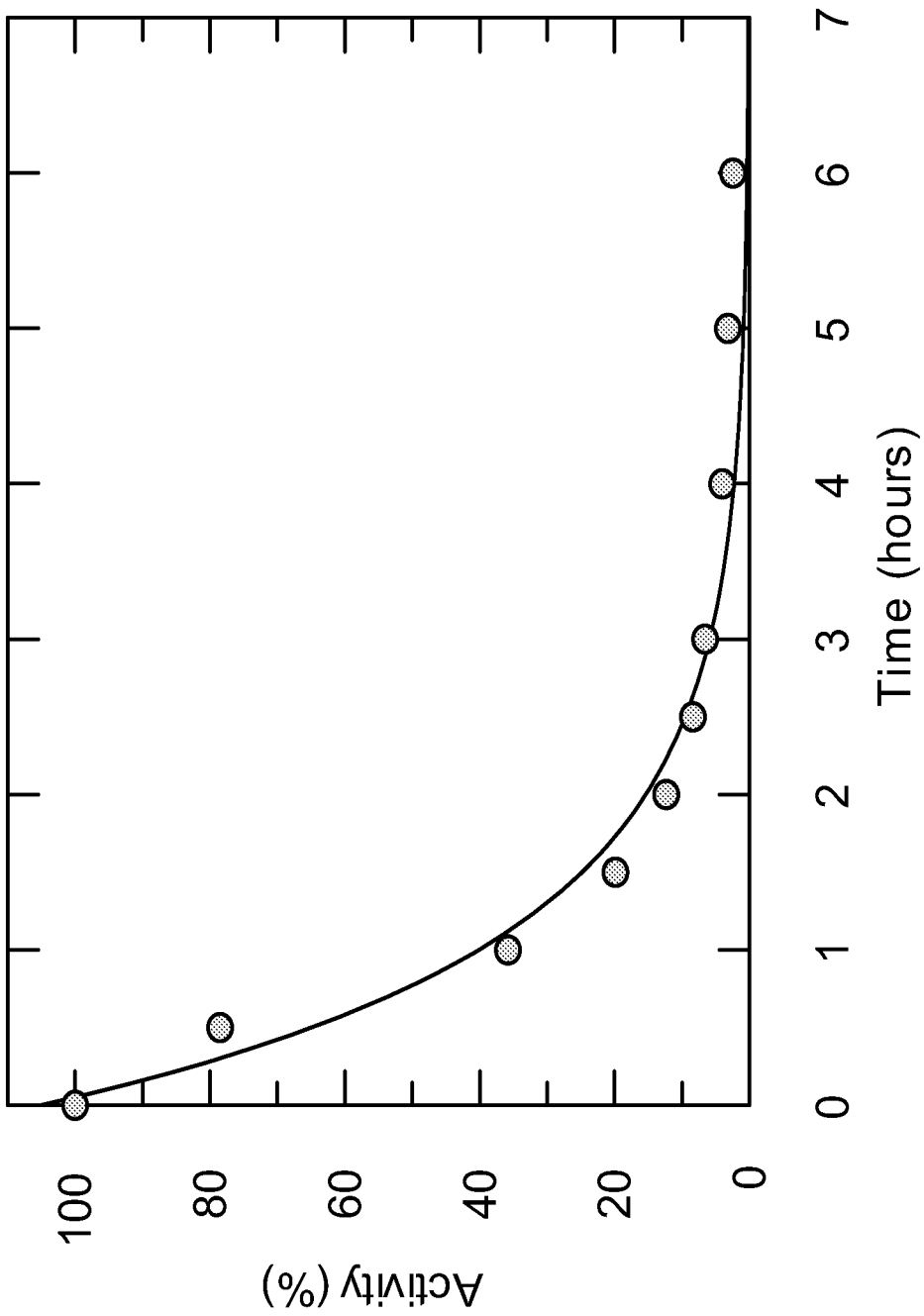


Figure 91

Figure 92A

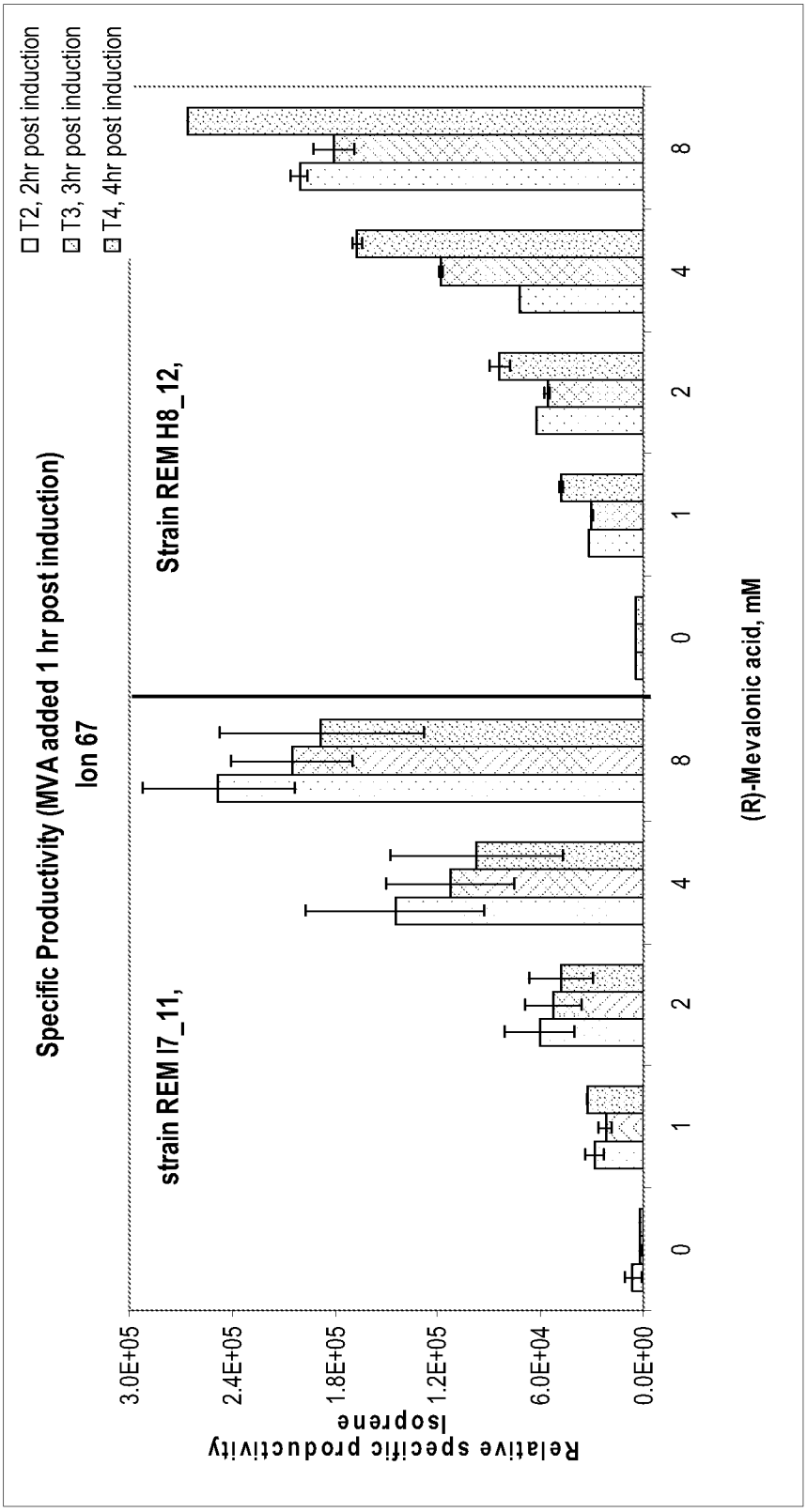
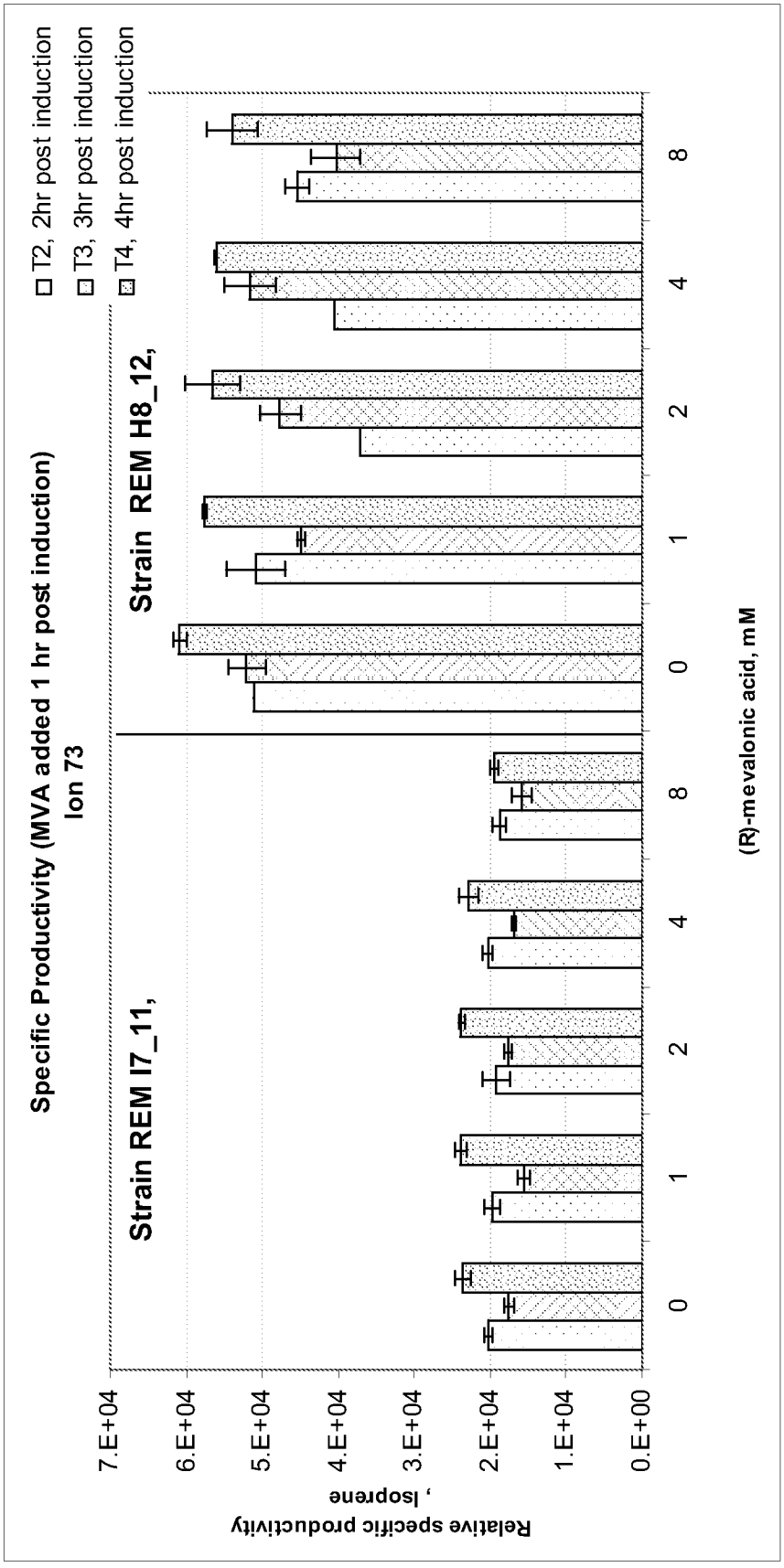

T2, 2hr post induction
T3, 3hr post induction
T4, 4hr post induction
Specific Productivity (MVA added 1 hr post induction)
Ion 67
strain REM I7_11,
Strain REM H8_12,
Relative specific productivity
Isoprene
3.0E+05
2.4E+05
1.8E+05
1.2E+05
6.0E+04
0.0E+00
0
1
2
4
8
0
1
2
4
8
(R)-Mevalonic acid, mM

Figure 92B


Specific Productivity (MVA added 1 hr post induction)
Ion 73
T2, 2hr post induction
T3, 3hr post induction
T4, 4hr post induction
Strain REM I7_11,
Strain REM H8_12,
Relative specific productivity
, Isoprene
7.E+04
6.E+04
5.E+04
4.E+04
3.E+04
2.E+04
1.E+04
0.E+00
0
1
2
4
8
0
1
2
4
8
(R)-mevalonic acid, mM

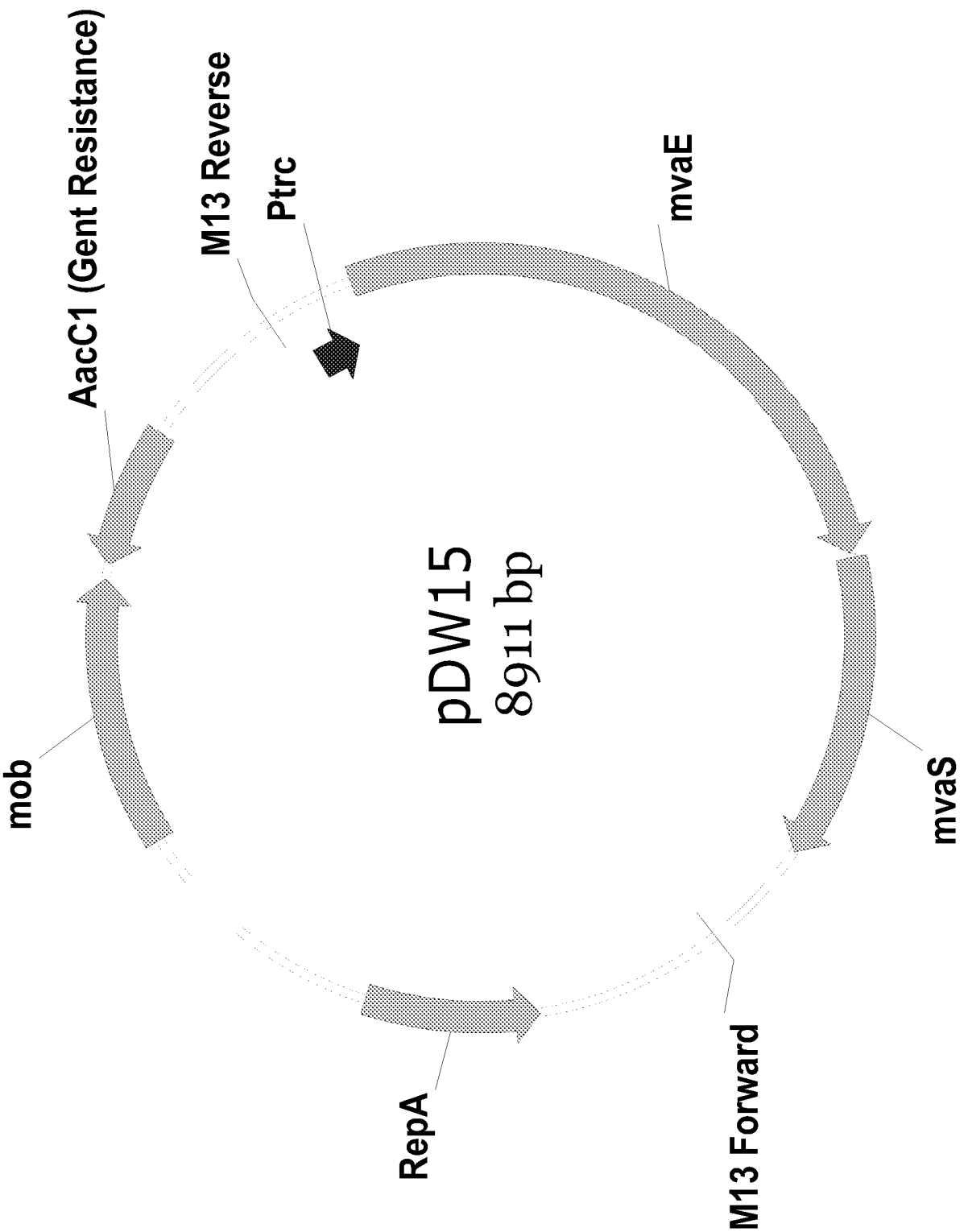

AacC1 (Gent Resistance)
M13 Reverse
Ptrc
mvaE
pDW15
8911 bp
mob
mvaS
RepA
M13 Forward

Figure 93

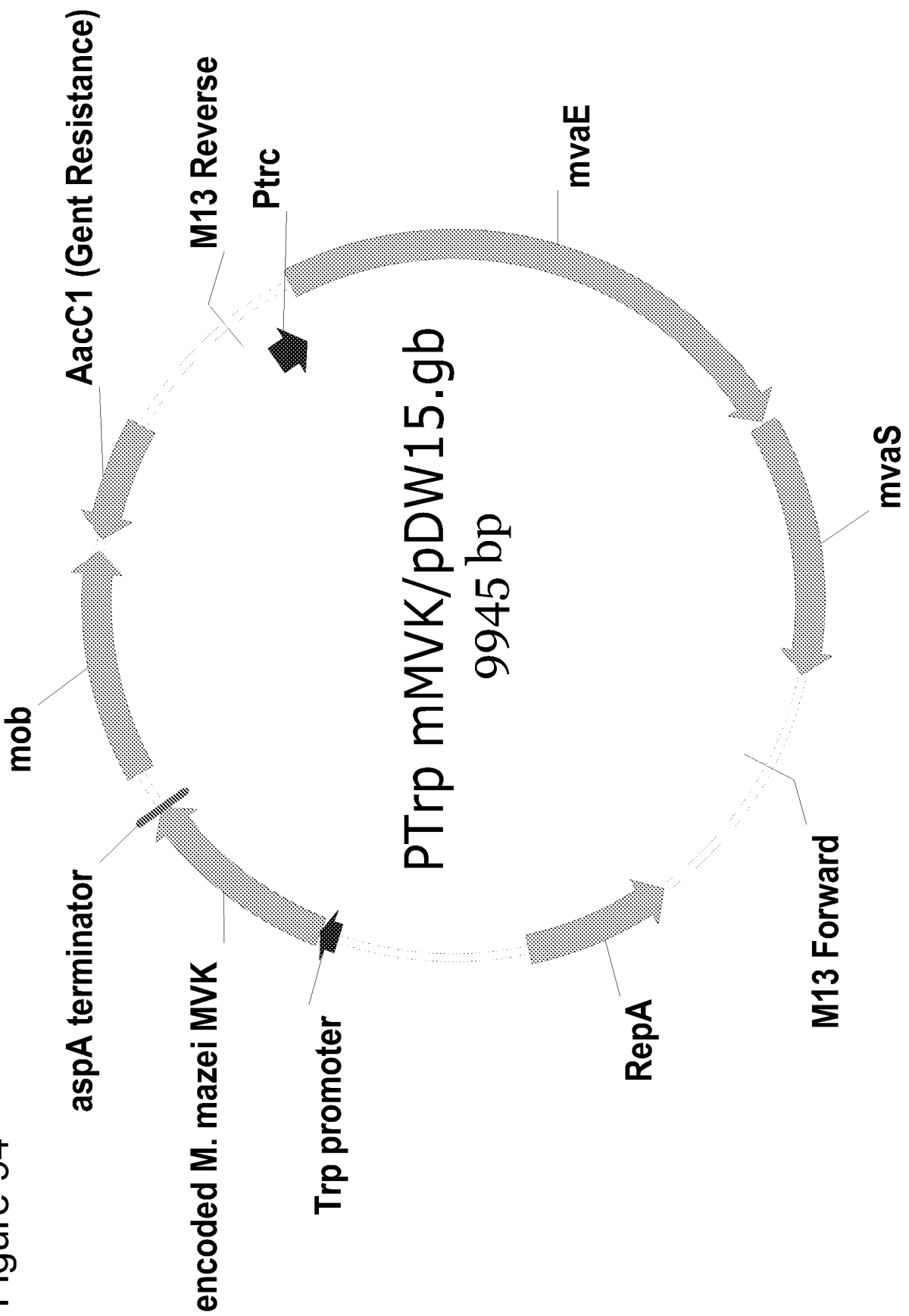

PTrp mMVK/pDW15.gb
9945 bp
AacC1 (Gent Resistance)
M13 Reverse
Ptrc
mvaE
mvaS
M13 Forward
RepA
Trp promoter
encoded M. mazei MVK
aspA terminator
mob

Figure 94

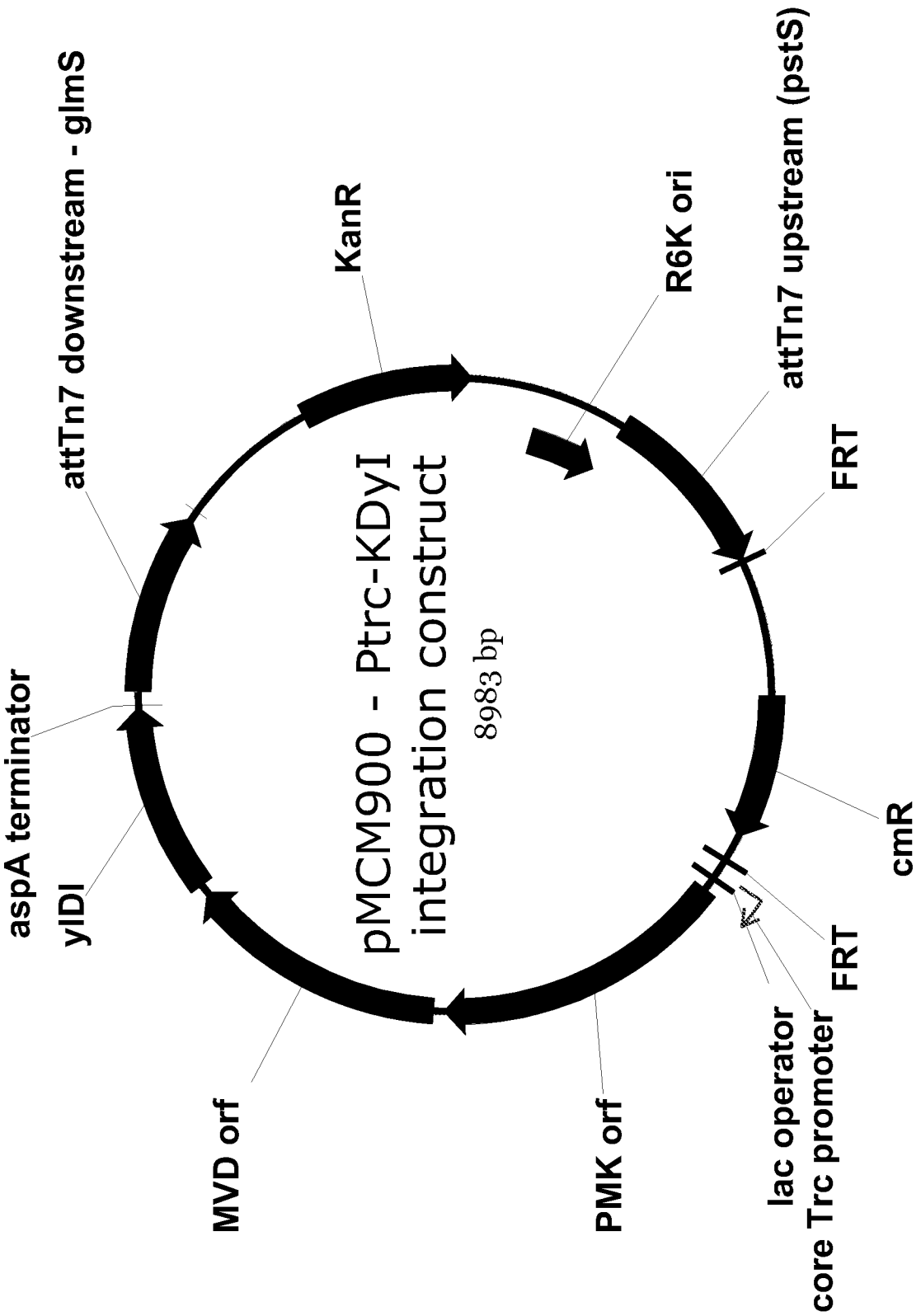

pMCM900 - Ptrc-KDyI
integration construct
8983 bp
attTn7 downstream - glmS
KanR
R6K ori
attTn7 upstream (pstS)
FRT
cmR
FRT
lac operator
core Trc promoter
PMK orf
MVD orf
yIDI
aspA terminator

Figure 95

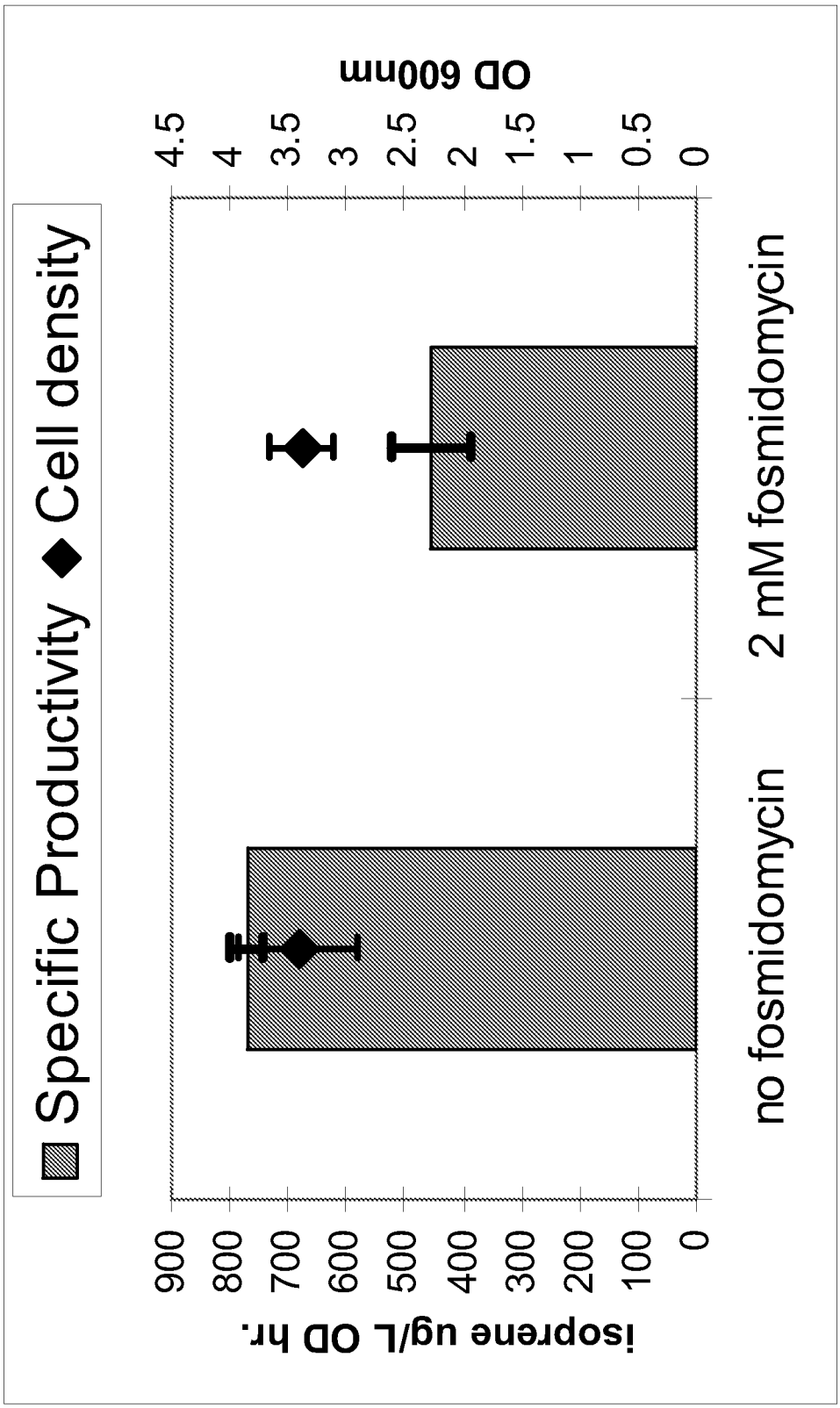

Specific Productivity
Cell density
isoprene ug/L OD hr.
OD 600nm
0
100
200
300
400
500
600
700
800
900
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
no fosmidomycin
2 mM fosmidomycin

Figure 96

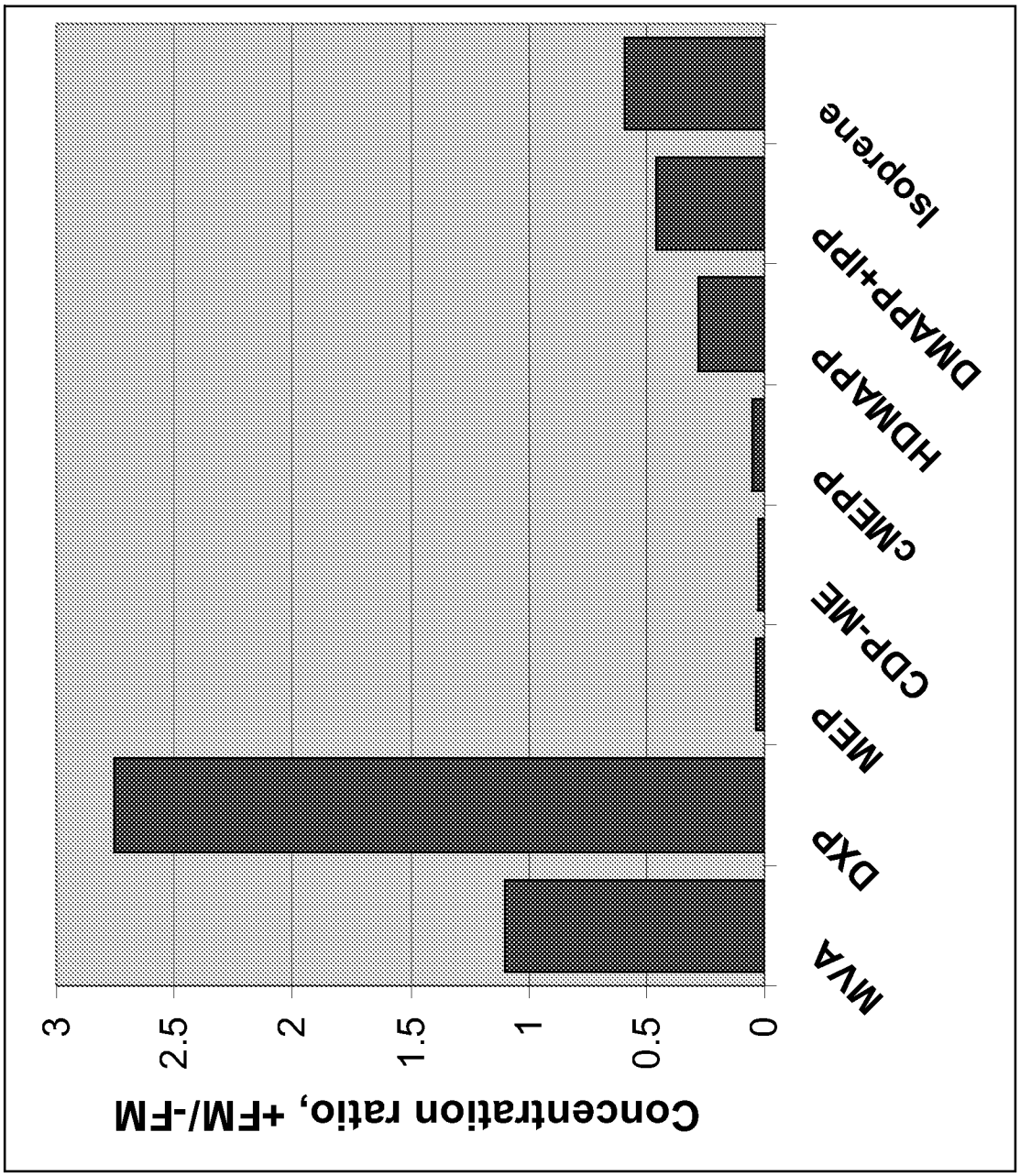
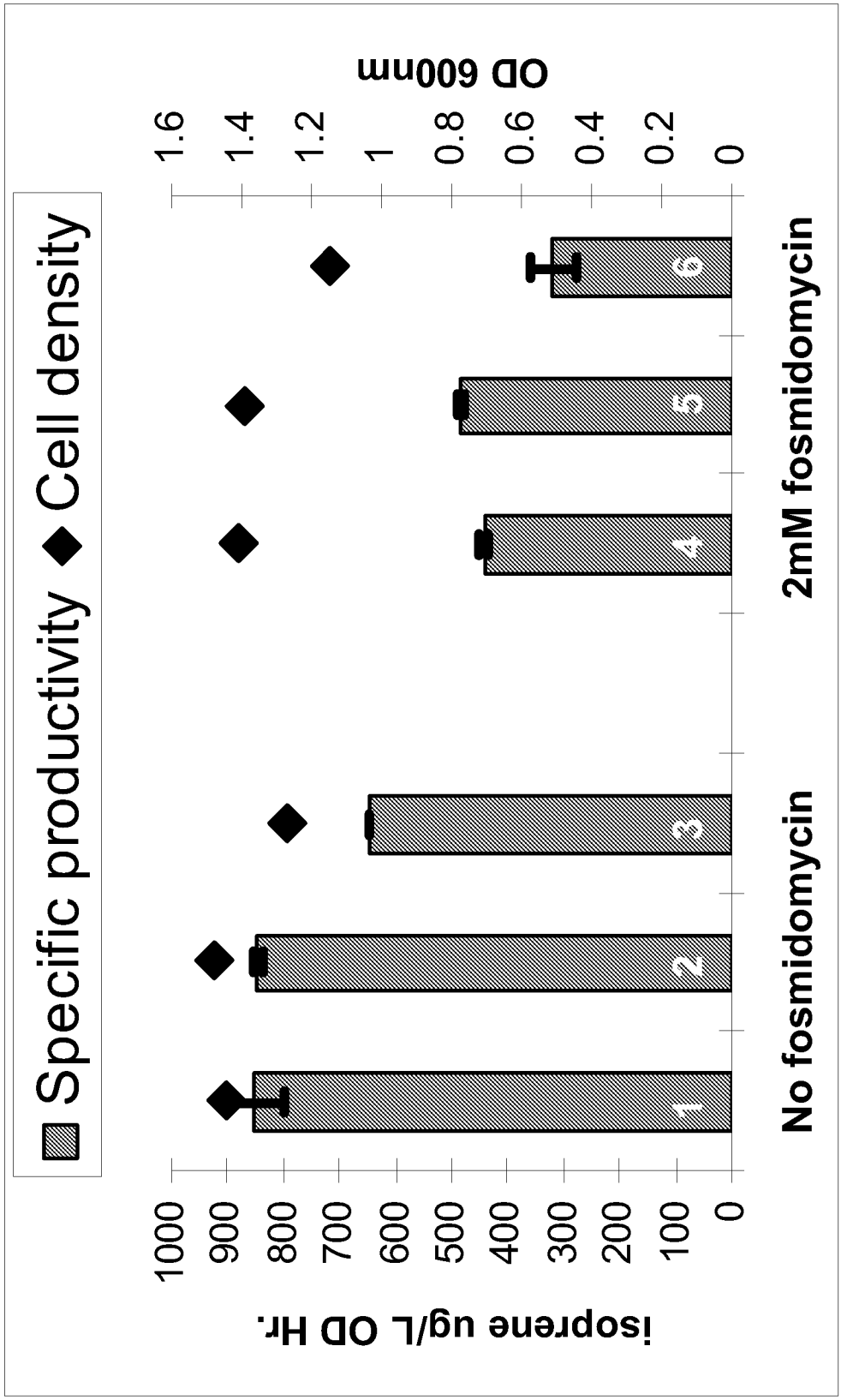

Concentration ratio, +FM/-FM
0
0.5
1
1.5
2
2.5
3
MVA
DXP
MEP
CDP-ME
CMEPP
HDMAPP
DMAPP+IPP
isoprene

Figure 97

Figure 98


Specific productivity
Cell density
isoprene ug/L OD Hr.
OD 600nm
0
100
200
300
400
500
600
700
800
900
1000
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1
2
3
4
5
6
No fosmidomycin
2mM fosmidomycin

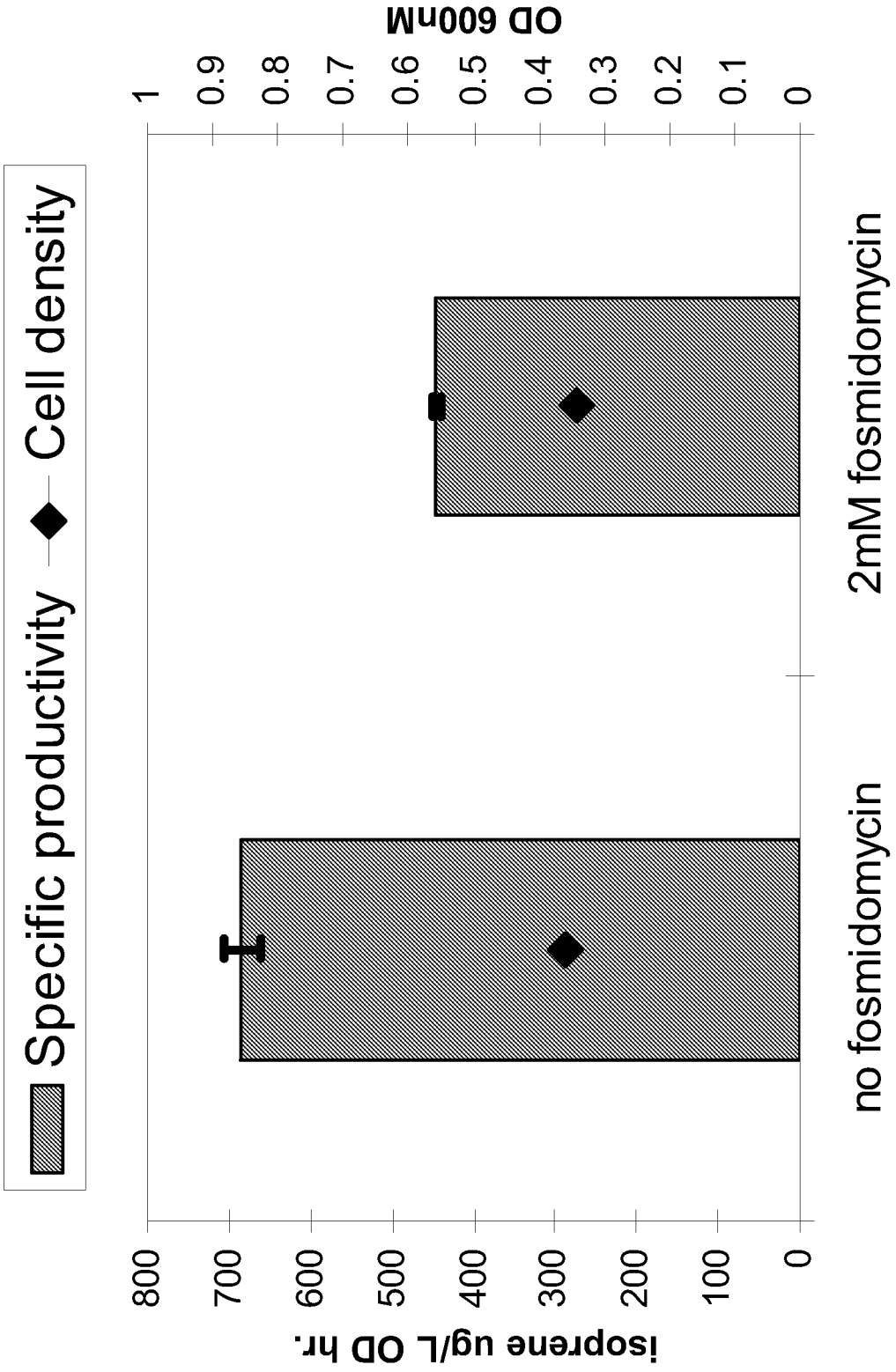

Specific productivity
Cell density
isoprene ug/L OD hr.
OD 600nM
0
100
200
300
400
500
600
700
800
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
no fosmidomycin
2mM fosmidomycin

Figure 99

Figure 100

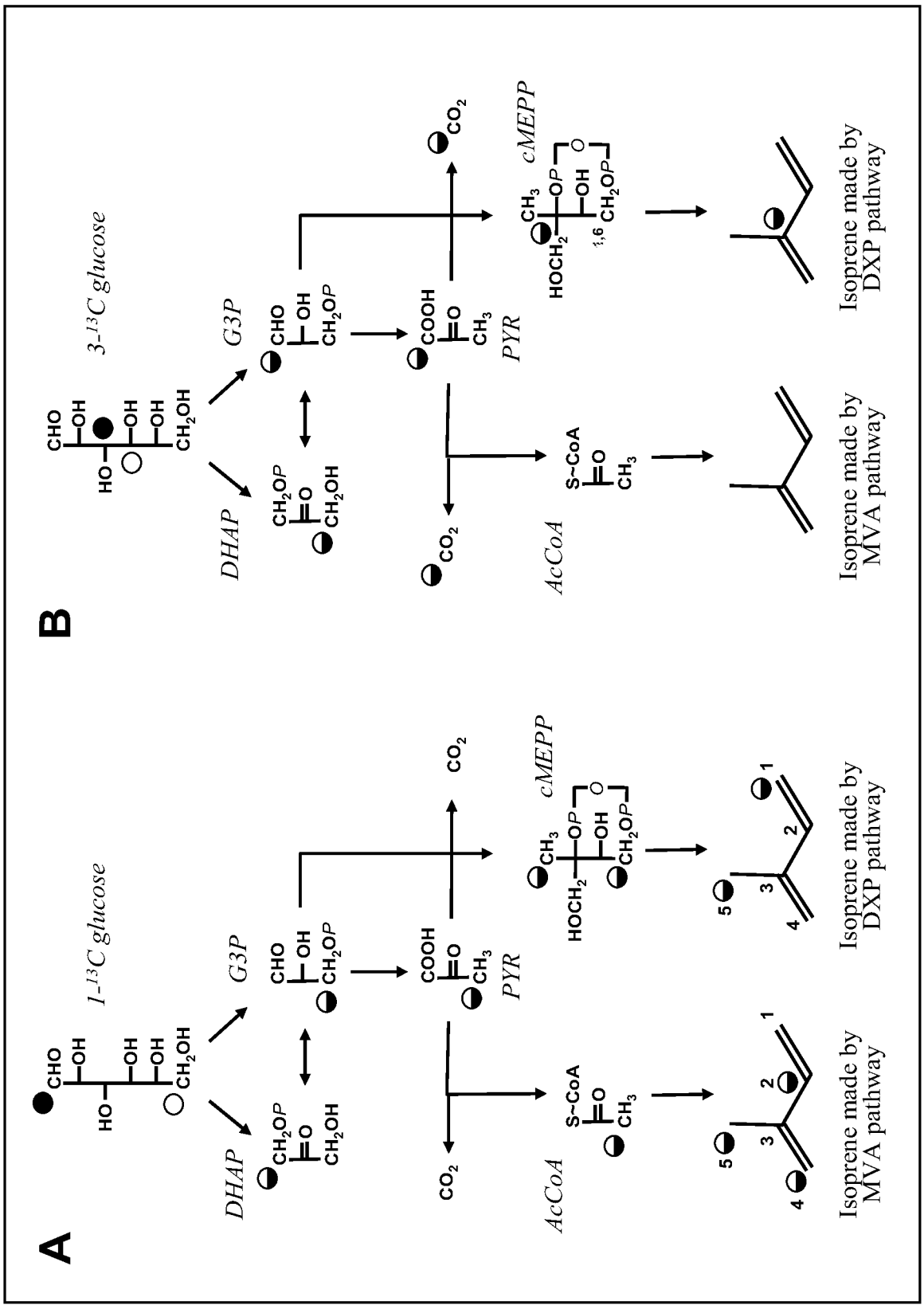

A
1-13C glucose
DHAP
G3P
PYR
AcCoA
cMEPP
Isoprene made by MVA pathway
Isoprene made by DXP pathway
B
3-13C glucose
DHAP
G3P
PYR
AcCoA
cMEPP
Isoprene made by MVA pathway
Isoprene made by DXP pathway

Figure 101 panel A

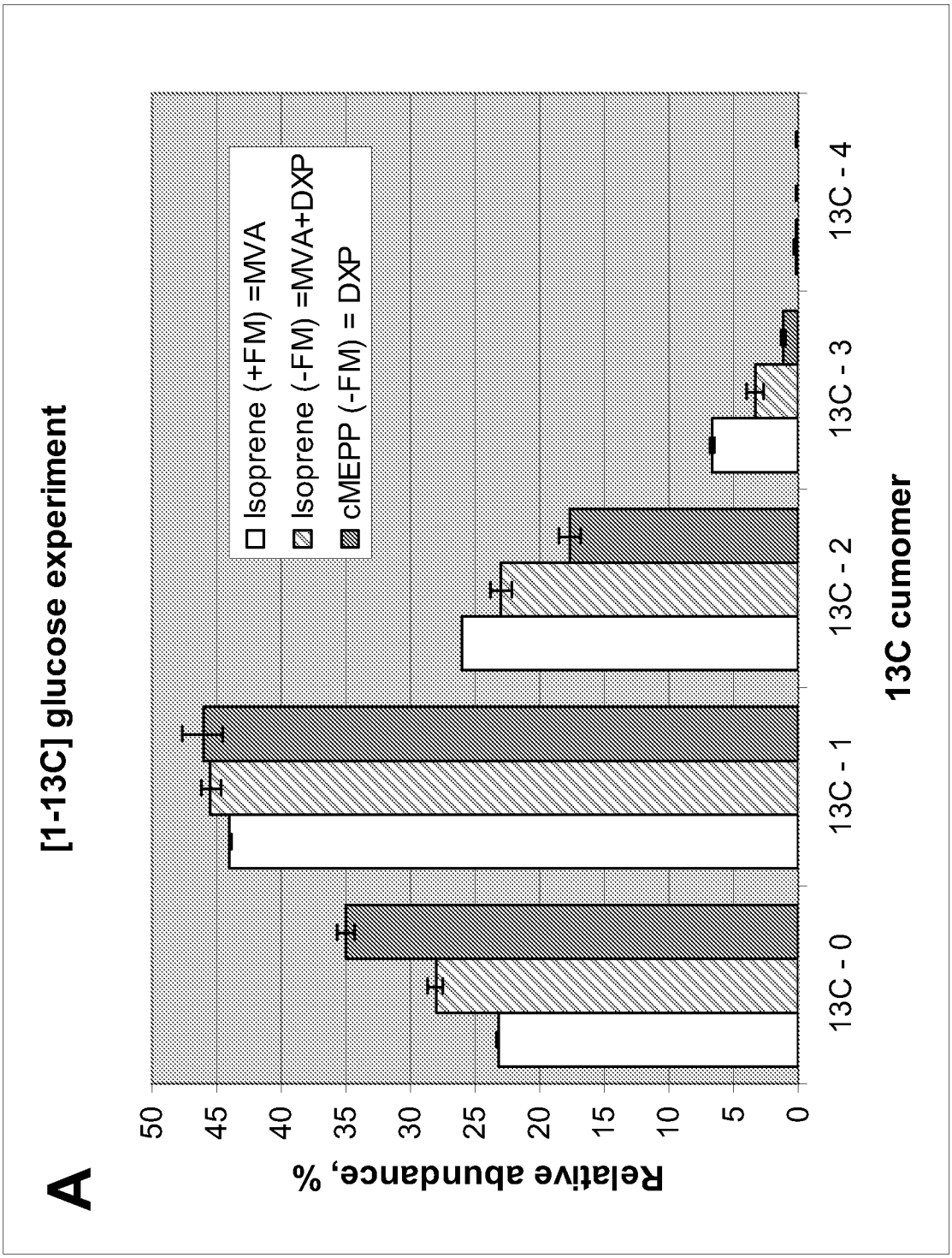


Figure 101 panel B

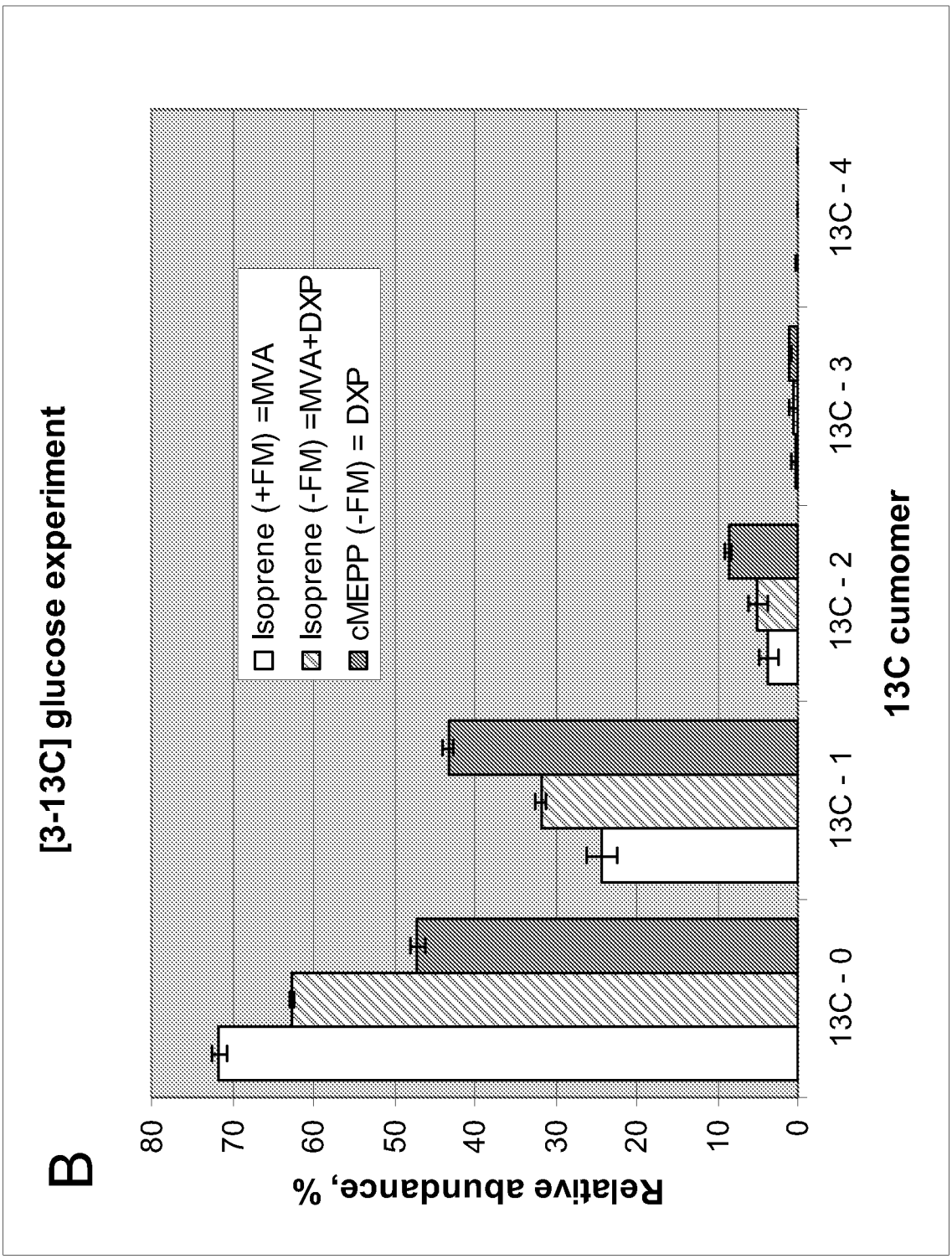


Figure 102

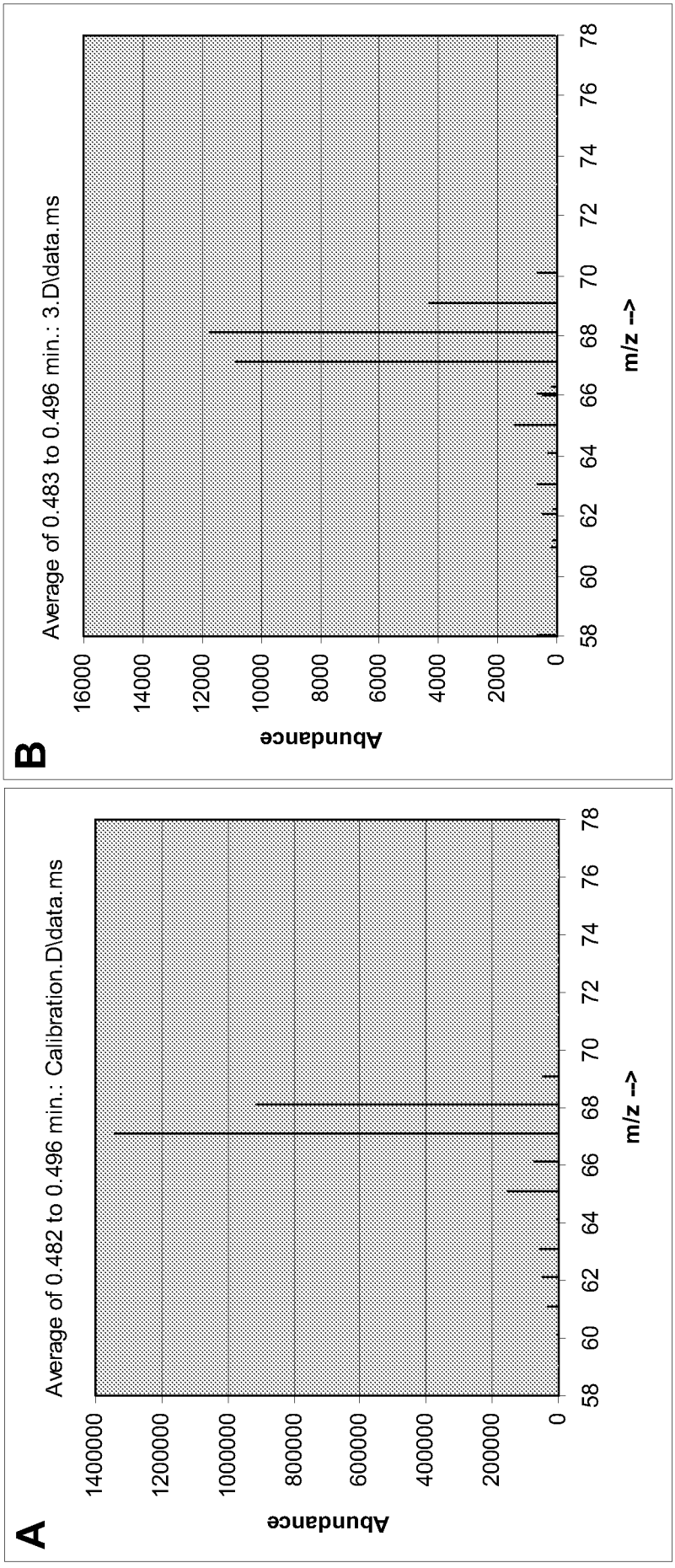

A
Average of 0.482 to 0.496 min.: Calibration.D\data.ms
Abundance
m/z -->
B
Average of 0.483 to 0.496 min.: 3.D\data.ms
Abundance
m/z -->

Figure 103

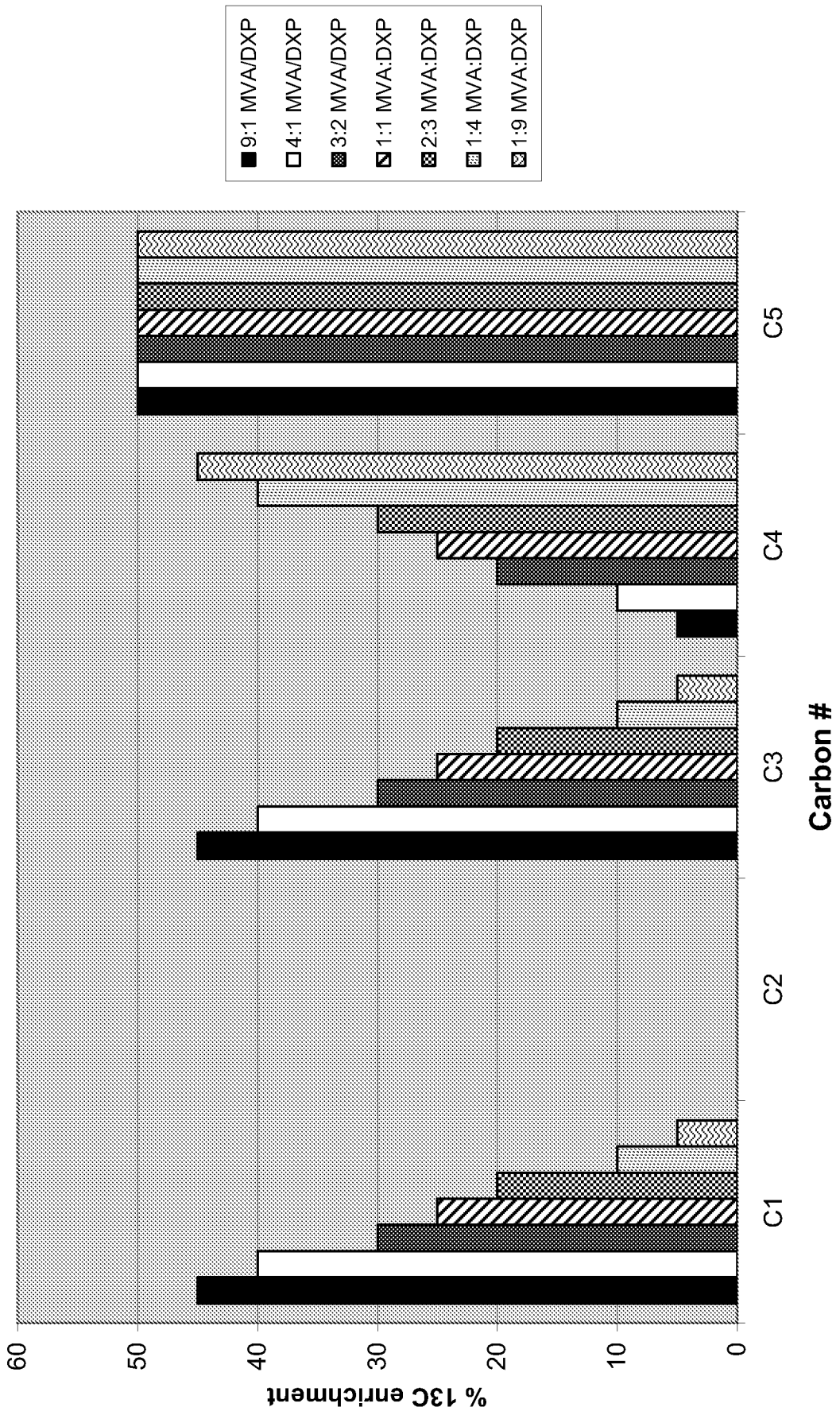

Isoprene ^{13}C Isotope enrichment vs. MVA/DXP ratio
9:1 MVA/DXP
4:1 MVA/DXP
3:2 MVA/DXP
1:1 MVA:DXP
2:3 MVA:DXP
1:4 MVA:DXP
1:9 MVA:DXP
% 13C enrichment
0
10
20
30
40
50
60
C1
C2
C3
C4
C5
Carbon #

Figure 104

Step 1: Grow strain on 1-^{13}C-glucose and collect Biolsoprene on small carbon filter.

Step 2: Desorb Biolsoprene with $CDCl_3$ into an NMR tube and record ^{13}C NMR spectrum

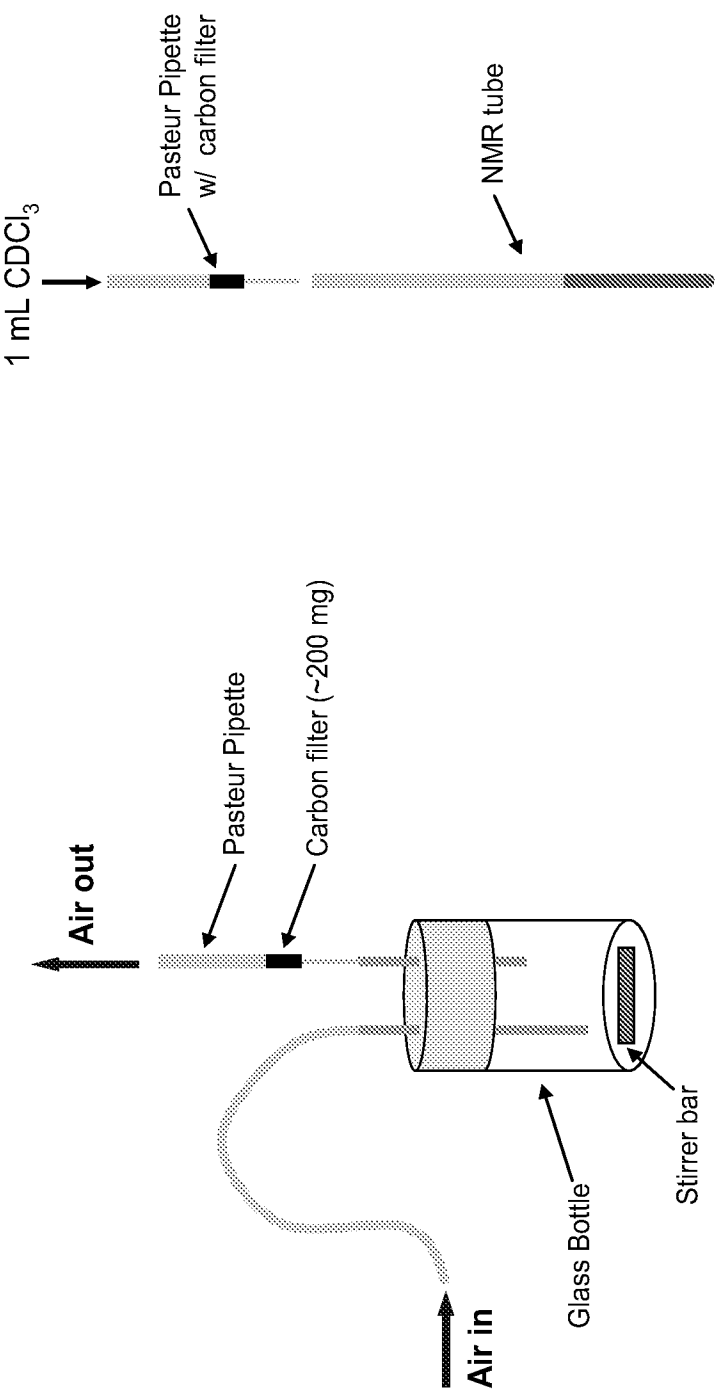


Figure 105

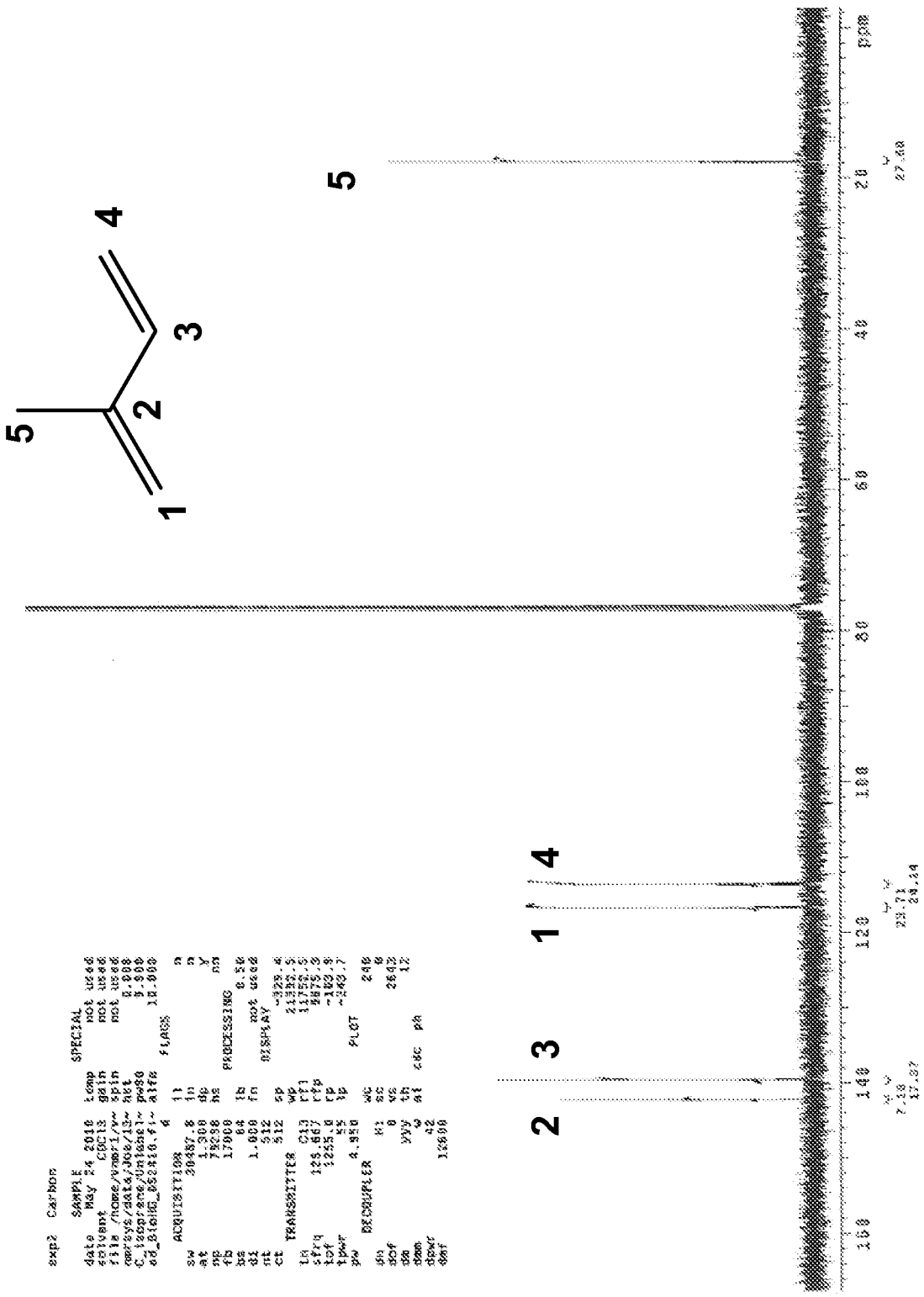


Figure 106

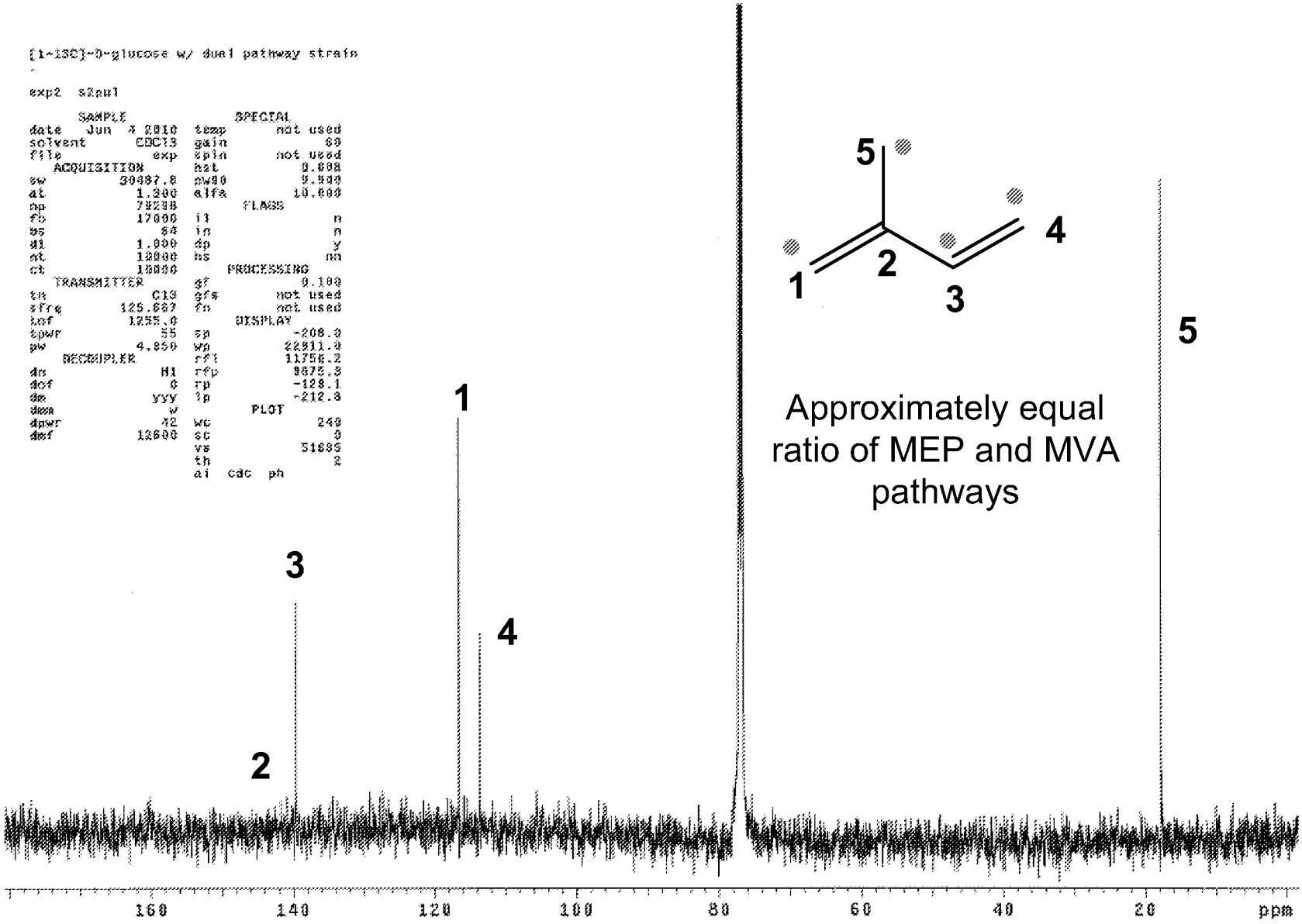
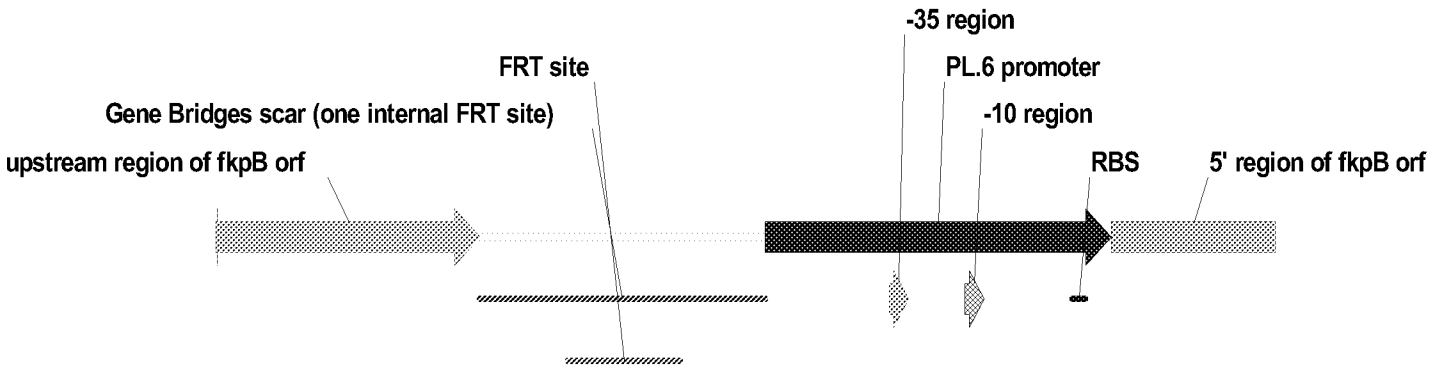

[1-13C]-D-glucose w/ dual pathway strain
exp2 s2pul
SAMPLE
date Jun * 2010
solvent CDCl3
file exp
ACQUISITION
sw 30487.8
at 1.200
np 73238
fb 17000
bs 64
d1 1.000
nt 12000
ct 12000
TRANSMITTER
tn C13
sfrq 125.687
tof 1255.0
tpwr 55
pw 4.850
RECEIVER
dr H1
dof 0
dm yyy
dmm w
dpwr 42
def 12600
SPECIAL
temp not used
gain 60
spin not used
hzt 0.008
pw90 9.300
alfa 10.000
FLAGS
il n
in n
dp y
hs nn
PROCESSING
gf 0.100
gfs not used
fn not used
DISPLAY
sp -208.0
wp 22811.8
rf1 11756.2
rfp 5875.8
rp -128.1
lp -212.8
PLOT
wc 240
sc 0
vs 51885
th 2
ai cdc ph
1
2
3
4
5
Approximately equal ratio of MEP and MVA pathways
160
140
120
100
80
60
40
20
ppm

Figure 107

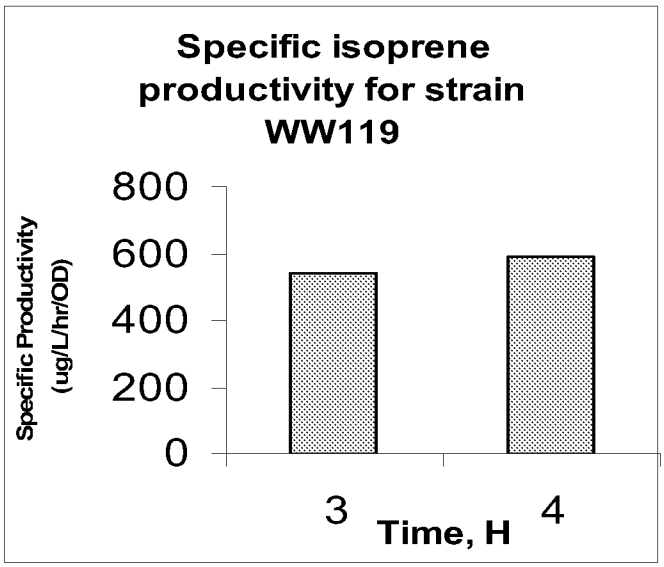


PL.6 fkpB locus

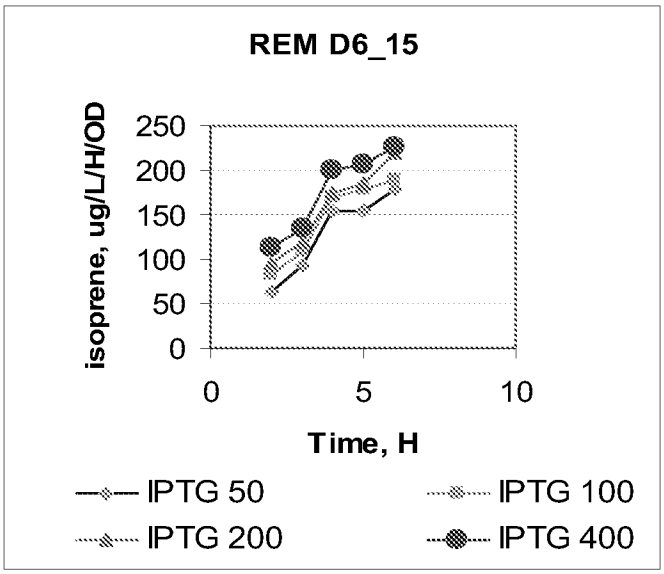
323 bp

agattgctgcgaaatcgtaggccggataaggcgtttacgccgcatccggcaaaaaatccttaaatataa
gagcaaacctgc**aattaaccctcactaaagggcggccgcgaagttcctattctctagaaagtat**
aggaacttcctcgagccctatagtgagtcgtattaagataaccatctgcggtgataaattatctctgg
cggtgttgacataaataccactggcggtgatactgagcacatcagcaggacgcactgcaaaggagg
taaaaacatgtctgaatctgtacagagcaatagcgccgtcctggtgcacttcacgct

A



B



C

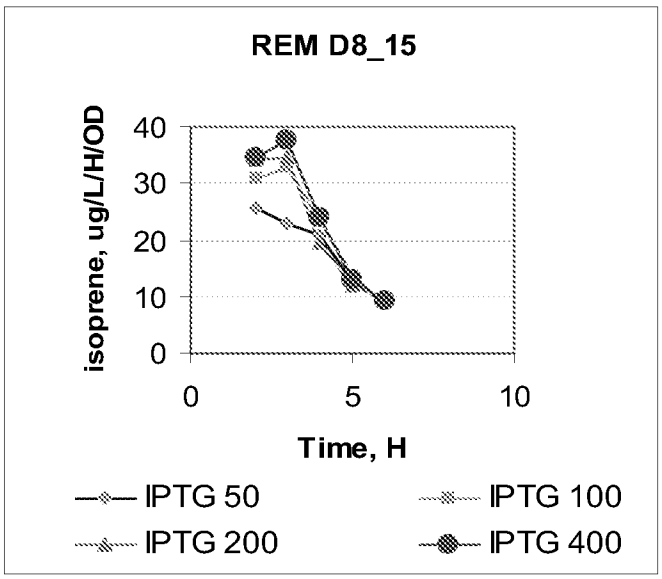


Figure 108

Figure 108 (con't)

D

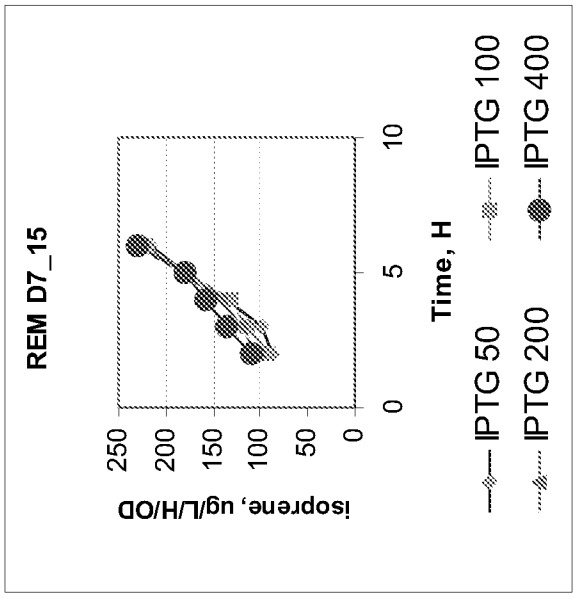


Figure 109

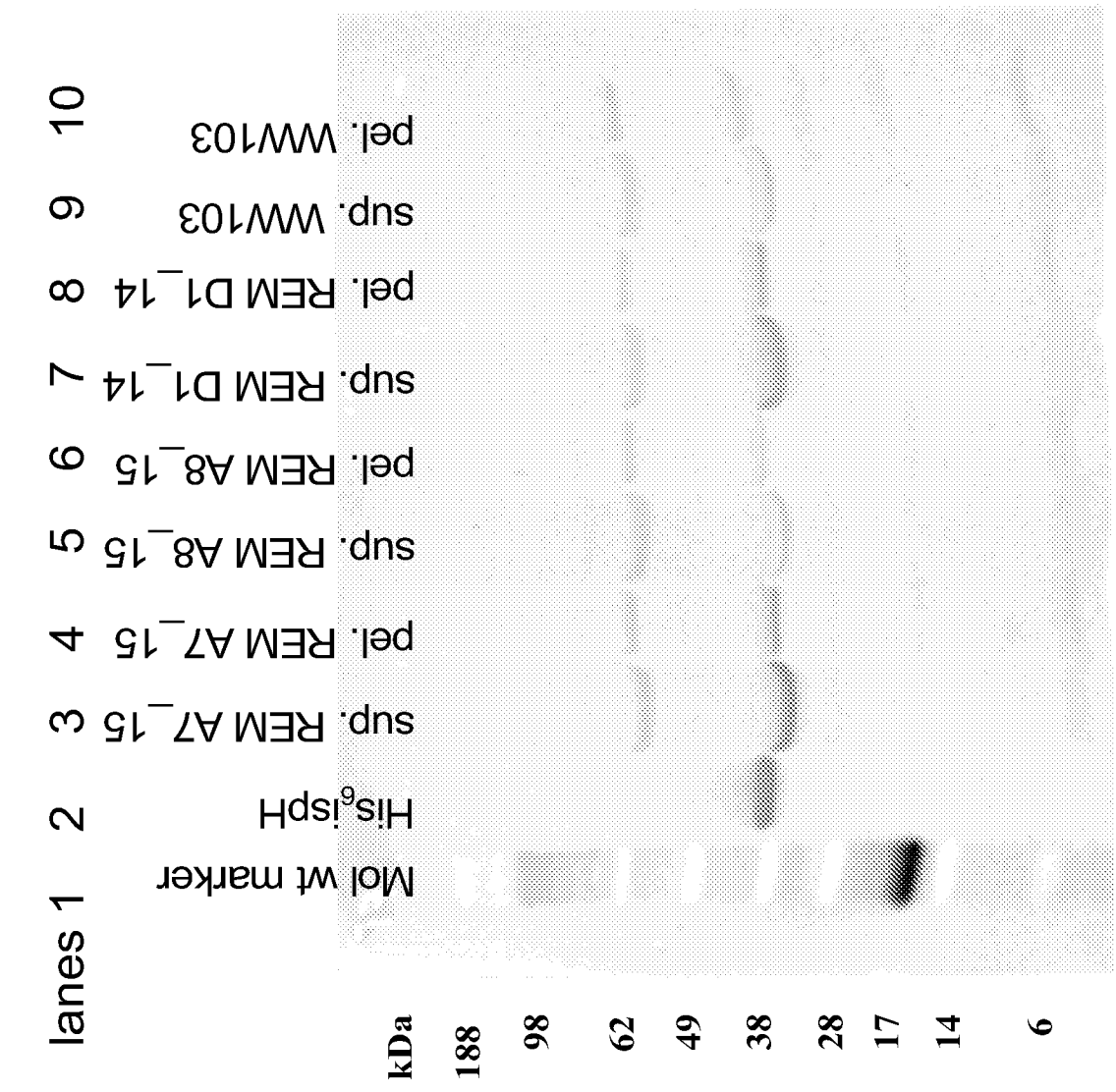
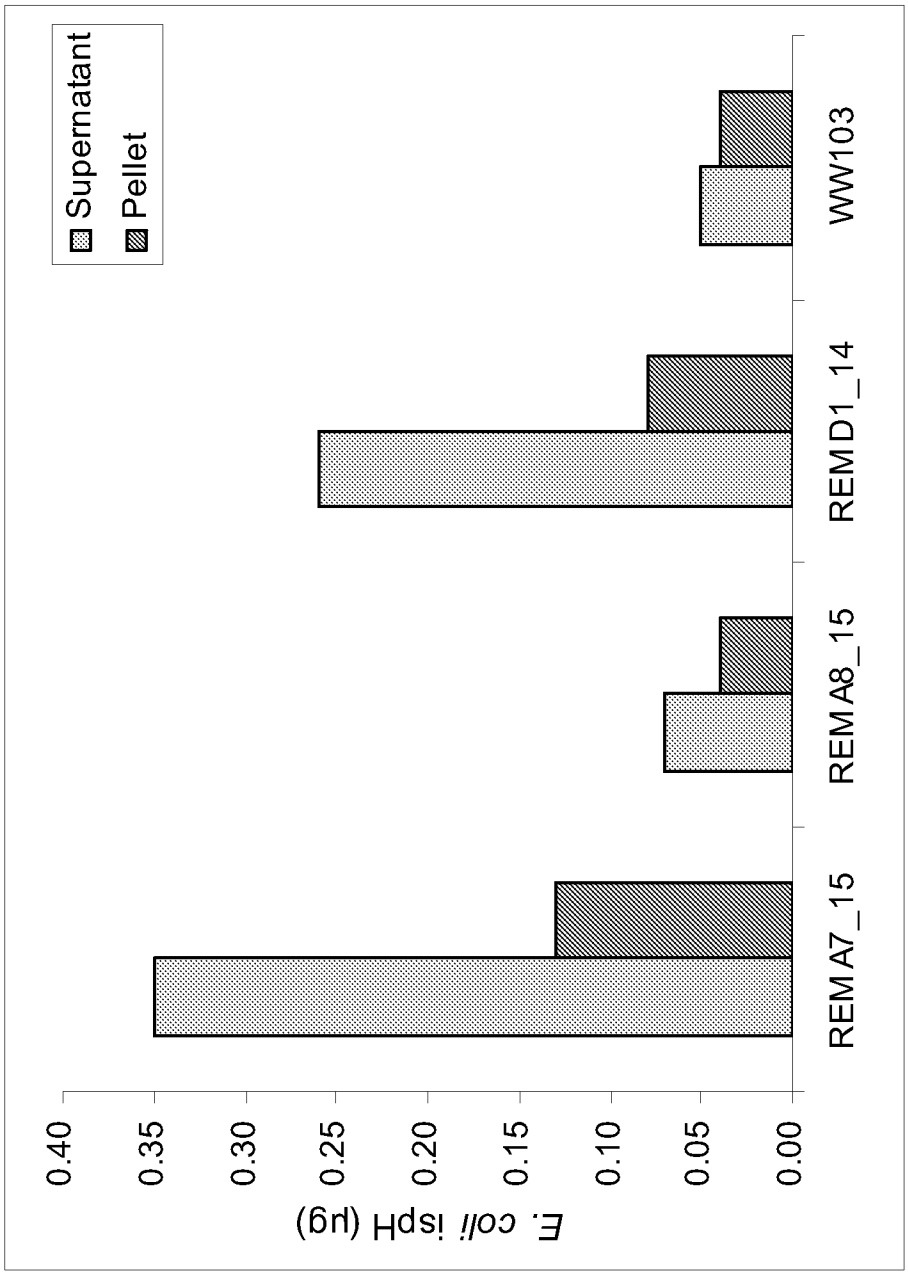

lanes 1
Mol wt marker
2
His6IspH
3
sup. REM A7_15
4
pel. REM A7_15
5
sup. REM A8_15
6
pel. REM A8_15
7
sup. REM D1_14
8
pel. REM D1_14
9
sup. WW103
10
pel. WW103
kDa
188
98
62
49
38
28
17
14
6

Figure 110


Supernatant
Pellet
E. coli ispH (µg)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
REMA7_15
REMA8_15
REMD1_14
WW103

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/38904

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 5/02 (2010.01)
USPC - 435/167
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 435/167

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/252.3, 435/69.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Databases: PubWEST: DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=NO; OP=ADJ; Google Patents; Google Scholar
Search terms used: DXP, MVA, pathway, isoprene synthase, DXS, DXR, MCT, CMK, MCS, HDR, IDI, AA-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, MVK, MVD, PMK, PMDC, IPK, Beck, McAuliffe, Miller

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JULSING et al., Functional analysis of genes involved in the biosynthesis of isoprene in Bacillus subtilis. Appl Microbiol Biotechnol, July 2007, Vol 75, No 6, pp 1377-1384. esp: abstract, pg 1379 first column second line-fourth line, pg 1383 last seven lines of the second column, pg 1383 second column, first paragraph,pg 1380-1381 section entitled "Results", Fig. 1, Fig. 2, Table I, Table 2, Table 3.	1-8
Y	MILLER et al., *First isolation of an isoprene synthase gene from poplar and successful* expression of the gene in Escherichia coli. Planta. July 2001, Vol 213, No 3, pp 483-7. abstract only.	1-8
A	WITHERS et al., Identification of Isopentenol Biosynthetic Genes from Bacillus subtilis by a Screening Method Based on Isoprenoid Precursor Toxicity. Appl Environ Microbiol, October 2007, Vol 73, No 19, pp 6277-6283.	1-8
A	WAGNER et al., Isoprene Biosynthesis in Bacillus subtilis via the Methylerythritol Phosphate Pathway. J. Nat. Prod., January 2000, Vol 63, No 1, pp 37-40.	1-8
A	SIVY et al., Isoprene synthase activity parallels fluctuations of isoprene release during growth of Bacillus subtilis. Biochem Biophys Res Commun. 31 May 2002, Vol 294, No 1, pp 71-5.	1-8
A	SASAKI et al., Gene expression and characterization of isoprene synthase from Populus alba. FEBS Lett, 25 April 2005, Vol 579, No 11, pp 2514-2518.	1-8
A	US 7129392 B2 (HAHN et al.) 31 October 2006 (31.10.2006) entire document.	1-8
A	US 6316695 B1 (HAN et al.) 13 November 2001 (13.11.2001) entire document.	1-8

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
28 August 2010 (28.08.2010)	09 SEP 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/38904

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6998471 B2 (HALLAHAN et al.) 14 February 2006 (14.02.2006) entire document.	1-8
Y, P	US 2010/0003716 A1 (CERVIN et al.) 7 January 2010 (07.01.2010) entire document.	1-8
Y, P	US 2010/0086978 A1 (BECK et al.) 8 April 2010 (08.04.2010) entire document.	1-8
A	US 2009/0137014 A1 (TSURUTA et al.) 28 May 2009 (28.05.2009) entire document.	1-8
A	US 2008/0274523 A1 (RENNINGER et al.) 6 November 2008 (06.11.2008) entire document.	1-8
A	KAJIWARA et al., Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in Escherichia coli. Biochem. J., 1 June 1997, Vol 324, Pt 2, pp 421-426.	1-8
A	MARTIN et al., Engineering a mevalonate pathway in Escherichia coli for production of terpenoids. Nature Biotechnology July 2003, Vol 21, No 7, pp 796 - 802.	1-8