(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 December 2010 (23.12.2010)

PCT

(10) International Publication Number
WO 2010/148256 A1

(51) International Patent Classification:
C07C 5/02 (2006.01)

(21) International Application Number:
PCT/US2010/039088

(22) International Filing Date:
17 June 2010 (17.06.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/187,959 17 June 2009 (17.06.2009) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

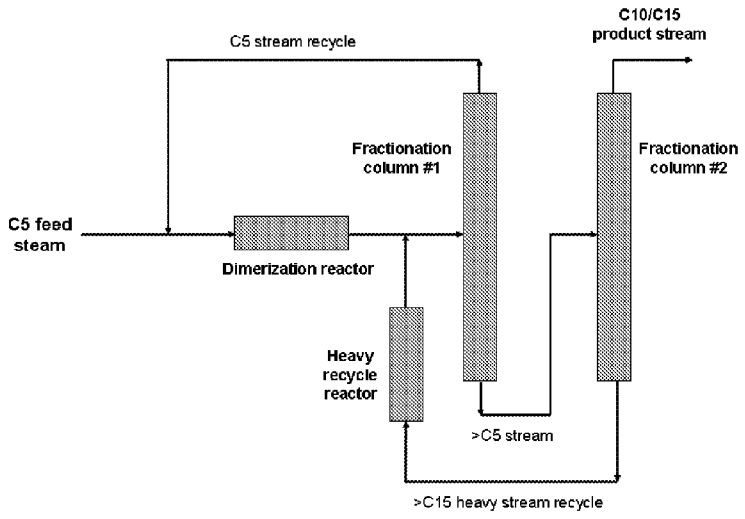
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: FUEL COMPOSITIONS COMPRISING ISOPRENE DERIVATIVES

Figure 173



(57) Abstract: The invention provides for methods, compositions and systems using bioisoprene derived from renewable carbon for production of a variety of hydrocarbon fuels and fuel additives.

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FUEL COMPOSITIONS COMPRISING ISOPRENE DERIVATIVES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/187,959, filed June 17, 2009, the disclosure of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The development of renewable transportation fuels is one of the key challenges of the twenty-first century. The current market is dominated by ethanol derived from yeast fermentation of sucrose and starch, and to a lesser extent by biodiesel (fatty acid esters) derived from triglycerides. Ethanol has limitations as a liquid fuel with a lower energy density relative to hydrocarbons. In addition, ethanol cannot be transported in conventional infrastructure due to its affinity for water and corrosive nature. Processes for the conversion of renewable carbon sources (biomass, sugars, oils) to hydrocarbon fuels offer an attractive alternative to bioethanol.

[0003] Isoprene (2-methyl-1,3-butadiene) is a key industrial chemical used primarily for the production of synthetic rubber. Currently isoprene is derived from petrochemical sources either directly by cracking of naphtha and other light petroleum fractions, or indirectly through chemical synthesis (See, for examples, H. Pommer and A. Nurrenbach, Industrial Synthesis of Terpene Compounds, *Pure Appl. Chem.*, **1975**, *43*, 527-551; H. M. Weitz and E. Loser, Isoprene, in *Ullmann's Encyclopedia of Industrial Chemistry*, Seventh Edition, Electronic Release, Wiley-VCH Verlag GMBH, Weinheim, **2005**; and H.M. Lybarger, Isoprene in *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed., Wiley, New York (1995), *14*, 934–952.) The resulting crude isoprene streams are typically subjected to extensive purification processes in order to remove numerous chemically similar impurities, many of which can interfere with subsequent transformation of isoprene to polymers and other chemicals.

[0004] In contrast, isoprene derived from biological sources contains very few hydrocarbon impurities and instead contains a number of oxygenated compounds such as ethanol, acetaldehyde and acetone. Many of these compounds can be easily removed by contact with water or passage through alumina or other adsorbents.

[0005] Industry relies on petrochemical feedstocks for isoprene production and extensive purification trains are needed before isoprene can be converted to polymers and other chemicals. Cost effective methods are desirable for converting biologically produced isoprene to valuable

chemical products taking advantage of the high purity and/or the unique impurity profiles of bioisoprene.

[0006] All patents, patent applications, documents, and articles cited herein are herein incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

[0007] Disclosed are methods and systems for producing fuel constituents from highly pure isoprene and fuel compositions produced from highly pure isoprene.

[0008] In one aspect, the invention provides a method for producing a fuel constituent from a bioisoprene composition comprising chemically transforming a substantial portion of the isoprene in the bioisoprene composition to non-isoprene compounds. In one embodiment, the bioisoprene composition is chemically transformed by subjecting the bioisoprene composition to heat or catalytic conditions suitable for isoprene dimerization to produce an isoprene dimer and then catalytically hydrogenating the isoprene dimer to form a saturated C10 fuel constituent. In another embodiment, the bioisoprene composition is chemically transformed by (i) partially hydrogenating the bioisoprene composition to produce an isoamylene, (ii) dimerizing the isoamylene with a mono-olefin selected from the group consisting of isoamylene, propylene and isobutene to form a dimate and (iii) completely hydrogenating the dimate to produce a fuel constituent. In some embodiments, at least about 95% of isoprene in the bioisoprene composition is converted to non-isoprene compounds during the chemical transformation. In some embodiments, the bioisoprene composition is heated to about 150 °C to about 250 °C to produce an unsaturated cyclic isoprene dimer and the unsaturated cyclic isoprene dimer is hydrogenated catalytically to produce a saturated cyclic isoprene dimer fuel constituent. In some embodiments, the method comprises: (i) contacting the bioisoprene composition with a catalyst for catalyzing cyclo-dimerization of isoprene to produce an unsaturated cyclic isoprene dimer and the unsaturated cyclic isoprene dimer is hydrogenated catalytically to produce a saturated cyclic isoprene dimer fuel constituent. In some embodiments, the catalyst for catalyzing cyclo-dimerization of isoprene comprising a catalyst selected from the group consisting of a nickel catalyst, iron catalysts and chromium catalysts. In some embodiments, the step of partially hydrogenating the bioisoprene composition comprises contacting the bioisoprene composition with hydrogen gas and a catalyst for catalyzing partial hydrogenation of isoprene. In some embodiments, the catalyst for catalyzing partial hydrogenation of isoprene comprises a palladium catalyst. In some embodiments, the step of dimerizing the isoamylene with a mono-

olefin comprises contacting the isoamylene with the mono-olefin in the presence of a catalyst for catalyzing dimerization of mono-olefin. In some embodiments, the catalyst for catalyzing dimerization of mono-olefin comprises an acid catalyst. In some embodiments, the method further comprises purifying the isoprene from the bioisoprene composition prior to chemically transforming the bioisoprene composition to a fuel constituent.

[0009] In one aspect, the invention provides a system for producing a fuel constituent from a bioisoprene composition, wherein a substantial portion of the isoprene in the bioisoprene composition is chemically converted to non-isoprene compounds, the system comprising a bioisoprene composition and (a) (i) one or more chemicals capable of dimerizing isoprene in the bioisoprene composition or a source of heat capable of dimerizing isoprene in the bioisoprene composition; and (ii) a catalyst capable of hydrogenating the isoprene dimer to form a saturated C10 fuel constituent; or (b) (i) a chemical capable of partially hydrogenating isoprene in the bioisoprene composition to produce an isoamylene, (ii) a chemical capable of dimerizing the isoamylene with mono-olefins selected from the group consisting of isoamylene, propylene and isobutene to form a dimate and (iii) a chemical capable of completely hydrogenating the dimate to produce a fuel constituent.

[0010] In some embodiments of the system, the bioisoprene composition comprising greater than about 2 mg of isoprene and comprising greater than or about 99.94% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments of the system, the one or more chemicals capable of dimerizing isoprene comprises catalyst for catalyzing cyclo-dimerization of isoprene comprising a catalyst selected from the group consisting of ruthenium catalysts, nickel catalysts, iron catalysts and chromium catalysts. In some embodiments of the system, the catalyst for hydrogenating the unsaturated isoprene dimers comprises a catalyst selected from the group consisting of palladium catalysts, nickel catalysts, ruthenium catalysts and rhodium catalysts. In some embodiments of the system, the chemical capable of partially hydrogenating isoprene comprises a palladium catalyst. In some embodiments of the system, the chemical capable of dimerizing the isoamylene with mono-olefins comprises an acid catalyst.

[0011] In one aspect, the invention provides a fuel composition comprising a fuel constituent produced by the methods described herein. In some embodiments, the fuel composition is substantially free of isoprene. In some embodiments, the fuel composition has $\delta^{13}C$ value which is greater than -22‰ or within the range of -32‰ to -24‰.

[0012] In some aspects, the invention provides a system for producing a fuel constituent from isoprene comprising: (a) a commercially beneficial amount of highly pure isoprene; and (b) a fuel constituent produced from at least a portion of the highly pure isoprene; wherein at least a portion of the commercially beneficial amount of highly pure isoprene undergoes a chemical transformation.

[0013] In some embodiments of the system, the commercially beneficial amount of highly pure isoprene comprises greater than about 2 mg of isoprene and comprising greater than or about 99.94% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the commercially beneficial amount of highly pure isoprene comprises greater than about 2 mg of isoprene and comprising one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms. In some embodiments, the commercially beneficial amount of highly pure isoprene comprises greater than about 2 mg of isoprene and comprising one or more second compounds selected from the group consisting of ethanol, acetone, methanol, acetaldehyde, methacrolein, methyl vinyl ketone, 2-methyl-2-vinyloxirane, *cis*- and *trans*-3-methyl-1,3-pentadiene, a C5 prenyl alcohol, 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine; wherein the amount of the second compound relative to the amount of the isoprene is greater than or about 0.01 % (w/w). In some embodiments, the commercially beneficial amount of highly pure isoprene comprises greater than about 2 mg of isoprene and comprising less than or about 0.5 μg/L per compound for any compound in the composition that inhibits the polymerization of isoprene. In some preferred embodiments, the commercially beneficial amount of highly pure isoprene is produced by a biological process.

[0014] In some embodiments of the system, the fuel constituent comprises one or more compounds selected from the group consisting of cyclic isoprene dimers and trimers, linear isoprene oligomers, aromatic and alicyclic isoprene derivatives, and oxygenated isoprene

derivatives. In some embodiments, the oxygenated isoprene derivatives are compounds selected from the group consisting of alcohols, ketones, esters and ethers derived from isoprene.

[0015] In some embodiments, the fuel constituent comprises cyclic isoprene dimers and the chemical transformation comprises a dimerization reaction of isoprene. In some embodiments, the dimerization reaction is carried out by heating the commercially beneficial amount of highly pure isoprene. In some embodiments, the dimerization reaction of isoprene produces a product comprising unsaturated isoprene dimers and the chemical transformation further comprises a hydrogenation reaction of the unsaturated isoprene dimers. In some embodiments, the system further comprises a catalyst for catalyzing the hydrogenation reaction of the unsaturated isoprene dimers. In some embodiments, the catalyst for catalyzing the hydrogenation reaction of the unsaturated isoprene dimers comprising a catalyst selected from the group consisting of palladium catalysts, nickel catalysts, ruthenium catalysts and rhodium catalysts.

[0016] In some embodiments, the dimerization reaction is carried out by contacting the commercially beneficial amount of highly pure isoprene with a catalyst for catalyzing cyclo-dimerization of isoprene. In some embodiments, the catalyst for catalyzing cyclo-dimerization of isoprene comprising a catalyst selected from the group consisting of ruthenium catalysts, nickel catalysts, iron catalysts and chromium catalysts. In some embodiments, the catalyst for catalyzing cyclo-dimerization of isoprene is a nickel catalyst and the fuel constituent comprising one or more eight-membered ring dimers of isoprene.

[0017] In some embodiments, the fuel constituent comprises linear and/or cyclic trimers of isoprene and the chemical transformation comprises catalytic trimerization of isoprene.

[0018] In one aspect, the invention provides a method for producing a fuel constituent from isoprene comprising: (a) obtaining a commercially beneficial amount of highly pure isoprene; and (b) chemically transforming at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent. In some embodiments, the commercially beneficial amount of highly pure isoprene comprises bioisoprene.

[0019] In some embodiments, the commercially beneficial amount of highly pure isoprene is obtained by the steps comprising: (i) culturing cells comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide under suitable culture conditions for the production of isoprene, wherein the cells (1) produce greater than about 400 nmole/g_{wcm}/hr of isoprene, (2) convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, or (3) have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr of isoprene, and (ii) producing isoprene. In some embodiments, the

cells further comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide or an MVA pathway polypeptide.

[0020] In some embodiments of the methods described herein, chemically transforming at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent comprises: (i) heating the commercially beneficial amount of highly pure isoprene to about 150 °C to about 250 °C; (ii) converting at least a portion of the commercially beneficial amount of highly pure isoprene to unsaturated cyclic isoprene dimers; (iii) hydrogenating the unsaturated cyclic isoprene dimers to produce saturated cyclic isoprene dimers; and (iv) producing the fuel constituent. In some embodiments, at least about 20% to about 100% of isoprene in the commercially beneficial amount of highly pure isoprene is converted to unsaturated cyclic isoprene dimers.

[0021] In some embodiments of the method, chemically transforming at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent comprises: (i) contacting the commercially beneficial amount of highly pure isoprene with a catalyst for catalyzing cyclo-dimerization of isoprene, (ii) converting at least a portion of the commercially beneficial amount of highly pure isoprene to cyclic isoprene dimers; and (iii) producing the fuel constituent.

[0022] In some embodiments of the method, chemically transforming at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent comprises: (i) contacting the commercially beneficial amount of highly pure isoprene with a catalyst for catalyzing cyclo-dimerization of isoprene, (ii) converting at least a portion of the commercially beneficial amount of highly pure isoprene to unsaturated cyclic isoprene dimers; (iii) hydrogenating the unsaturated cyclic isoprene dimers to produce saturated cyclic isoprene dimers; and (iv) producing the fuel constituent. In some embodiments of the method, the catalyst for catalyzing cyclo-dimerization of isoprene comprising a catalyst selected from the group consisting of a nickel catalyst, iron catalysts and chromium catalysts.

[0023] In some embodiments of the method, chemically transforming at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent comprises: (i) contacting the highly pure isoprene composition with a catalyst system; (ii) converting at least a portion of the starting isoprene composition to unsaturated isoprene dimers and/or trimers; and (iii) hydrogenating the unsaturated dimers and/or trimers to produce saturated C10 and/or C15 hydrocarbons.

[0024] In some embodiments, the method for producing a fuel constituent from isoprene comprises: (a) obtaining a commercially beneficial amount of any of the highly pure isoprene starting composition described herein; (b) converting at least a portion of the starting isoprene composition to oxygenated isoprene derivatives; and optionally (c) hydrogenating any unsaturated oxygenated isoprene derivatives to produce saturated oxygenates. In some embodiments, the oxygenated isoprene derivatives are compounds selected from the group consisting of alcohols, ketones, esters and ethers derived from isoprene.

[0025] In some embodiments, any of the methods described herein further comprises purifying the commercially beneficial amount of highly pure isoprene prior to chemically transforming at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent.

[0026] In one aspect, provided is a continuous process for producing a fuel constituent from isoprene comprising: (a) continuously producing a commercially beneficial amount of highly pure isoprene; and (b) continuously transforming chemically at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent. In some embodiments, the commercially beneficial amount of highly pure isoprene comprising a gas phase comprising isoprene. In some embodiments, the method further comprises passing the gas phase comprising isoprene to a reactor for chemically transforming at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent. In a preferred embodiment, the commercially beneficial amount of highly pure isoprene comprises bioisoprene.

[0027] Also provided is a fuel composition comprising a fuel constituent produced by any of the methods described herein. In some embodiments, the fuel constituent comprises less than or about 0.5 μg/L a product from a C5 hydrocarbon other than isoprene after undergoing the steps according to the methods described herein. In some embodiments, the fuel constituent comprises one or more product from one or more compound selected from the group consisting of ethanol, acetone, methanol, acetaldehyde, methacrolein, methyl vinyl ketone, 2-methyl-2-vinyloxirane, *cis*- and *trans*-3-methyl-1,3-pentadiene, a C5 prenyl alcohol, 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl

acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene and 2,3-cycloheptenolpyridine after undergoing the steps according to the methods described herein.

[0028] In some embodiments, the fuel composition comprises a fuel composition having $\delta^{13}C$ value which is greater than -22‰. In some embodiments, the fuel composition has $\delta^{13}C$ value which is within the range of -22‰ to -10‰ or -34‰ to -24‰. In some embodiments, the fuel composition has f_M value which is greater than 0.9. Also provided is a blend of any of the fuel compositions described herein with a petroleum based fuel in the amount of from about 1% to about 95% by weight or volume, based on the total weight or volume of the total fuel composition.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] 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.

[0030] Figure 2 is a map of pTrcKudzu.

[0031] Figures 3A-C are the nucleotide sequence of pTrcKudzu (SEQ ID NO:2). The RBS is underlined, the kudzu isoprene synthase start codon is in bold capitol letters and the stop codon is in bold, capital letters. The vector backbone is pTrcHis2B.

[0032] Figure 4 is a map of pETNHisKudzu.

[0033] Figures 5A-C are the nucleotide sequence of pETNHisKudzu (SEQ ID NO:3).

[0034] Figure 6 is a map of pCL-lac-Kudzu.

[0035] Figures 7A-C are the nucleotide sequence of pCL-lac-Kudzu (SEQ ID NO:4).

[0036] Figure 8A is a graph showing the production of isoprene in *E. coli* BL21 cells with no vector.

[0037] Figure 8B is a graph showing the production of isoprene in *E. coli* BL21 cells with pCL-lac-Kudzu

[0038] Figure 8C is a graph showing the production of isoprene in *E. coli* BL21 cells with pTrcKudzu.

[0039] Figure 8D is a graph showing the production of isoprene in *E. coli* BL21 cells with pETN-HisKudzu.

[0040] Figure 9A is a graph showing OD over time of fermentation of *E. coli* BL21/pTrcKudzu in a 14 liter fed batch fermentation.

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

[0042] 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}.

[0043] 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}.

[0044] 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}.

[0045] Figure 11 is a graph showing the production of isoprene in *Bacillus subtilis* expressing recombinant isoprene synthase. BG3594comK is a *B. subtilis* strain without plasmid (native isoprene production). CF443 is *B. subtilis* strain BG3594comK with pBSKudzu (recombinant isoprene production). IS on the y-axis indicates isoprene.

[0046] Figures 12A-C are the nucleotide sequence of pBS Kudzu #2 (SEQ ID NO:5).

[0047] Figure 13 is the nucleotide sequence of kudzu isoprene synthase codon-optimized for expression in *Yarrowia* (SEQ ID NO:6).

[0048] Figure 14 is a map of pTrex3g comprising a kudzu isoprene synthase gene codon-optimized for expression in *Yarrowia.*

[0049] Figures 15A-C are the nucleotide sequence of vector pSPZ1(MAP29Spb) (SEQ ID NO:7).

[0050] Figure 16 is the nucleotide sequence of the synthetic kudzu (*Pueraria montana*) isoprene gene codon-optimized for expression in *Yarrowia* (SEQ ID NO:8).

[0051] Figure 17 is the nucleotide sequence of the synthetic hybrid poplar (*Populus alba x Populus tremula*) isoprene synthase gene (SEQ ID NO:9). The ATG start codon is in bold and the stop codon is underlined.

[0052] Figure 18A (Figures 18A1 and 18A2) shows a schematic outlining construction of vectors pYLA 1, pYL1 and pYL2 (SEQ ID NO:75, 73, 72, 71, 70, 69).

[0053] Figure 18B shows a schematic outlining construction of the vector pYLA(POP1) (SEQ ID NO:68, 69).

[0054] Figure 18C shows a schematic outlining construction of the vector pYLA(KZ1)

[0055] Figure 18D shows a schematic outlining construction of the vector pYLI(KZ1) (SEQ ID NO:66, 67)

[0056] Figure 18E shows a schematic outlining construction of the vector pYLI(MAP29)

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

[0058] Figure 19A shows the MVA and DXP metabolic pathways for isoprene (based on F. Bouvier *et al.*, Progress in Lipid Res. 44: 357-429, 2005). The following description includes alternative names for each polypeptide in the pathways and a reference that discloses an assay for measuring the activity of the indicated polypeptide (each of these references are each hereby incorporated by reference in their entireties, particularly with respect to assays for polypeptide activity for polypeptides in the MVA and DXP pathways). **Mevalonate Pathway: AACT**; Acetyl-CoA acetyltransferase, MvaE, EC 2.3.1.9. Assay: J. Bacteriol., 184: 2116–2122, 2002; **HMGS**; Hydroxymethylglutaryl-CoA synthase, MvaS, EC 2.3.3.10. Assay: J. Bacteriol., 184: 4065–4070, 2002; **HMGR**; 3-Hydroxy-3-methylglutaryl-CoA reductase, MvaE, EC 1.1.1.34. Assay: J. Bacteriol., 184: 2116–2122, 2002; **MVK**; Mevalonate kinase, ERG12, EC 2.7.1.36. Assay: Curr Genet 19:9-14, 1991. **PMK**; Phosphomevalonate kinase, ERG8, EC 2.7.4.2, Assay: Mol Cell Biol., 11:620–631, 1991; **DPMDC**; Diphosphomevalonate decarboxylase, MVD1, EC 4.1.1.33. Assay: Biochemistry, 33:13355-13362, 1994; **IDI**; Isopentenyl-diphosphate delta-isomerase, IDI1, EC 5.3.3.2. Assay: J. Biol. Chem. 264:19169-19175, 1989. **DXP Pathway: DXS**; 1-Deoxyxylulose-5-phosphate synthase, dxs, EC 2.2.1.7. Assay: PNAS, 94:12857-62, 1997; **DXR**; 1-Deoxy-D-xylulose 5-phosphate reductoisomerase, dxr, EC 2.2.1.7. Assay: Eur. J. Biochem. 269:4446–4457, 2002; **MCT**; 4-Diphosphocytidyl-2C-methyl-D-erythritol synthase, IspD, EC 2.7.7.60. Assay: PNAS, 97: 6451–6456, 2000; **CMK**; 4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase, IspE, EC 2.7.1.148. Assay: PNAS, 97:1062-1067, 2000; **MCS**; 2C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase, IspF, EC 4.6.1.12. Assay: PNAS, 96:11758-11763, 1999; **HDS**; 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, ispG, EC 1.17.4.3. Assay: J. Org. Chem., 70:9168 -9174, 2005; **HDR**; 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, IspH, EC 1.17.1.2. Assay: JACS, 126:12847-12855, 2004.

[0059] Figure 19B illustrates the classical and modified MVA pathways. 1, acetyl-CoA acetyltransferase (**AACT**); 2, HMG-CoA synthase (**HMGS**); 3, HMG-CoA reductase (**HMGR**); 4, mevalonate kinase **(MVK)**; 5, phosphomevalonate kinase (**PMK**); 6, diphosphomevalonate decarboxylase **(MVD or DPMDC)**; 7, isopentenyl diphosphate isomerase **(IDI)**; 8, phosphomevalonate decarboxylase **(PMDC)**; 9, isopentenyl phosphate kinase **(IPK)**. The classical MVA pathway proceeds from reaction 1 through reaction 7 via reactions 5 and 6, while a modified MVA pathway goes through reactions 8 and 9. P and PP in the structural formula are phosphate and pyrophosphate, respectively. This figure was taken from Koga and Morii, *Microbiology and Mol. Biology Reviews*, 71:97-120, 2007, which is incorporated by reference in

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

[0060] Figure 20 (Figures 20A and 20B) 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.

[0061] Figure 21 is a map of pTrcKudzu yIDI DXS Kan.

[0062] Figures 22A-D are the nucleotide sequence of pTrcKudzu yIDI DXS Kan (SEQ ID NO:10).

[0063] 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).

[0064] 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).

[0065] 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).

[0066] 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).

[0067] 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).

[0068] 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).

[0069] 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).

[0070] 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).

[0071] Figure 24 is a map of pTrcKKDyIkIS kan.

[0072] Figures 25A-D are a nucleotide sequence of pTrcKKDyIkIS kan (SEQ ID NO:11).

[0073] Figure 26 is a map of pCL PtrcUpperPathway.

[0074] Figures 27A-27D is a nucleotide sequence of pCL PtrcUpperPathway (SEQ ID NO:12).

[0075] Figure 28 shows a map of the cassette containing the lower MVA pathway and yeast idi for integration into the *B. subtilis* chromosome at the nprE locus. nprE upstream/downstream indicates 1 kb each of sequence from the nprE locus for integration. aprE promoter (alkaline serine protease promoter) indicates the promoter (-35, -10, +1 transcription start site, RBS) of the *aprE* gene. MVK1 indicates the yeast mevalonate kinase gene. RBS-PMK indicates the yeast

phosphomevalonate kinase gene with a *Bacillus* RBS upstream of the start site. RBS-MPD indicates the yeast diphosphomevalonate decarboxylase gene with a *Bacillus* RBS upstream of the start site. RBS-IDI indicates the yeast idi gene with a *Bacillus* RBS upstream of the start site. Terminator indicates the terminator alkaline serine protease transcription terminator from *B. amyliquefaciens*. SpecR indicates the spectinomycin resistance marker. "nprE upstream repeat for amp." indicates a direct repeat of the upstream region used for amplification.

[0076] Figures 29A-D are a nucleotide sequence of cassette containing the lower MVA pathway and yeast idi for integration into the *B. subtilis* chromosome at the *nprE* locus (SEQ ID NO:13).

[0077] Figure 30 is a map of p9796-poplar.

[0078] Figures 31A-B are a nucleotide sequence of p9796-poplar (SEQ ID NO:14).

[0079] Figure 32 is a map of pTrcPoplar.

[0080] Figures 33A-C are a nucleotide sequence of pTrcPoplar (SEQ ID NO:15).

[0081] Figure 34 is a map of pTrcKudzu yIDI Kan.

[0082] Figures 35A-C are a nucleotide sequence of pTrcKudzu yIDI Kan (SEQ ID NO:16).

[0083] Figure 36 is a map of pTrcKudzuDXS Kan.

[0084] Figures 37A-C are a nucleotide sequence of pTrcKudzuDXS Kan (SEQ ID NO:17).

[0085] Figure 38 is a map of pCL PtrcKudzu.

[0086] Figures 39A-C are a nucleotide sequence of pCL PtrcKudzu (SEQ ID NO:18).

[0087] Figure 40 is a map of pCL PtrcKudzu A3.

[0088] Figures 41A-C are a nucleotide sequence of pCL PtrcKudzu A3 (SEQ ID NO:19).

[0089] Figure 42 is a map of pCL PtrcKudzu yIDI.

[0090] Figures 43A-C are a nucleotide sequence of pCL PtrcKudzu yIDI (SEQ ID NO:20).

[0091] Figure 44 is a map of pCL PtrcKudzu DXS.

[0092] Figures 45A-D are a nucleotide sequence of pCL PtrcKudzu DXS (SEQ ID NO:21).

[0093] Figures 46A-E show graphs representing isoprene production from biomass feedstocks. Panel A shows isoprene production from corn stover, Panel B shows isoprene production from bagasse, Panel C shows isoprene production from softwood pulp, Panel D shows isoprene production from glucose, and Panel E shows isoprene production from cells with no additional feedstock. Grey squares represent OD_{600} measurements of the cultures at the indicated times post-inoculation and black triangles represent isoprene production at the indicated times post-inoculation.

[0094] Figure 47A shows a graph representing isoprene production by BL21 (λDE3) pTrcKudzu yIDI DXS (kan) in a culture with no glucose added. Squares represent OD_{600}, and triangles represent isoprene produced (μg/ml).

[0095] Figure 47B shows a graph representing isoprene production from 1% glucose feedstock invert sugar by BL21 (λDE3) pTrcKudzu yIDI DXS (kan). Squares represent OD_{600}, and triangles represent isoprene produced (μg/ml).

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

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

[0098] Figures 48A-C show graphs demonstrating the effect of yeast extract of isoprene production. Panel A shows the time course of optical density within fermentors fed with varying amounts of yeast extract. Panel B shows the time course of isoprene titer within fermentors fed with varying amounts of yeast extract. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Panel C shows the effect of yeast extract on isoprene production in *E. coli* grown in fed-batch culture.

[0099] Figures 49A-C show graphs demonstrating isoprene production from a 500 L bioreactor with *E. coli* cells containing the pTrcKudzu + yIDI + DXS plasmid. Panel A shows the time course of optical density within the 500-L bioreactor fed with glucose and yeast extract. Panel B shows the time course of isoprene titer within the 500-L bioreactor fed with glucose and yeast extract. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Panel C shows the time course of total isoprene produced from the 500-L bioreactor fed with glucose and yeast extract.

[0100] Figure 50 is a map of pJMupperpathway2.

[0101] Figures 51A-C are the nucleotide sequence of pJMupperpathway2 (SEQ ID NO:22).

[0102] Figure 52 is a map of pBS Kudzu #2.

[0103] Figure 53A is a graph showing growth during fermentation time of *Bacillus* expressing recombinant kudzu isoprene synthase in 14 liter fed batch fermentation. Black diamonds represent a control strain (BG3594comK) without recombinant isoprene synthase (native isoprene production) and grey triangles represent CF443, *Bacillus* strain BG3594comK with pBSKudzu (recombinant isoprene production).

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

[0105] Figure 54 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0106] Figure 55 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0107] Figure 56 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0108] Figure 57 is a time course of optical density within the 15-L bioreactor fed with glycerol.

[0109] Figure 58 is a time course of isoprene titer within the 15-L bioreactor fed with glycerol. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0110] Figure 59 is a time course of total isoprene produced from the 15-L bioreactor fed with glycerol.

[0111] Figures 60A-60C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 150-L bioreactor fed with glucose.

[0112] Figures 61A-61C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0113] Figures 62A-62C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0114] Figure 63A-63C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0115] Figures 64A-64C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0116] Figures 65A-65C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0117] Figures 66A-66C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0118] Figure 67A-67C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0119] Figure 68 is a graph of the calculated adiabatic flame temperatures for Series A as a function of fuel concentration for various oxygen levels. The figure legend lists the curves in the order in which they appear in the graph. For example, the first entry in the figure legend (isoprene in air at 40 °C) corresponds to the highest curve in the graph.

[0120] Figure 69 is a graph of the calculated adiabatic flame temperatures for Series B as a function of fuel concentration for various oxygen levels with 4% water. The figure legend lists the curves in the order in which they appear in the graph.

[0121] Figure 70 is a graph of the calculated adiabatic flame temperatures for Series C as a function of fuel concentration for various oxygen levels with 5% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0122] Figure 71 is a graph of the calculated adiabatic flame temperatures for Series D as a function of fuel concentration for various oxygen levels with 10% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0123] Figure 72 is a graph of the calculated adiabatic flame temperatures for Series E as a function of fuel concentration for various oxygen levels with 15% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0124] Figure 73 is a graph of the calculated adiabatic flame temperatures for Series F as a function of fuel concentration for various oxygen levels with 20% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0125] Figure 74 is a graph of the calculated adiabatic flame temperatures for Series G as a function of fuel concentration for various oxygen levels with 30% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0126] Figure 75A is a table of the conversion of the CAFT Model results from weight percent to volume percent for series A.

[0127] Figure 75B is a graph of the flammability results from the CAFT model for Series A in Figure 68 plotted as volume percent.

[0128] Figure 76A is a table of the conversion of the CAFT Model results from weight percent to volume percent for series B.

[0129] Figure 76B is a graph of the flammability results from the CAFT model for Series B in Figure 69 plotted as volume percent.

[0130] Figure 77 is a figure depicting the flammability test vessel.

[0131] Figure 78A is a graph of the flammability Curve for Test Series 1: 0% Steam, 0 psig, and 40°C.

[0132] Figure 78B is a table summarizing the explosion and non-explosion data points for Test Series 1.

[0133] Figure 78C is a graph of the flammability curve for Test Series 1 compared with the CAFT Model.

[0134] Figure 79A is a graph of the flammability curve for Test Series 2: 4% Steam, 0 psig, and 40°C.

[0135] Figure 79B is a table summarizing the explosion and non-explosion data points for Test Series 2.

[0136] Figure 79C is a graph of the flammability curve for Test Series 2 compared with the CAFT Model.

[0137] Figures 80A-B are a table of the detailed experimental conditions and results for Test Series 1.

[0138] Figure 81 is a table of the detailed experimental conditions and results for Test Series 2.

[0139] Figure 82 is a graph of the calculated adiabatic flame temperature plotted as a function of fuel concentration for various nitrogen/oxygen ratios at 3 atmospheres of pressure.

[0140] Figure 83 is a graph of the calculated adiabatic flame temperature plotted as a function of fuel concentration for various nitrogen/oxygen ratios at 1 atmosphere of pressure.

[0141] Figure 84 is a graph of the flammability envelope constructed using data from Figure 82 and following the methodology described in Example 13. The experimental data points (circles) are from tests described herein that were conducted at 1 atmosphere initial system pressure.

[0142] Figure 85 is a graph of the flammability envelope constructed using data from Figure 83 and following the methodology described in Example 13. The experimental data points (circles) are from tests described herein that were conducted at 1 atmosphere initial system pressure.

[0143] Figure 86A is a GC/MS chromatogram of fermentation off-gas.

[0144] Figure 86B is an expansion of Fig 86A to show minor volatiles present in fermentation off-gas.

[0145] Figure 87A is a GC/MS chromatogram of trace volatiles present in off-gas following cryo-trapping at -78°C.

[0146] Figure 87B is a GC/MS chromatogram of trace volatiles present in off-gas following cryo-trapping at -196°C.

[0147] Figure 87C is an expansion of Figure 87B.

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

[0149] Figures 88A-B are GC/MS chromatogram comparing C5 hydrocarbons from petroleum-derived isoprene (Figure 88A) and biologically produced isoprene (Figure 88B). The standard contains three C5 hydrocarbon impurities eluting around the main isoprene peak (Figure 88A). In contrast, biologically produced isoprene contains amounts of ethanol and acetone (run time of 3.41 minutes) (Figure 88A).

[0150] Figure 89 is a graph of the analysis of fermentation off-gas of an *E. coli* BL21 (DE3) pTrcIS strain expressing a Kudzu isoprene synthase and fed glucose with 3 g/L yeast extract.

[0151] Figure 90 shows the structures of several impurities that are structurally similar to isoprene and may also act as polymerization catalyst poisons.

[0152] Figure 91 is a map of pTrcHis2AUpperPathway (also called pTrcUpperMVA).

[0153] Figures 92A-92C are the nucleotide sequence of pTrcHis2AUpperPathway (also called pTrcUpperMVA) (SEQ ID NO:23).

[0154] Figure 93 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0155] Figure 94 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0156] Figure 95 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0157] Figure 96 is a time course of optical density within the 15-L bioreactor fed with invert sugar.

[0158] Figure 97 is a time course of isoprene titer within the 15-L bioreactor fed with invert sugar. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0159] Figure 98 is a time course of total isoprene produced from the 15-L bioreactor fed with invert sugar.

[0160] Figure 99 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0161] Figure 100 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0162] Figure 101 is a time course of isoprene specific activity from the 15-L bioreactor fed with glucose.

[0163] Figure 102 is a map of pCLPtrcUpperPathwayHGS2.

[0164] Figures 103A-103C are the nucleotide sequence of pCLPtrcUpperPathwayHGS2 (SEQ ID NO:24).

[0165] Figure 104 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0166] Figure 105 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0167] Figure 106 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0168] Figure 107 is a map of plasmid MCM330 (FRT-cm-FRT-gi1.2-KKDy at attTn7).

[0169] Figures 108A-108C are the nucleotide sequence of plasmid MCM330 (SEQ ID NO:25).

[0170] Figure 109 is a map of pET24D-Kudzu.

[0171] Figures 110A-B are the nucleotide sequence of pET24D-Kudzu (SEQ ID NO:26).

[0172] Figure 111A is a time course of optical density within the 15-L bioreactor fed with glucose.

[0173] Figure 111B is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0174] Figure 111C is a time course of specific productivity of isoprene in the 15-L bioreactor fed with glucose.

[0175] Figure 112A is a map of the *M. mazei* archaeal Lower Pathway operon.

[0176] Figures 112B-C are the nucleotide sequence of the *M. mazei* archaeal lower Pathway operon (SEQ ID NO:27).

[0177] Figure 113A is a map of MCM382 – pTrcKudzuMVK(mazei).

[0178] Figures 113B-C are the nucleotide sequence of MCM382 – pTrcKudzuMVK(mazei) (SEQ ID NO:28).

[0179] Figure 114A is a map of MCM376 - MVK from *M. mazei* archaeal Lower in pET200D.

[0180] Figures 114B-C are the nucleotide sequence of MCM376 - MVK from *M. mazei* archaeal Lower in pET200D (SEQ ID NO:29).

[0181] Figures 115A-115D demonstrate that over-expression of MVK and isoprene synthase results in increased isoprene production. Accumulated isoprene and CO_2 from MCM401 and MCM343 during growth on glucose in 100 mL bioreactors with 100 and 200 μM IPTG induction of isoprene production was measured over a 22 hour time course. Figure 115A is a

graph of the accumulated isoprene (%) from MCM343. Figure 115B is a graph of the accumulated isoprene (%) from MCM401. Figure 115C is a graph of the accumulated CO_2 (%) from MCM343. Figure 115D is a graph of the accumulated CO_2 (%) from MCM401.

[0182] Figure 116 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0183] Figure 117 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0184] Figure 118 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0185] Figure 119 is a graph of the total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

[0186] Figure 120 is a graph of the cell viability during isoprene production within the 15-L bioreactor fed with glucose. TVC/OD is the total viable counts (colony forming units) in 1 mL of broth per optical density unit (OD_{550}).

[0187] Figure 121 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0188] Figure 122 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0189] Figure 123 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0190] Figure 124 is a time course of volumetric productivity within the 15-L bioreactor fed with glucose. The volumetric productivity is defined as the amount of isoprene produced per liter of broth per hour.

[0191] Figure 125 is a time course of instantaneous yield within the 15-L bioreactor fed with glucose. The instantaneous yield is defined as the amount of isoprene (gram) produced per amount of glucose (gram) fed to the bioreactor (w/w) during the time interval between the data points.

[0192] Figure 126 is a graph of the total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

[0193] Figure 127 is cell viability during isoprene production within the 15-L bioreactor fed with glucose. TVC/OD is the total viable counts (colony forming units) in 1 mL of broth per optical density unit (OD_{550}).

[0194] Figure 128 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0195] Figure 129 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0196] Figure 130 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0197] Figure 131 is a graph of total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

[0198] Figure 132 is a graph showing that a transient decrease in the airflow to the bioreactor caused a spike in the concentration of isoprene in the off-gas that did not cause a dramatic decrease in metabolic activity (TCER). TCER, or metabolic activity, is the total carbon dioxide evolution rate.

[0199] Figure 133 is a graph of the cell viability during isoprene production within the 15-L bioreactor fed with glucose. TVC/OD is the total viable counts (colony forming units) in 1 mL of broth per optical density unit (OD_{550}).

[0200] Figure 134 is a time course of optical density within the 15-L bioreactor fed with glucose. Dotted vertical lines denote the time interval when isoprene was introduced into the bioreactor at a rate of 1 g/L/hr.

[0201] Figure 135 is total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose. Dotted vertical lines denote the time interval when isoprene was introduced into the bioreactor at a rate of 1 g/L/hr.

[0202] Figure 136 is cell viability during isoprene production within the 15-L bioreactor fed with glucose. TVC/OD is the total viable counts (colony forming units) in 1 mL of broth per optical density unit (OD_{550}). Dotted vertical lines denote the time interval when isoprene was introduced into the bioreactor at a rate of 1 g/L/hr.

[0203] Figures 137A-B are the sequence of *Populus alba* pET24a: isoprene synthase gene highlighted in bold letters (SEQ ID NO:30).

[0204] Figures 137C-D are the sequence of *Populus nigra* pET24a: isoprene synthase gene highlighted in bold letters (SEQ ID NO:31).

[0205] Figures 137E-F are the sequence of *Populus tremuloides* pET24a (SEQ ID NO:32).

[0206] Figure 137G is the amino acid sequence of *Populus tremuloides* isoprene synthase gene (SEQ ID NO:33).

[0207] Figures 137H-I are the sequence of *Populus trichocarpa* pET24a: isoprene synthase gene highlighted in bold letters (SEQ ID NO:34).

[0208] Figures 137J-K are the sequence of *Populus tremula* × *Populus alba* pET24a: isoprene synthase gene highlighted in bold letters (SEQ ID NO:35).

[0209] Figure 137L is a map of MCM93 which contains the kudzu IspS coding sequence in a pCR2.1 backbone.

[0210] Figures 137M-N are the sequence of MCM93 (SEQ ID NO:36).

[0211] Figure 137O is a map of pET24D-Kudzu.

[0212] Figures 137P-Q are the sequence of pET24D-Kudzu (SEQ ID NO:37).

[0213] Figure 138 is isoprene synthase expression data for various poplar species as measured in the whole cell head space assay. Y-axis is μg/L/OD of isoprene produced by 0.2 mL of a culture induced with IPTG.

[0214] Figure 139 is relative activity of Poplar isoprene synthase enzymes as measured by DMAPP assay. Poplar enzymes have significantly higher activity than the isoprene synthase from Kudzu. Poplar [*alba x tremula*] only had traces (< 1%) of activity and is not shown in the plot.

[0215] Figure 140 is a map of pDONR221:19430 - hybrid_HGS.

[0216] Figure 141 is the nucleotide sequence of pDONR221:19430 - hybrid_HGS, the sequence of Kudzu isoprene synthase codon-optimized for yeast (SEQ ID NO:38).

[0217] Figure 142A is a map of pDW14.

[0218] Figures 142B-C are the complete nucleotide sequence of pDW14 (SEQ ID NO:39).

[0219] Figure 143 shows induced INVSc-1 strains harboring pDW14 or pYES-DEST52. Figure 143A. A 4-12% bis tris gel (Novex, Invitrogen) of lysates generated from INVSc-1 strains induced with galactose and stained with SimplyBlue SafeStain (Invitrogen). Figure 143B. Western blot analysis of the same strains using the WesternBreeze kit (Invitrogen). Lanes are as follows: 1, INVSc-1 + pYES-DEST52; 2, INVSc-1 + pDW14 (isolate 1); 3, INVSc-1 + pDW14 (isolate 2). MW (in kDa) is indicated (using the SeeBlue Plus2 molecular weight standard).

[0220] Figure 144 (Figures 144A and 144B) shows induced INVSc-1 strains harboring pDW14 or pYES-DEST52. Figure 144A. OD_{600} of galactose-induced strains prior to lysis. The y-axis is OD_{600}. Figure 144B. DMAPP assay of isoprene synthase headspace in control and isoprene synthase-harboring strains. Specific activity was calculated as μg HG/L/OD. Samples are as follows: Control, INVSc-1 + pYES-DEST52; HGS-1, INVSc-1 + pDW14 (isolate 1); HGS-2, INVSc-1 + pDW14 (isolate 2).

[0221] Figure 145A is a map of codon optimized isoprene synthase fluo-opt2v2.

[0222] Figure 145B is the nucleotide sequence of codon optimized isoprene synthase fluo-opt2v2 (SEQ ID NO:40).

[0223] Figure 146A is a map of pBBR1MCS5.

[0224] Figures 146B-C are the nucleotide sequence of pBBR1MCS5 (SEQ ID NO:41).

[0225] Figure 147A is a map of pBBR5HGSOpt2_2.

[0226] Figures 147B-C are the nucleotide sequence of pBBR5HGSOpt2_2 (SEQ ID NO:42).

[0227] Figure 148 is a graph of CER versus fermentation time for strain MCM401, uninduced, induced with IPTG (4 x 50 μmol) or IPTG (2 x 100 μmol).

[0228] Figure 149 shows concentration of glucose in sugar cane solutions, pH adjusted or not, as a function of the number of autoclaving cycles (one cycle = 30 min).

[0229] Figure 150 shows growth curves (OD_{600} as a function of time) of *Pseudomonas putida* F1 and *Pseudomonas fluorescens* ATCC13525 on glucose, sugar cane, and inverted sugar cane.

[0230] Figure 151 shows growth curves (OD_{600} as a function of time) of *E. coli* BL21(DE3), MG1655, ATCC11303 and B REL 606 on glucose, sugar cane, and inverted sugar cane.

[0231] Figure 152 is a map of plasmid pET24 P. alba HGS.

[0232] Figure 153A-B are the nucleotide sequence of plasmid pET24 P. alba HGS (SEQ ID NO:43).

[0233] Figure 154 is a schematic diagram showing restriction sites used for endonuclease digestion to construct plasmid EWL230 and compatible cohesive ends between BspHI and NcoI sites.

[0234] Figure 155 is a map of plasmid EWL230.

[0235] Figures 156A-B are the nucleotide sequence of plasmid EWL230 (SEQ ID NO:44).

[0236] Figure 157 is a schematic diagram showing restriction sites used for endonuclease digestion to construct plasmid EWL244 and compatible cohesive ends between NsiI and PstI sites.

[0237] Figure 158 is a map of plasmid EWL244.

[0238] Figures 159A-B are the nucleotide sequence of plasmid EWL244 (SEQ ID NO:45).

[0239] Figure 160A is a map of the *M. mazei* archaeal Lower Pathway operon.

[0240] Figures 160B-C are the nucleotide sequence of the *M. mazei* archaeal Lower Pathway operon (SEQ ID NO:46).

[0241] Figure 161A is a map of MCM376-MVK from *M. mazei* archaeal Lower in pET200D.

[0242] Figures 161B-C are the nucleotide sequence of MCM376-MVK from *M. mazei* archaeal Lower in pET200D (SEQ ID NO:47).

[0243] Figure 162 is a map of plasmid pBBRCMPGI1.5-pgl.

[0244] Figures 163A-B are the nucleotide sequence of plasmid pBBRCMPGI1.5-pgl (SEQ ID NO:48).

[0245] Figures 164A-F are graphs of isoprene production by *E. coli* strain expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, and *pgl* (RHM111608-2), and grown in fed-batch culture at the 15-L scale. Figure 164A shows the time course of optical density within the 15-L bioreactor fed with glucose. Figure 164B shows the time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Method for calculating isoprene: cumulative isoprene produced in 59 hrs, g/Fermentor volume at 59 hrs, L [=] g/L broth. Figure 164C also shows the time course of isoprene titer within the 15-L bioreactor fed with glucose. Method for calculating isoprene: $\int$(Instantaneous isoprene production rate, g/L/hr)dt from t = 0 to 59 hours [=] g/L broth. Figure 164D shows the time course of total isoprene produced from the 15-L bioreactor fed with glucose. Figure 164E shows volumetric productivity within the 15-L bioreactor fed with glucose. Figure 164F shows carbon dioxide evolution rate (CER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

[0246] Figures 165A-B are graphs showing analysis of off-gas from fermentation in 15L bioreactors. Sample A is strain RM111608-2 sampled at 64.8 hours. Sample B is strain EWL256 was *E. coli* BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK sampled at 34.5 hours. Hydrogen is detected above the baseline (0.95×10^{-8} torr) for both samples.

[0247] Figure 166A shows an exemplary Bioisoprene™ recovery unit.

[0248] Figure 166B shows an exemplary Bioisoprene™ desorption/condensation setup.

[0249] Figure 167 shows a GC/FID chromatogram of a Bioisoprene™ product. The material was determined to be 99.7% pure.

[0250] Figure 168A-C show the GC/FID chromatograms of a Bioisoprene™ sample before (A) and after treatment with alumina (B) or silica (C). The isoprene peak is not shown in these chromatograms.

[0251] Figure 169 shows a diagram of a process and associated apparatus for purifying isoprene from a fermentation off-gas.

[0252] Figure 170 shows GC/FID chromatogram of partially hydrogenated BioIsoprene™ monomer. Compound 1 (RT = 12.30 min) = 3-methyl-1-butene, compound 2 (RT = 12.70 min)

= 2-methylbutane, compound 3 (RT = 13.23 min) = 2-methyl-1-butene, compound 4 (RT = 13.53 min) = isoprene, compound 5 (RT = 14.01 min) = 2-methyl-2-butene).

[0253] Figure 171 shows the GC/MS Total Ion Chromatogram for products derved from the Amberlyst-15 acid resin-catalyzed dimerization of 2-methyl-2-butene.

[0254] Figure 172 shows the GC/MS Total Ion Chramatogram for products derived from Amberlyst 15 acid resin-catalyzed oligomerization of BioIsoprene™ monomer.

[0255] Figure 173 shows a process flow diagram for the conversion of a C5 stream into a C10/C15 product stream using a dimerization reactor. The C5 stream comprises BioIsoprene™ monomer and/or C5 derivatives of BioIsoprene™ monomer.

DETAILED DESCRIPTION OF THE INVENTION

[0256] The invention provides, *inter alia*, compositions and methods for producing a fuel constituent from isoprene. Provided herein are fuel constituents or additives, for example, cyclic isoprene dimers and trimers, linear isoprene oligomers, aromatic and alicyclic isoprene derivatives, and oxygenated isoprene derivatives. The fuel constituent can be produced by chemical transformations of a starting material comprising a commercially beneficial amount of highly pure isoprene. In one aspect, the commercially beneficial amount of highly pure isoprene comprises bioisoprene. In another aspect, a commercially beneficial amount of highly pure isoprene can be bioisoprene. In another aspect, a commercially beneficial amount of highly pure isoprene can be highly pure isoprene compositions produced by culturing cells expressing a heterologous isoprene synthase enzyme. In other aspects, highly pure isoprene undergoes oligomerization to form unsaturated isoprene oligomers such as cyclic dimers or trimers and linear oligomers. The unsaturated oligomers may be hydrogenated to produce saturated hydrocarbon fuel constituent. In some embodiment, reaction of highly pure isoprene with alcohols in the presence of an acid catalyst produces fuel oxygenates. In another aspect, the highly pure isoprene is partially hydrogenated to produce isoamylenes. In some embodiments, an isoamylene product derived from the highly pure isoprene undergoes dimerization to form isodecenes. In some embodiments, isoamylene products derived from the highly pure isoprene react with alcohols in the presence of an acid catalyst to produce fuel oxygenates.

[0257] Bioisoprene derived from renewable carbon can be converted to a variety of hydrocarbon fuels by chemical catalysis. Provided herein are methods for recovering isoprene from fermentation and subsequent conversion to hydrocarbon fuels by chemical catalysis to compounds of higher molecular weight. These methods include, but are not limited to,

recovering and purifying isoprene from fermentation off-gas and subsequent gas or liquid phase catalysis to provide compounds with fuel value. Both continuous and batch mode processes are contemplated within the scope of the invention.

[0258] As further detailed herein, a bioisoprene composition is distinguished from a petro-isoprene composition in that a bioisoprene composition is substantially free of any contaminating unsaturated C5 hydrocarbons that are usually present in petro-isoprene compositions, such as, but not limited to, 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, and *cis*-pent-3-ene-1-yne. If any contaminating unsaturated C5 hydrocarbons are present in the bioisoprene starting material described herein, they are present in lower levels than that in petro-isoprene compositions. Accordingly, any fuel products derived from bioisoprene compositions described herein is essentially free of, or contains at lower levels than that in fuel products derived from petro-isoprene, any contaminating unsaturated C5 hydrocarbons or products derived from such contaminating unsaturated C5 hydrocarbons. In addition, the sulfur levels in a bioisoprene composition are lower than the sulfur levels in petro-isoprene compositions. Fuels products derived from bioisoprene compositions contain lower levels of sulfur than that in fuel products derived from petro-isoprene.

[0259] Bioisoprene is distinguished from petro-isoprene in that bioisoprene is produced with other bio-byproducts (compounds derived from the biological sources and/or associated the biological processes that are obtained together with bioisoprene) that are not present or present in much lower levels in petro-isoprene compositions, such as alcohols, aldehydes, ketone and the like. The bio-byproducts may include, but are not limited to, ethanol, acetone, methanol, acetaldehyde, methacrolein, methyl vinyl ketone, 2-methyl-2-vinyloxirane, *cis*- and *trans*-3-methyl-1,3-pentadiene, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). Fuel products

derived from bioisoprene contain one or more of the bio-byproducts or compounds derived from any of the bio-byproducts. In addition, fuel products derived from bioisoprene may contain compounds formed from these bio-byproducts during subsequent chemical conversion. Examples of such compounds include those derived from Diels-Alder cycloaddition of dienophiles to isoprene or fuel derivatives thereof, the oxidation of isoprene or fuel derivatives.

[0260] Further, bioisoprene is distinguished from petro-isoprene by carbon finger-printing. In one aspect, bioisoprene has a higher radioactive carbon-14 (^{14}C) content or higher $^{14}C/^{12}C$ ratio than petro-isoprene. Bioisoprene is produced from renewable carbon sources, thus the ^{14}C content or the $^{14}C/^{12}C$ ratio in bioisoprene is the same as that in the present atmosphere. Petro-isoprene, on the other hand, is derived from fossil fuels deposited thousands to millions of years ago, thus the ^{14}C content or the $^{14}C/^{12}C$ ratio is diminished due to radioactive decay. As discussed in greater detail herein, the fuel products derived from bioisoprene has higher ^{14}C content or $^{14}C/^{12}C$ ratio than fuel products derived from petro-isoprene. In one embodiment, a fuel product derived from bioisoprene described herein has a ^{14}C content or $^{14}C/^{12}C$ ratio similar to that in the atmosphere. In another aspect, bioisoprene can be analytically distinguished from petro-isoprene by the stable carbon isotope ration ($^{13}C/^{12}C$), which can be reported as "delta values" represented by the symbol $d^{13}C$. For examples, for isoprene derived from extractive distillation of C_5 streams from petroleum refineries, $\delta^{13}C$ is about -22‰ to about -24‰. This range is typical for light, unsaturated hydrocarbons derived from petroleum, and products derived from petroleum-based isoprene typically contain isoprenic units with the same $\delta^{13}C$. Bioisoprene produced by fermentation of corn-derived glucose ($\delta^{13}C$ -10.73‰) with minimal amounts of other carbon-containing nutrients (e.g., yeast extract) produces isoprene which can be polymerized into polyisoprene with $\delta^{13}C$ -14.66‰ to -14.85‰. Products produced from such bioisoprene are expected to have $\delta^{13}C$ values that are less negative than those derived from petroleum-based isoprene.

[0261] Compounds made by these methods include cyclic isoprene dimers and trimers, linear oligomers, aromatic and alicyclic derivatives. Diisoamylenes are made by methods comprising partial hydrogenation of bioisoprene compositons. These chemical derivatives of isoprene are useful as liquid transportation fuels (IsoFuels™) and as fuel additives.

[0262] Also provided herein are methods for the production of oxygenated derivatives of isoprene including alcohols, ketones, esters and ethers. Methods for the synthesis of oxygenated derivatives of isoprene can also be performed in liquid or gas phase, using homogeneous and heterogeneous catalysts. Compounds of this chemical class are also useful as liquid

transportation fuels, and can be used in fuel blends as fuel oxygenates for emissions reduction and as fuel modifiers, for example as cetane boosters for diesel.

[0263] 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. Isoprene is also 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. Genetically engineered cell cultures in bioreactors have produced isoprene more efficiently, in larger quantities, in higher purities and/or with unique impurity profiles, e.g. as described in U.S. provisional patent application Nos. 61/013,386 and 61/013,574, filed on December 13, 2007, WO 2009/076676, U.S. provisional patent application Nos. 61/134,094, 61/134,947, 61/134,011 and 61/134,103, filed on July 2, 2008, WO 2010/003007, U.S. provisional patent application No. 61/097,163, filed on September 15, 2008, WO 2010/031079, U.S. provisional patent application No. 61/097,186, filed on September 15, 2008, WO 2010/031062, U.S. provisional patent application No. 61/097,189, filed on September 15, 2008, WO 2010/031077, U.S. provisional patent application No. 61/097,200, filed on September 15, 2008, WO 2010/031068, U.S. provisional patent application No. 61/097,204, filed on September 15, 2008, WO 2010/031076, U.S. provisional patent application No.61/141,652, filed on December 30, 2008, PCT/US09/069862, U.S. patent application No. 12/335,071, filed December 15, 2008 (US 2009/0203102 A1) and U.S. patent application No. 12/429,143, filed April 23, 2009 (US 2010/0003716 A1), which are incorporated by reference in their entireties.

Definitions

[0264] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, the preferred methods and materials are described herein. Accordingly, the terms defined immediately below are more fully described by reference to the Specification as a whole. All documents cited are, in relevant part, incorporated herein by reference. However, the citation of any document is not to be construed as an admission that it is prior art with respect to the present invention.

[0265] As used herein, the singular terms "a," "an," and "the" include the plural reference unless the context clearly indicates otherwise.

[0266] It is intended that every maximum numerical limitation given throughout this specification includes every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

[0267] The term "isoprene" refers to 2-methyl-1,3-butadiene (CAS# 78-79-5), which is 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 [an] IPP molecule(s) to [a] DMAPP molecule(s). The term "isoprene" is not generally intended to be limited to its method of production unless indicated otherwise herein.

[0268] As used herein, "biologically produced isoprene" or "bioisoprene" is isoprene produced by any biological means, such as produced by genetically engineered cell cultures, natural microbials, plants or animals.

[0269] A "bioisoprene composition" refers to a composition that can be produced by any biological means, such as systems (e.g., cells) that are engineered to produce isoprene. It contains isoprene and other compounds that are co-produced (including impurities) and/or isolated together with isoprene. A bioisoprene composition usually contains fewer hydrocarbon impurities than isoprene produced from petrochemical sources and often requires minimal treatment in order to be of polymerization grade. As detailed herein, bioisoprene composition also has a different impurity profile from a petrochemically produced isoprene composition.

[0270] As used herein, "at least a portion of the isoprene starting composition" can refer to at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9%, or 100% of the isoprene starting composition undergoing chemical transformation.

[0271] As used herein, IsoFuels™ refers to fuels including liquid transportation fuels that are derived from isoprene. BioIsoFuels™ refers to fuels including liquid transportation fuels that are derived from bioisoprene.

[0272] The term "oligomerization" as used herein refers to a chemical process for combining two or more monomer units. "Oligomerization" of isoprene produces a derivative of isoprene

derived from two or more molecules of isoprene, such as linear dimers of isoprene, cyclic dimers of isoprene, linear trimers of isoprene, cyclic trimers of isoprene and the like.

[0273] "Complete hydrogenation", "Completely hydrogenate" or "Fully hydrogenate" is defined as the addition of hydrogen (H_2), typically in the presence of a hydrogenation catalyst, to all unsaturated functional groups, such as carbon-carbon double bonds, within a precursor compound to give fully saturated product compounds. For example, complete hydrogenation of isoprene forms isopentane whereby 2 moles of H_2 is comsumed per mole of isoprene.

[0274] "Partial hydrogenation" or "Partially hydrogenate" is defined as the addition of hydrogen (H_2), typically in the presence of a hydrogenation catalyst, to at least one, but not all unsaturated functional groups, such as carbon-carbon double bonds, within a precursor compound. The product(s) of partial hydrogenation can be further completely hydrogenated to give fully saturated product compounds. Partial hydrogenation of a diene forms one or more mono-olefins. For example, partial hydrogenation of isoprene can give 3 isomeric isopentenes (2-methylbut-1-ene, 2-methylbut-2-ene and 3-methylbut-1-ene) whereby 1 mole of H_2 is comsumed per mole of isoprene.

[0275] "Selective hydrogenation" or "Selectively hydrogenate" is defined as the addition of hydrogen (H_2), typically in the presence of a hydrogenation catalyst, to at least one, but not all unsaturated functional groups, such as carbon-carbon double bonds, within a precursor compound whereby certain unsaturated functional groups are preferentially hydrogenated over other unsaturated groups under the chosen conditions. For example, selective hydrogenation of isoprene may form preferentially 2-methyl-2-butene, 2-methyl-1-butene, 3-methyl-1-butene or a mixture thereof.

[0276] As used herein, the term "polypeptides" includes polypeptides, proteins, peptides, fragments of polypeptides, and fusion polypeptides.

[0277] 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.

[0278] By "heterologous polypeptide" is meant a polypeptide whose amino acid sequence is not identical to that of another polypeptide naturally expressed in the same host cell. In particular, a heterologous polypeptide is not identical to a wild-type polypeptide that is found in the same host cell in nature.

[0279] "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.

[0280] As used herein, a "nucleic acid" refers to two or more deoxyribonucleotides and/or ribonucleotides covalently joined together in either single or double-stranded form.

[0281] By "recombinant nucleic acid" is meant a nucleic acid of interest that is free of one or more nucleic acids (*e.g.*, genes) which, in the genome occurring in nature of the organism from which the nucleic acid of interest is derived, flank the nucleic acid of interest. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA, a genomic DNA fragment, or a cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences.

[0282] By "heterologous nucleic acid" is meant a nucleic acid whose nucleic acid sequence is not identical to that of another nucleic acid naturally found in the same host cell. In particular, a heterologous nucleic acid is not identical to a wild-type nucleic acid that is found in the same host cell in nature.

[0283] 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.

[0284] 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.

[0285] The term "selective marker" or "selectable marker" refers to a nucleic acid capable of expression in a host cell that allows for ease of selection of those host cells containing an introduced nucleic acid or vector. Examples of selectable markers include, but are not limited to,

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

Compositions and Systems

[0286] Isoprene derived from petrochemical sources usually is an impure C5 hydrocarbon fraction which requires extensive purification before the material is suitable for polymerization or other chemical transformations. Several impurities are particularly problematic given their structural similarity to isoprene and the fact that they can act as polymerization catalyst poisons. Such compounds include, but are not limited to, 1,3-cyclopentadiene, *cis*- and *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, and *cis*-pent-3-ene-1-yne. As detailed below, biologically produced isoprene can be substantially free of any contaminating unsaturated C5 hydrocarbons without undergoing extensive purification. Some biologically produced isoprene compositions contain ethanol, acetone, and C5 prenyl alcohols. These components are more readily removed from the isoprene stream than the isomeric C5 hydrocarbon fractions that are present in isoprene compositions derived from petrochemical sources. Further, these impurities can be managed in the bioprocess, for example by genetic modification of the producing strain, carbon feedstock, alternative fermentation conditions, recovery process modifications and additional or alternative purification methods.

[0287] In one aspect, the invention features compositions and systems for producing a fuel constituent from isoprene comprising: (a) a commercially beneficial amount of highly pure isoprene starting composition; and (b) a fuel constituent produced from at least a portion of the highly pure isoprene starting material; where at least a portion of the commercially beneficial amount of highly pure isoprene starting composition undergoes a chemical transformation. A highly pure isoprene starting material is subjected to chemical reactions to produce a commercially beneficial amount of product that is useful for making fuels. In one aspect, a commercially beneficial amount of highly pure isoprene comprises bioisoprene. In one aspect, a commercially beneficial amount of highly pure isoprene can be bioisoprene.

Exemplary starting isoprene compositions

[0288] In some embodiments, the commercially beneficial amount of highly pure isoprene starting 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 starting isoprene 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 starting isoprene composition comprises greater than or about 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 kg of isoprene. In some embodiments, the amount of isoprene in the starting 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 starting 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, the amount of isoprene in the starting composition is between about 2 to about 5,000 g, such as between about 2 to about 100 g, about 100 to about 500 g, about 500 to about 1,000 g, about 1,000 to about 2,000 g, or about 2,000 to about 5,000 g. In some embodiments, the amount of isoprene in the starting composition is between about 2 to about 5,000 kg, about 10 to about 2,000 kg, about 20 to about 1,000 kg, about 20 to about 500 kg, about 30 to about 200 kg, or about 40 to about 100 kg. In some embodiments, greater than or about 20, 25, 30, 40, 50, 60, 70, 80, 90, or 95% (w/w) of the volatile organic fraction of the starting composition is isoprene.

[0289] In some embodiments, the highly pure isoprene starting composition comprises greater than or about 98.0, 98.5, 99.0, 99.5, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the starting composition. In some embodiments, the highly pure isoprene starting 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 starting composition. In some embodiments, the starting composition has a relative detector response of greater than or about 98.0, 98.5, 99.0, 99.5, or 100% for isoprene compared to the detector response for all C5 hydrocarbons in the starting composition. In some embodiments, the starting 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 starting composition. In some embodiments, the starting isoprene composition comprises between about 98.0 to about 98.5, about 98.5 to about 99.0,

about 99.0 to about 99.5, about 99.5 to about 99.8, about 99.8 to 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the starting composition. In some embodiments, the starting 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 starting composition.

[0290] In some embodiments, the highly pure isoprene starting composition comprises less than or about 2.0, 1.5, 1.0, 0.5, 0.2, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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 starting composition. In some embodiments, the starting composition has a relative detector response of less than or about 2.0, 1.5, 1.0, 0.5, 0.2, 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 starting composition. In some embodiments, the starting composition has a relative detector response of less than or about 2.0, 1.5, 1.0, 0.5, 0.2, 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, 1-pentene, 2-methyl-1-butene, 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 starting composition. In some embodiments, the highly pure isoprene starting composition comprises between about 0.02 to about 0.04%, about 0.04 to about 0.06%, about 0.06 to 0.08%, about 0.08 to 0.10%, or about 0.10 to about 0.12% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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 starting composition.

[0291] In some embodiments, the highly pure isoprene starting composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the starting composition that inhibits the polymerization of isoprene. In some embodiments, the starting 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 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the starting composition that inhibits the polymerization of isoprene. In some embodiments, the starting 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 μg/L of a hydrocarbon other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne). In some embodiments, the starting 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 μg/L of a hydrocarbon other than isoprene. In some embodiments, the starting 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 μg/L of a protein or fatty acid (such as a protein or fatty acid that is naturally associated with natural rubber).

[0292] In some embodiments, the highly pure isoprene starting 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 starting 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 starting isoprene composition comprises less than or about 30, 20, 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of all acetylenes (such as 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, and *cis*-pent-3-ene-1-yne). In some embodiments, the starting isoprene composition comprises less than or about 2000, 1000, 500, 200, 100, 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of isoprene dimers, such as cyclic isoprene dimers (*e.g.*, cyclic C10 compounds derived from the dimerization of two isoprene units).

[0293] In some embodiments, the highly pure isoprene starting composition includes ethanol, acetone, methanol, acetaldehyde, methacrolein, methyl vinyl ketone, 2-methyl-2-vinyloxirane, *cis*- and *trans*-3-methyl-1,3-pentadiene, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the starting isoprene composition comprises greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 μg/L of ethanol, acetone, methanol, acetaldehyde, methacrolein, methyl vinyl ketone, 2-methyl-2-vinyloxirane, *cis*- and *trans*-3-methyl-1,3-pentadiene, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In some embodiments, the isoprene composition comprises

between about 0.005 to about 120, such as about 0.01 to about 80, about 0.01 to about 60, about 0.01 to about 40, about 0.01 to about 30, about 0.01 to about 20, about 0.01 to about 10, about 0.1 to about 80, about 0.1 to about 60, about 0.1 to about 40, about 5 to about 80, about 5 to about 60, or about 5 to about 40 μg/L of ethanol, acetone, methanol, acetaldehyde, methacrolein, methyl vinyl ketone, 2-methyl-2-vinyloxirane, *cis*- and *trans*-3-methyl-1,3-pentadiene, a C5 prenyl alcohol, or any two or more of the foregoing.

[0294] In some embodiments, the highly pure isoprene starting composition includes one or more of the following components: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In various embodiments, the amount of one of these components relative to amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w). In some embodiments, the relative detector response for the second compound compared to the detector response for isoprene is greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110%. In various embodiments, the amount of one of these components relative to amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is between about 0.01 to about 105 % (w/w), such as about 0.01 to about 90, about 0.01 to about 80, about 0.01 to about 50, about 0.01 to about 20, about 0.01 to about 10, about 0.02 to about 50, about 0.05 to about 50, about 0.1 to about 50, or 0.1 to about 20% (w/w).

[0295] In some embodiments, at least a portion of the highly pure isoprene starting composition is in a gas phase. In some embodiments, at least a portion of the highly pure isoprene starting composition is in a liquid phase (such as a condensate). In some embodiments, at least a portion of the highly pure isoprene starting composition is in a solid phase. In some embodiments, at least a portion of the highly pure isoprene starting composition is absorbed to a

solid support, such as a support that includes silica and/or activated carbon. In some embodiments, the starting isoprene composition is mixed with one or more solvents. In some embodiments, the starting isoprene composition is mixed with one or more gases.

[0296] In other embodiments, the commercially beneficial amount of highly pure isoprene starting composition is produced by a biological process. In some preferred embodiments, the highly pure isoprene starting composition is a bioisoprene composition produced by culturing cells 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 one embodiment, the bioisoprene composition is produced by culturing cells that convert more than about 0.002% of the carbon in a cell culture medium into isoprene. In other embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide, e.g. a naturally-occurring polypeptide from a plant such as *Pueraria*, and (ii) is operably linked to a promoter, e.g. a T7 promoter. Other isoprene synthase polypeptides, for example, from poplar and variants of naturally-occurring as well as parent isoprene synthase, can be used to produce bioisoprene. Examples of isoprene synthase and its variants that can be used are described in U.S. Appl. No. 12/429,143, which is incorporated herein in its entirety.

[0297] 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. In some embodiment, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding an MDV pathway polypeptide. In some embodiment, the starting isoprene composition is an isoprene composition as descried or is produced by culturing any of the cells described in U.S. provisional patent application Nos. 61/134,094, filed on July 2, 2008, WO 2010/003007, and U.S. patent application No. 12/335,071, filed December 15, 2008 (US 2009/0203102 A1), which are incorporated by reference in their entireties.

[0298] In some embodiments, the highly pure isoprene starting composition comprises a gas phase (off-gas) produced by cells in culture that produces isoprene. In some embodiments, the gas phase has a nonflammable concentration of isoprene. 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.

[0299] In some embodiments, the highly pure isoprene starting composition includes one or more of the following: an alcohol, an aldehyde, a ketone, or an ester (such as any of the alcohols, aldehydes, ketones or esters described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone. In some embodiments, any of the isoprene compositions further includes an ester.

[0300] In some embodiments, the highly pure isoprene starting composition derived from a biological source (such as a cell culture) contains one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole. In some embodiments, the starting isoprene composition contains 1 ppm or more of one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole. In some embodiments, the concentration of more of one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole, is between about 1 to about 10,000 ppm in a starting isoprene composition (such as off-gas before it is purified). In some embodiments, the starting isoprene composition (such as off-gas after it has undergone one or more purification steps) includes one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole, at a concentration between about 1 to about 100 ppm, such as about 1 to about 10 ppm, about 10 to about 20 ppm, about 20 to about 30 ppm, about 30 to about 40 ppm, about 40 to about 50 ppm, about 50 to about 60 ppm, about 60 to about 70 ppm, about 70 to about 80 ppm, about 80 to about 90 ppm, or about 90 to about 100 ppm. In some embodiments, the starting isoprene composition contains less than 1 ppm of methanethiol (a potent catalyst poison and a source of sulfur in the final fuel product) Volatile organic compounds from cell cultures (such as volatile organic compounds in the headspace of cell cultures) can be analyzed

using standard methods such as those described herein or other standard methods such as proton transfer reaction-mass spectrometry (*see*, for example, Bunge *et al.*, *Applied and Environmental Microbiology*, 74(7):2179-2186, 2008 which is hereby incorporated by reference in its entirety, particular with respect to the analysis of volatile organic compounds).

[0301] The invention also contemplates the use of highly pure isoprene starting composition that is derived from a biological source (such as a cell culture) the co-produces isoprene and hydrogen. In some embodiments, the starting bioisoprene compositions comprise isoprene and hydrogen in ratios ranging from at least one molar percent of isoprene for every three molar percent of hydrogen to at least one molar percent of isoprene for every four molar percent of hydrogen. In some embodiments, the starting bioisoprene compositions comprise isoprene and hydrogen in molar ratios of about 1 to 9, 2 to 8, 3 to 7, 4 to 6, 5 to 5, 6 to 4, 7 to 3, 8 to 2, or 9 to 1. In some embodiments, the composition further comprises from 1 to 11 molar percent isoprene and from 4 to 44 molar percent hydrogen. In some embodiments, the composition further comprises oxygen, carbon dioxide, or nitrogen. In some embodiments, the composition further comprises from 0 to 21 molar percent oxygen, from 18 to 44 molar percent carbon dioxide, and from 0 to 78 molar percent nitrogen. In some embodiments, the composition further comprises 1.0×10^{-4} molar percent or less of non-methane volatile impurities. In some embodiments, the non-methane volatile impurities comprise one or more of the following: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In some embodiments, the non-methane volatile impurities comprise one or more of the following: the isoprene composition includes one or more of the following: an alcohol, an aldehyde, or a ketone (such as any of the alcohols, aldehydes, or ketones described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone. In some embodiments, the non-methane volatile impurities comprise one or more of the

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

[0302] Techniques for producing isoprene in cultures of cells that produce isoprene are described in U.S. provisional patent application Nos. 61/013,386 and 61/013,574, filed on December 13, 2007, WO 2009/076676, U.S. provisional patent application Nos. 61/134,094, 61/134,947, 61/134,011 and 61/134,103, filed on July 2, 2008, WO 2010/003007, U.S. provisional patent application No. 61/097,163, filed on September 15, 2008, WO 2010/031079, U.S. provisional patent application No. 61/097,186, filed on September 15, 2008, WO 2010/031062, U.S. provisional patent application No. 61/097,189, filed on September 15, 2008, WO 2010/031077, U.S. provisional patent application No. 61/097,200, filed on September 15, 2008, WO 2010/031068, U.S. provisional patent application No. 61/097,204, filed on September 15, 2008, WO 2010/031076, U.S. provisional patent application No.61/141,652, filed on December 30, 2008, PCT/US09/069862, U.S. patent application No. 12/335,071, filed December 15, 2008 (US 2009/0203102 A1) and U.S. patent application No. 12/429,143, filed April 23, 2009 (US 2010/0003716 A1), the teachings of which are incorporated herein by reference for the purpose of teaching techniques for producing and recovering isoprene by such a process. In any case, U.S. provisional patent application Nos. 61/013,386 and 61/013,574, filed on December 13, 2007, WO 2009/076676, U.S. provisional patent application Nos. 61/134,094, 61/134,947, 61/134,011 and 61/134,103, filed on July 2, 2008, WO 2010/003007, U.S. provisional patent application No. 61/097,163, filed on September 15, 2008, WO 2010/031079, U.S. provisional patent application No. 61/097,186, filed on September 15, 2008, WO 2010/031062, U.S. provisional patent application No. 61/097,189, filed on September 15, 2008, WO 2010/031077, U.S. provisional patent application No. 61/097,200, filed on September 15, 2008, WO 2010/031068, U.S. provisional patent application No. 61/097,204, filed on September 15, 2008, WO 2010/031076, U.S. provisional patent application No.61/141,652, filed on December 30, 2008, PCT/US09/069862, U.S. patent application No. 12/335,071, filed December 15, 2008 (US 2009/0203102 A1) and U.S. patent application No. 12/429,143, filed April 23, 2009 (US 2010/0003716 A1) teach compositions and methods for the production of increased amounts of isoprene in cell cultures. U.S. patent application No. 12/335,071, filed December 15, 2008 and US 2009/0203102 A1 further teaches compositions and methods for co-production of isoprene and hydrogen from cultured cells. In particular, these compositions and methods compositions and methods increase the rate of isoprene production and increase the total amount of isoprene that is produced. For example, cell culture systems that generate 4.8 x

10^4 nmole/g_{wcm}/hr of isoprene have been produced (Table 1). The efficiency of these systems is demonstrated by the conversion of about 2.2% of the carbon that the cells consume from a cell culture medium into isoprene. As shown in the Examples and Table 2, approximately 3 g of isoprene per liter of broth was generated. If desired, even greater amounts of isoprene can be obtained using other conditions, such as those described herein. In some embodiments, a renewable carbon source is used for the production of isoprene. In some embodiments, the production of isoprene is decoupled from the growth of the cells. In some embodiments, the concentrations of isoprene and any oxidants are within the nonflammable ranges to reduce or eliminate the risk that a fire may occur during production or recovery of isoprene. The compositions and methods 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 products such as rubber and provides a desirable, low-cost alternative to using natural rubber.

[0303] As discussed further below, the amount of isoprene produced by cells can be greatly increased by introducing a heterologous nucleic acid encoding an isoprene synthase polypeptide (*e.g.*, a plant isoprene synthase polypeptide) into the cells. Isoprene synthase polypeptides convert dimethyl allyl diphosphate (DMAPP) into isoprene. As shown in the Examples, a heterologous *Pueraria Montana* (kudzu) isoprene synthase polypeptide was expressed in a variety of host cells, such as *Escherichia coli, Panteoa citrea, Bacillus subtilis, Yarrowia lipolytica,* and *Trichoderma reesei.* All of these cells produced more isoprene than the corresponding cells without the heterologous isoprene synthase polypeptide. As illustrated in Tables 1 and 2, large amounts of isoprene are produced using the methods described herein. For example, *B. subtilis* cells with a heterologous isoprene synthase nucleic acid produced approximately 10-fold more isoprene in a 14 liter fermentor than the corresponding control *B. subtilis* cells without the heterologous nucleic acid (Table 2). The production of 300 mg of isoprene per liter of broth (mg/L, wherein the volume of broth includes both the volume of the cell medium and the volume of the cells) by *E. coli* and 30 mg/L by *B. subtilis* in fermentors indicates that significant amounts of isoprene can be generated (Table 2). If desired, isoprene can be produced on an even larger scale or other conditions described herein can be used to further increase the amount of isoprene. The vectors listed in Tables 1 and 2 and the experimental conditions are described in further detail below and in the Examples section.

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

Strain	Isoprene Production in a Headspace vial*	
	Headspace concentration $\mu g/L_{gas}$	**Specific Rate** $\mu g/L_{broth}/hr/OD$ $(nmol/g_{wcm}/hr)$
E. coli BL21/ pTrcKudzu IS	1.40	53.2 (781.2)
E. coli BL21/ pCL DXS yidi Kudzu IS	7.61	289.1 (4.25×10^3)
E. coli BL21/MCM127 with kudzu IS and entire MVA pathway	23.0	874.1 (1.28×10^4)
E. coli BL21/ pET N-HisKudzu IS	1.49	56.6 (831.1)
Pantoea citrea /pTrcKudzu IS	0.66	25.1 (368.6)
E. coli w/ Poplar IS [Miller (2001)]	-	5.6 (82.2)
Bacillis licheniformis Fall US 5849970	-	4.2 (61.4)
Yarrowia lipolytica with kudzu isoprene synthase	~0.05 µg/L	~2 (~30)
Trichoderma reesei with kudzu isoprene synthase	~0.05 µg/L	~2 (~30)
E. coli BL21/ pTrcKKD$_y$I$_k$IS with	85.9	3.2×10^3

Strain	Isoprene Production in a Headspace vial*	
kudzu IS and lower MVA pathway		(4.8×10^4)

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

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

Strain	Isoprene Production in Fermentors		
	Peak Headspace concentration ($\mu g/L_{gas}$)**	**Titer (mg/L_{broth})**	**Peak Specific rate $\mu g/L_{broth}/hr/OD$ ($nmol/g_{wcm}/hr$)**
E. coli BL21 /pTrcKudzu with Kudzu IS	52	41.2	37 (543.3)
E. coli FM5/pTrcKudzu IS	3	3.5	21.4 (308.1)
E. coli BL21/ triple strain (DXS, yidi, IS)	285	300	240 (3.52×10^3)
E. coli FM5/ triple strain (DXS, yidi, IS)	50.8	29	180.8 (2.65×10^3)
E. coli/MCM127 with Kudzu IS and entire MVA pathway	3815	3044	992.5 (1.46×10^4)
E. coli BL21/pCLPtrc Upper Pathway gi1.2 integrated lower pathway pTrcKudzu	2418	1640	1248 (1.83×10^4)
E. coli BL21/MCM401 with 4 x 50 μM IPTG	13991	23805	3733 (5.49×10^4)
E. coli BL21/MCM401 with 2 x 1000 μM IPTG	22375	19541	5839.5 (8.59×10^4)

Strain	Isoprene Production in Fermentors		
E. coli BL21/pCLPtrc UpperPathwayHGS2 - pTrcKKDyIkIS	3500	3300	1088 (1.60×10^4)
Bacillus subtilis wild-type	1.5	2.5	0.8 (11.7)
Bacillus pBS Kudzu IS	16.6	~30 (over 100 hrs)	5 (73.4)
Bacillus Marburg 6051 [Wagner and Fall (1999)]	2.04	0.61	24.5 (359.8)
Bacillus Marburg 6051 Fall US 5849970	0.7	0.15	6.8 (100)
E. coli BL21/pCLPtrcUpperPathway and gil.2KKDyI and pTrcAlba-mMVK	2.03×10^4	3.22×10^4	5.9×10^3 (8.66×10^4)
E. coli BL21/pCLPtrcUpper Pathway and gi1.2KKDyI and pTrcAlba-mMVK plus pBBRCMPGI1.5pgl	3.22×10^4	6.05×10^4	1.28×10^4 (1.88×10^5)

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

[0304] Additionally, isoprene production by cells that contain a heterologous isoprene synthase nucleic acid can be enhanced by increasing the amount of a 1-deoxy-D-xylulose-5-phosphate synthase (DXS) polypeptide and/or an isopentenyl diphosphate isomerase (IDI) polypeptide expressed by the cells. For example, a DXS nucleic acid and/or an IDI nucleic acid can be introduced into the cells. The DXS nucleic acid may be a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid. Similarly, the IDI nucleic acid may be a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid. In some embodiments, the amount of DXS and/or IDI polypeptide is increased by replacing the endogenous DXS and/or IDI promoters or regulatory regions with other promoters and/or regulatory regions that result in greater transcription of the DXS and/or IDI nucleic acids. In

some embodiments, the cells contain both a heterologous nucleic acid encoding an isoprene synthase polypeptide (*e.g.*, a plant isoprene synthase nucleic acid) and a duplicate copy of an endogenous nucleic acid encoding an isoprene synthase polypeptide.

[0305] The encoded DXS and IDI polypeptides are part of the DXP pathway for the biosynthesis of isoprene (Figure 19A). DXS polypeptides convert pyruvate and D-glyceraldehyde-3-phosphate into 1-deoxy-D-xylulose-5-phosphate. While not intending to be bound by any particular theory, it is believed that increasing the amount of DXS polypeptide increases the flow of carbon through the DXP pathway, leading to greater isoprene production. IDI polypeptides catalyze the interconversion of isopentenyl diphosphate (IPP) and dimethyl allyl 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.

[0306] For example, fermentation of *E. coli* cells with a kudzu isoprene synthase, *S. cerevisia* IDI, and *E. coli* DXS nucleic acids was used to produce isoprene. The levels of isoprene varied from 50 to 300 μg/L over a time period of 15 hours (Example 7, part VII).

[0307] In some embodiments, the presence of heterologous or extra endogenous isoprene synthase, IDI, and DXS nucleic acids causes cells to grow more reproducibly or remain viable for longer compared to the corresponding cell with only one or two of these heterologous or extra endogenous nucleic acids. For example, cells containing heterologous isoprene synthase, IDI, and DXS nucleic acids grew better than cells with only heterologous isoprene synthase and DXS nucleic acids or with only a heterologous isoprene synthase nucleic acid. Also, heterologous isoprene synthase, IDI, and DXS nucleic acids were successfully operably linked to a strong promoter on a high copy plasmid that was maintained by *E. coli* cells, suggesting that large amounts of these polypeptides could be expressed in the cells without causing an excessive amount of toxicity to the cells. While not intending to be bound to a particular theory, it is believed that the presence of heterologous or extra endogenous isoprene synthase and IDI nucleic acids may reduce the amount of one or more potentially toxic intermediates that would otherwise accumulate if only a heterologous or extra endogenous DXS nucleic acid was present in the cells.

[0308] In some embodiments, the production of isoprene by cells that contain a heterologous isoprene synthase nucleic acid is augmented by increasing the amount of a MVA polypeptide expressed by the cells (Figures 19A and 19B). Exemplary MVA pathways polypeptides include any of the following polypeptides: acetyl-CoA acetyltransferase (AA-CoA thiolase)

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

[0309] For example, *E. coli* cells containing a nucleic acid encoding a kudzu isoprene synthase polypeptide and nucleic acids encoding *Saccharomyces cerevisiae* MVK, PMK, MVD, and IDI polypeptides generated isoprene at a rate of 6.67 x 10^{-4} mol/L_{broth}/OD_{600}/hr (*see* Example 8). Additionally, a 14 liter fermentation of *E. coli* cells with nucleic acids encoding *Enterococcus faecalis* AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase polypeptides produced 22 grams of mevalonic acid (an intermediate of the MVA pathway). A shake flask of these cells produced 2-4 grams of mevalonic acid per liter. These results indicate that heterologous MVA pathways nucleic acids are active in *E. coli*. *E. coli* cells that contain nucleic acids for both the upper MVA pathway and the lower MVA pathway as well as a kudzu isoprene synthase (strain MCM 127) produced significantly more isoprene (874 μg/L) compared to *E.*

coli cells with nucleic acids for only the lower MVA pathway and the kudzu isoprene synthase (strain MCM 131) (*see* Table 3 and Example 8, part VIII).

[0310] In some embodiments, at least a portion of the cells maintain the heterologous isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid for at least about 5, 10, 20, 50, 75, 100, 200, 300, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects described herein, the nucleic acid comprising the heterologous or duplicate copy of an endogenous isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid also comprises a selective marker, such as a kanamycin, ampicillin, carbenicillin, gentamicin, hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol antibiotic resistance nucleic acid.

[0311] As indicated in Example 7, part VI, the amount of isoprene produced can be further increased by adding yeast extract to the cell culture medium. In this example, the amount of isoprene produced was linearly proportional to the amount of yeast extract in the cell medium for the concentrations tested (Figure 48C). Additionally, approximately 0.11 grams of isoprene per liter of broth was produced from a cell medium with yeast extract and glucose (Example 7, part VIII). Both of these experiments used *E. coli* cells with kudzu isoprene synthase, *S. cerevisia* IDI, and *E. coli* DXS nucleic acids to produce isoprene. Increasing the amount of yeast extract in the presence of glucose resulted in more isoprene being produced than increasing the amount of glucose in the presence of yeast extract. Also, increasing the amount of yeast extract allowed the cells to produce a high level of isoprene for a longer length of time and improved the health of the cells.

[0312] Isoprene production was also demonstrated using three types of hydrolyzed biomass (bagasse, corn stover, and soft wood pulp) as the carbon source (Figures 46A-C). *E. coli* cells with kudzu isoprene synthase, *S. cerevisia* IDI, and *E. coli* DXS nucleic acids produced as much isoprene from these hydrolyzed biomass carbon sources as from the equivalent amount of glucose (*e.g.*, 1% glucose, w/v). If desired, any other biomass carbon source can be used in the compositions and methods described herein. Biomass carbon sources are desirable because they are cheaper than many conventional cell mediums, thereby facilitating the economical production of isoprene.

[0313] Additionally, invert sugar was shown to function as a carbon source for the generation of isoprene (Figures 47C and 96-98). For example, 2.4 g/L of isoprene was produced from cells expressing MVA pathway polypeptides and a Kudzu isoprene synthase (Example 8, part XV). Glycerol was as also used as a carbon source for the generation of 2.2 mg/L of isoprene from

cells expressing a Kudzu isoprene synthase (Example 8, part XIV). Expressing a DXS nucleic acid, an IDI nucleic acid, and/or one or more MVA pathway nucleic acids (such as nucleic acids encoding the entire MVA pathway) in addition to an isoprene synthase nucleic acid may increase the production of isoprene from glycerol.

[0314] In some embodiments, an oil is included in the cell medium. For example, *B. subtilis* cells containing a kudzu isoprene synthase nucleic acid produced isoprene when cultured in a cell medium containing an oil and a source of glucose (Example 4, part III). As another example, *E. coli* fadR atoC mutant cells containing the upper and lower MVA pathway plus kudzu isoprene synthase produced isoprene when cultured in a cell medium containing palm oil and a source of glucose (Example 27, part II). In some embodiments, more than one oil (such as 2, 3, 4, 5, or more oils) is included in the cell medium. While not intending to be bound to any particular theory, it is believed that (i) the oil may increase the amount of carbon in the cells that is available for conversion to isoprene, (ii) the oil may increase the amount of acetyl-CoA in the cells, thereby increasing the carbon flow through the MVA pathway, and/or (ii) the oil may provide extra nutrients to the cells, which is desirable since much of the carbon in the cells is converted to isoprene rather than other products. In some embodiments, cells that are cultured in a cell medium containing oil naturally use the MVA pathway to produce isoprene or are genetically modified to contain nucleic acids for the entire MVA pathway. In some embodiments, the oil is partially or completely hydrolyzed before being added to the cell culture medium to facilitate the use of the oil by the host cells.

[0315] One of the major hurdles to commercial production of small molecules such as isoprene in cells (*e.g.*, bacteria) is the decoupling of production of the molecule from growth of the cells. In some embodiments for the commercially viable production of isoprene, a significant amount of the carbon from the feedstock is converted to isoprene, rather than to the growth and maintenance of the cells ("carbon efficiency"). In various embodiments, the cells convert greater than or about 0.0015, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, or 8.0% of the carbon in the cell culture medium into isoprene. In particular embodiments, a significant portion of the carbon from the feedstock that is converted to downstream products is converted to isoprene. As described further in Example 11, *E. coli* cells expressing MVA pathway and kudzu isoprene synthase nucleic acids exhibited decoupling of the production of isoprene or the intermediate mevalonic acid from growth, resulting in high carbon efficiency. In particular, mevalonic acid was formed from cells expressing the upper MVA pathway from *Enterococcus faecalis*.

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

Exemplary Polypeptides and Nucleic Acids

[0316] Various isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptides and nucleic acids can be used in the compositions and methods described herein.

[0317] In some embodiments, the fusion polypeptide includes part or all of a first polypeptide (*e.g.*, an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide or catalytically active fragment thereof) and may optionally include part or all of a second polypeptide (*e.g.*, a peptide that facilitates purification or detection of the fusion polypeptide, such as a His-tag). In some embodiments, the fusion polypeptide has an activity of two or more MVA pathway polypeptides (such as AA-CoA thiolase and HMG-CoA reductase polypeptides). In some embodiments, the polypeptide is a naturally-occurring polypeptide (such as the polypeptide encoded by an *Enterococcus faecalis mvaE* nucleic acid) that has an activity of two or more MVA pathway polypeptides.

[0318] In various embodiments, a polypeptide has at least or about 50, 100, 150, 175, 200, 250, 300, 350, 400, or more amino acids. In some embodiments, the polypeptide fragment contains at least or about 25, 50, 75, 100, 150, 200, 300, or more contiguous amino acids from a full-length polypeptide and has at least or about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% of an activity of a corresponding full-length polypeptide. In particular embodiments, the polypeptide includes a segment of or the entire amino acid sequence of any naturally-occurring isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide. In some embodiments, the polypeptide has one or more mutations compared to the sequence of a wild-type (*i.e.*, a sequence occurring in nature) isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide.

[0319] In some embodiments, the polypeptide is an isolated polypeptide. In some embodiments, the polypeptide is a heterologous polypeptide.

[0320] In some embodiments, the nucleic acid is a recombinant nucleic acid. In some embodiments, an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid is operably linked to another nucleic acid encoding all or a portion of another polypeptide such that the recombinant nucleic acid encodes a fusion polypeptide that includes an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide and all or part of another polypeptide (*e.g.*, a peptide that facilitates purification or detection of the fusion polypeptide, such as a His-tag). In some embodiments, part or all of a recombinant nucleic acid is chemically synthesized. It is to be understood that mutations, including single nucleotide mutations, can occur within a nucleic acid as defined herein.

[0321] In some embodiments, the nucleic acid is a heterologous nucleic acid. In particular embodiments, the nucleic acid includes a segment of or the entire nucleic acid sequence of any naturally-occurring isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid. In some embodiments, the nucleic acid includes at least or about 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, or more contiguous nucleotides from a naturally-occurring isoprene synthase nucleic acid DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid. In some embodiments, the nucleic acid has one or more mutations compared to the sequence of a wild-type (*i.e.*, a sequence occurring in nature) isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid. In some embodiments, the nucleic acid has one or more mutations (*e.g.*, a silent mutation) that increase the transcription or translation of isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, or transcription factor nucleic acid. In some embodiments, the nucleic acid is a degenerate variant of any nucleic acid encoding an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide.

[0322] The accession numbers of exemplary isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids are listed in Appendix 1 (the accession numbers of Appendix 1 and their corresponding sequences are herein incorporated by reference in their entireties, particularly with respect to the amino acid and nucleic acid sequences of isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids). The Kegg database also contains the amino acid and nucleic acid sequences of numerous exemplary isoprene

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

Exemplary Isoprene Synthase Polypeptides and Nucleic Acids

[0323] As noted above, isoprene synthase polypeptides convert dimethyl allyl 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 re-suspended in 5 ml of PEB without glycerol. The cells are lysed using a French Pressure cell using standard procedures. Alternatively the cells are treated with lysozyme (Ready-Lyse lysozyme solution; EpiCentre) after a freeze/thaw at -80 °C.

[0324] 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 re-hydrated 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.

[0325] 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.

[0326] In some embodiments, the isoprene synthase polypeptide or nucleic acid is from the family Fabaceae, such as the Faboideae subfamily. In some embodiments, the isoprene synthase polypeptide or nucleic acid is a polypeptide or nucleic acid from *Pueraria montana* (kudzu) (Sharkey *et al.*, Plant Physiology 137: 700-712, 2005), *Pueraria lobata*, poplar (such as *Populus alba*, *Populus nigra*, *Populus trichocarpa*, or *Populus alba x tremula* (CAC35696) Miller *et al.*, Planta 213: 483-487, 2001) aspen (such as *Populus tremuloides*) Silver *et al.*, JBC 270(22): 13010-1316, 1995), or English Oak (*Quercus robur*) (Zimmer *et al.*, WO 98/02550), which are each hereby incorporated by reference in their entireties, particularly with respect to isoprene synthase nucleic acids and the expression of isoprene synthase polypeptides. Suitable isoprene synthases include, but are not limited to, those identified by Genbank Accession Nos. AY341431, AY316691, AY279379, AJ457070, and AY182241, which are each hereby incorporated by reference in their entireties, particularly with respect to sequences of isoprene synthase nucleic acids and polypeptides. In some embodiments, the isoprene synthase polypeptide or nucleic acid is not a naturally-occurring polypeptide or nucleic acid from *Quercus robur* (*i.e.*, the isoprene synthase polypeptide or nucleic acid is an isoprene synthase polypeptide or nucleic acid other than a naturally-occurring polypeptide or nucleic acid from *Quercus robur*). In some embodiments, the isoprene synthase nucleic acid or polypeptide is a naturally-occurring polypeptide or nucleic acid from poplar. In some embodiments, the isoprene synthase nucleic acid or polypeptide is not a naturally-occurring polypeptide or nucleic acid from poplar.

Exemplary DXS Polypeptides and Nucleic Acids

[0327] As noted above, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) polypeptides convert pyruvate and D-glyceraldehyde-3-phosphate into 1-deoxy-D-xylulose-5-phosphate. Exemplary DXS polypeptides include polypeptides, fragments of polypeptides, peptides, and

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

Exemplary IDI Polypeptides and Nucleic Acids

[0328] Isopentenyl diphosphate isomerase polypeptides (isopentenyl-diphosphate delta-isomerase or IDI) catalyses the interconversion of isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP) (*e.g.*, converting IPP into DMAPP and/or converting DMAPP into IPP). Exemplary IDI polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of an IDI polypeptide. Standard methods (such as those described herein) can be used to determine whether a polypeptide has IDI polypeptide activity by measuring the ability of the polypeptide to interconvert IPP and DMAPP *in vitro*, in a cell extract, or *in vivo*. Exemplary IDI nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of an IDI polypeptide. Exemplary IDI polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

Exemplary MVA Pathway Polypeptides and Nucleic Acids

[0329] 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, diphosphomevalonate decarboxylase (MVD) polypeptides, phosphomevalonate decarboxylase (PMDC) polypeptides, isopentenyl phosphate kinase (IPK) polypeptides, IDI polypeptides, and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more MVA pathway polypeptides. In particular, MVA pathway polypeptides include polypeptides, fragments of

polypeptides, peptides, and fusions polypeptides that have at least one activity of an MVA pathway polypeptide. Exemplary MVA pathway nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of an MVA pathway polypeptide. Exemplary MVA pathway polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

[0330] In particular, acetyl-CoA acetyltransferase polypeptides (AA-CoA thiolase or AACT) convert two molecules of acetyl-CoA into acetoacetyl-CoA. Standard methods (such as those described herein) can be used to determine whether a polypeptide has AA-CoA thiolase polypeptide activity by measuring the ability of the polypeptide to convert two molecules of acetyl-CoA into acetoacetyl-CoA *in vitro*, in a cell extract, or *in vivo*.

[0331] 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase or HMGS) polypeptides convert acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA. Standard methods (such as those described herein) can be used to determine whether a polypeptide has HMG-CoA synthase polypeptide activity by measuring the ability of the polypeptide to convert acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA *in vitro*, in a cell extract, or *in vivo*.

[0332] 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase or HMGR) polypeptides convert 3-hydroxy-3-methylglutaryl-CoA into mevalonate. Standard methods (such as those described herein) can be used to determine whether a polypeptide has HMG-CoA reductase polypeptide activity by measuring the ability of the polypeptide to convert 3-hydroxy-3-methylglutaryl-CoA into mevalonate *in vitro*, in a cell extract, or *in vivo*.

[0333] Mevalonate kinase (MVK) polypeptides phosphorylates mevalonate to form mevalonate-5-phosphate. Standard methods (such as those described herein) can be used to determine whether a polypeptide has MVK polypeptide activity by measuring the ability of the polypeptide to convert mevalonate into mevalonate-5-phosphate *in vitro*, in a cell extract, or *in vivo*.

[0334] Phosphomevalonate kinase (PMK) polypeptides phosphorylates mevalonate-5-phosphate to form mevalonate-5-diphosphate. Standard methods (such as those described herein) can be used to determine whether a polypeptide has PMK polypeptide activity by measuring the ability of the polypeptide to convert mevalonate-5-phosphate into mevalonate-5-diphosphate *in vitro*, in a cell extract, or *in vivo*.

[0335] Diphosphomevalonate decarboxylase (MVD or DPMDC) polypeptides convert mevalonate-5-diphosphate into isopentenyl diphosphate (IPP). Standard methods (such as those described herein) can be used to determine whether a polypeptide has MVD polypeptide activity by measuring the ability of the polypeptide to convert mevalonate-5-diphosphate into IPP *in vitro*, in a cell extract, or *in vivo*.

[0336] Phosphomevalonate decarboxylase (PMDC) polypeptides convert mevalonate-5-phosphate into isopentenyl phosphate (IP). Standard methods (such as those described herein) can be used to determine whether a polypeptide has PMDC polypeptide activity by measuring the ability of the polypeptide to convert mevalonate-5-phosphate into IP *in vitro*, in a cell extract, or *in vivo*.

[0337] Isopentenyl phosphate kinase (IPK) polypeptides phosphorylate isopentyl phosphate (IP) to form isopentenyl diphosphate (IPP). Standard methods (such as those described herein) can be used to determine whether a polypeptide has IPK polypeptide activity by measuring the ability of the polypeptide to convert IP into IPP *in vitro*, in a cell extract, or *in vivo*.

[0338] Exemplary IDI polypeptides and nucleic acids are described above.

Exemplary Hydrogenase Polypeptides and Nucleic Acids

[0339] Hydrogenase polypeptides catalyze the reaction: $2H^+ + 2e^- \leftrightarrow H_2$. *In vitro* that reaction is reversible, but certain hydrogenases may work in only one direction *in vivo*, either oxidizing H_2 or reducing H^+. Hydrogenase polypeptides can be oxygen-sensitive, contain complex metal cofactors as part of their catalytic center and sometimes consist of multiple subunits, with hydrogenase gene expression sometimes involving additional accessory polypeptides, such as 'maturation' factors or transcription regulatory factors (*i.e.*, activators or repressors). Hydrogenases are classified into at least three broad groups based upon the type of metal cofactor in their catalytic center: (1) nickel-iron ("NiFe") hydrogenases have a nickel/iron cofactor; (2) iron-iron hydrogenases ("FeFe") have an iron/iron cofactor; and (3) iron/sulfur-free ("Fe") hydrogenases, which lack the 4Fe4S clusters found in groups (1) and (2), have an iron cofactor and a methenyl-tetrahydromethanopterin electron carrier. *See, e.g.*, Chung-Jung Chou et al., "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels," *Metabol. Eng.* 10:394-404 (2008), and Gönül Vardar-Schara et al., "Metabolically engineered bacteria for producing hydrogen via fermentation," *Microbial Biotechnol.* 1(2):107-125 (2008), both of which are incorporated herein by reference in their entireties, particularly with respect to

the various types and classes of hydrogenases. Although many organisms contain multiple hydrogenases, few contain genes for both NiFe and FeFe hydrogenases.

[0340] The catalytic center of NiFe hydrogenases consists of a nickel atom and an iron atom, each with two carbon monoxide (CO) and two cyanide (CN^-) ligands. The NiFe hydrogenases all comprise at least a second subunit containing multiple iron-sulfur (Fe-S) centers for the transfer of electrons to and from the catalytic center. The NiFe hydrogenases can be subdivided into four main classes: (1) respiratory enzymes, which are part of multi-enzyme systems that couple the oxidation of H_2 to reduction of terminal electron acceptors such as SO_4^{2-} or NO_3^- under anaerobic conditions, or to O_2 in aerobic microorganisms; (2) H_2 sensors, which activate expression of the metabolically active NiFe hydrogenases; (3) cytoplasmic hydrogenases, containing multiple subunits able to utilize $NADP^+$, which are readily reversible in *vitro*, but *in vivo* may only oxidize H_2; and (4) membrane-bound, energy-conserving multi-enzyme complexes also found in bacteria and Archaea. Chung-Jung Chou et al., "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels," *Metabol. Eng.* 10:394-404 (2008).

[0341] The catalytic center of FeFe hydrogenases contains a catalytic "H cluster" which coordinates a binuclear (FeFe) site bridged to a [4Fe-4S] center by a single protein (cysteine) ligand. The two iron atoms of the binuclear center each have two carbon monoxide (CO) and two cyanide (CN^-) ligands, and are also bridged by two sulfur atoms which are part of a small organic molecule. Most FeFe hydrogenases are monomeric enzymes of about 50 kilodaltons (kDa), and appear to function *in vivo* primarily to dispose of excess reducing equivalents by reducing protons to hydrogen gas. Chung-Jung Chou et al., "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels," *Metabol. Eng.* 10:394-404 (2008).

[0342] The catalytic center of Fe hydrogenases was originally thought to have an active site based on an organic cofactor with no metals involved, but was later shown to contain a mononuclear Fe atom. Despite the phylogenetic differences between the three types of hydrogenase, in addition to at least one iron atom, all three groups of hydrogenases also contain at least one carbon monoxide (CO) ligand to the iron atom in their active sites, which facilitates the catalytic oxidation of H_2 and the reduction of protons. Chung-Jung Chou et al., "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels," *Metabol. Eng.* 10:394-404 (2008).

[0343] Exemplary hydrogenase polypeptides include, but are not limited to, the *E. coli* hydrogenase-1 (Hyd-1) polypeptides, *E. coli* hydrogenase-2 (Hyd-2) polypeptides, *E. coli*

hydrogenase-3 (Hyd-3) polypeptides, *E. coli* hydrogenase-4 (Hyd-4) polypeptides, *E. coli* formate hydrogen lyase (FHL) complex, which produces hydrogen gas from formate and CO_2 under anaerobic conditions at acidic pH (*see, e.g.*, Akihito Yoshida et al., "Efficient induction of formate hydrogen lyase of aerobically grown *Escherichia coli* in a three-step biohydrogen production process," *Appl. Microbiol. Biotechnol.* 74:754-760 (2007), which is incorporated herein by reference in its entirety, particularly with respect to the induction of expression of formate hydrogen lyase in *E. coli*), *Ralstonia eutropha* H16 hydrogenase (*R. eutropha* HoxH) *Rhodococcus opacus MR11* hydrogenase (*R. opacus* HoxH) polypeptides, *Synechosystis* sp. PCC 6803 hydrogenase (*Syn.* PCC 6803 HoxH) polypeptides, *Desulfovibrio gigas* hydrogenase (*D. gigas*) polypeptides, and *Desulfovibrio desulfuricans* ATCC 7757 hydrogenase (*D. desulfuricans*) polypeptides (*see, e.g.*, Gönül Vardar-Schara et al., "Metabolically engineered bacteria for producing hydrogen via fermentation," *Microbial Biotechnol.* 1(2):107-125 (2008), which is incorporated herein by reference in its entirety, particularly with respect to the various types and classes of hydrogenases) and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more hydrogenase polypeptides. In particular, hydrogenase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusion polypeptides that have at least one activity of a hydrogenase polypeptide. Exemplary hydrogenase nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of a hydrogenase polypeptide, or at least one activity necessary for expression, processing, or maturation of a hydrogenase polypeptide. Exemplary hydrogenase polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

[0344] *E. coli* Hyd-3, which is part of the anaerobic formate hydrogen lyase (FHL) complex, is encoded by the *hyc* operon (comprising the *hycA*, *hycB*, *hycC*, *hycD*, *hycE*, *hycF*, *hycG*, *hycH*, and *hycI* genes). *E. coli* Hyd-4 is encoded by the *hyf* operon (comprising the *hyfA*, *hyfB*, *hyfC*, *hyfD*, *hyfE*, *hyfF*, *hyfG*, *hyfH*, *hyfI*, *hyfJ*, and *hyfR* genes). *E. coli* FHL is encoded by six genes from the *hyc* operon (*hycB*, *hycC*, *hycD*, *hycE*, *hycF* and *hycG*) and the *fdhF* gene (encoding formate dehydrogenase H (Fdh-H)). Expression of the FHL complex can further involve expression of pyruvate formate lyase (*pfl*), FhlA, a transcription factor that activates transcription of *fdhF* and the *hyc* operon, or deletion/inactivation of HycA, a transcription factor encoded by the *hycA* gene that negatively regulates transcription of FHL. Co-production of isoprene and hydrogen can be improved by expression or inactivation/deletion of additional

proteins involved in the regulation of gene expression for hydrogenases and other enzymes, such as, for example, iron-sulfur complex transcriptional regulator (*iscR*) (Kalim-Akhtar et al., "Deletion of iscR stimulates recombinant Clostridial Fe/Fe hydrogenase activity and H_2-accumulation in *Escherichia coli* BL21(DE3)," *Appl. Microbiol. Biotechnol.* 78:853–862 (2008), which is incorporated herein by reference in its entirety, particularly with reference to stimulation of Clostridial Fe/Fe hydrogenase activity and hydrogen accumulation in *E. coli* by deleting the *iscR* gene).

[0345] Exemplary ferredoxin-dependent hydrogenase polypeptides include, but are not limited to, *Clostridium acetobutulicum* hydrogenase A (HydA) (*see, e.g.*, P.W. King et al., "Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system," *J. Bacteriol.* 188(6):163-172 (2006), which is incorporated herein by reference in its entirety, particularly with respect to production of hydrogen by HydA and three HydA-associated maturation enzymes (HydE, HydG, and HydF), which may be expressed alone or in conjunction with one or more of: (1) *Bacillus subtilis* NADPH ferredoxin oxidoreductase (NFOR) (*see, e.g.*, Viet et al., (2008)), which is incorporated herein by reference in its entirety, particularly with respect to production of hydrogen by NFOR; *see also* PCT Publication No. WO/2007/089901, which is incorporated herein by reference in its entirety, particularly with respect to optimization of *E. coli* strains for production of hydrogen), *Clostridium kluyveri* NADH ferredoxin oxidoreductase (RnfCDGEAB) (Henning Seedorf et al., "The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features," *Proc. Nat'l Acad. Sci. U.S.A.* 105(6):2128-2133 (2008), which is incorporated herein by reference in its entirety, particular with reference to NADH ferredoxin oxidoreductase, and with reference to components of the anaerobic ethanol-acetate fermentation pathway), or *Clostridium pasteuranium* ferredoxin oxidoreductase (Fdx); (2) glyceraldehyde-6-phosphate ferredoxin oxidoreductase ("GAPOR"); or (3) pyruvate ferredoxin oxidoreductase ("POR"), and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more hydrogenase polypeptides or of one or more hydrogenase polypeptides and an activity of one or more ferredoxin-dependent oxidoreductases. In particular, ferredoxin-dependent hydrogenase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusion polypeptides that have at least one activity of a ferredoxin-dependent hydrogenase polypeptide.

[0346] Exemplary NADPH-dependent hydrogenase polypeptides include, but are not limited to thermophilic hydrogenase polypeptides such as *Pyrococcus furiosus* hydrogenase (*see, e.g.*, J. Woodward et al., "Enzymatic production of biohydrogen," *Nature* 405(6790):1014-1015

(2000)), and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more NADPH-dependent hydrogenase polypeptides. In particular, NADPH-dependent hydrogenase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusion polypeptides that have at least one activity of a NADPH-dependent hydrogenase polypeptide.

[0347] Exemplary oxygen-tolerant or oxygen-insensitive hydrogenases include, but are not limited to, *Rubrivivax gelatinosus* hydrogenase (*see, e.g.*, P.C. Maness et al., "Characterization of the oxygen tolerance of a hydrogenase linked to a carbon monoxide oxidation pathway in *Rubrivivax gelatinosus*," *Appl. Environ. Microbiol.* 68(6):2633-2636 (2002), which is incorporated herein by reference in its entirety, particularly with respect to *R. gelatinosus* hydrogenase), and *Ralstonia eutropha* hydrogenase polypeptides (*see, e.g.*, T. Burgdorf et al., "[NiFe]-hydrogenases of *Ralstonia eutropha* H16: modular enzymes for oxygen-tolerant biological hydrogen oxidation," *J. Mol. Microbiol. Biotechnol.* 10(2-4):181-196 (2005), which is incorporated herein by reference in its entirety, particularly with respect to *R. eutropha* hydrogenase polypeptides). Alternatively, heterologous nucleic acids encoding hydrogenase polypeptides can be mutagenized and screened for O_2-tolerance or O_2-insensitivity using standard methods and assays (*see, e.g.*, L.E. Nagy et al., "Application of gene-shuffling for the rapid generation of novel [FeFe]-hydrogenase libraries," *Biotechnol. Letts.* 29(3)421-430 (2007), which is incorporated herein by reference, particularly with respect to mutagenesis and screening for oxygen tolerant hydrogenase polypeptides).

[0348] Standard methods (such as those described herein) can be used to determine whether a polypeptide has hydrogenase activity by measuring the ability of the polypeptide to produce hydrogen gas *in vitro*, in a cell extract, or *in vivo*.

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

[0349] In addition to expressing or over-expressing heterologous or native hydrogenases in *E. coli*, co-production of isoprene and hydrogen can be improved by inactivation of anaerobic biosynthetic pathways, thereby blocking the carbon flow to a variety of metabolites (*i.e.*, fermentation side products) produced under oxygen-limited or anaerobic conditions, including, but not limited to, lactate, acetate, pyruvate, ethanol, succinate, and glycerol. Exemplary polypeptides involved in the production of fermentation side products include formate dehydrogenase N, alpha subunit (*fdnG*), formate dehydrogenase O, large subunit (*fdoG*), nitrate reductase (*narG*), formate transporter A (*focA*), formate transporter B (*focB*), pyruvate oxidase

(*poxB*), pyruvate dehydrogenase E1 component ackA/pta (*aceE*), alcohol dehydrogenase (*adhE*), fumarate reductase membrane protein (*frdC*), and lactate dehydrogenase (*ldh*A). *See, e.g.*, Toshinori Maeda et al., "Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli*," *Appl. Microbiol. Biotechnol.* 77(4):879-890 (2007), which is incorporated by reference in its entirety, particularly with respect to production of *E. coli* strains with modified glucose metabolism. Exemplary polypeptides involved in the regulation or expression of genes involved in the production of fermentation side products that may also be inactivated to improve co-production of isoprene and hydrogen include, but are not limited to, repressor of formate hydrogen lyase (*hycA*), fumarate reductase regulator (*fnr*), acetyl-coenzyme A synthetase (*acs*), and formate dehydrogenase regulatory protein (*hycA*), which regulates expression of the transcriptional regulator fhlA (formate hydrogen lyase transcriptional activator).

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

[0350] Exemplary polypeptides involved in hydrogen re-uptake that may also be inactivated to improve co-production of isoprene and hydrogen include, but are not limited to, *E. coli* hydrogenase-1 (Hyd-1) (*hya* operon) and *E. coli* hydrogenase-2 (Hyd-2) (*hyb* operon). *E. coli* Hyd-1 is encoded by the *hya* operon (comprising the *hyaA*, *hyaB*, *hyaC*, *hyaD*, *hyaE*, and *hyaF* genes). *E. coli* Hyd-2 is encoded by the *hyb* operon (comprising the *hybA*, *hybB*, *hybC*, *hybD*, *hybE*, *hybF*, *hybG*, and *hybO* genes).

Exemplary Methods for Isolating Nucleic Acids

[0351] Isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids can be isolated using standard methods. Methods of obtaining desired nucleic acids from a source organism of interest (such as a bacterial genome) are common and well known in the art of molecular biology (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to the isolation of nucleic acids of interest). For example, if the sequence of the nucleic acid is known (such as any of the known nucleic acids described herein), suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes complementary to the desired nucleic acid sequence. Once the sequence is isolated, the DNA may be amplified using standard primer directed amplification methods such as polymerase chain reaction (PCR) (U.S. Patent No. 4,683,202,

which is incorporated by reference in its entirety, particularly with respect to PCR methods) to obtain amounts of DNA suitable for transformation using appropriate vectors.

[0352] Alternatively, isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids (such as any isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids with a known nucleic acid sequence) can be chemically synthesized using standard methods.

[0353] Additional isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides and nucleic acids which may be suitable for use in the compositions and methods described herein can be identified using standard methods. For example, cosmid libraries of the chromosomal DNA of organisms known to produce isoprene naturally can be constructed in organisms such as *E. coli*, and then screened for isoprene production. In particular, cosmid libraries may be created where large segments of genomic DNA (35-45 kb) are packaged into vectors and used to transform appropriate hosts. Cosmid vectors are unique in being able to accommodate large quantities of DNA. Generally cosmid vectors have at least one copy of the cos DNA sequence which is needed for packaging and subsequent circularization of the heterologous DNA. In addition to the cos sequence, these vectors also contain an origin of replication such as ColEI and drug resistance markers such as a nucleic acid resistant to ampicillin or neomycin. Methods of using cosmid vectors for the transformation of suitable bacterial hosts are well described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to transformation methods.

[0354] 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.

[0355] Additional methods for obtaining isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids include screening a metagenomic library by assay (such as the headspace assay described herein) or by PCR using

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

[0356] Additionally, standard sequence alignment and/or structure prediction programs can be used to identify additional DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides and nucleic acids based on the similarity of their primary and/or predicted polypeptide secondary structure with that of known DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides and nucleic acids. Standard databases such as the swissprot-trembl database (world-wide web at "expasy.org", Swiss Institute of Bioinformatics Swiss-Prot group CMU - 1 rue Michel Servet CH-1211 Geneva 4, Switzerland} can also be used to identify isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription regulatory polypeptides and nucleic acids. The secondary and/or tertiary structure of an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptide can be predicted using the default settings of standard structure prediction programs, such as PredictProtein (630 West, 168 Street, BB217, New York, N.Y. 10032, USA). Alternatively, the actual secondary and/or tertiary structure of an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptide can be determined using standard methods. Additional isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids can also be identified by hybridization to probes generated from known isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids.

Exemplary Promoters and Vectors

[0357] Any of the isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids described herein can be included in one or more vectors. Accordingly, also described herein are vectors with one more nucleic acids encoding any of the isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides that are described herein. In some embodiments, the vector contains a nucleic acid under the control of an expression control sequence.

[0358] In some embodiments, the vector contains a selective marker or selectable marker. Markers useful in vector systems for transformation of *Trichoderma* are known in the art (*see, e.g.*, Finkelstein, Chapter 6 in Biotechnology of Filamentous Fungi, Finkelstein *et al., Eds.* Butterworth-Heinemann, Boston, MA, Chap. 6., 1992; and Kinghorn *et al.*, Applied Molecular Genetics of Filamentous Fungi, Blackie Academic and Professional, Chapman and Hall, London, 1992, which are each hereby incorporated by reference in their entireties, particularly with respect to selective markers). In some embodiments, the selective marker is the *amd*S nucleic acid, which encodes the enzyme acetamidase, allowing transformed cells to grow on acetamide as a nitrogen source. The use of an *A. nidulans amd*S nucleic acid as a selective marker is described in Kelley *et al.*, *EMBO J.* 4:475 – 479, 1985 and Penttila *et al.*, *Gene* 61:155-164, 1987 (which are each hereby incorporated by reference in their entireties, particularly with respect to selective markers). In some embodiments, an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation, or transcription regulatory nucleic acid integrates into a chromosome of the cells without a selective marker.

[0359] 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).

[0360] Promoters are well known in the art. Any promoter that functions in the host cell can be used for expression of an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid in the host cell. Initiation control regions or promoters, which are useful to drive expression of isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids in various host cells are numerous and familiar to those skilled in the art (*see*, for example, WO

2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to vectors for the expression of nucleic acids of interest). Virtually any promoter capable of driving these nucleic acids can be used 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*).

[0361] 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).

[0362] In various embodiments, an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is contained in a low copy plasmid (*e.g.*, a plasmid that is maintained at about 1 to about 4 copies per cell), medium copy plasmid (*e.g.*, a plasmid that is maintained at about 10 to about 15 copies per cell), or high copy plasmid (*e.g.*, a plasmid that is maintained at about 50 or more copies per cell). In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to a T7 promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid operably linked to a T7 promoter is contained in a medium or high copy plasmid. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to a Trc promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid operably linked to a Trc promoter is contained in a medium or high copy plasmid. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to a Lac promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA

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

[0363] 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.

[0364] In some embodiments, an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to a suitable promoter that shows transcriptional activity in a fungal host cell. The promoter may be derived from one or more nucleic acids encoding a polypeptide that is either endogenous or heterologous to the host cell. In some embodiments, the promoter is useful in a *Trichoderma* host. Suitable non-limiting examples of promoters include *cbh*1, *cbh*2, *egl*1, *egl*2, *pepA*, *hfb*1, *hfb*2, *xyn*1, and *amy*. In some embodiments, the promoter is one that is native to the host cell. For example, in some embodiments when *T. reesei* is the host, the promoter is a native *T. reesei* promoter. In

some embodiments, the promoter is *T. reesei cbh1*, which is an inducible promoter and has been deposited in GenBank under Accession No. D86235, which is incorporated by reference in its entirety, particularly with respect to promoters. In some embodiments, the promoter is one that is heterologous to the fungal host cell. Other examples of useful promoters include promoters from the genes of *A. awamori* and *A. niger* glucoamylase (glaA) (Nunberg *et al.*, *Mol. Cell Biol.* 4:2306-2315, 1984 and Boel *et al.*, *EMBO J.* 3:1581-1585, 1984, which are each hereby incorporated by reference in their entireties, particularly with respect to promoters); *Aspergillus niger* alpha amylases, *Aspergillus oryzae* TAKA amylase, *T. reesei xln*1, and the *T. reesei cellobiohydrolase* 1 (EP 137280, which is incorporated by reference in its entirety, particularly with respect to promoters).

[0365] 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.

[0366] In some embodiments, the promoter, coding, region, and terminator all originate from the isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid to be expressed. In some embodiments, the coding region for an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is inserted into a general-purpose expression vector such that it is under the transcriptional control of the expression construct promoter and terminator sequences. In some embodiments, genes or part thereof are inserted downstream of the strong *cbh*1 promoter.

[0367] An isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid can be incorporated into a vector, such as an expression vector, using standard techniques (Sambrook *et al.*, Molecular Cloning: A Laboratory

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

[0368] In some embodiments, it may be desirable to over-express isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids at levels far higher than currently found in naturally-occurring cells. This result may be accomplished by the selective cloning of the nucleic acids encoding those polypeptides into multicopy plasmids or placing those nucleic acids under a strong inducible or constitutive promoter. Methods for over-expressing desired polypeptides are common and well known in the art of molecular biology and examples may be found in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to cloning techniques.

[0369] In some embodiments, it may be desirable to under-express (*e.g.*, mutate, inactivate, or delete) isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation, or transcription factor polypeptide-encoding nucleic acids at levels far below that those currently found in naturally-occurring cells. This result may be accomplished by the mutation or inactivation of transcriptional regulatory proteins required for expression of isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids, by deletion of the isoprene synthase, DXS, IDI, MVA pathway, hydrogenase,

hydrogenase maturation and/or transcription factor nucleic acids, or by placing those nucleic acids under the control of a strong repressible promoter. Methods for mutating, inactivating, or deleting desired polypeptides are common and well known in the art of molecular biology and examples may be found in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to cloning and mutagenesis techniques.

[0370] The following resources include descriptions of additional general methodology useful in accordance with the compositions and methods described herein: 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

[0371] Isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids (and their encoded polypeptides) can be obtained from any organism that naturally contains isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids. As noted above, isoprene is formed naturally by a variety of organisms, such as bacteria, yeast, plants, and animals. Organisms contain the MVA pathway, DXP pathway, or both the MVA and DXP pathways for producing isoprene (Figures 19A and 19B). Thus, DXS nucleic acids can be obtained, *e.g.*, from any organism that contains the DXP pathway or contains both the MVA and DXP pathways. IDI and isoprene synthase nucleic acids can be obtained, *e.g.*, from any organism that contains the MVA pathway, DXP pathway, or both the MVA and DXP pathways. MVA pathway nucleic acids can be obtained, *e.g.*, from any organism that contains the MVA pathway or contains both the MVA and DXP pathways. Hydrogenase nucleic acids can be obtained, *e.g.*, from any organism that oxidizes hydrogen or reduces hydrogen ions. Fermentation side product genes can be obtained or identified, *e.g.*, from any organism that undergoes oxygen-limited or anaerobic respiration, such as glycolysis.

[0372] In some embodiments, the nucleic acid sequence of the isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic is identical to the sequence of a nucleic acid that is produced by any of the following organisms in nature. In some embodiments, the amino acid sequence of the isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptide is

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

[0373] 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.*

[0374] 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.

[0375] In some embodiments, the source organism is a yeast, such as *Saccharomyces sp.*, *Schizosaccharomyces sp.*, *Pichia sp.*, or *Candida sp*. In some embodiments, the *Saccharomyces sp*. is *Saccharomyces cerevisiae*.

[0376] In some embodiments, the source organism is a bacterium, such as strains of *Bacillus* such as *B. lichenformis* or *B. subtilis,* strains of *Pantoea* such as *P. citrea*, strains of *Pseudomonas* such as *P. alcaligenes, P. putida, or P. fluorescens,* strains of *Streptomyces* such as *S. lividans* or *S. rubiginosus,* strains of *Corynebacterium* sp. such as *Corynebacterium glutamicum*, strains of *Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris*, or strains of *Escherichia* such as *E. coli*.

[0377] 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*.

[0378] In some embodiments, the source organism is a gram-positive bacterium. Non-limiting examples include strains of *Streptomyces* (*e.g.*, *S. lividans*, *S. coelicolor*, or *S. griseus*) and *Bacillus*. In some embodiments, the source organism is a gram–negative bacterium, such as *E.*

coli., Rhodopseudomonas sp. such as *Rhodopseudomonas palustris,* or *Pseudomonas sp.*, such as *P. alcaligenes, P. putida, or P. fluorescens.*

[0379] In some embodiments, the source organism is a plant, such as a plant from the family Fabaceae, such as the Faboideae subfamily. In some embodiments, the source organism is kudzu, poplar (such as *Populus alba x tremula* CAC35696), aspen (such as *Populus tremuloides*), or *Quercus robur*.

[0380] In some embodiments, the source organism is an algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates.

[0381] In some embodiments, the source organism is a cyanobacteria, such as cyanobacteria classified into any of the following groups based on morphology: *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales*, or *Stigonematales*.

[0382] In some embodiments, the source organism is an anaerobic organism. Anaerobic organisms can include, but are not limited to, obligate anaerobes, facultatitive anaerobes, and aerotolerant anaerobes. Such organisms can be any of the organisms listed above, bacteria, yeast, etc. In one embodiment, the obligate anaerobes can be any one or combination selected from the group consisting of *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Eurobacterium limosum*, *Clostridium carboxydivorans*, *Peptostreptococcus productus*, and *Butyribacterium methylotrophicum*. It is to be understood that any combination of any of the source organisms described herein can be used for other embodiments of the invention.

Exemplary Host Cells

[0383] A variety of host cells can be used to express isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides and to co-produce isoprene and hydrogen in the methods described herein. Exemplary host cells include cells from any of the organisms listed in the prior section under the heading "*Exemplary Source Organisms.*" The host cell may be a cell that naturally produces isoprene or a cell that does not naturally produce isoprene. In some embodiments, the host cell naturally produces isoprene using the DXP pathway, and an isoprene synthase, DXS, and/or IDI nucleic acid is added to enhance production of isoprene using this pathway. In some embodiments, the host cell naturally produces isoprene using the MVA pathway, and an isoprene synthase and/or one or more MVA pathway nucleic acids are added to enhance production of isoprene using this pathway. In some embodiments, the host cell naturally produces isoprene using the DXP pathway and one or more MVA pathway nucleic acids are added to produce isoprene using part

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

[0384] In some embodiments, the host cell naturally produces isoprene using both the DXP and MVA pathways, and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways, one or more hydrogenase nucleic acids are added to enhance hydrogen production and one or more fermentation side product-producing genes are inactivated or deleted to limit production of fermentation side products. In some embodiments, the host cell naturally co-produces isoprene and hydrogen using both the DXP and MVA pathways and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways, one or more hydrogenase nucleic acids are added to enhance hydrogen production, one or more fermentation side product-producing genes are inactivated or deleted to limit production of fermentation side products, and one or more hydrogen reuptake genes are inactivated or deleted to increase hydrogen production. In some embodiments, the host cell naturally co-produces isoprene and hydrogen using both the DXP and MVA pathways and a hydrogenase, and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways, one or more hydrogenase nucleic acids are added to enhance hydrogen production, one or more hydrogenase maturation nucleic acids are added to enhance hydrogen production, one or more fermentation side product-producing genes are inactivated or deleted to limit production of fermentation side products, and one or more hydrogen reuptake genes are inactivated or deleted to increase hydrogen production. In some embodiments, the host cell naturally co-produces isoprene and hydrogen using both the DXP and MVA pathways and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways, one or more hydrogenase nucleic acids are added to enhance hydrogen production, one or more hydrogenase maturation nucleic acids are added to enhance hydrogen production, one or more transcription factor nucleic acids are added or inactivated or deleted to enhance hydrogenase production, one or more fermentation side product-producing genes are inactivated or deleted to limit production of fermentation side products, and one or more hydrogen reuptake genes are inactivated or deleted to increase hydrogen production.

Exemplary Transformation Methods

[0385] Isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids or vectors containing them can be inserted into a host cell (*e.g.*, a plant cell, a fungal cell, a yeast cell, or a bacterial cell described herein) using standard techniques for expression of the encoded isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptide. Introduction of a DNA construct or vector into a host cell can be performed using techniques such as transformation, electroporation, nuclear microinjection, transduction, transfection (*e.g.*, lipofection mediated or DEAE-Dextrin mediated transfection or transfection using a recombinant phage virus), incubation with calcium phosphate DNA precipitate, high velocity bombardment with DNA-coated microprojectiles, and protoplast fusion. General transformation techniques are known in the art (*see*, *e.g.*, Current Protocols in Molecular Biology (F. M. Ausubel *et al.* (eds) Chapter 9, 1987; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989; and Campbell *et al.*, *Curr. Genet.* 16:53-56, 1989, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods). The expression of heterologous polypeptide in *Trichoderma* is described in U.S. Patent No. 6,022,725; U.S. Patent No. 6,268,328; U.S. Patent No. 7,262,041;WO 2005/001036; Harkki *et al.*, *Enzyme Microb. Technol.* 13:227-233, 1991; Harkki *et al.*, *Bio Technol.* 7:596-603, 1989; EP 244,234; EP 215,594; and Nevalainen *et al.*, "*The Molecular Biology of Trichoderma and its Application to the Expression of Both Homologous and Heterologous Genes*," in Molecular Industrial Mycology, *Eds.* Leong and Berka, Marcel Dekker Inc., NY pp. 129 – 148, 1992, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation and expression methods). Reference is also made to Cao *et al.*, (*Sci.* 9:991–1001, 2000; EP 238023; and Yelton *et al.*, *Proceedings. Natl. Acad. Sci.* USA 81:1470-1474, 1984 (which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods) for transformation of *Aspergillus* strains. The introduced nucleic acids may be integrated into chromosomal DNA or maintained as extrachromosomal replicating sequences.

[0386] 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.

[0387] In some embodiments, fungal cells are transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a known manner. In one specific embodiment, the preparation of *Trichoderma sp.* for transformation involves the preparation of protoplasts from fungal mycelia (*see*, Campbell *et al.*, *Curr. Genet.* 16:53-56, 1989, which is incorporated by reference in its entirety, particularly with respect to transformation methods). In some embodiments, the mycelia are obtained from germinated vegetative spores. The mycelia are treated with an enzyme that digests the cell wall resulting in protoplasts. The protoplasts are then protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate, and the like. Usually the concentration of these stabilizers varies between 0.8 M and 1.2 M. It is desirable to use about a 1.2 M solution of sorbitol in the suspension medium.

[0388] 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.

[0389] 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).

[0390] 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.

[0391] 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

[0392] Also described herein is a cell or a population of cells in culture that co-produce isoprene and hydrogen. By "cells in culture" is meant two or more cells in a solution (*e.g.*, a cell growth medium) that allows the cells to undergo one or more cell divisions. "Cells in culture" do not include plant cells that are part of a living, multicellular plant containing cells that have differentiated into plant tissues. In various embodiments, the cell culture includes at least or about 10, 20, 50, 100, 200, 500, 1,000, 5,000, 10,000 or more cells.

[0393] By "cells in oxygen-limited culture" is meant two or more cells in a solution (*e.g.*, a cell growth medium) that allows the cell to under go one or more cell divisions, wherein the solution contains a limiting amount of oxygen. The term "oxygen-limited culture" means that the culture is either anoxic or contains less than the required amount of oxygen to support respiration via the biological transfer of reducing equivalents to oxygen, and also encompasses anaerobic cultures. Under oxygen-limited culture conditions, some electrons derived from carbon metabolism cannot be accepted because oxygen concentrations are too low, causing cells to switch to hydrogen production if they comprise the appropriate metabolic pathways for doing

so. Oxygen-limited culture conditions occur when the oxygen transfer rate ("OTR") is less than the oxygen uptake rate ("OUR") indicated by dissolved oxygen concentrations of close to zero in culture medium.

[0394] 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.

[0395] In some embodiments, the carbon source is a carbohydrate (such as monosaccharide, disaccharide, oligosaccharide, or polysaccharides), invert sugar (*e.g.*, enzymatically treated sucrose syrup), glycerol, glycerine (*e.g.*, a glycerine byproduct of a biodiesel or soap-making process), dihydroxyacetone, one-carbon source, oil (*e.g.*, a plant or vegetable oil such as corn, palm, or soybean oil), animal fat, animal oil, fatty acid (*e.g.*, a saturated fatty acid, unsaturated fatty acid, or polyunsaturated fatty acid), lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, polypeptide (*e.g.,* a microbial or plant protein or peptide), renewable carbon source (*e.g.*, a biomass carbon source such as a hydrolyzed biomass carbon source), yeast extract, component from a yeast extract, polymer, acid, alcohol, aldehyde, ketone, amino acid, succinate, lactate, acetate, ethanol, or any combination of two or more of the foregoing. In some embodiments, the carbon source is a product of photosynthesis, including, but not limited to, glucose.

[0396] 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.

[0397] 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.

[0398] 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.

[0399] 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.

[0400] Exemplary lipids are any substance containing one or more fatty acids that are C4 and above fatty acids that are saturated, unsaturated, or branched.

[0401] 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.

[0402] Exemplary fatty acids include compounds of the formula RCOOH, where "R" is a hydrocarbon. Exemplary unsaturated fatty acids include compounds where "R" includes at least one carbon-carbon double bond. Exemplary unsaturated fatty acids include, but are not limited to, oleic acid, vaccenic acid, linoleic acid, palmitoleic acid, and arachidonic acid. Exemplary polyunsaturated fatty acids include compounds where "R" includes a plurality of carbon-carbon double bonds. Exemplary saturated fatty acids include compounds where "R" is a saturated aliphatic group. In some embodiments, the carbon source includes one or more C_{12}-C_{22} fatty acids, such as a C_{12} saturated fatty acid, a C_{14} saturated fatty acid, a C_{16} saturated fatty acid, a C_{18} saturated fatty acid, a C_{20} saturated fatty acid, or a C_{22} saturated fatty acid. In an exemplary embodiment, the fatty acid is palmitic acid. In some embodiments, the carbon source is a salt of a fatty acid (*e.g.*, an unsaturated fatty acid), a derivative of a fatty acid (*e.g.*, an unsaturated fatty acid), or a salt of a derivative of fatty acid (*e.g.*, an unsaturated fatty acid). Suitable salts include, but are not limited to, lithium salts, potassium salts, sodium salts, and the like. Di- and triglycerols are fatty acid esters of glycerol.

[0403] 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.

[0404] 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.

[0405] 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.

[0406] 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.

[0407] 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.

[0408] 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.

[0409] 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).

[0410] 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.

[0411] 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).

[0412] 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.

[0413] In addition to an appropriate carbon source, the cell medium desirably contains suitable minerals, salts, cofactors, buffers, and other components known to those skilled in the art suitable for the growth of the cultures or the enhancement of isoprene production (*see*, for example, WO 2004/033646 and references cited therein and WO 96/35796 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect cell medias and cell culture conditions). In some embodiments where an isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid is under the control of an inducible promoter, the inducing agent (*e.g.*, a sugar, metal salt or antimicrobial), is desirably added to the medium at a concentration effective to induce expression of an isoprene synthase, DXS, IDI, and/or MVA pathway polypeptide. In some embodiments, cell medium has an antibiotic (such as kanamycin) that corresponds to the antibiotic resistance nucleic acid (such as a kanamycin resistance nucleic acid) on a vector that has one or more DXS, IDI, or MVA pathway nucleic acids.

Exemplary Cell Culture Conditions

[0414] Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Exemplary techniques may be found in *Manual of Methods for General Bacteriology* Gerhardt *et al.*, *eds*), American Society for Microbiology, Washington, D.C. (1994) or Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA, which are each hereby incorporated by reference in their entireties, particularly with respect to cell culture techniques. In some embodiments, the cells are cultured in a culture medium under conditions permitting the expression of one or more isoprene synthase, DXS, IDI, or MVA pathway polypeptides encoded by a nucleic acid inserted into the host cells.

[0415] Standard cell culture conditions can be used to culture the cells (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to cell culture and fermentation conditions). Cells are grown and maintained at an appropriate temperature, gas mixture, and pH (such as at about 20°C to about 37°C, at about 6% to about 84% CO_2, and at a pH between about 5 to about 9). In some embodiments, cells are grown at 35°C in an appropriate cell medium. In some embodiments, *e.g.*, cultures are cultured at approximately 28ºC in appropriate medium in shake cultures or fermentors until the desired amount of isoprene and hydrogen co-production is achieved. In some embodiments, the pH ranges for fermentation are between about pH 5.0 to about pH 9.0

(such as about pH 6.0 to about pH 8.0 or about 6.5 to about 7.0). Reactions may be performed under aerobic, anoxic, or anaerobic conditions based on the requirements of the host cells. In some embodiments, the cells are cultured under oxygen-limited conditions. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are cultured under anaerobic conditions. Exemplary culture conditions for a given filamentous fungus are known in the art and may be found in the scientific literature and/or from the source of the fungi such as the American Type Culture Collection and Fungal Genetics Stock Center.

[0416] 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.

[0417] 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.

[0418] 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.

[0419] 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.

[0420] In some embodiments, cells are immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isoprene production.

[0421] 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.

[0422] 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).

[0423] In some embodiments, a constitutive or leaky promoter (such as a Trc promoter) is used and a compound (such as IPTG) is not added to induce expression of the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid(s) operably linked to the promoter. In some embodiments, a compound (such as IPTG) is added to induce expression of the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid(s) operably linked to the promoter.

Exemplary Methods for Decoupling Isoprene Production from Cell Growth

[0424] Desirably, carbon from the feedstock is converted to isoprene rather than to the growth and maintenance of the cells. In some embodiments, the cells are grown to a low to medium OD_{600}, then production of isoprene is started or increased. This strategy permits a large portion of the carbon to be converted to isoprene.

[0425] In some embodiments, cells reach an optical density such that they no longer divide or divide extremely slowly, but continue to make isoprene for several hours (such as about 2, 4, 6, 8, 10, 15, 20, 25, 30, or more hours). For example, Figs. 60A-67C illustrate that cells may continue to produce a substantial amount of mevalonic acid or isoprene after the cells reach an optical density such that they no longer divide or divide extremely slowly. In some cases, the optical density at 550 nm decreases over time (such as a decrease in the optical density after the cells are no longer in an exponential growth phase due to cell lysis), and the cells continue to produce a substantial amount of mevalonic acid or isoprene. In some embodiments, the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%) over a certain time period (such as greater than or about 5, 10, 15, 20, 25, 30, 40, 50 or 60 hours), and the cells produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr) during this time period. In some embodiments, the amount of isoprene is between about 2 to about 5,000 nmole/g_{wcm}/hr, such as between about 2 to about 100 nmole/g_{wcm}/hr, about 100 to about 500 nmole/g_{wcm}/hr, about 150 to about 500 nmole/g_{wcm} /hr, about 500 to about 1,000 nmole/g_{wcm}/hr, about 1,000 to about 2,000 nmole/g_{wcm}/hr, or about

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

[0426] 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}.

[0427] 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%.

[0428] 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.

[0429] In some embodiments, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

Production of Isoprene within Safe Operating Ranges

[0430] 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, described herein is a method for the production of isoprene within the nonflammable range of isoprene concentrations (outside the flammability envelope of isoprene).

[0431] 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.

[0432] 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

[0433] 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.

[0434] 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.

[0435] 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.

[0436] 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.

[0437] 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).

[0438] 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.

[0439] 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.

[0440] 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 60̃ 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.%).

[0441] In various embodiments, the amount of oxidant (such as oxygen) is below the LOC of any fuel in the system (such as isoprene or ethanol). In various embodiments, the amount of oxidant (such as oxygen) is less than about 60, 40, 30, 20, 10, or 5% of the LOC of isoprene or ethanol. In various embodiments, the amount of oxidant (such as oxygen) is less than the LOC

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

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

[0442] Methods are provided herein of producing isoprene comprising a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the liquid phase concentration of isoprene is less than about 200 mg/L. In some embodiments, the liquid phase concentration of isoprene in the culture is less than about any of 175 mg/L, 150 mg/L, 125 mg/L, 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, or 2.5 mg/L. In some embodiments, the liquid phase concentration of isoprene in culture is between about any of 0.1 mg/L to 200 mg/L, 1 mg/L to 200 mg/L, 1 mg/L to 150 mg/L, 1 mg/L to 100 mg/L, 1 mg/L to 50 mg/L, 1 mg/L to 25 mg/L, 1 mg/L to 20 mg/L, or 10 mg/L to 20 mg/L. In some embodiments, the isoprene produced is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the liquid phase concentration is below the solubility limit of isoprene.

[0443] In some embodiments of the methods, the cells produce greater than about 400 nmole/gwcm/hour of isoprene. In some embodiments, the amount of isoprene is between about any of 400 nmole/g_{wcm}/hour to 1 mole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 1 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 40 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 4 mmole/g_{wcm}/hour, 1 mmole/g_{wcm}/hour to 1.5 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour to 3 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour to 5 mmole/g_{wcm}/hour, 5 mmole/g_{wcm}/hour to 25 mmole/g_{wcm}/hour, 25 mmole/g_{wcm}/hour to 100 mmole/g_{wcm}/hour, 100 mmole/g_{wcm}/hour to 500 mmole/g_{wcm}/hour, or 500 mmole/g_{wcm}/hour to 1000 mmole/g_{wcm}/hour. In some embodiments, the amount of isoprene is about any of 1 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour, 2 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour, 4 mmole/g_{wcm}/hour, or 5 mmole/g_{wcm}/hour.

[0444] The low value for Henry's coefficient (in M atm^{-1} units) means that isoprene can be recovered from fermentation broth by gas stripping at low sparging rates, for example 0.01 vvm to 2 vvm. In some embodiments, the gas sparging rate is between about any of 0.1 vvm to 1 vvm, 0.01 vvm to 0.5 vvm, 0.2 vvm to 1 vvm, or 0.5 vvm to 1 vvm. In some embodiments, the gas sparging rate is about any of 0.1 vvm, 0.25 vvm, 0.5 vvm, 0.75 vvm, 1 vvm, 1.25 vvm, 1.5

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

[0445] In some embodiments, the oxygen, CO_2, and isoprene are any of the amounts or concentrations discussed in the section entitled "Production of Isoprene with Safe Operating Ranges." In some embodiments, all the oxygen is consumed by the cells while maintaining fully aerobic metabolism. In some embodiments, an excess of oxygen is used in order to satisfy the oxygen demands of the cells. Desirable ranges of oxygen in the off-gas are less than 20%, or less than 15% or less than 10% (v/v). Levels of oxygen below the limiting oxygen concentration required for combustion of isoprene (9.5% v/v at 1 atm) are particularly desirable. In some embodiments, oxygen-enriched air is utilized with the purpose of allowing minimal gas sweep rates while satisfying the cellular oxygen demand. In some embodiments, the portion of the gas phase of the gas sweep comprises between about 0.1% to about 10%, about 10% to about 20%, or about 20% to about 30% (volume) oxygen. In some embodiments, isoprene fermentations are performed under high pressure in order minimize the amount of excess oxygen required to maintain the required dissolved oxygen levels in the liquid phase.

[0446] In some embodiments, the reduction of the gas sweep rate through the fermentor is advantageous for an integrated isoprene production process in that such conditions enrich the off-gas isoprene levels up to about 30,000 μg/L (about 1% v/v) without adversely affecting the physiology of the cells.

[0447] In some embodiments, reduced gas-sparge rates do not significantly adversely affect the physiology of the cells. In some embodiments, the carbon dioxide evolution rate of cells in culture with reduced gas-sparge rates is between about any of 1×10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25

mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour. In some embodiments, cell viability with reduced gas-sparge rates is reduced by less than about any of 1.75-fold, 1.5-fold, 1.25-fold, 1-fold, 0.75-fold, 0.5-fold, or 0.25-fold. In some embodiments, cell viability with reduced gas-sparge rates is reduced by about 2-fold. In some embodiments, cell viability with reduced gas-sparge rates of a cell expressing a MVA pathway and/or DXP pathway RNA and/or protein from one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid is compared to a control cell lacking one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid with reduced gas-sparge rates. In some embodiments, cell viability with reduced gas-sparge rates of a cell expressing a MVA pathway and/or DXP pathway RNA and/or protein from one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid under the control of an inducible promoter, wherein the promotor is induced, is compared to a control cell containing one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid under the control of an inducible promoter, wherein the promotor is not induced (uninduced) with reduced gas-sparge rates. In some embodiments, the inducible promoter is a beta-galactosidase promotor.

[0448] In some embodiments, the fermentation of a genetically modified host organism that converts at least 5% of the total carbon consumed by the organism into a volatile, unsaturated hydrocarbon. In some embodiments, the production of an unsaturated hydrocarbon at such a rate as to be present in the fermentation off-gas at a level of at least about any of 100 μg/L, 500 μg/L, 1000 μg/L, 2, 500 μg/L, 5,000 μg/L, 7,500 μg/L, or 10,000 μg/L.

[0449] In some embodiments, the unsaturated hydrocarbon is recovered from the off-gas stream in a manner that is suited to high-rates of production, which correspond to concentrations in the off-gas of at least about any of 100 μg/L, 500 μg/L, 1000 μg/L, 2,500 μg/L, 5,000 μg/L, 7,500 μg/L, or 10,000 μg/L. In some embodiments, the continuous extraction and recovery of an unsaturated hydrocarbon from the fermentation off-gas in particular at low gas sweep rates such that the resulting off-gas is enriched in the volatile component of interest. In some embodiments, recovery of the volatile hydrocarbon by methods that depend on elevated concentrations of the volatile. For example, efficient capture of isoprene in fermentation off-gas through the use of compression/condensation or extractive distillation technologies. Also contemplated is the use of

activated carbon cartridges in addition to silica gel adsorbants, desorption and concentration of isoprene from carbon cartridges, and/or construction and fermentation of host organisms such as E. coli strains that can convert about 5% or more of the glucose substrate to isoprene and result in off-gas concentrations of greater than about 15,000 μg/L isoprene. Recovery methods include any of the methods described herein.

[0450] Also provided herein are methods of producing a compound, wherein the compound has one or more characteristics selected from the group consisting of (a) a Henry's law coefficient of less than about 250 M/atm and (b) a solubility in water of less than about 100 g/L. In some embodiments, the method comprises: a) culturing cells under suitable conditions for production of the compound, wherein gas is added (such as the addition of gas to a system such as a fermentation system) at a gas sparging rate between about 0.01 vvm to about 2 vvm; and b) producing the compound.

[0451] In some embodiments, the amount of the compound that partitions into the cell mass is not included in the liquid phase solubility values. In some embodiments, the liquid phase concentration is below the solubility limit of compound.

[0452] In some embodiments, the compounds can be continuously recovered from fermentation broth by gas stripping at moderate to low gas sparging rates, in particular those compounds with Henry's law coefficients of about any of less than 250 M/atm, 200 M/atm, 150 M/atm, 100 M/atm, 75 M/atm, 50 M/atm, 25 M/atm, 10 M/atm, 5 M/atm, or 1 M/atm. Examples include aldehydes such as acetaldehyde (15 M/atm), ketones such as acetone (30 M/atm) or 2-butanone (20 M/atm), or alcohols including methanol (220 M/atm), ethanol (200 M/atm), 1-butanol (120 M/atm) or C5 alcohols including 3-methyl-3-buten-1-ol, and 3-methyl-2-buten-1-ol (50- 100 M/atm). Esters of alcohols generally have lower Henry's constants than the respective alcohols, for example ethyl acetate (6-9 M/atm) or the acetyl esters of C5 alcohols (<5 M/atm).Compounds with Henry's law coefficients of less than 1 M/atm are particularly desirable. Examples include hemiterpenes, monoterpenes, or sesquiterpenes, in addition to other hydrocarbons such as C1 to C5 hydrocarbons (*e.g.*, methane, ethane, ethylene, or propylene). In some embodiments, the hydrocarbons such as C1 to C5 hydrocarbons are saturated, unsaturated, or branched.

[0453] In general, there is a correlation between Henry's law coefficient and water solubility in that compounds with very low coefficients are sparingly soluble in water (substantially water insoluble). Although volatiles with infinite solubilities in water (*e.g.* acetone or ethanol) can be

removed by gas stripping, desirable solubility limits are less than about any of 100 g/L, 75 g/L, 50 g/L, 25 g/L, 10 g/L, 5 g/L, or 1g/L.

[0454] In some embodiments of any of the methods of producing any of the compounds described above, the gas sparging rate is between about any of 0.1 vvm to 1 vvm, 0.2 vvm to 1 vvm, or 0.5 vvm to 1 vvm. In some embodiments, the gas sparging rate is about any of 0.1 vvm, 0.25 vvm, 0.5 vvm, 0.75 vvm, 1 vvm, 1.25 vvm, 1.5 vvm, 1.75 vvm, or 2 vvm. In some embodiments, the low sparging rates are maintained for the entire course of the fermentation run, during growth phase, or during stationary phase. In some embodiments, the low sparging rates are maintained for between about any of 1 hour to 5 hours, 5 hours to 10 hours, 10 hours to 20 hours, 20 hours to 30 hours, 30 hours to 40 hours, 40 hours to 50 hours, or 50 hours to 60 hours.

[0455] Any of the systems described herein can be used in the methods of producing a compound described above. Standard methods would be used to purify such as those described in the section entitled "Exemplary Purification Methods." Separation can be performed post-recovery for example, by distillation or selective adsorption techniques.

Exemplary Production of BioIsoprene

[0456] In some embodiments, the cells are cultured in a culture medium under conditions permitting the production of isoprene by the cells.

[0457] 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.

[0458] By "peak specific productivity" is meant the maximum amount of isoprene produced per cell during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak specific productivity time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per cell is at a maximum. The peak specific productivity is determined by dividing the total productivity by the amount of cells, as determined by optical density at 600nm (OD_{600}). In some embodiments, the

isoprene amount is measured at the peak specific productivity time point. In some embodiments, the peak specific productivity for the cells is about any of the isoprene amounts per cell disclosed herein.

[0459] By "peak volumetric productivity" is meant the maximum amount of isoprene produced per volume of broth (including the volume of the cells and the cell medium) during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak specific volumetric productivity time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per volume of broth is at a maximum. The peak specific volumetric productivity is determined by dividing the total productivity by the volume of broth and amount of time. In some embodiments, the isoprene amount is measured at the peak specific volumetric productivity time point. In some embodiments, the peak specific volumetric productivity for the cells is about any of the isoprene amounts per volume per time disclosed herein.

[0460] By "peak concentration" is meant the maximum amount of isoprene produced during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak concentration time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per cell is at a maximum. In some embodiments, the isoprene amount is measured at the peak concentration time point. In some embodiments, the peak concentration for the cells is about any of the isoprene amounts disclosed herein.

[0461] By "average volumetric productivity" is meant the average amount of isoprene produced per volume of broth (including the volume of the cells and the cell medium) during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). The average volumetric productivity is determined by dividing the total productivity by the volume of broth and amount of time. In some embodiments, the average specific volumetric productivity for the cells is about any of the isoprene amounts per volume per time disclosed herein.

[0462] 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.

[0463] As used herein, "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.

[0464] In some embodiments, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 12,500, 20,000, 30,000, 40,000, 50,000, 75,000, 100,000, 125,000, 150,000, 188,000, or more nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr). In some embodiments, the amount of isoprene is between about 2 to about 200,000 nmole/g_{wcm}/hr, such as between about 2 to about 100 nmole/g_{wcm}/hr, about 100 to about 500 nmole/g_{wcm}/hr, about 150 to about 500 nmole/g_{wcm} /hr, about 500 to about 1,000 nmole/g_{wcm}/hr, about 1,000 to about 2,000 nmole/g_{wcm}/hr, or about 2,000 to about 5,000 nmole/g_{wcm}/hr, about 5,000 to about 10,000 nmole/g_{wcm}/hr, about 10,000 to about 50,000 nmole/g_{wcm}/hr, about 50,000 to about 100,000 nmole/g_{wcm}/hr, about 100,000 to about 150,000 nmole/g_{wcm}/hr, or about 150,000 to about 200,000 nmole/g_{wcm}/hr. In some embodiments, the amount of isoprene is between about 20 to about 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, about 1,000 to about 5,000 nmole/g_{wcm}/hr, about 2,000 to about 20,000 nmole/g_{wcm}/hr, about 5,000 to about 50,000 nmole/g_{wcm}/hr, about 10,000 to about 100,000 nmole/g_{wcm}/hr, about 20,000 to about 150,000 nmole/g_{wcm}/hr, or about 20,000 to about 200,000 nmole/g_{wcm}/hr.

[0465] 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.

[0466] 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).

[0467] 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}.

[0468] 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.

[0469] In some embodiments, the cells in culture have an average volumetric productivity of isoprene at greater than or about 0.1, 1.0, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1100, 1200, 1300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400, 2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, or more mg of isoprene/L of broth/hr (mg/L_{broth}/hr, wherein the volume of broth includes the volume of the cells and the cell medium). In some embodiments, the average volumetric productivity of isoprene is between about 0.1 to about 3,500 mg/L_{broth}/hr, such as between about 0.1 to about 100 mg/L_{broth}/hr, about 100 to about 500 mg/L_{broth}/hr, about 500 to about 1,000 mg/L_{broth}/hr, about 1,000 to about 1,500 mg/L_{broth}/hr, about 1,500 to about 2,000 mg/L_{broth}/hr, about 2,000 to about 2,500 mg/L_{broth}/hr, about 2,500 to about 3,000 mg/L_{broth}/hr, or about 3,000 to about 3,500 mg/L_{broth}/hr. In some embodiments, the average volumetric productivity of isoprene is between about 10 to about 3,500 mg/L_{broth}/hr, about 100 to about 3,500 mg/L_{broth}/hr, about 200 to about 1,000 mg/L_{broth}/hr, about 200 to about 1,500 mg/L_{broth}/hr, about 1,000 to about 3,000 mg/L_{broth}/hr, or about 1,500 to about 3,000 mg/L_{broth}/hr.

[0470] In some embodiments, the cells in culture have a peak volumetric productivity of isoprene at greater than or about 0.5, 1.0, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600,

700, 800, 900, 1,000, 1100, 1200, 1300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400, 2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, 3,750, 4,000, 4,250, 4,500, 4,750, 5,000, 5,250, 5,500, 5,750, 6,000, 6,250, 6,500, 6,750, 7,000, 7,250, 7,500, 7,750, 8,000, 8,250, 8,500, 8,750, 9,000, 9,250, 9,500, 9,750, 10,000, 12,500, 15,000, or more mg of isoprene/L of broth/hr (mg/L_{broth}/hr, wherein the volume of broth includes the volume of the cells and the cell medium). In some embodiments, the peak volumetric productivity of isoprene is between about 0.5 to about 15,000 mg/L_{broth}/hr, such as between about 0.5 to about 10 mg/L_{broth}/hr, about 1.0 to about 100 mg/L_{broth}/hr, about 100 to about 500 mg/L_{broth}/hr, about 500 to about 1,000 mg/L_{broth}/hr, about 1,000 to about 1,500 mg/L_{broth}/hr, about 1,500 to about 2,000 mg/L_{broth}/hr, about 2,000 to about 2,500 mg/L_{broth}/hr, about 2,500 to about 3,000 mg/L_{broth}/hr, about 3,000 to about 3,500 mg/L_{broth}/hr, about 3,500 to about 5,000 mg/L_{broth}/hr, about 5,000 to about 7,500 mg/L_{broth}/hr, about 7,500 to about 10,000 mg/L_{broth}/hr, about 10,000 to about 12,500 mg/L_{broth}/h, or about 12,500 to about 15,000 mg/L_{broth}/hr. In some embodiments, the peak volumetric productivity of isoprene is between about 10 to about 15,000 mg/L_{broth}/hr, about 100 to about 2,500 mg/L_{broth}/hr, about 1,000 to about 5,000 mg/L_{broth}/hr, about 2,500 to about 7,500 mg/L_{broth}/hr, about 5,000 to about 10,000 mg/L_{broth}/hr, about 7,500 to about 12,500 mg/L_{broth}/hr, or about 10,000 to about 15,000 mg/L_{broth}/hr.

[0471] 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}.

[0472] 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, 2.2, 2.4, 2.6, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0, 15.0, 16.0, 17.0,

18.0, 19.0, 20.0, 21.0, 22.0, 23.0, 23.2, 23.4, 23.6, 23.8, 24.0, 25.0, 30.0, 31.0, 32.0, 33.0, 35.0, 37.5, 40.0, 45.0, 47.5, 50.0, 55.0, 60.0, 65.0, 70.0, 75.0, 80.0, 85.0, or 90.0 molar % of the carbon in the cell culture medium into isoprene. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about 90.0 molar %, such as about 0.002 to about 0.005%, about 0.005 to about 0.01%, about 0.01 to about 0.05%, about 0.05 to about 0.15%, 0.15 to about 0.2%, about 0.2 to about 0.3%, about 0.3 to about 0.5%, about 0.5 to about 0.8%, about 0.8 to about 1.0%, about 1.0 to about 1.6%, about 1.6 to about 3.0%, about 3.0 to about 5.0%, about 5.0 to about 8.0%, about 8.0 to about 10.0%, about 10.0 to about 15.0%, about 15.0 to about 20.0%, about 20.0 to about 25.0%, about 25.0% to 30.0%, about 30.0% to 35.0%, about 35.0% to 40.0%, about 45.0% to 50.0%, about 50.0% to 55.0%, about 55.0% to 60.0%, about 60.0% to 65.0%, about 65.0% to 70.0%, about 75.0% to 80.0%, about 80.0% to 85.0%, or about 85.0% to 90.0%. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about 0.4 molar %, 0.002 to about 0.16 molar %, 0.04 to about 0.16 molar %, about 0.005 to about 0.3 molar %, about 0.01 to about 0.3 molar %, about 0.05 to about 0.3 molar %, about 0.1 to 0.3 molar %, about 0.3 to about 1.0 molar %, about 1.0 to about 5.0 molar %, about 2 to about 5.0 molar %, about 5.0 to about 10.0 molar %, about 7 to about 10.0 molar %, about 10.0 to about 20.0 molar %, about 12 to about 20.0 molar %, about 16 to about 20.0 molar %, about 18 to about 20.0 molar %, about 18 to 23.2 molar %, about 18 to 23.6 molar %, about 18 to about 23.8 molar %, about 18 to about 24.0 molar %, about 18 to about 25.0 molar %, about 20 to about 30.0 molar %, about 30 to about 40.0 molar %, about 30 to about 50.0 molar %, about 30 to about 60.0 molar %, about 30 to about 70.0 molar %, about 30 to about 80.0 molar %, or about 30 to about 90.0 molar %.

[0473] 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

[0474] 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%

[0475] For the two 500 liter fermentations described herein (Example 7, parts VII and VIII), the percent conversion of carbon into isoprene was between 0.04-0.06%. A 0.11-0.16% carbon yield has been achieved using 14 liter systems as described herein. Example 11, part V describes the 1.53% conversion of carbon to isoprene using the methods described herein.

[0476] 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 µg isoprene/L_{gas} isoprene in off-gas = 60 µg 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)

[0477] 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

[0478] If desired, Equation 11 can be used to convert between units of ppm and μg/L. In particular, "ppm" means parts per million defined in terms of μg/g (w/w). Concentrations of gases can also be expressed on a volumetric basis using "ppmv" (parts per million by volume), defined in terms of μL/L (vol/vol). Conversion of μg/L to ppm (*e.g.*, μg of analyte per g of gas) can be performed by determining the mass per L of off-gas (*i.e.*, the density of the gas). For example, a liter of air at standard temperature and pressure (STP; 101.3 kPa (1 bar) and 273.15K). has a density of approximately 1.29 g/L. Thus, a concentration of 1 ppm (μg/g) equals 1.29 μg/L at STP (equation 11). The conversion of ppm (μg/g) to μg/L is a function of both pressure, temperature, and overall composition of the off-gas.

Equation 11

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

[0479] Conversion of μg/L to ppmv (*e.g.*, μL of analyte per L of gas) can be performed using the Universal Gas Law (equation 12). For example, an off-gas concentration of 1000 μg/L_{gas} corresponds to 14.7 μmol/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 μmol of HG at STP is equal to 0.329 mL. Therefore, the concentration of 1000 μg/L HG is equal to 329 ppmv or 0.0329% (v/v) at STP.

Equation 12

[0480] 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.

[0481] The amount of impurities in isoprene compositions are typically measured herein on a weight per volume (w/v) basis in units such as μg/L. If desired, measurements in units of μg/L can be converted to units of mg/m^3 using equation 13.

Equation 13

1 μg/L = 1 mg/m^3

[0482] In some embodiments described herein, 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.

[0483] In some embodiments described herein, a cell comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide and one or more heterologous nucleic acids encoding a DXS, IDI, and/or MVA pathway polypeptide produces an amount of isoprene that is at least or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 150-fold, 200-fold, 400-fold, or greater than the amount of isoprene produced from a corresponding cell grown under essentially the same conditions without the heterologous nucleic acids.

[0484] 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.

[0485] 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, cis-1,3-pentadiene, trans-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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, cis-1,3-pentadiene, trans-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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, cis-1,3-pentadiene, trans-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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.

[0486] 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 μg/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 μg/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 μg/L of a hydrocarbon other than isoprene (such as 1,3-cyclopentadiene, cis-1,3-pentadiene, trans-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-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 μg/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 μg/L of a protein or fatty acid (such as a protein or fatty acid that is naturally associated with natural rubber).

[0487] In some embodiments, the isoprene composition comprises less than or about 10, 5, 1, 0.8, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of alpha acetylenes, piperylenes, acetonitrile, or 1,3-cyclopentadiene. In some embodiments, the isoprene composition comprises less than or about 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of sulfur or allenes. In some embodiments, the isoprene composition comprises less than or about 30, 20, 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of all acetylenes (such as 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, and *cis*-pent-3-ene-1-yne). 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).

[0488] In some embodiments, the isoprene composition includes ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the isoprene composition comprises greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 μg/L of ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In some embodiments, the isoprene composition comprises between about 0.005 to about 120, such as about 0.01 to about 80, about 0.01 to about 60, about 0.01 to about 40, about 0.01 to about 30, about 0.01 to about 20, about 0.01 to about 10, about 0.1 to about 80, about 0.1 to about 60, about 0.1 to about 40, about 5 to about 80, about 5 to about 60, or about 5 to about 40 μg/L of ethanol, acetone, a C5 prenyl alcohol, or any two or more of the foregoing.

[0489] In some embodiments, the isoprene composition includes one or more of the following components: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In various embodiments, the amount of one of these components relative to

amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w). In some embodiments, the relative detector response for the second compound compared to the detector response for isoprene is greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110%. In various embodiments, the amount of one of these components relative to amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is between about 0.01 to about 105 % (w/w), such as about 0.01 to about 90, about 0.01 to about 80, about 0.01 to about 50, about 0.01 to about 20, about 0.01 to about 10, about 0.02 to about 50, about 0.05 to about 50, about 0.1 to about 50, or 0.1 to about 20% (w/w).

[0490] In some embodiments, the isoprene composition includes one or more of the following: an alcohol, an aldehyde, or a ketone (such as any of the alcohols, aldehydes, or ketones described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone.

[0491] In some embodiments, the isoprene composition contains one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole. In some embodiments, the isoprene composition contains 1 ppm or more of one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole. In some embodiments, the concentration of more of one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole, is between about 1 to about 10,000 ppm in an isoprene composition (such as off-gas before it is purified). In some embodiments, the isoprene composition (such as off-gas after it has undergone one or more purification steps) includes one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole, at a concentration between about 1 to about 100 ppm, such as about 1 to about 10 ppm, about 10 to about 20 ppm, about 20 to about 30 ppm, about 30 to about 40 ppm, about 40 to about 50 ppm, about 50 to about 60 ppm, about 60 to about 70 ppm, about 70 to about 80 ppm, about 80 to about 90 ppm, or about 90 to about 100 ppm. Volatile organic compounds from cell cultures (such as volatile organic compounds in the headspace of cell cultures) can be analyzed using standard methods such as those described herein or other

standard methods such as proton transfer reaction-mass spectrometry (*see*, for example, Bunge *et al.*, *Applied and Environmental Microbiology*, 74(7):2179-2186, 2008 which is hereby incorporated by reference in its entirety, particular with respect to the analysis of volatile organic compounds).

[0492] 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.

[0493] 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.

[0494] In some embodiments described herein, a cell comprising one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, DXS polypeptide, IDI polypeptide, and/or MVA pathway polypeptide produces an amount of an isoprenoid compound (such as a compound with 10 or more carbon atoms that is formed from the reaction of one or more IPP molecules with one or more DMAPP molecules) that is greater than or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 150-fold, 200-fold, 400-fold, or greater than the amount of the isoprenoid compound produced from a corresponding cell grown under essentially the same conditions without the one or more heterologous nucleic acids. In some embodiments described herein, a cell comprising one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, DXS polypeptide, IDI polypeptide, and/or MVA pathway polypeptide produces an amount of a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-

1-ol) that is greater than or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 150-fold, 200-fold, 400-fold, or greater than the amount of the C5 prenyl alcohol produced from a corresponding cell grown under essentially the same conditions without the one or more heterologous nucleic acids.

Exemplary Co-Production of BioIsoprene and Hydrogen

[0495] In some embodiments, any of the isoprene-producing cells described herein that comprise one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a DXS polypeptide, an IDI polypeptide, and/or an MVA pathway polypeptide operably linked to a promoter further comprise a heterologous nucleic acid also operably linked to a promoter encoding one or more hydrogenase polypeptides or one or more polypeptides involved in the regulation or expression of hydrogenase polypeptides (*e.g.*, hydrogenase maturation proteins or transcription factors). In some embodiments, any of the isoprene-producing cells described herein that comprise one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a DXS polypeptide, an IDI polypeptide, an MVA pathway polypeptide, one or more hydrogenase polypeptides or one or more polypeptides involved in the regulation or expression of hydrogenase polypeptides operably linked to a promoter further comprise a mutation or deletion inactivating one or more polypeptides involved in the production of fermentation side products, one or more polypeptides involved in the regulation or expression of genes for the production of fermentation side products, or one or more polypeptides involved in hydrogen reuptake. Such cells can co-produce isoprene and hydrogen.

[0496] In some embodiments of any of the aspects described herein, the cells are bacterial cells, such as gram-positive bacterial cells (*e.g.*, *Bacillus* cells such as *Bacillus subtilis* cells or *Streptomyces* cells such as *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus* cells). In some embodiments of any of the aspects described herein, the cells are gram-negative bacterial cells (*e.g.*, *Escherichia* cells such as *Escherichia coli* cells, *Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris* cells, *Pseudomonas sp.* such as *Pseudomonas fluorescens* cells or *Pseudomonas putida* cells, or *Pantoea* cells such as *Pantoea citrea* cells). In some embodiments of any of the aspects described herein, the cells are fungal, cells such as filamentous fungal cells (*e.g.*, *Trichoderma* cells such as *Trichoderma reesei* cells or *Aspergillus* cells such as *Aspergillus oryzae* and *Aspergillus niger*) or yeast cells (*e.g.*, *Yarrowia* cells such as *Yarrowia lipolytica* cells or *Sacchraomyces* cells such as *Saccaromyces cerevisiae*).

[0497] In some embodiments of any of the aspects described herein, 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*).

[0498] In some embodiments of any of the aspects described herein, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects described herein, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects described herein, the cells further comprise a heterologous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects described herein, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects described herein, the cells further comprise one or more nucleic acids encoding an IDI polypeptide and a DXS polypeptide. In some embodiments of any of the aspects described herein, one nucleic acid encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments of any of the aspects described herein, one vector encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments, the vector comprises a selective marker or a selectable marker, such as an antibiotic resistance nucleic acid.

[0499] In some embodiments of any of the aspects described herein, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects described herein, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects described herein, the cells comprise an isoprene synthase, DXS, and MVA pathway nucleic acid. In some embodiments of any of the aspects described herein, the cells comprise an isoprene synthase nucleic acid, a DXS nucleic acid, an IDI nucleic acid, and a MVA pathway nucleic acid.

[0500] In some embodiments, the isoprene-producing cells described herein further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide operably linked to a promoter. In some embodiments, the hydrogenase polypeptide comprises *E. coli* hydrogenase-1 (Hyd-1), *E. coli* hydrogenase-2 (Hyd-2), *E. coli* hydrogenase-3 (Hyd-3), *E. coli* hydrogenase-4 (Hyd-4), *E. coli* formate hydrogen lyase (FHL) complex, which produces hydrogen gas from formate and

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

[0501] In some embodiments, the hydrogenase polypeptide encodes a ferredoxin-dependent hydrogenase polypeptide. In some embodiments, the ferredoxin-dependent hydrogenase polypeptide comprises *Clostridium acetobutulicum* hydrogenase A (HydA), which can be expressed in conjunction with one or more of: (1) *Bacillus subtilis* NADPH ferredoxin oxidoreductase (NFOR) or *Clostridium kluyveri* NADH ferredoxin oxidoreductase (RnfCDGEAB), *Clostridium pasteuranium* ferredoxin oxidoreductase (Fdx); (2) glyceraldehyde-6-phosphate ferredoxin oxidoreductase (GAPOR); or (3) pyruvate ferredoxin oxidoreductase (POR). In some embodiments, the ferredoxin-dependent hydrogenase polypeptide *Clostridium acetobutulicum* hydrogenase A (HydA) is expressed with three HydA-associated maturation enzymes (HydE, HydG, and HydF), and further in conjunction with one or more of: (1) *Bacillus subtilis* NADPH ferredoxin oxidoreductase (NFOR) or *Clostridium kluyveri* NADH ferredoxin oxidoreductase (RnfCDGEAB), *Clostridium pasteuranium* ferredoxin oxidoreductase (Fdx); (2) glyceraldehyde-6-phosphate ferredoxin oxidoreductase (GAPOR); or (3) pyruvate ferredoxin oxidoreductase (POR).

[0502] In some embodiments, the hydrogenase polypeptide encodes an NADPH-dependent hydrogenase polypeptide. In some embodiments, the NADPH-dependent hydrogenase polypeptide comprises *Pyrococcus furiosus* hydrogenase. In some embodiments, the hydrogenase polypeptide encodes an oxygen-tolerant hydrogenase. In some embodiments, the oxygen-tolerant hydrogenase comprises *Rubrivivax gelatinosus* hydrogenase, and *Ralstonia eutropha* hydrogenase.

[0503] In some embodiments, the isoprene-producing cells described herein further comprise a mutation or deletion inactivating a gene involved in regulation of hydrogenase activity, such as iron-sulfur complex transcriptional regulator (*iscR*) (Kalim-Akhtar et al., "Deletion of iscR stimulates recombinant Clostridial Fe/Fe hydrogenase activity and H_2-accumulation in *Escherichia coli* BL21(DE3)," *Appl. Microbiol. Biotechnol.* 78:853–862 (2008), which is incorporated herein by reference in its entirety, particularly with reference to stimulation of

Clostridial Fe/Fe hydrogenase activity and hydrogen accumulation in *E. coli* by deleting the *iscR* gene).

[0504] In some embodiments, the isoprene-producing cells described herein further comprise a mutation or deletion inactivating a gene encoding one or more cellular polypeptides involved in production of fermentation side products, such as lactate, acetate, pyruvate, ethanol, succinate, and glycerol. In some embodiments, the inactivated polypeptides involved in production of fermentation side products comprise one or more polypeptides encoding formate dehydrogenase N, alpha subunit (*fdnG*), formate dehydrogenase O, large subunit (*fdoG*), nitrate reductase (*narG*), formate transporter A (*focA*), formate transporter B (*focB*), pyruvate oxidase (*poxB*), pyruvate dehydrogenase E1 component ackA/pta (*aceE*), alcohol dehydrogenase (*adhE*), fumarate reductase membrane protein (*frdC*), or lactate dehydrogenase (*ldh*A).

[0505] In some embodiments, the isoprene-producing cells described herein further comprise a mutation or deletion inactivating a gene encoding one or more cellular polypeptides involved in the regulation or expression of genes involved in production of fermentation side products. In some embodiments, the inactivated polypeptides involved in the regulation or expression of genes involved in production of fermentation side products comprise repressor of formate hydrogen lyase (*hycA*), fumarate reductase regulator (*fnr*), acetyl-coenzyme A synthetase (*acs*), and formate dehydrogenase regulatory protein (*hycA*).

[0506] In some embodiments, the isoprene-producing cells described herein further comprise a mutation or deletion inactivating a gene encoding one or more cellular polypeptides involved in hydrogen re-uptake. In some embodiments, the inactivated polypeptides involved in hydrogen re-uptake comprise *E. coli* hydrogenase-1 (Hyd-1) (*hya* operon) and *E. coli* hydrogenase-2 (Hyd-2) (*hyb* operon).

[0507] In some embodiments of any of the aspects described herein, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide or nucleic acid is operably linked to a T7 promoter, such as a T7 promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects described herein, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid is operably linked to a Trc promoter, such as a Trc promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects described herein, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid is operably

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

[0508] In some embodiments, one or more MVA pathway, IDI, DXS, isoprene synthase, hydrogenase, hydrogenase maturation or transcription factor nucleic acids are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more MVA pathway, IDI, DXS, isoprene synthase, hydrogenase, hydrogenase maturation or transcription factor nucleic acids may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more MVA pathway, IDI, DXS, isoprene synthase, hydrogenase, hydrogenase maturation or transcription factor nucleic acids are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

[0509] In some embodiments of any of the aspects described herein, at least a portion of the cells maintain the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid for at least or about 5, 10, 20, 40, 50, 60, 65, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects described herein, the nucleic acid comprising the isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid also comprises a selective marker or a selectable marker, such as an antibiotic resistance nucleic acid.

[0510] In some embodiments of any of the aspects described herein, cells that co-produce isoprene and hydrogen are cultured in any of the culture media described herein, under oxygen-limited conditions to facilitate the co-production of isoprene and hydrogen by the cells. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are grown in the presence of 0.5 moles of oxygen per mole of isoprene. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0511] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and hydrogen. In some embodiments, the cells in oxygen-

limited culture produce isoprene at a rate greater than about 400 nmole/g_{wcm}/hr, and produce hydrogen at a rate greater than about 125 nmole/g_{wcm}/hr. In some embodiments, the cells in oxygen-limited culture produce isoprene at a rate between about 400 nmole/g_{wcm}/hr to about 2.0 $\times 10^5$ nmole/g_{wcm}/hr and hydrogen at a rate between about 125 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr. In some embodiments, the cells in oxygen-limited culture produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0 $\times 10^5$ nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr and about 1.5 $\times 10^5$ nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr and about 1 $\times 10^5$ nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr and about 1 $\times 10^5$ nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr and about 1 $\times 10^5$ nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr and about 1 $\times 10^5$ nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr and about 1 $\times 10^5$ nmole/g_{wcm}/hr, and between about 1 $\times 10^4$ nmole/g_{wcm}/hr and about 1 $\times 10^5$ nmole/g_{wcm}/hr. In some embodiments, the cells in oxygen-limited culture produce greater than about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments, the cells in oxygen-limited culture produce hydrogen at a rate between about 125 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, between about 250 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, between about 1250 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr, and between about 1.00 $\times 10^4$ nmole/g_{wcm}/hr to about 1.25 $\times 10^4$ nmole/g_{wcm}/hr. In some embodiments, the cells in oxygen-limited culture produce greater than about 125, 250, 500, 750, 1000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 7,500, 10,000, or more nmole/g_{wcm}/hr hydrogen.

[0512] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and hydrogen. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 1000 mg/L_{broth}/hr and a peak volumetric productivity of hydrogen greater than about 5 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 3000 mg/L_{broth}/hr and

a peak volumetric productivity of hydrogen greater than about 5 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 5000 mg/L_{broth}/hr and a peak volumetric productivity of hydrogen greater than about 5 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 0.1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of hydrogen between about 0.005 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 5 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 10 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 25 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 50 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 100 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 250 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 1000 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and between about 2500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of hydrogen between about 0.01 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.025 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.05 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.1 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.25 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.5 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 1 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, and between about 2.5 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr.

[0513] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and hydrogen. In some embodiments, the cells in oxygen-limited culture convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium. In some embodiments, the cells in oxygen-limited culture convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 400 molar percent of the carbon that the cells consumer from a cell culture medium.

[0514] In some embodiments, any of the cells described herein that co-produce isoprene and hydrogen are grown in oxygen-limited culture. In some embodiments, the cells in oxygen-limited culture co-produce isoprene and hydrogen in a ratio ranging from at least one molar percent of isoprene for every three molar percent of hydrogen to at least one molar percent of

isoprene for every four molar percent of hydrogen. In some embodiments, the cells in oxygen-limited culture produce an off-gas containing from 1 to 11 molar percent isoprene and from 3 to 33 molar percent hydrogen. In some embodiments, the cells produce from 1 to 11 molar percent isoprene and from 4 to 44 molar percent hydrogen. In some embodiments, the cells in oxygen-limited culture also produce an off-gas containing oxygen, carbon dioxide, or nitrogen. In some embodiments, the cells in oxygen limited culture produce an off-gas containing from 0 to 21 molar percent oxygen, from 18 to 44 molar percent carbon dioxide, and from 0 to 78 molar percent nitrogen.

[0515] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen, comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the cells: (i) produce isoprene at a rate greater than about 400 nmole/g_{wcm}/hr and produce hydrogen at a rate greater than about 125 nmole/g_{wcm}/hr; (ii) have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr; or (iii) convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium.

[0516] In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells produce greater than about 400 nmole/g_{wcm}/hr of isoprene and greater than about 125 nmole/g_{wcm}/hr of hydrogen. In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium into hydrogen. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide.

[0517] In some embodiments, the cells in oxygen-limited culture comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr and about 1.5×10^5 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and between about 1×10^4 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and produce hydrogen at a rate between about 125 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, and between about 1.00×10^4 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr.

[0518] In some embodiments, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and wherein the cells produce greater than about 125 nmole/g_{wcm}/hr of hydrogen.

[0519] In some embodiments, the cells in oxygen-limited culture comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr and about 1.5×10^5 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and between about 1×10^4 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and produce hydrogen at a rate between about 125 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr,

between about 1250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, and between about 1.00×10^4 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr.

[0520] In some embodiments, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr.

[0521] In some embodiments, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium.

[0522] In some embodiments, provided herein are compositions comprising isoprene and hydrogen in a ratio ranging from at least one molar percent of isoprene for every three molar percent of hydrogen to at least one molar percent of isoprene for every four molar percent of hydrogen, and 0.1 molar percent or less of volatile impurities. In some embodiments, the compositions further comprise from 1 to 11 molar percent isoprene and from 4 to 44 molar percent hydrogen. In some embodiments, the compositions further comprise oxygen, carbon dioxide, or nitrogen. In some embodiments, the compositions further comprise from 0 to 21 molar percent oxygen, from 18 to 44 molar percent carbon dioxide, and from 0 to 78 molar percent nitrogen. In some embodiments, the composition further comprises 1.0×10^{-4} molar percent or less of non-methane volatile impurities. In some embodiments, the non-methane volatile impurities comprise one or more of the following: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, or a

linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In some embodiments, the non-methane volatile impurities comprise one or more of the following: the isoprene composition includes one or more of the following: an alcohol, an aldehyde, or a ketone (such as any of the alcohols, aldehydes, or ketones described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone. In some embodiments, the non-methane volatile impurities comprise one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole.

[0523] Also provided herein are methods of co-producing isoprene and hydrogen, the methods comprising: a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and b) co-producing isoprene and hydrogen, wherein the peak concentration of the isoprene produced by the cells in oxygen-limited culture is greater than about 10 ng/L_{broth} and the hydrogen evolution rate of the cells is greater than about 0.0025 mmol/L_{broth}/hour. In some embodiments of any of these methods, the hydrogen evolution rate is between about any of 0.0025 mmol/L_{broth}/hr and about 10 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 2.5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 1 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.25 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.025 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 0.5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 1 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 2.5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 10 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 1 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 2.5 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 2.5 mmol/L_{broth}/hr, and between about 0.25 mmol/L_{broth}/hr and 10 mmol/L_{broth}/hr.

[0524] Provided herein are also methods of co-producing isoprene and hydrogen comprising a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and b) co-producing isoprene and hydrogen, wherein the liquid phase concentration of isoprene is less than about 200 mg/L, the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and the hydrogen evolution rate of the cells is greater than about 0.0025 mmol/L/hour. In some

embodiments, the liquid phase concentration of isoprene in the culture is less than about any of 175 mg/L, 150 mg/L, 125 mg/L, 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, or 2.5 mg/L. In some embodiments, the liquid phase concentration of isoprene in culture is between about any of 0.1 mg/L to 200 mg/L, 1 mg/L to 200 mg/L, 1 mg/L to 150 mg/L, 1 mg/L to 100 mg/L, 1 mg/L to 50 mg/L, 1 mg/L to 25 mg/L, 1 mg/L to 20 mg/L, or 10 mg/L to 20 mg/L. In some embodiments of any of these methods, the hydrogen evolution rate is between about any of 0.0025 mmol/L_{broth}/hr and about 10 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 2.5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 1 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.25 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.025 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 0.5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 1 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 2.5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 10 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 1 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 2.5 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 2.5 mmol/L_{broth}/hr, and between about 0.25 mmol/L_{broth}/hr and 10 mmol/L_{broth}/hr.

[0525] In one aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the cells in oxygen-limited culture produce greater than about 400 nmole/g_{wcm}/hr of isoprene and greater than about 125 nmole/g_{wcm}/hr of hydrogen. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes 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.

[0526] In some embodiments, provided herein are cells in oxygen-limited culture that convert more than about 0.002% of the carbon in a cell culture medium into isoprene and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon in a cell culture

medium. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes 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.

[0527] In some embodiments, provided herein are cells in oxygen-limited culture that comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes 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.

[0528] In one aspect, provided herein are methods of co-producing isoprene with another compound, such as methods of using any of the cells described herein to co-produce isoprene and hydrogen. In some embodiments, the method involves culturing cells under oxygen-limited conditions sufficient to produce greater than about 400 nmole/g_{wcm}/hr of isoprene and greater than about 125 nmole/g_{wcm}/hr of hydrogen. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the method also includes recovering the isoprene and hydrogen produced by the cells. In some embodiments, the method further includes purifying the isoprene and the hydrogen produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes 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. 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.

[0529] In some embodiments, the method includes culturing cells under oxygen-limited conditions sufficient to convert more than about 0.002% of the carbon (mol/mol) in a cell culture medium into isoprene and to produce hydrogen equivalent to more than about 0.024 molar percent of the carbon in a cell culture medium. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the method also includes recovering isoprene and hydrogen produced by the cells. In some embodiments, the method further includes purifying isoprene and hydrogen produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes 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.

[0530] In some embodiments of any of the aspects described herein, the microbial polypeptide carbon source includes one or more polypeptides from yeast or bacteria. In some embodiments of any of the aspects described herein, 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.

[0531] In some embodiments, isoprene and hydrogen are only co-produced in stationary phase. In some embodiments, isoprene and hydrogen are co-produced in both the growth phase and stationary phase. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of isoprene produced during the growth phase for the same length of time. In

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

[0532] In some embodiments, the compositions provided herein comprise hydrogen and greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such as 1,3-cyclopentadiene, cis-1,3-pentadiene, trans-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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, cis-1,3-pentadiene, trans-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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. In some embodiments, the composition has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In particular embodiments, the composition also comprises greater than about 2 mg of isoprene and greater than about 0.48 mg of hydrogen.

[0533] In some embodiments, the volatile organic fraction of the gas phase has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the volatile organic fraction of the gas phase that inhibits the polymerization of isoprene. In particular embodiments, the volatile organic fraction of the gas phase also has greater than about 2 mg of isoprene and greater than about 0.48 mg of hydrogen.

[0534] In some embodiments, the systems include any of the cells and/or compositions described herein. In some embodiments, the system includes a reactor that chamber comprises

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

[0535] In one aspect, featured herein is a product produced by any of the compositions or methods described herein.

Cell Viability at High Isoprene Titer

[0536] Isoprene is a hydrophobic molecule secreted by many plants, animals, and microbes. Bacteria, such as Bacillus, produce isoprene at fairly low levels. While there is some evidence that plants secrete isoprene to help with thermoprotection, it has been hypothesized that isoprene may act antagonistically to cyanobacteria or fungi, or as an antimicrobial agent. *See, e.g.,* Ladygina et al., *Process Biochemistry* 41:1001-1014 (2006), which is incorporated by reference in its entirety, particularly with respect to isoprene acting antagonistically. Since the very low production levels happening in nature are sufficient to be anti-microbial, it was of great concern that the titers and productivity levels of isoprene necessary for commercialization of isoprene would kill the host microbe.

[0537] We have found methods for producing titers and productivity levels of isoprene for commercialization of isoprene while maintaining cell viability and/or metabolic activity as indicated by carbon dioxide evolution rate or total carbon dioxide evolution rate.

[0538] Provided herein are methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the isoprene produced is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the amount of isoprene is between about any of 400 nmole/g_{wcm}/hour to 1 mole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 1 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 40 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 4 mmole/g_{wcm}/hour, 1 mmole/g_{wcm}/hour to 1.5 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour to 3 mmole/g_{wcm}/hour, 3

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

[0539] Provided herein are also methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and cell viability is reduced by less than about two-fold. In some embodiments, the isoprene produced is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the amount of isoprene is between about any of 400 nmole/g_{wcm}/hour to 1 mole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 1 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 40 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 4 mmole/g_{wcm}/hour, 1 mmole/g_{wcm}/hour to 1.5 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour to 3 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour to 5 mmole/g_{wcm}/hour, 5 mmole/g_{wcm}/hour to 25 mmole/g_{wcm}/hour, 25 mmole/g_{wcm}/hour to 100 mmole/g_{wcm}/hour, 100 mmole/g_{wcm}/hour to 500 mmole/g_{wcm}/hour, or 500 mmole/g_{wcm}/hour to 1000 mmole/g_{wcm}/hour. In some embodiments, the amount of isoprene is about any of 1 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour, 2 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour, 4 mmole/g_{wcm}/hour, or 5 mmole/g_{wcm}/hour. In some embodiments, cell viability is reduced by less than about any of 1.75-fold, 1.5-fold, 1.25-fold, 1-fold, 0.75-fold, 0.5-fold, or 0.25-fold. In some embodiments, cell viability is reduced by about 2-fold.

[0540] Further provided herein are methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the cumulative total productivity of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In

some embodiments, the cumulative total productivity of the isoprene is between about any of 0.2 mg/L_{broth}/hour to 5 g/L_{broth}/hour, 0.2 mg/L_{broth}/hour to 1 g/L_{broth}/hour, 1 g/L_{broth}/hour to 2.5 g/L_{broth}/hour, 2.5 g/L_{broth}/hour to 5 g/L_{broth}/hour. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0541] Provided herein are methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and cell viability is reduced by less than about two-fold. In some embodiments, the cumulative total productivity of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the cumulative total productivity of the isoprene is between about any of 0.2 mg/L_{broth}/hour to 5 g/L_{broth}/hour, 0.2 mg/L_{broth}/hour to 1 g/L_{broth}/hour, 1 g/L_{broth}/hour to 2.5 g/L_{broth}/hour, 2.5 g/L_{broth}/hour to 5 g/L_{broth}/hour. In some embodiments, cell viability is reduced by less than about any of 1.75-fold, 1.5-fold, 1.25-fold, 1-fold, 0.75-fold, 0.5-fold, or 0.25-fold.

[0542] Methods of producing isoprene are also provided herein comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the peak concentration of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the peak concentration of isoprene is between about any of 10 ng/L_{broth} to 500 ng/L_{broth}, 500 ng/L_{broth} to 1 μg/L_{broth}, 1 μg/L_{broth} to 5 μg/L_{broth}, 5 μg/L_{broth} to 50 μg/L_{broth}, 5 μg/L_{broth} to 100 μg/L_{broth}, 5 μg/L_{broth} to 250 μg/L_{broth}, 250 μg/L_{broth} to 500 μg/L_{broth}, 500 μg/L_{broth} to 1 mg/L_{broth}, 1 mg/L_{broth} to 50 mg/L_{broth}, 1 mg/L_{broth} to 100 mg/L_{broth}, 1 mg/L_{broth} to 200 mg/L_{broth}, 10 ng/L_{broth} to 200 mg/L_{broth}, 5 μg/L_{broth} to 100 mg/L_{broth}, or 5 μg/L_{broth} to 200 mg/L_{broth}. In some embodiments, the peak concentration is any of about 10 ng/L_{broth}, 100 ng/L_{broth}, 1 μg/L_{broth}, 5 μg/L_{broth}, 1 mg/L_{broth}, 30 mg/ L_{broth}, 100 mg/L_{broth}, or 200 mg/L_{broth}. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour

to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0543] In addition, methods of producing isoprene are also provided herein comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and cell viability is reduced by less than about two-fold. In some embodiments, the peak concentration of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the peak concentration of isoprene is between about any of 10 ng/L_{broth} to 500 ng/L_{broth}, 500 ng/L_{broth} to 1 μg/L_{broth}, 1 μg/L_{broth} to 5 μg/L_{broth}, 5 μg/L_{broth} to 50 μg/L_{broth}, 5 μg/L_{broth} to 100 μg/L_{broth}, 5 μg/L_{broth} to 250 μg/L_{broth}, 250 μg/L_{broth} to 500 μg/L_{broth}, 500 μg/L_{broth} to 1 mg/L_{broth}, 1 mg/L_{broth} to 50 mg/L_{broth}, 1 mg/L_{broth} to 100 mg/L_{broth}, 1 mg/L_{broth} to 200 mg/L_{broth}, 10 ng/L_{broth} to 200 mg/L_{broth}, 5 μg/L_{broth} to 100 mg/L_{broth}, or 5 μg/L_{broth} to 200 mg/L_{broth}. In some embodiments, the peak concentration is any of about 10 ng/L_{broth}, 100 ng/L_{broth}, 1 μg/L_{broth}, 5 μg/L_{broth}, 1 mg/L_{broth}, 30 mg/ L_{broth}, 100 mg/L_{broth}, or 200 mg/L_{broth}. In some embodiments, cell viability is reduced by less than about any of 1.75-fold, 1.5-fold, 1.25-fold, 1-fold, 0.75-fold, 0.5-fold, or 0.25-fold. In some embodiments, cell viability is reduced by about 2-fold.

[0544] Cells in culture are also provided herein comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the isoprene produced is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the amount of isoprene is between about any of 400 nmole/g_{wcm}/hour to 1 mole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 1 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 40 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 4 mmole/g_{wcm}/hour, 1 mmole/g_{wcm}/hour to 1.5 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour to 3 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour to 5 mmole/g_{wcm}/hour, 5 mmole/g_{wcm}/hour to 25 mmole/g_{wcm}/hour, 25 mmole/g_{wcm}/hour to 100 mmole/g_{wcm}/hour, 100 mmole/g_{wcm}/hour to 500 mmole/g_{wcm}/hour, or 500 mmole/g_{wcm}/hour to 1000 mmole/g_{wcm}/hour. In some embodiments, the amount of isoprene is about any of 1

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

[0545] Provided herein are also cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the cumulative total productivity of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the cumulative total productivity of the isoprene is between about any of 0.2 mg/L_{broth}/hour to 5 g/L_{broth}/hour, 0.2 mg/L_{broth}/hour to 1 g/L_{broth}/hour, 1 g/L_{broth}/hour to 2.5 g/L_{broth}/hour, 2.5 g/L_{broth}/hour to 5 g/L_{broth}/hour. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0546] In addition, provided herein are cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the peak concentration of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the peak concentration of isoprene is between about any of 10 ng/L_{broth} to 500 ng/L_{broth}, 500 ng/L_{broth} to 1 µg/L_{broth}, 1 µg/L_{broth} to 5 µg/L_{broth}, 5 µg/L_{broth} to 50 µg/L_{broth}, 5 µg/L_{broth} to 100 µg/L_{broth}, 5 µg/L_{broth} to 250 µg/L_{broth}, 250 µg/L_{broth} to 500 µg/L_{broth}, 500 µg/L_{broth} to 1 mg/L_{broth}, 1 mg/L_{broth} to 50 mg/L_{broth}, 1 mg/L_{broth} to 100 mg/L_{broth}, 1 mg/L_{broth} to 200 mg/L_{broth}, 10 ng/L_{broth} to 200 mg/L_{broth}, 5 µg/L_{broth} to 100 mg/L_{broth}, or 5 µg/L_{broth} to 200

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

[0547] In some embodiments of any of the methods and cells described herein, carbon dioxide evolution rate and/or cell viability of a cell expressing a MVA pathway and/or DXP pathway RNA and/or protein from one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid is compared to a control cell lacking one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid. In some embodiments, carbon dioxide evolution rate and/or cell viability of a cell expressing a MVA pathway and/or DXP pathway RNA and/or protein from one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid under the control of an inducible promoter, wherein the promotor is induced, is compared to a control cell containing one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid under the control of an inducible promoter, wherein the promotor is not induced (uninduced). In some embodiments, the inducible promoter is a beta-galactosidase promoter.

[0548] In some embodiments, the methods of producing isoprene comprise: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. Further provided herein are methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. Methods of producing isoprene are also provided herein comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments of

any of these methods, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about 50 mmol/L/hour or about 500 mmol/L/hour.

[0549] Further provided herein are cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. Provided herein are also cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In addition, provided herein are cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments of any of these cells in culture, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about 50 mmol/L/hour or about 500 mmol/L/hour.

[0550] Provided herein are also methods of producing isoprene comprising a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the liquid phase concentration of isoprene is less than about 200 mg/L and the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene. In some embodiments, the liquid phase concentration of isoprene in the culture is less than about any of 175 mg/L, 150 mg/L, 125 mg/L, 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, or 2.5 mg/L. In some embodiments, the liquid phase concentration of isoprene in culture is between about any of 0.1 mg/L to 200 mg/L, 1 mg/L to 200 mg/L, 1 mg/L to 150 mg/L, 1 mg/L to 100 mg/L, 1 mg/L to 50 mg/L, 1 mg/L to 25 mg/L, 1 mg/L to 20 mg/L, or 10 mg/L to 20 mg/L.

[0551] Also provided herein are methods of producing a compound, wherein the compound has one or more characteristics selected from the group consisting of (a) a Henry's law coefficient of less than about 250 M/atm and (b) a solubility in water of less than about 100 g/L.

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

[0552] In one aspect, cells in culture are used to produce isoprene. In some embodiments, the cells in culture produce greater than about 400 nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr) of isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes a carbon source, such as, but not limited to, a carbohydrate, 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.

[0553] In some embodiments, the cells in culture convert more than about 0.002% of the carbon in a cell culture medium into isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, 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.

[0554] In some embodiments, the cells in culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, 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.

[0555] In one aspect, described herein are methods of producing isoprene, such as methods of using any of the cells described herein to produce isoprene. In some embodiments, the method involves culturing cells under conditions sufficient to produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the method also includes recovering isoprene produced by the cells. In some embodiments, the method includes purifying isoprene produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing. In some embodiments, the cells are cultured under limited glucose conditions. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2 or more times the amount of isoprene produced during the growth phase for the same length of time. In some embodiments, the gas phase comprises greater than or about 9.5 % (volume) oxygen, and the concentration of isoprene in the gas phase is less than the

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

[0556] In some embodiments, the method includes culturing cells under conditions sufficient to convert more than about 0.002% of the carbon (mol/mol) in a cell culture medium into isoprene. In some embodiments, the method also includes recovering isoprene produced by the cells. In some embodiments, the method includes purifying isoprene produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, 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.

[0557] 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.

[0558] In one aspect, described herein are 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.

[0559] 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, 1-pentene, 2-methyl-1-butene, 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, cis-1,3-pentadiene, trans-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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.

[0560] In some embodiments, the composition has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In particular embodiments, the composition also has greater than about 2 mg of isoprene.

[0561] In some embodiments, the composition has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms. In some embodiments, the composition has greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 μg/L of ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the composition has greater than about 2 mg of isoprene and has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms.

[0562] In some embodiments, the composition includes isoprene and one or more second compounds selected from the group consisting of 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine. In various embodiments, the amount of one of these second components relative to the amount of

isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is at greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w).

[0563] 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 μg/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 μg/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, 1-pentene, 2-methyl-1-butene, 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, 1-pentene, 2-methyl-1-butene, 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.

[0564] In some embodiments, the volatile organic fraction of the gas phase has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the volatile organic fraction of the gas phase that inhibits the polymerization of isoprene. In particular embodiments, the volatile organic fraction of the gas phase also has greater than about 2 mg of isoprene.

[0565] In some embodiments, the volatile organic fraction of the gas phase has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms. In some embodiments, the volatile organic fraction of the gas phase has greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 μg/L of ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the volatile organic fraction of the gas phase has greater than about 2 mg of isoprene and has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms.

[0566] In some embodiments, the volatile organic fraction of the gas phase has includes isoprene and one or more second compounds selected from the group consisting of 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine. In various embodiments, the amount of one of these second components relative to amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is at greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w) in the volatile organic fraction of the gas phase.

[0567] In some embodiments of any of the compositions described herein, 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.

[0568] In some embodiments, the systems 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.

[0569] In some embodiments of any of the compositions, systems, and methods described herein, 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.

[0570] In some embodiments of any of the aspects described herein, 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 described herein, 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 described herein, 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 described herein, 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.

[0571] In some embodiments of any of the aspects described herein, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects described herein, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects described herein, the cells further comprise a heterologous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects described herein, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects described herein, the cells further comprise one or more nucleic acids encoding an IDI polypeptide and a DXS polypeptide. In some embodiments of any of the aspects described herein, one nucleic acid encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments of any of the aspects described herein, one vector encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments, the vector comprises a selective marker, such as an antibiotic resistance nucleic acid.

[0572] In some embodiments of any of the aspects described herein, 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 described herein, 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 described herein, 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 described herein, 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.

[0573] In some embodiments, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more MVA pathway, IDI, DXP, or isoprene synthase

nucleic acids are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

[0574] In some embodiments of any of the aspects described herein, at least a portion of the cells maintain the heterologous isoprene synthase nucleic acid for at least or about 5, 10, 20, 40, 50, 60, 65, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects described herein, the nucleic acid comprising the isoprene synthase, IDI, or DXS nucleic acid also comprises a selective marker, such as an antibiotic resistance nucleic acid.

[0575] In some embodiments of any of the aspects described herein, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects described herein, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects described herein, the cells comprise an isoprene synthase, DXS, and MVA pathway nucleic acid. In some embodiments of any of the aspects described herein, the cells comprise an isoprene synthase nucleic acid, a DXS nucleic acid, an IDI nucleic acid, and a MVA pathway nucleic (in addition to the IDI nucleic acid).

[0576] In some embodiments of any of the aspects described herein, 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*).

[0577] In some embodiments of any of the aspects described herein, the cells are bacterial cells, such as gram-positive bacterial cells (*e.g.*, *Bacillus* cells such as *Bacillus subtilis* cells or *Streptomyces* cells such as *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus* cells). In some embodiments of any of the aspects described herein, the cells are gram-negative bacterial cells (*e.g.*, *Escherichia* cells such as *Escherichia coli* cells, *Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris* cells, *Pseudomonas sp.* such as *Pseudomonas fluorescens* cells or *Pseudomonas putida* cells, or *Pantoea* cells such as *Pantoea citrea* cells). In some embodiments of any of the aspects described herein, the cells are fungal, cells such as filamentous fungal cells (*e.g.*, *Trichoderma* cells such as *Trichoderma reesei* cells or *Aspergillus* cells such as *Aspergillus oryzae* and *Aspergillus niger*) or yeast cells (*e.g.*,

Yarrowia cells such as *Yarrowia lipolytica* cells or *Sacchraomyces* cells such as *Saccaromyces cerevisiae*).

[0578] In some embodiments of any of the aspects described herein, the microbial polypeptide carbon source includes one or more polypeptides from yeast or bacteria. In some embodiments of any of the aspects described herein, 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.

Production of isoprene in anaerobic microorganisms using synthesis gas as an energy source

[0579] In some embodiements, the bioisoprene composition is produced in anaerobic microorganisms using synthesis gas as an energy source as described in US Provisional Patent Application Nos. 61/289,347 and 61/289,355, filed on December 22, 2009, the disclosures of which are incorporated herein by reference in their entireties.

[0580] Production of isoprene from syngas by anaerobic organisms may provide a number of advantages over production of isoprene from sugars by aerobic organisms. First, the maximum theoretical mass yield of isoprene can be greater for the aerobic organisms, as discussed further below. Second, the anaerobic organisms do not have excess reducing power in the form of NAD(P)H that must be turned over via cell growth, formation of byproducts (such as glycerol, lactic acid, or ethanol) or oxidation using molecular oxygen. Without this NAD(P)H turnover requirement, anaerobic organisms can have higher energy yield, lower oxygen demand, lower heat of fermentation, and lower utility costs to run the process. Third, due to the lack of oxygen in the system, anaerobic organisms can have greater isoprene concentration in the offgas, lower probability of creating a flammable isoprene-oxygen mixture, easier recovery, and higher isoprene quality. Fourth, the anaerobic organisms can be more easily grown by using existing infrastructure, such as existing plants designed for production of bioethanol.

[0581] Anaerobic organisms useful for isoprene production can include obligate anaerobes, facultatitive anaerobes, and aerotolerant anaerobes. The obligate anaerobes can be any one or combination selected from the group consisting of *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Eurobacterium limosum*, *Clostridium carboxydivorans*, *Peptostreptococcus productus*, and *Butyribacterium methylotrophicum*. Syngas useful as energy source for production of isoprene can be derived from a feedstock by a variety of processes, including

methane reforming, coal liquefaction, co-firing, fermentative reactions, enzymatic reactions, and biomass gasification.

Exemplary Purification Methods

[0582] In some embodiments, any of the methods described herein further include recovering the co-produced compounds. In some embodiments, any of the methods described herein further include recovering the isoprene. In some embodiments, any of the methods described herein further include recovering the hydrogen by cryogenic membrane, adsorption matrix-based separation methods.

[0583] The isoprene and hydrogen produced using the compositions and methods described herein can be recovered using standard techniques. such as gas stripping, membrane enhanced separation, fractionation, adsorption/desorption, pervaporation, thermal or vacuum desorption of isoprene from a solid phase, or extraction of isoprene immobilized or absorbed to a solid phase with a solvent (see, for example, U.S. Patent Nos. 4,703,007, 4,570,029, and 4,740,222 ("Recovery and Purification of Hydrogen from Refinery and Petrochemical Off-gas Streams") which are each hereby incorporated by reference in their entireties, particularly with respect to isoprene recovery and purification methods ('007 and '029 patents) and with respect to hydrogen recovery and purification methods ('222 patent)). In particular embodiments, extractive distillation with an alcohol (such as ethanol, methanol, propanol, or a combination thereof) is used to recover the isoprene. In some embodiments, the recovery of isoprene involves the isolation of isoprene in a liquid form (such as a neat solution of isoprene or a solution of isoprene in a solvent). Gas stripping involves the removal of isoprene vapor from the fermentation off-gas stream in a continuous manner. Such removal can be achieved in several different ways including, but not limited to, adsorption to a solid phase, partition into a liquid phase, or direct condensation (such as condensation due to exposure to a condensation coil or do to an increase in pressure). In some embodiments, membrane enrichment of a dilute isoprene vapor stream above the dew point of the vapor resulting in the condensation of liquid isoprene. In some embodiments, the isoprene is compressed and condensed.

[0584] 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.

[0585] The recovery of hydrogen may involve one step or multiple steps. In some embodiments, the removal of hydrogen gas from the fermentation off-gas and the conversion of hydrogen to a liquid phase are performed simultaneously. In some embodiments, the removal of hydrogen gas from the fermentation off-gas and the conversion of hydrogen to a liquid phase are performed sequentially. For example, hydrogen may be adsorbed to a solid phase and then desorbed from the solid phase by a pressure swing. In some embodiments, recovered hydrogen gas is concentrated and compressed.

[0586] In some embodiments, any of the methods described herein further include purifying the isoprene. For example, the isoprene produced using the compositions and methods described herein 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.

[0587] In some embodiments, any of the methods described herein further include purifying the hydrogen. For example, the hydrogen produced using the compositions and methods described herein can be purified using standard techniques. Purification refers to a process through which hydrogen is separated from one or more components that are present when the hydrogen is produced. In some embodiments, the hydrogen is obtained as a substantially pure gas. In some embodiments, the hydrogen is obtained as a substantially pure liquid. Examples of purification methods include (i) cryogenic condensation and (ii) solid matrix adsorption. As used herein, "purified hydrogen" means hydrogen that has been separated from one or more components that are present when the hydrogen is produced. In some embodiments, the hydrogen is at least about 20%, by weight, free from other components that are present when the hydrogen is produced. In various embodiments, the hydrogen is at least or about 25%, 30%,

40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, or 99%, by weight, pure. Purity can be assayed by any appropriate method, *e.g.*, by column chromatography or GC-MS analysis.

[0588] 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.

[0589] Methods and apparatus for the purification of a bioisoprene composition from fermentor off-gas is described in US Provisional Patent Application No. 61/88,142, filed December 18, 2009, which is incorporated herein by reference in its entirety.

[0590] A bioisoprene composition from a fermentor off-gas may contain bioisoprene with volatile impurities and bio-byproduct impurities. In some embodiments, a bioisoprene composition from a fermentor off-gas is purified using a method comprising: (a) contacting the fermentor off-gas with a solvent in a first column to form: an isoprene-rich solution comprising the solvent, a major portion of the isoprene and a major portion of the bio-byproduct impurity; and a vapor comprising a major portion of the volatile impurity; (b) transferring the isoprene-rich solution from the first column to a second column; and (c) stripping isoprene from the isoprene-rich solution in the second column to form: an isoprene-lean solution comprising a major portion of the bio-byproduct impurity; and a purified isopene composition.

[0591] Figure 169 illustrates an exemplary method of purifying isoprene and an exemplary apparatus. Fermentor off-gas comprising isoprene may be generated from renewable resources (*e.g.*, carbon sources) by any method in the art for example, as described in U.S. provisional patent application Nos. 61/187,944, the content of which is hereby incorporated by reference, particularly with respect to the methods of generating fermentor off-gas comprising isoprene. The fermentor off-gas generated from one or more individual fermentors **12** (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or more fermentors connected in series and/or in parallel) may be directed to a first column **14**. As described below, the fermentor off-gas may be directed through an isolation unit **16** and/or compressed by a compression means, such as compression system **18**. Additionally, the temperature of the fermentor off-gas may optionally be reduced at any point, for example, to form a condensate or partial condensate prior to contact with the solvent (which may aid in solubiliztion of one or more off-gas components, such as isoprene). The fermentor off-gas may be contacted (*e.g.*, absorbed) at column **14** with a solvent (*e.g.*, any solvent described herein, such as a non-polar high boiling-point solvent). The volatile impurities having less propensity

for absorption in the solvent (particularly with non-polar high boiling-point solvents) are separated from the remaining solvent/fermentor off-gas mixture, resulting in a vapor comprising a major portion of the volatile impurity (*e.g.*, exiting at port **20**), and an isoprene-rich solution having a major portion of the isoprene and a major portion of the bio-byproduct impurity (*e.g.*, at port **22**). The solvent may optionally be heated by any suitable means (*e.g.*, by steam) prior to, simultaneously, and/or after contact with the fermentor off-gas, which may aid in separation of the volatile impurity from the remaining solution. Steam may be directed through the column (at any suitable location, such as near entry of the off-gas and/or the opposite end of the volatile impurity exit as shown in Figure 169) to provide a sweeping vapor phase which may aid in the removal of the volatile impurity.

[0592] The isoprene-rich solution having a major portion of the isoprene and a major portion of the bio-byproduct impurity (*e.g.*, at port **22**) may be directed to a second column **24**. The second column may be isolated from the first column 14 (as shown in Figure 169) or may be part of a single column comprising both the first and second columns (*e.g.*, a tandem column wherein the solvent enters the first column at or near one end, and exits the second column at or near an opposite end). The isoprene may be stripped from the isoprene-rich solution in the second column to generate a purified isopene composition (*e.g.*, at port **26**) and an isoprene-lean solution comprising a major portion of the bio-byproduct impurity (*e.g.*, at port **28**). The isoprene-rich solution may be heated by any suitable means (*e.g.*, by steam), which may aid in stripping of the isoprene from the remaining solution. Steam may be directed through the column (at any suitable location, such as the opposite end of the entry point of the isoprene-rich solution and/or the near the end of the isoprene-lean solution exit as shown in Figure 169).

[0593] As described herein, the columns may be conventional and of any suitable size. Exemplary types of columns are commercially available from manufacturers including Koch Modular Process Systems (Paramus, NJ), Fluor Corporation (Irving, TX), Kuhni USA (Mount Holly, NC). In general, columns are designed to maximize vapor/liquid contact in order to achieve the desired efficiency. This is achieved by filling the column with either a packing material, or trays spaced at regular intervals along the column. Suitable packing materials include both random and structured types based on metal, glass, polymer and ceramic materials. Exemplary random packing types include Raschig rings, Pall rings, A-PAK rings, Saddle rings, Pro-Pak, Heli-Pak, Ceramic saddles and FLEXIRINGS®. Structured packings include wire mesh and perforated metal plate type materials. Manfacturers specializing in column packings include ACS Separations & Mass-Transfer Products (Houston, TX), Johnson Bros. Metal Forming Co.

(Berkeley, IL) and Koch Glitsch, Inc. Knight Div. (East Canton, OH). The efficiency of a gas stripping column is expressed in terms of the theoretical plate height and the total number of plates in the column. In general, the greater the number of theoretical plates present, the greater the efficiency of the column. Laboratory scale columns can be purchased from Ace Glass (Vineland, NJ), Sigma-Aldrich (St. Louis, MO) and Chemglass (Vineland, NJ). Suitable types of glass column include Vigreux, Snyder, Hemple and Perforated-plate type columns. Columns can include packing materals, or contain features designed to maximize vapor/liquid contact. A laboratory scale gas scrubber unit (part # CG-1830-10) is available from Chemglass and consists of a packed glass column, solvent reservoir and solvent recirculation pump.

[0594] The purified isoprene composition from the second column **24** (*e.g.*, exiting at port **26**) may be further purified by any suitable means (*e.g.*, by using a reflux condenser **34** and/or an adsorption system **36**, such as a silica adsorption system). The reflux reduces the solvent composition in the isoprene product. The isoprene-lean solution may be recycled back to the first column for reuse (*e.g.*, as shown in Figure 169 at port **30**). The isoprene-lean solution may be purified by any suitable means (*e.g.*, by liquid-liquid extraction and/or an adsorption system **32**, such as a silica adsorption system) prior to recycling to the first column **14** to reduce to amount of bio-byproduct. Additionally, the temperature of the isoprene-lean solution may be reduced by any suitable means prior to recycling to the first column **14** (*e.g.*, prior to, simultaneously, and/or after optionally purifying the isoprene solution). Figure 169 shows an example of reducing the temperature of the isoprene-lean solution at port **40** prior to purification of the isoprene-lean solution (in this case, using coolant for temperature reduction).

[0595] The vapor comprising a major portion of the volatile impurity (*e.g.*, the vapor exiting at port **20** in Figure 169) may comprise a minor portion of isoprene (*e.g.*, residual isoprene not remaining in the isoprene-rich solution). The residual isoprene may be recollected for use from the vapor comprising a major portion of the volatile impurity by any suitable means (*e.g.*, an adsorption system **38**, such as an activated carbon adsorption system) and in some cases, as shown in Figure 169, may be combined with the purified isoprene composition (*e.g.*, prior to, during, or after additional purification, such as an adsorption system similar to system **36**). Figure 169 also shows an optional capture device **42** (*e.g.*, a thermal oxidizer and/or CO_2 capture system) capable of reducing the amount of undesirable components released into the atmosphere (*e.g.*, CO_2) from the vapor.

Exemplary chemical transformations of isoprene

[0596] Although current industrial use of isoprene is predominantly in the production of synthetic rubber, isoprene is a reactive conjugated diene and undergoes a varieties of chemical transformations to form oxygenates and higher molecular weight hydrocarbons. For example, Palladium(0) complexes ($Pd(acac)_2$-Ph_3P and $Pd(OAc)_2$-Ph_3P) catalyze dimerization and telomerization of isoprene in alcohol solvents to give linear isoprene dimers (e.g. 2,7-dimethyl-1,3,7-octatriene) and methoxydimethyloctadienes (Zakharkin, L. I. and Babich, S. A. *Russ. Chem. Bull.* (**1976**), pp 1967-1968.) Adams, J. M. and Clapp, T. V. (*Clay and Clay Minerals* (**1986**), 34(3), 287-294) reported reactions of isoprene over divalent and trivalent transition metal-exchanged montmorillonites (e.g. Cr^{3+}-montmorillonite) to give isoprene dimers and adducts with methanol. The linear dimerization of isoprene catalyzed by Ni(0)-aminophosphinite systems resulted in regioselective tail-to-tail linear dimers, accompanied by a competitive cyclodimerization reaction (Denis, Philippe; Croizy, Jean Francois; Mortreux, Andre; Petit, Francis, *Journal of Molecular Catalysis* (**1991**), 68(2), 159-75. Denis, Philippe; Jean, Andre; Croizy, Jean Francois; Mortreux, Andre; Petit, Francis, *Journal of the American Chemical Society* (**1990**), 112(3), 1292-4.) New chiral aminophosphinite ligands, e.g., (+)-$MeCH_2CHMeCH(NH_2)CH_2OPPh_2$ was investigated as homogeneous catalysts in the linear dimerization of isoprene, leading to a conversion rate above 50% (Masotti, Henriette; Peiffer, Gilbert; Siv, Chhan; Courbis, Pierre; Sergent, Michelle; Phan Tan Luu, Roger, *Bulletin des Societes Chimiques Belges* (**1991**), 100(1), 63-77.)

[0597] Thermal dimerization of isoprene at 110-250° in presence of dinitrocresol as polymerization inhibitor gives high yields of dimers and little polymer (U.S. Patent No. 4,973,787.) Ni-catalyzed dimerization of isoprene yields a dimethyl-1,5-cyclooctadiene mixture consisting of 80% 1,5-dimethyl-1,5-cyclooctadiene and 20% 1,6-dimethyl-1,5-cyclooctadiene (Doppelt, Pascal; Baum, Thomas H.; Ricard, Louis, *Inorganic Chemistry* (**1996**), 35(5), 1286-91.) Isoprene is converted to dimethylcyclooctadienes with a catalytic amt. of Cp*Ru(**η**4-isoprene)Cl and AgOTf (Itoh, Kenji; Masuda, Katsuyuki; Fukahori, Takahiko; Nakano, Katsumasa; Aoki, Katsuyuki; Nagashima, Hideo, *Organometallics* (**1994**), 13(3), 1020-9.) JP59065026A (1984) reported preparation of 1,6-dimethyl-1,5-cyclooctadiene by cyclic dimerization of isoprene in the presence of catalysts comprising Fe carboxylates or ß-diketone compounds, organo-Al or Mg compounds, and 2,2'-dipyridyl derivatives having electron-donating groups. Dimethylcyclooctadiene was prepared by cyclodimerization of isoprene over 3-component catalysts containing Ni carboxylates or ß-ketones, organoaluminum or

organomagnesium compounds and substituted triphenylphosphite (JP58055434A, 1983.) 1,5-Dimethyl-1,5-cyclooctadiene was prepared by cyclodimerization of isoprene at 100-300° in an inert organic solvent in the presence of a homogeneous catalyst containing Fe(3) salt, organoaluminum compound and an activator (SU615056A1, 1978), in the presence of a homogeneous catalyst containing Ni acetylacetonate, a triarylphosphite and perhydroalumophenolene (SU493455A1, 1975), in the presence of a catalyst containing a mixture of a Ni carboxylate or carboxylate or chelate compounds of Ni and 1-hydroxy-3-carbonyl compounds, trialkylaluminum, dialkylmagnesium or active organo-Mg compounds obtained from conjugated dienes and Mg, triaryl phosphites and tertiary amines (JP48064049A, 1973), or in the presence of a catalyst composed of Ni naphthenate, Et_3Al, and tri-o-cresyl phosphate (Suga, K.; Watanabe, S.; Fujita, T.; Shimada, T., *Israel Journal of Chemistry* (**1972**), 10(1), 15-18.) U.S. Patent No. 3,954,665 disclosed dimerization of isoprene in the presence of reaction products of $[(\eta 3\text{-}C6H5)NiBr]_2$ or $[M(NO)_2X]_2$ (M = Fe, Co; X = Cl, I, Br) with Fe, Co, or Ni carbonyls. European Patent No. 2411(1981) disclosed cyclodimerization of isoprene over a $Fe(NO)_2Cl$-bis(1,5-cyclooctadiene)nickel catalyst at from -5° to +20° to give 1-methyl- and 2-methyl-4-isopropenyl-1-cyclohexene and 1,4- and 2,4-dimethyl-4-vinyl-1-cyclohexene. U.S. Patent No. 4,189,403 disclosed preparation of 1,5-dimethyl-1,5-cyclooctadiene and 1,4-dimethyl-4-vinyl-1-cyclohexene by contacting isoprene with a mixed catalyst of a tris(substituted hydrocarbyl) phosphite, arsenite, or antimonite and a Group VIII metal(0) compound (e.g. Ni acetylacetonate). Jackstell, R.; Grotevendt, A.; Michalik, D.; El Firdoussi, L.; Beller, M. *J. Organometallic Chem.* (**2007**) 692(21), 4737-4744 cites the use of palladium/carbene catalysts for isoprene dimerization. Bowen, L.; Charernsuk, M.; Wass, D.F. *Chem. Commun.* (**2007**) 2835-2837 describes the use of a chromium *N,N*-bis(diarylphosphino)amine catalyst for the production of linear and cyclic trimers of isoprene.

[0598] Isoprene was reportedly dimerized in the presence of a Ni catalyst to yield cis-2-isopropenyl-1-methylvinylcyclobutane (Billups, W. E.; Cross, J. H.; Smith, C. V., *Journal of the American Chemical Society* (**1973**), 95(10), 3438-9.) The oligomerization of isoprene [78-79-5] catalyzed by nickel naphthenate and isoprenemagnesium in the presence of various phosphites as electron donors gave cyclic dimers containing dimethylcyclooctadiene [39881-79-3]; in particular 1,1,1-tris(hydroxymethyl)propane phosphite [39865-19-5] gave trimethylcyclododecatriene [39881-80-6] selectively (Suga, Kyoichi; Watanabe, Shoji; Fujita, Tsutomu; Shimada, Takashi, *Journal of Applied Chemistry & Biotechnology* (**1973**), 23(2), 131-8.) WO2006/051011 discloses preparation of trimethylcyclododecatriene, useful in perfumes

and fragrances, by the trimerization of isoprene in the presence of a catalyst system comprising Ni and/or Ti, one or more organometallic compound, and a Group VA compound, and that the reaction is conducted in a hydroxyl group-containing solvent. Ligabue, R. A.; Dupont, J.; de Souza, R. F., Alegre, R.S. *J. Mol. Cat. A: Chem.* (**2001**), 169(1-2), 11-17, describes the selective dimerization of isoprene to six-membered dimers using an iron nitrosyl catalyst in an ionic liquid. Huchette, D.; Nicole, J.; Petit, F., Tetrahedron Letters (**1979**), (12), 1035-8, describes the electrochemical generation of an iron nitrosyl catalyst and subsequent use for the dimerization of isoprene to cyclohexene dimers. Zakharkin, L. I.; Zhigareva, G. G.; Pryanishnikov, A. P. *Zhurnal Obshchei Khimii* (**1987**), 57(11), 2551-6, describes the cyclooligomerization of isoprene on complex nickel and iron catalysts.

[0599] The highly pure isoprene starting compositions described herein are chemically transformed using each catalyst systems and reaction conditions disclosed in the references cited. Other catalysts and reaction conditions known in the art such as catalysts and reaction conditions applied to chemical transformations of 1,3-butadiene can be adapted to the isoprene starting compositions by one skilled in the art.

Dimerization and Trimerization

[0600] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material undergoes catalytic chemical transformation to give dimers and trimers. Catalyst systems are identified using methods known in the art. Preferred catalysts include those known to convert isoprene to dimers and trimers with high efficiency. Examples include those based upon Palladium, Nickel, Cobalt, Iron and Chromium.

[0601] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material undergoes thermal or catalytic dimerization. The isoprene starting composition is converted to a mixture of dimers that includes unsaturated 6 and 8-membered rings by heating the starting composition under pressure. In some embodiments, the starting isoprene composition is heated to over or about 100, 125, 150, 175, 200, 225 or 250 °C under pressure. In some embodiments, the starting isoprene composition is heated in the presence of an antioxidant (e.g. 2,6-di-tert-butyl-4-methylphenol) to prevent radical-mediated polymerization. In some embodiments, the reaction is accelerated by one or more catalysts selected from catalysts known in the art for catalyzing cyclic dimerization of isoprene, e.g., iron nitrosyl halide catalysts described in US patents 4,144,278, 4,181,707, 5,545,789 and European patent EP0397266A2. The use of iron nitrosyl catalysts in ionic liquids is described by Ligabue *et al.*

(2001) *J. Mol. Catalysis A: Chemical*, 169 (2), 11-17. Examples of catalysts include but are not limited to $Fe(NO)_2Cl_2$ and Cr^{3+}-montmorillonite. In another example, an isoprene starting composition is converted to C10 cyclic dimers (e.g. a mixture of dimethyl-cyclooctadienes) using a ruthenium catalyst (Itoh, Kenji; Masuda, Katsuyuki; Fukahori, Takahiko; Nakano, Katsumasa; Aoki, Katsuyuki; Nagashima, Hideo, *Organometallics* (**1994**), 13(3), 1020-9.)

[0602] In a specific embodiment, a isoprene starting composition in a liquid state is heated to over 100 °C under pressure and in the presence of an antioxidant to produce a mixture of dimers that includes 6 and 8-membered rings, for examples, limonene (1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene), 1-methyl-5-(prop-1-en-2-yl)cyclohex-1-ene, 1,4-dimethyl-4-vinylcyclohexene, 2,4-dimethyl-4-vinylcyclohexene, 1,5-dimethylcycloocta-1,5-diene, 1,6-dimethylcycloocta-1,5-diene, 2,6-dimethyl-1,3-cyclooctadiene, 2,6-dimethyl-1,4-cyclooctadiene, 3,7-dimethyl-1,5-cyclooctadiene, 3,7-dimethyl-1,3-cyclooctadiene, 3,6-dimethyl-1,3-cyclooctadiene and other 8-membered ring isoprene dimers as described in *Organometallics* (**1994**), 13(3), 1020-9. All stereoisomers of these compounds are contemplated. Conversion of isoprene to dimers is best performed in the absence of oxygen so as to avoid the production of undesirable reaction products.

[0603] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material undergoes photo-dimerization to give 4-membered ring dimers. The isoprene starting composition is converted by light irradiation to a mixture comprising one or more of 1,2-di(prop-1-en-2-yl)cyclobutane, 1,3-di(prop-1-en-2-yl)cyclobutane, 1-methyl-1-vinyl-3-(prop-1-en-2-yl)cyclobutane, 1-methyl-1-vinyl-2-(prop-1-en-2-yl)cyclobutane, 1,3-dimethyl-1,3-divinylcyclobutane, 1,2-dimethyl-1,2-divinylcyclobutane and stereoisomers thereof . In some embodiments, the highly pure isoprene starting material is dimerized in the presence of a catalyst (e.g. a nickel catalyst) or a photosensitizer (e.g. benzophenone) to yield 4-membered ring dimers, e.g. cis-2-isopropenyl-1-methylvinylcyclobutane, in addition to 6- and 8-membered rings [See: Hammond, J.S. ;Turro, N.J. ; Liu, R.S.H. (**1963**) "Mechanisms of Photochemical Reactions in Solution. XVI. Photosensitized Dimerization of Conjugated Dienes. *J. Org. Chem.*, 28, 3297-3303.]

[0604] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material is converted to a cyclic trimer in the presence of a catalyst system. In some embodiments, the cyclic trimer is trimethylcyclododecatriene. In some embodiments, the catalyst system comprises a nickel catalyst. In some embodiments, the catalyst system comprises a titanium compound. In some embodiments, the catalyst system comprises a nickel

catalyst and a titanium compound. In some embodiments, the catalyst system comprises one or more organometallic compound and a Group VA compound. In some embodiments, the catalytic transformation is conducted in a hydroxyl group-containing solvent. In a particular embodiment, a starting isoprene composition is converted to trimethylcyclododecatriene in the presence of a catalyst system comprising Ni and/or Ti, one or more organometallic compound, and a Group VA compound, and that the reaction is conducted in a hydroxyl group-containing solvent.

[0605] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material undergoes thermal or catalytic conversion to hydrocarbons in the C7 to C14 range by treatment with a C2 to C5 unsaturated hydrocarbon. The C2 to C5 hydrocarbon can be an alkene, a diene or an alkyne. For example, in some embodiments, isoprene is contacted with ethylene and an appropriate catalyst to produce C7 compounds. In another embodiment, isoprene is contacted with 1,3-butadiene to form cyclic C9 hydrocarbons. Hydrogenation of these C7 to C14 hydrocarbons results in compositions suitable for use as jet fuels and other aviation fuels. In yet another embodiment, unsaturated C7 to C14 hydrocarbons derived from isoprene and one or more unsaturated hydrocarbons undergo dehydrogenation to form aromatic derivatives suitable for use as jet fuels and other aviation fuels, or as blendstocks for such fuels.

[0606] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material is converted to a mixture of linear dimers and trimers using a catalyst system in alcohol solvents. This reaction also produces alkoxy derivatives when performed in methanol or ethanol. In some embodiments, the catalyst system is a palladium-based catalyst system, e.g., a palladium acetylacetonate – triphenylphosphine system or a palladium acetate – triphenylphosphine system. In some embodiments, the alcohol solvent is methanol, ethanol or isopropyl alcohol. The nature of the products depends on the catalyst and the solvent used. For example, when a palladium-based catalyst system, e.g., $Pd(acac)_2$-Ph_3P or $Pd(OAc)_2$-Ph_3P, is used in isopropyl alcohol solvent, linear dimers of isoprene is formed, e.g. 2,7-dimethyl-1,3,7-octatriene and 2,7-dimethyl-2,4,6-octatriene. When this reaction is performed in methanol, methoxydimethyloctadienes (e.g., 1-methoxy-2,7-dimethyl-2,7-octadiene and 3-methoxy-2,7-dimethyl-1,7-octadiene) are formed in addition to linear isoprene dimers such as dimethyloctatrienes. Some reactions produce linear trimers of isoprene, such as a-farnesene (3,7,11-trimethyl-1,3,6,10-dodecatetraene), ß-farnesene (7,11-dimethyl-3-methylene-1,6,10-dodecatriene) and other positional isomers (e.g. from tail to tail and head to head addition of isoprene units). In another example, isoprene is converted to linear and cyclic C15 trimers using

a chromium *N,N*-bis(diarylphosphino)amine catalyst (Bowen, L.; Charernsuk, M.; Wass, D.F. *Chem. Commun.* (**2007**) 2835-2837.)

Conversion to Oxygenates

[0607] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material is converted to fuel oxygenates by reaction with ethanol and other alcohols in the presence of an acid catalyst. In one embodiment, the acid catalyst is sulfuric acid. In another embodiment, the acid catalyst is a solid phase sulfuric acid (e.g., Dowex Marathon®). Other catalysts include both liquid and solid-phase fluorosulfonic acids, for example, trifluoromethanesulfonic acid and Nafion-H (DuPont). Zeolite catalysts can also been used, for example beta-zeolite, under conditions similar to those described by Hensel *et al.* [Hensen, K.; Mahaim, C.; Holderich, W.F., *Applied Catalysis A: General* (**1997**) 140(2), 311-329.] for the methoxylation of limonene and related monoterpenes. In some embodiments, a highly pure isoprene starting material is converted to alcohols and esters by a hydroxylation/esterification process or other known reaction of alkenes in the art, for example peroxidation to epoxides with peracids such as peracetic acid and 3-chloroperbenzoic acid; and hydration to give alcohols and diols with i) water and acid catalysts and ii) hydroboration methods. Such reactions are described in, for example, Michael B. Smith and Jerry March, *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, Sixth Edition, John Wiley & Sons, 2007. Figures 3 and 4 show examples of alcohols and oxygenates that can be produced from the starting isoprene compositions.

Partial Hydrogenation

[0608] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material is partially hydrogenated to a mono-olefin (e.g. 2-methylbut-1-ene, 3-methyl-but-1-ene and 2-methylbut-2-ene). In some embodiments, the mono-olefin undergoes dimerization or reaction with other olefins using traditional hydrocarbon cationic catalysis, such as that used to covert isobutylene to isooctane. See, for example, H.M. Lybarger. Isoprene in *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed., Wiley, New York (1995), *14*, 934–952. In some preferred embodiments, the highly pure isoprene starting material is a bioisoprene composition.

[0609] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material undergoes partial hydrogenation to form mono-olefins (e.g. 2-methylbut-1-ene, 3-methyl-but-1-ene and 2-methylbut-2-ene). In some preferred embodiments, the highly pure

isoprene starting material is a bioisoprene composition. In some embodiments, partial hydrogenation of the isoprene starting material produces high yields of mono-olefins with minimal conversion to isopentane and low residual isoprene levels. In some embodiments, a mixture of mono-olefin and isopentane is produced, with low levels of residual isoprene. In some embodiments, the partial hydrogenation is selective hydrogenation where a particular mono-olefin such as 2-methylbut-2-ene, 2-methylbut-1-ene, or 3-methylbut-1-ene is preferentially produced. Preferred hydrogenation catalysts give high catalytic activity, maintain catalytic activity over time, and are highly selective for the conversion of isoprene to mono-olefins.

[0610] Suitable catalysts for partial hydrogenation of the isoprene starting composition may contain a platinum-group metal such as platinum, palladium, rhodium, or ruthenium, or a transition metal such as nickel, cobalt, copper, iron, molybdenum, or a noble metal such as silver or gold in elemental form, or a salt or complex with organic and inorganic ligands. Alloys of these metals can also be used in some circumatances. These metals and their derivatives can be pure or mixed with other active and inert materials. Catalysts may be adsorbed to a support material (such as activated carbon, alumina, or silica) in order to maximize the effective surface area. The physical morphology of hydrogenation catalysts is known to have a considerable influence on their performance. Preferred hydrogenation catalysts include heterogeneous palladium catalysts such as palladium on carbon (Pd/C), palladium on alumina (Pd/Al_2O_3), or palladium on silica (Pd/SiO_2) in grades ranging from 0.1% Pd to 20% Pd (w/w) relative to the support material. An example of a suitable selective hydrogenation catalyst is LD 2773, a sulfur tolerant promoted Pd on alumina (Axens, Rueil-Malmaison Cedex, France). Partial hydrogenation catalysts that allow the conversion of isoprene into isoamylenes with minimal conversion to isopentane include the Lindlar catalyst ($Pd/BaSO_4$ treated with quinoline), Pd/C treated with the triphenyl derivative of a group 15 element (N, P, As), Pd/C treated with a sulfur-containing compound, molybdenum sulfide, and Pd/Fe alloys. One preferred catalyst comprises palladium adsorbed to egg-shell alumina ($d\text{-}Al_2O_3$). Catalysts used in the refining industry for the removal of diolefins and alkynes from pyrolysis gasoline are particularly preferred, for example Ni/Al_2O_3 and Pd/Al_2O_3 based catalysts. Another suitable class of catalyst for the conversion of isoprene to isoamylenes are those used in industry for the hydrotreating of pyrolysis gasoline. See for example US Patent Nos. 7,014,750 and 6,9949,686, and references cited therein. In general, the catalysts used in pyrolysis gas hydrotreament allow for selective conversion of acetlyenes and diolefins (e.g. isoprene and piperylenes) into monoolefins.

[0611] The hydrogen source can be hydrogen gas or a hydrogen source including, but not limited to, hydrogen gas (H_2), formic acid, hydrazine, or isopropanol. The hydrogen source can be either chemically or biologically derived. In some embodiments the hydrogen used for hydrogenation is co-produced with isoprene during fermentation. The hydrogenation can be performed at hydrogen pressures ranging from 0.5 atm to 200 atm, or higher. The temperature can range from 0 °C to 200 °C.

[0612] Products from partial hydrogenation of a bioisoprene composition are expected to contain certain impurities originally present in the starting isoprene composition and/or hydrogenated derivatives of the imputities such as acetone, ethanol, amyl alcohol, ethyl acetate, isoamyl acetate, methyl ethyl ketone and other saturated polar impurities.

[0613] In some embodiments, the isoprene starting composition undergoes partial hydrogenation or selective hydrogenation in the presence of a palladium catalyst. For example, palladium catalysts, such as $Pd/CaCO_3$, $Pd/BaSO_4$, Pd/C, Pd black, Pd/SiO3, Pd/Al_2O_3, or Pd/SiO_2, were shown to convert isoprene to mono-olefins with a selectivity of greater than 95% over fully reduced C5 alkanes. Use of these palladium catalysts gave a mixture of mono-olefin products, with 5%Pd/CaCO3 having the greatest selectivity for 3-methylbut-1-ene and 5% Pd/SiO_2 having the greatest selectivity for 2-methylbut-2-ene. (See G.C. Bond and A.F. Rawle. J. Mol. Catalysis A: Chemical 109 (1996) 261-271.) Bond and Rawle have also shown that palladium-gold and palladium-silver catalysts (e.g., $Pd\text{-}Au/SiO_2$ and $Pd\text{-}Ag/SiO_2$) have high selectivity for reducing isoprene to mono-olefins over fully reduced C5 alkanes. In some embodiments, silica-supported polyamidoamine (PAMAM) dendrimer-palladium complexes have been used to selectively catalyze the reduction of cyclic and acyclic dienes to a mixture of mono-olefin isomers. Selectivity for the various mono-olefin isomers was dependent on the precise PAMAM ligand used in the catalyst. (See P.P. Zweni and H. Alper. Adv. Synth. Catal. 348 (2006) 725-731.) In some embodiments, reduction of isoprene to mono-olefins can be carried out using a Group VIB metal on an inorganic support (e.g., a metal zeolite) as a catalyst. For example, a Mo/Al_2O_3 catalyst was used to selectively reduce 1,3-butadiene to the respective mono-olefins. (See US 6,235,954 B1.) In some embodiment, isoprene can be reduced to mono-olefins using a Group VIII metal catalyst promoted by a metal from Group IB, VIB, VIIB, or zinc to reduce poisoning of the catalyst. (See US 6,949,686 B2). In some embodiments, isoprene can be reduced to mono-olefins using a monolithic catalyst bed, which may be in a honeycomb configuration. Catalyst support materials for the monolithic catalyst bed may include metals such as nickel, platinum, palladium, rhodium, ruthenium, silver, iron, copper,

cobalt, chromium, iridium, tin, and alloys or mixtures thereof. (See US 7,014,750 B2). In some embodiments, isoprene can be reduced to mono-olefins using an eggshell Pd/d-Al_2O_3 catalyst, particularly if the reaction is free of water. The eggshell Pd/d-Al_2O_3 catalyst is selective for mono-olefins over fully reduced alkanes and 2-methylbut-2-ene is the thermodynamically favorable isomer. (See J.-R. Chang and C.-H. Cheng. Ind. Eng. Chem. Res. 36 (1997) 4094-4099.)

[0614] Light olefins (C3-C6) can be readily dimerized using acid catalysts to give higher olefins (C6-C12) also known as dimate. For example, the conversion of isobutylene (2-methylpropene) to isooctene is well described in the art using a variety of acid catalysts including sulfuric, phosphoric and other mineral acids, sulfonic acids, fluorosulfonic acids, zeolites and acidic clays.

[0615] In some embodiments, the isoprene starting composition undergoes partial hydrogenation or selective hydrogenation to form a mono-olefin, and the mono-olefin undergoes dimerization or reaction with other olefins using traditional hydrocarbon cationic catalysis, such as that used to covert isobutylene to isooctane, e.g. sulfuric, phosphoric and other mineral acids, sulfonic acids, fluorosulfonic acids, zeolites and acidic clays. See, for example, H.M. Lybarger. Isoprene in Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed., Wiley, New York (1995), 14, 934–952. In some preferred embodiments, the highly pure isoprene starting material is a bioisoprene composition. Acid catalyst in both liquid and solid forms can be used. Acid resins are preferred and include Amberlyst 15, 35, XE586, XN1010 (Rohm and Haas) and similar acidic ion-exchange resins. Acidic molecular sieves are also preferred catalysts including a medium-pore acid molecular sieve such as ZSM-5, ferrierite, ZSM-22 and ZSM-23.

[0616] In some embodiments, the isoprene starting composition undergoes partial hydrogenation or selective hydrogenation to form isoamylenes. In some embodiments, the isoamylenes are dimerized to give C10 dimates such as isodecenes. In some embodiments, the isoamylenes are dimerized with an olefin such as propylene, butane or isobutene to give C8-C10 dimates. In some embodiments, the mono-olefin undergoes dimerization using a resin catalyst (e.g., Amberlyst, Amberlyst 35, Amberlyst 15, Amberlyst XN1010, Amberlyst XE586) to produce diisoamylenes. Use of such resin catalysts has been shown to minimize cracking and further oligomerization reactions, and under optimal conditions the resin catalysts provide selectivity for dimers of greater than 92%, with trimer formation of less than 8%. See, for example, M. Marchionna et al. Catalysis Today 65 (2001) 397-403. In some embodiments, the mono-olefin undergoes dimerization using a catalytic material containing an acidic mesoporous

molecular sieve, such as a mesoporous sieve embedded in a zeolite structure, ZSM-5, ferrierite, ZSM-22, or ZSM-23. In a particular embodiment, the catalytic material is thermally stable at high temperatures (e.g., at least 900 °C). In another particular embodiment, the mono-olefin is substantially free of multi-unsaturated hydrocarbons, such as isoprene. Use of a catalytic material containing an acidic mesoporous molecular sieve for dimerization can lead to selectivity for dimers in excess of 80%. See, for example, US 2007/0191662 A1. In some embodiments, the mono-olefin undergoes dimerization using a solid acidic catalyst (e.g., a solid phosphoric acid catalyst, acidic ion exchange resins). Selectivity for dimers using a solid phosphoric acid catalyst can be at least 75%, at least 85% or at least 90%. See, for example, US 2009/0099400 A1 and US 6,660,898 B1.

[0617] Efficient dimerization can sometimes require the presence of a polar component(s) such as water and oxygenated compound in the feed stream. Examples include alcohols such as methanol, ethanol and t-butanol, ethers such as methyl t-butyl ether (MTBE) and methyl t-amyl ether (TAME) or an ester such as C1 to C5 acetates. In some embodiments, isoamylenes can be converted to ethers such as TAME by treatment with alcohols in the presence of an acid catalyst.

[0618] In some embodiments, the C10 dimers of mono-olefins produced by any of the methods described herein can be reduced to fully saturated C10 alkylates (e.g., by hydrogenation). In a particular embodiment, isoprene or a mono-olefin can be reduced to an alkylate using a catalyst containing an acid component, such as sulfuric acid, a fluorosulfonic acid, a perhaloalkylsulfonic acid, an ionic liquid, or a mixture of Bronsted acids and Lewis acids, mixed with a polymer component, such as a polyacrylate. (See US 2010/0094072 A1).

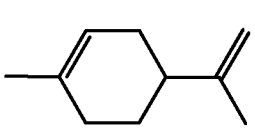
[0619] In some embodiments, a commercially beneficial amount of highly pure isoprene starting material undergoes oligomerization in the presence of an acid catalysts to produce dimers, trimers, higher oligomers, aromatic products, and/or polymeric products. Kinetic control of the reaction can favor certain products, for example lower oligomers, although gum formation and coking are known issues leading to catalyst deactivation.

Exemplary unsaturated isoprene derivatives

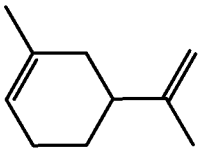
[0620] In some embodiments, the compositions and systems for producing a fuel constituent from isoprene further comprise an unsaturated hydrocarbon or oxygenate intermediate for chemical transformation of an isoprene starting composition to a fuel constituent. In some embodiment, the unsaturated hydrocarbon intermediate comprise one or more unsaturated dimers of isoprene selected from the group consisting of 1,2-di(prop-1-en-2-yl)cyclobutane, 1,3-

di(prop-1-en-2-yl)cyclobutane, 1-methyl-1-vinyl-3-(prop-1-en-2-yl)cyclobutane, 1-methyl-1-vinyl-2-(prop-1-en-2-yl)cyclobutane, 1,3-dimethyl-1,3-divinylcyclobutane, 1,2-dimethyl-1,2-divinylcyclobutane, 1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene), 1-methyl-5-(prop-1-en-2-yl)cyclohex-1-ene, 1,4-dimethyl-4-vinylcyclohexene, 2,4-dimethyl-4-vinylcyclohexene, 1,5-dimethylcycloocta-1,5-diene, 1,6-dimethylcycloocta-1,5-diene, 1,4-dimethyl-4-vinyl-1-cyclohexene, 2,4-dimethyl-4-vinyl-1-cyclohexene, 2,7-dimethyl-1,3,7-octatriene, 2,7-dimethyl-2,4,6-octatriene, 2,6-dimethyl-1,3-cyclooctadiene, 2,6-dimethyl-1,4-cyclooctadiene, 3,7-dimethyl-1,5-cyclooctadiene, 3,7-dimethyl-1,3-cyclooctadiene and 3,6-dimethyl-1,3-cyclooctadiene. In some embodiments, the unsaturated hydrocarbon intermediate comprises one or more unsaturated trimers such as a-farnesene, ß-farnesene, trimethylcyclododecatrienes (e.g. 1,5,9-trimethyl-(1*E*,5*E*,9*E*)-cyclododecatriene and positional and geometric isomers thereof) and trimethyldodecatetraenes and the like. In some embodiments, the unsaturated oxygenate intermediate comprise one or more unsaturated methyl ethers such as 1-methoxy-2,7-dimethyl-2,7-octadiene and 3-methoxy-2,7-dimethyl-1,7-octadiene and the like. In some embodiments, the unsaturated oxygenate intermediate comprise one or more unsaturated ethyl ethers such as ethyl 3-methyl-3-butenyl ether, ethyl 1,1-dimethyl-2-propenyl ether, ethyl 1,2-dimethyl-2-propenyl ether, ethyl 2-methyl-3-butenyl ether and the like. In some embodiments, the unsaturated oxygenate intermediate comprise one or more unsaturated alcohols such as 3-methyl-3-buten-1-ol, 2-methyl-3-buten-2-ol, 3-methyl-3-buten-2-ol, 2-methyl-3-buten-1-ol and the like. In some embodiments, the unsaturated oxygenate intermediate comprise one or more unsaturated esters such as 3-methyl-3-buten-1-yl acetate, 2-methyl-3-buten-2-yl acetate, 3-methyl-3-buten-2-yl acetate, 2-methyl-3-buten-1-yl acetate and esters of other C_3-C_{18} aliphatic carboxylic acids. In some embodiment, the unsaturated hydrocarbon intermediate comprise one or more isoamylenes, e.g. 2-methylbut-1-ene, 3-methyl-but-1-ene and 2-methylbut-2-ene. In some embodiments, the unsaturated hydrocarbon intermediate comprise on or more diisoamylenes derived from dimerization of isoamylenes.

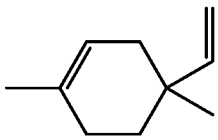
Scheme I. Exemplary unsaturated 6- and 8-membered ring intermediates



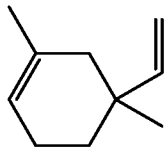
1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene



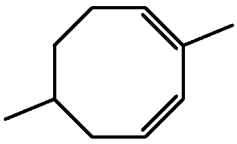
1-methyl-5-(prop-1-en-2-yl)cyclohex-1-ene



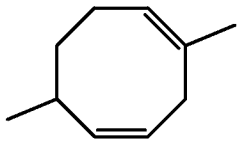
1,4-dimethyl-4-vinylcyclohexene



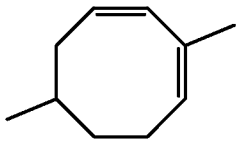
2,4-dimethyl-4-vinylcyclohexene



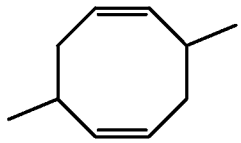
2,6-dimethyl-1,3-cyclooctadiene



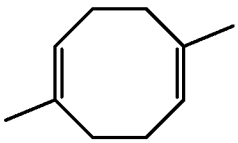
2,6-dimethyl-1,4-cyclooctadiene



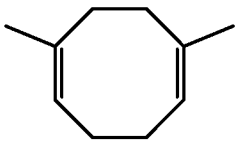
3,7-dimethyl-1,3-cyclooctadiene



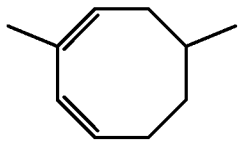
3,7-dimethyl-1,5-cyclooctadiene



1,5-dimethylcycloocta-1,5-diene

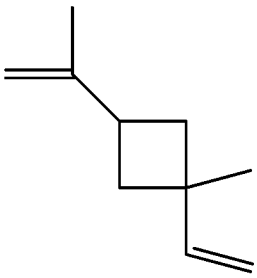


1,6-dimethylcycloocta-1,5-diene

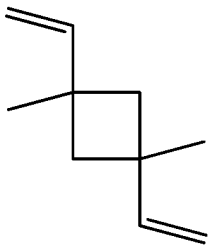


3,6-dimethyl-1,3-cyclooctadiene

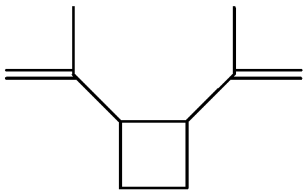
Scheme II. Exemplary unsaturated 4-membered ring intermediates



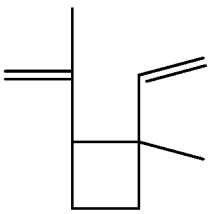
1-methyl-3-(prop-1-en-2-yl)-1-vinylcyclobutane



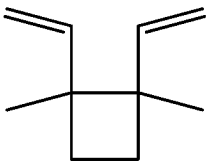
1,3-dimethyl-1,3-divinylcyclobutane



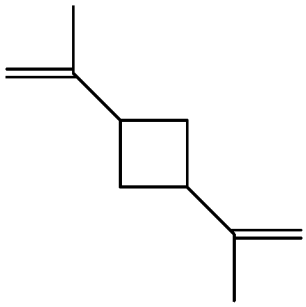
1,2-di(prop-1-en-2-yl)cyclobutane



1-methyl-2-(prop-1-en-2-yl)-1-vinylcyclobutane

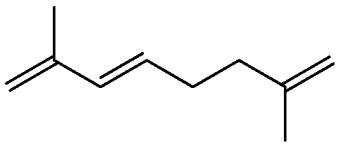


1,2-dimethyl-1,2-divinylcyclobutane

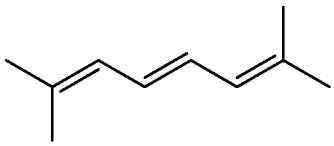


1,3-di(prop-1-en-2-yl)cyclobutane

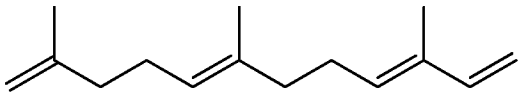
Scheme III. Exemplary unsaturated isoprene telomer and oxygenate intermediates



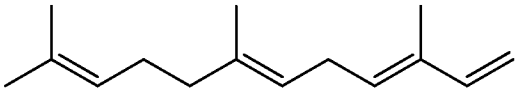
2,7-dimethylocta-1,3,7-triene



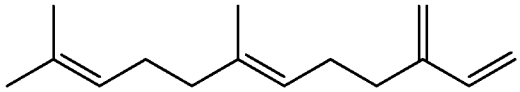
2,7-dimethylocta-2,4,6-triene



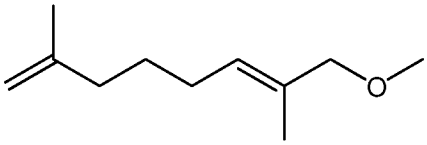
3,7,11-trimethyldodeca-1,3,7,11-tetraene



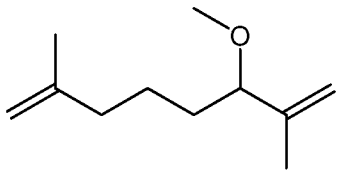
3,7,11-trimethyldodeca-1,3,6,10-tetraene



7,11-dimethyl-3-methylenedodeca-1,6,10-triene

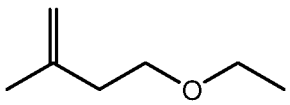


8-methoxy-2,7-dimethylocta-1,6-diene

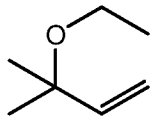


3-methoxy-2,7-dimethylocta-1,7-diene

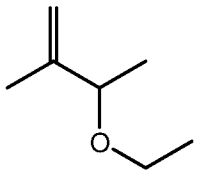
Scheme IV. Exemplary unsaturated ethyl ether intermediates



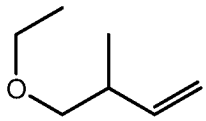
Ethyl 3-methyl-3-butenyl ether



Ethyl 1,1-dimethyl-2-propenyl ether

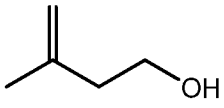


Ethyl 1,2-dimethyl-2-propenyl ether

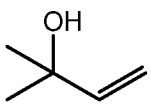


Ethyl 2-methyl-3-butenyl ether

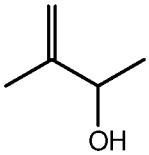
Scheme V. Exemplary unsaturated alcohol intermediates



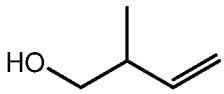
3-methyl-3-buten-1-ol



2-methyl-3-buten-2-ol

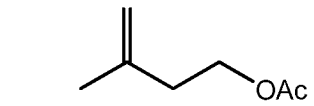


3-methyl-3-buten-2-ol

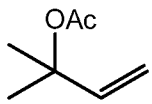


2-methyl-3-buten-1-ol

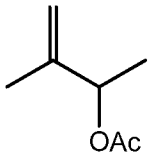
Scheme VI. Exemplary unsaturated ester intermediates



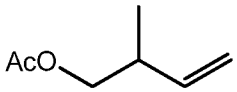
3-methyl-3-buten-1-yl acetate



2-methyl-3-buten-2-yl acetate



3-methyl-3-buten-2-yl acetate



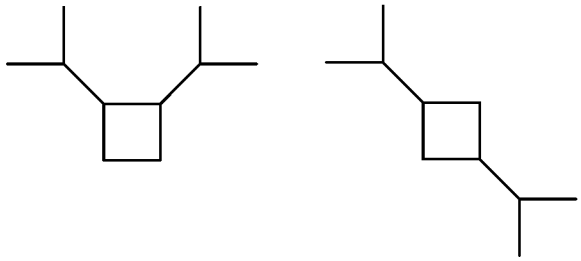
2-methyl-3-buten-1-yl acetate

Exemplary hydrogenation of unsaturated intermediates

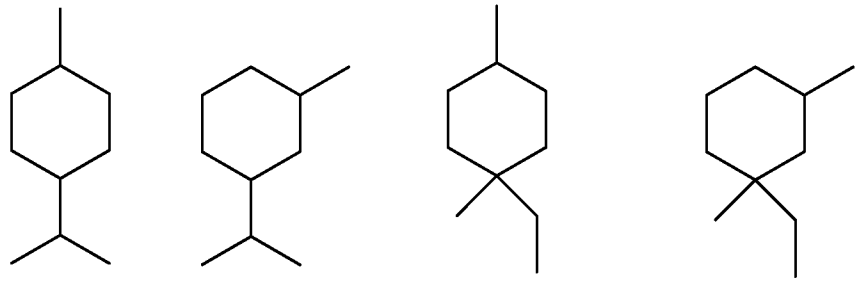
[0621] The unsaturated isoprene derivatives are subject hydrogenation in the presence of a hydrogenation catalyst to produce saturated compounds. The saturated compounds are characterized and their values as fuels are assessed. In some embodiments, the hydrogen source for hydrogenation is hydrogen gas. In some embodiments, the hydrogen gas is co-produced with the bioisoprene as described in U.S. provisional patent application No.61/141,652, filed on December 30, 2008 and US 2009/0203102 A1. In some embodiments, the hydrogenation catalyst is a palladium based catalyst such as Pd/C (e.g. 5% (wt.) Pd/C). In some embodiments, the hydrogenation catalyst is a Raney Nickel catalyst. In some embodiments, the hydrogenation catalyst is a homogenous catalyst such as ruthenium or rhodium based homogenous hydrogenation catalysts. In some embodiments, unsaturated isoprene dimers and trimers are hydrogenated to produce saturated C10 and C15 hydrocarbons suitable for making fuels. In some embodiments, unsaturated cyclic dimers are hydrogenated to produce saturated cyclic C10 hydrocarbons such as 1,2-bis(isopropyl)cyclobutane, 1,2-bis(isopropyl)cyclobutane, 1-methyl-4-isopropylcyclohexane, 1-methyl-3-isopropylcyclohexane, 1-ethyl-1,4-dimethylcyclohexane, 1-ethyl-1,3-dimethylcyclohexane, 1,5-dimethylcyclooctane and 1,4-dimethylcyclooctane. In some embodiments, unsaturated cyclic trimers are hydrogenated to produce saturated cyclic C15 hydrocarbons such as 1,5,9-trimethylcyclododecane and 1,5,10-trimethylcyclododecane (see Scheme VII). In some embodiments, unsaturated linear dimers are hydrogenated to produce saturated aliphatic C10 hydrocarbons such as 2,6-dimethyloctane, 2,7-dimethyloctane and 3,6-dimethyloctane. In some embodiments, unsaturated linear trimers are hydrogenated to produce saturated aliphatic C15 hydrocarbons such as 2,6,10-trimethyldodecane, 2,7,10-trimethyldodecane and 3,7,10-trimethyldodecane (see Scheme VIII). In some embodiments, the product of the dimerization of isoamylenes (diamylenes or C10 dimates) are fully hydrogenated

to isoparaffins (e.g. 2,3,4,4-tetramethylhexane, 2,2,3,4-tetramethylhexane, 2,3,3,4-tetramethylhexane and 3,3,5-trimethylheptane) (see Scheme VIIIa). In some embodiments, a commercially beneficial amount of highly pure isoprene starting composition is hydrogenated to produce a product comprising 2-methylbutane. In some embodiments, the unsaturated isoprene hydroxylates are hydrogenated to produce saturated hydroxylates such as C5 alcohols and diols (e.g. 3-methyl-butan-1-ol, 2-methyl-butan-1-ol and 2-methyl-butan-2-ol, 3-methyl-butan-1,3-diol and 2-methyl-butan-2,3-diol), C-10 alcohols and diols (e.g. 3,7-dimethyloctan-1-ol, 2,7-dimethyloctan-1-ol, 2,7-dimethyloctan-2-ol and 2,7-dimethyloctan-2,7-diol) and cyclic C-10 alcohols (e.g. 2-(4-methylcyclohexyl)propan-2-ol, 2-(4-methylcyclohexyl)propan-1-ol, 2-(1,4-dimethylcyclohexyl)ethanol and 4-ethyl-1,4-dimethylcyclohexanol) (see Scheme IX). In some embodiments, the unsaturated isoprene oxygenates are hydrogenated to produce saturated ethers such as 1,3-diethoxy-3-methylbutane, 1-ethoxy-3-methylbutane, 1-methoxy-2,7-dimethyloctane and 3-methoxy-2,7-dimethyloctane (see Scheme X). It is understood that when an alkene moiety is hydrogenated, one or more stereo isomers are produced. The relative ratios between the stereo isomers depend on the reaction conditions and the catalysts used. When applicable, each and every stereo isomer is intended for the saturated hydrocarbons and oxygenates described herein.

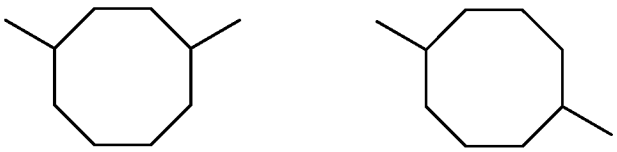
Scheme VII. Examples of cyclic hydrocarbons derived from isoprene



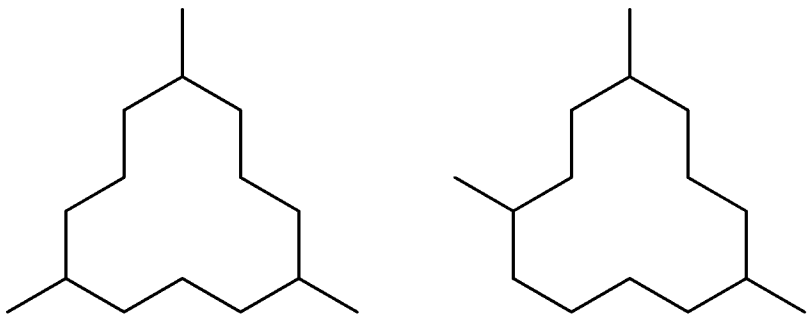
bis-(isopropyl)cyclobutanes
from 2+2 photodimerization



Cyclohexanes from thermal or catalytic cyclodimerization

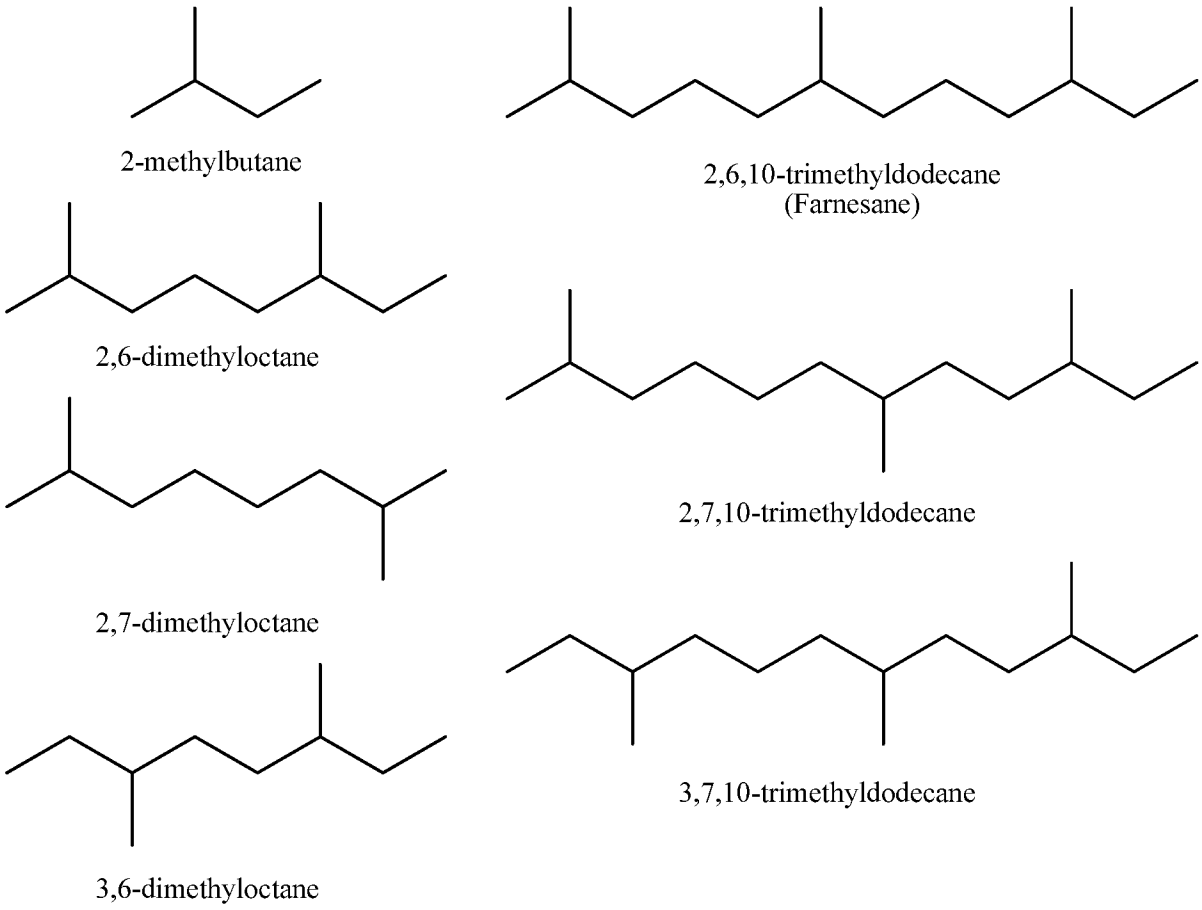


1,5-dimethyl- and 1,6-dimethylcyclooctanane from thermal or catalytic cyclodimerization

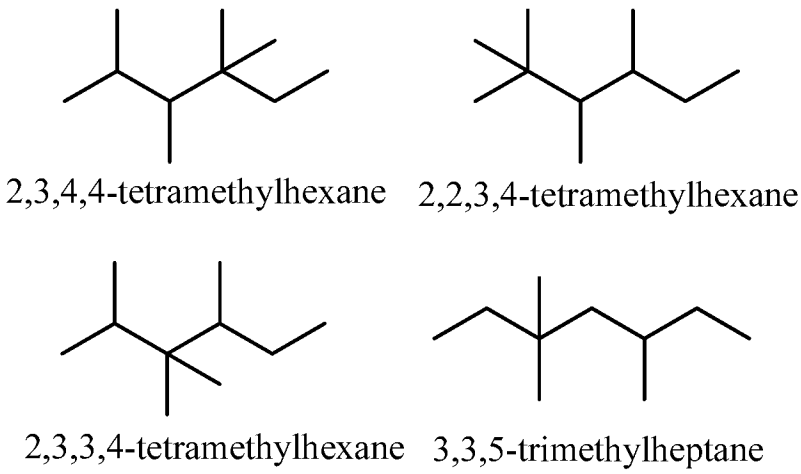


1,5,9- and 1,5,10-trimethylcyclododecane from cyclic trimerization of isoprene

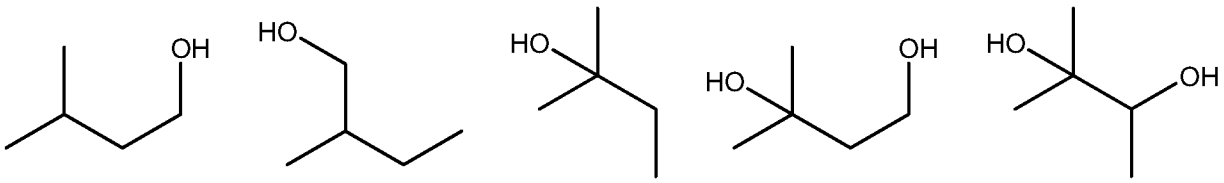
Scheme VIII. Examples of aliphatic hydrocarbons derived from isoprene



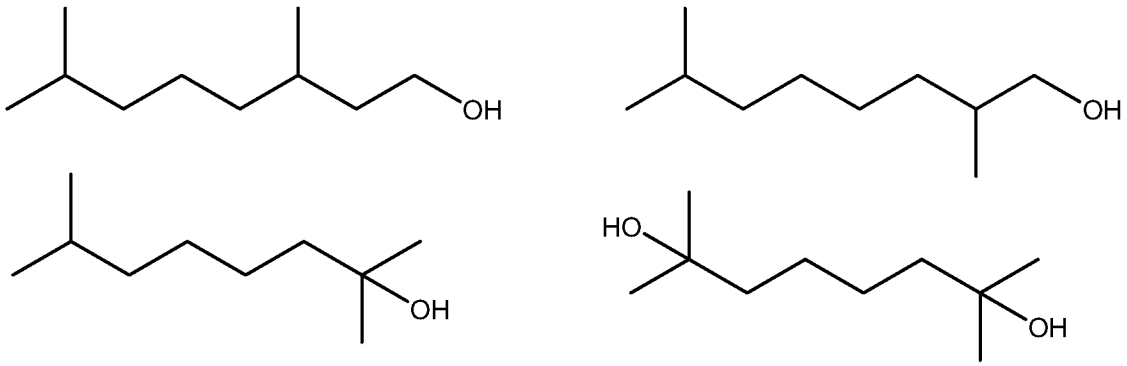
Scheme VIIIa. Examples of isodecanes derived from isoamylenes



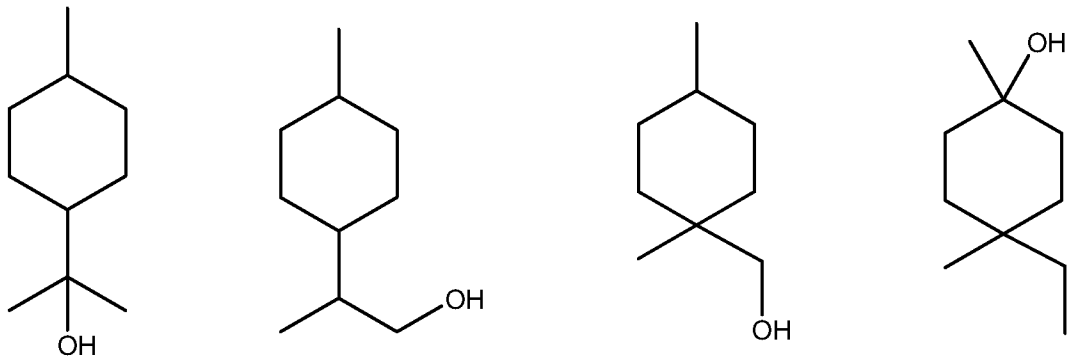
Scheme IX. Examples of IsoFuel™ alcohols derived from isoprene



C5 alcohols and diols

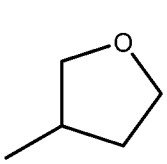


C10 alcohols and diols

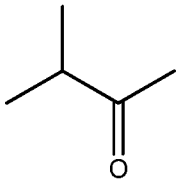


Cyclic C10 alcohols

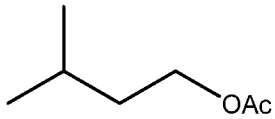
Scheme X. Examples of IsoFuel™ oxygenates derived from isoprene



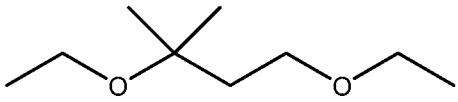
3-methyltetrahydrofuran

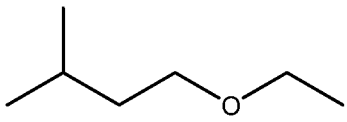


3-methyl-2-butanone

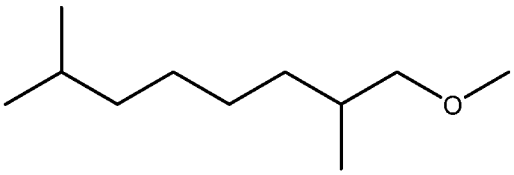


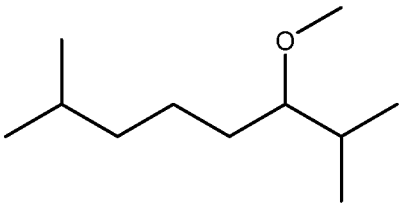
Isopentyl acetate





Reaction products of isoprene and ethanol
(see attached presentation from S. Reyer)





Alkoxy-derivatives produced by Pd-catalyzed telomerization of isoprene in methanol solvent.
(see Zakharkin (1976))

[0622] In some embodiment, the fuel constituent produced by chemical transformation of a commercially beneficial amount of highly pure isoprene starting composition comprises saturated isoprene derivatives. In some embodiments, the fuel constituent comprises saturated C10 and C15 hydrocarbons derived from isoprene. In some embodiments, the fuel constituent comprises one or more saturated cyclic C10 hydrocarbons selected from the group consisting of 1,2-bis(isopropyl)cyclobutane, 1,2-bis(isopropyl)cyclobutane, 1-methyl-4-isopropylcyclohexane, 1-methyl-3-isopropylcyclohexane, 1-ethyl-1,4-dimethylcyclohexane, 1-ethyl-1,3-dimethylcyclohexane, 1,5-dimethylcyclooctane and 1,4-dimethylcyclooctane. In some embodiments, the fuel constituent comprises one or more saturated cyclic C15 hydrocarbons selected from the group consisting of 1,5,9-trimethylcyclododecane and 1,5,10-trimethylcyclododecane. In some embodiments, the fuel constituent comprises one or more saturated aliphatic C10 hydrocarbons selected from the group consisting of 2,6-dimethyloctane, 2,7-dimethyloctane and 3,6-dimethyloctane. In some embodiments, the fuel constituent comprises one or more saturated aliphatic C15 hydrocarbons selected from the group consisting

of 2,6,10-trimethyldodecane, 2,7,10-trimethyldodecane and 3,7,10-trimethyldodecane. In some embodiments, the fuel constituent comprises 2-methylbutane. In some embodiments, the fuel constituent comprises one or more isoparaffins selected from the group consisting of 2,3,4,4-tetramethylhexane, 2,2,3,4-tetramethylhexane, 2,3,3,4-tetramethylhexane and 3,3,5-trimethylheptane. In some embodiments, the fuel constituent comprises one or more saturated hydroxylates selected from the group consisting of 3-methyl-butan-1-ol, 2-methyl-butan-1-ol and 2-methyl-butan-2-ol, 3-methyl-butan-1,3-diol, 2-methyl-butan-2,3-diol, 3,7-dimethyloctan-1-ol, 2,7-dimethyloctan-1-ol, 2,7-dimethyloctan-2-ol, 2,7-dimethyloctan-2,7-diol, 2-(4-methylcyclohexyl)propan-2-ol, 2-(4-methylcyclohexyl)propan-1-ol, 2-(1,4-dimethylcyclohexyl)ethanol and 4-ethyl-1,4-dimethylcyclohexanol. In some embodiments, the fuel constituent comprises one or more saturated ethers selected from the group consisting of 1,3-diethoxy-3-methylbutane, 1-ethoxy-3-methylbutane, 1-methoxy-2,7-dimethyloctane and 3-methoxy-2,7-dimethyloctane. In some embodiments, the fuel constituent comprises one or more oxygenates of isoprene selected from the group consisting of 3-methyltetrahydrofuran, 3-methyl-2-butanone and isopentyl acetate.

[0623] In some embodiments, the compositions and systems for producing a fuel constituent from isoprene further comprise catalysts for catalyzing the chemical transformation of an isoprene starting composition to a fuel constituent or an intermediate for making a fuel constituent. In some embodiments, the compositions and systems for producing a fuel constituent from isoprene further comprise catalysts for catalyzing hydrogenation of an unsaturated intermediate to produce a saturated fuel constituent. In some embodiments, the catalyst is any catalyst described or a combination of one or more of the catalyst described herein.

Method and/or Processes for Producing Fuels

[0624] The invention provides methods and/or processes for producing a fuel constituent from isoprene comprising: (a) obtaining a commercially beneficial amount of highly pure isoprene; and (b) chemically transforming at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent. In one embodiment, a highly pure isoprene composition is transformed to a fuel component in a continuous chemical process. In another embodiment, a highly pure isoprene is further purified before chemical transformation to a fuel composition. In yet another embodiment, a highly pure isoprene is chemically transformed to an intermediate composition; the intermediate composition undergoes further chemical

transformations to produce a fuel or a fuel component. In a further embodiment, the fuel component produced is mixed with a petroleum distillate and other optional additives to make a fuel. In some preferred embodiments, the highly pure isoprene is a bioisoprene composition.

[0625] In some embodiments, the method for producing a fuel constituent from isoprene comprises obtaining a commercially beneficial amount of highly pure isoprene composition. In some embodiments, the highly pure isoprene composition useful in the invention 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 highly pure isoprene 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 starting isoprene composition comprises greater than or about 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 kg of isoprene.

[0626] In some embodiments, the method for producing a fuel constituent from isoprene comprises obtaining a commercially beneficial amount of highly pure isoprene composition. In some embodiments, the highly pure isoprene starting composition comprises greater than or about 98.0, 98.5, 99.0, 99.5, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the starting composition. In some embodiments, the highly pure isoprene composition useful in the invention 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 highly pure isoprene composition comprises less than or about 2.0, 1.5, 1.0, 0.5, 0.2, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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 highly pure isoprene composition has less than or about 2.0, 1.5, 1.0, 0.5, 0.2, 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, 1-pentene, 2-methyl-1-butene, 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 highly pure isoprene composition has greater than about 2 mg of isoprene and has greater than or about 98.0, 98.5, 99.0, 99.5, 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.

[0627] In some embodiments, the method for producing a fuel constituent from isoprene comprises obtaining a commercially beneficial amount of highly pure isoprene composition. In some embodiments, the highly pure isoprene composition useful in the invention has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In particular embodiments, the composition also has greater than about 2 mg of isoprene.

[0628] In some embodiments, the method for producing a fuel constituent from isoprene comprises obtaining a commercially beneficial amount of highly pure isoprene composition. In some embodiments, the highly pure isoprene composition useful in the invention has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms. In some embodiments, the highly pure isoprene composition has greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 μg/L of ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the highly pure isoprene composition has greater than about 2 mg of isoprene and has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms.

[0629] In some embodiments, the method for producing a fuel constituent from isoprene comprises obtaining a commercially beneficial amount of highly pure isoprene composition. In some embodiments, the highly pure isoprene composition useful in the invention includes isoprene and one or more second compounds selected from the group consisting of 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine. In various embodiments, the amount of one of these second components relative to the amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is at greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w). In some

embodiments, the amount of methanethiol relative to the amount of isoprene in units of percentage by weight is at less than 0.01% (w/w).

[0630] In some embodiments, the method for producing a fuel constituent from isoprene comprises obtaining a commercially beneficial amount of highly pure isoprene composition. In some embodiments, the method comprises obtaining a commercially beneficial amount of highly pure isoprene composition from a biological process comprising: (a) culturing cells comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide under suitable culture conditions for the production of isoprene, wherein the cells (i) produce greater than about 400 nmole/g_{wcm}/hr of isoprene, (ii) convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, or (iii) have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr of isoprene, and (b) producing isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide, e.g. a naturally-occurring polypeptide from a plant such as *Pueraria*, and (ii) is operably linked to a promoter, e.g. a T7 promoter. In some embodiments, the cells are cultured in a culture medium that includes a carbon source, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, component from a yeast extract, or any combination of two or more of the foregoing. In some embodiments, the cells are cultured under limited glucose conditions. In some embodiment, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding an MDV pathway polypeptide. In some embodiment, the method comprises producing isoprene using the methods described in U.S. provisional patent application Nos. 61/134,094, filed on July 2, 2008, WO 2010/003007, and U.S. patent application No. 12/335,071, filed December 15, 2008 (US 2009/0203102 A1), which are incorporated by reference in their entireties.

[0631] In some embodiment, the method comprises obtaining a gas phase (off-gas) produced by cells in culture that produces isoprene. In some embodiments, the cells in culturing produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the gas phase comprises greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μg/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 2.0, 1.5, 1.0, 0.5, 0.2, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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 2.0, 1.5, 1.0, 0.5, 0.2, 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, 1-pentene, 2-methyl-1-butene, 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.

[0632] In some embodiment, the method comprises obtaining a gas phase (off-gas) produced by cells in culture that produces isoprene. In some embodiments, the volatile organic fraction of the gas phase has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the volatile organic fraction of the gas phase that inhibits the polymerization of isoprene. In particular embodiments, the volatile organic fraction of the gas phase also has greater than about 2 mg of isoprene. In some embodiments, the volatile organic fraction of the gas phase has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms. In some embodiments, the volatile organic fraction of the gas phase has greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 μg/L of ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the volatile organic fraction of the gas phase has greater than about 2 mg of isoprene and has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms. In some embodiments, the volatile organic fraction of the gas phase has includes isoprene and one or more second compounds selected from the group consisting of 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol,

diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine. In various embodiments, the amount of one of these second components relative to amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is at greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w) in the volatile organic fraction of the gas phase. In some embodiments, the method further includes recovering the isoprene from the gas phase. For example, the isoprene in the gas phase comprising isoprene 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 some 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 recovered isoprene is compressed and condensed.

[0633] The recovery of isoprene may involve one step or multiple steps. In some embodiments, the removal of isoprene vapor from a 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.

[0634] In some embodiments, a method is provided for producing a cyclic dimer of isoprene comprising heating neat bioisoprene. The cyclic dimers of isoprene produced may be 6-membered ring dimers (e.g. a [2+4] electrocyclization product such as limonene) or 8-membered ring dimers or a mixture thereof. Examples of 6-membered ring dimers include but not limited to 1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene, 1-methyl-5-(prop-1-en-2-yl)cyclohex-1-ene, 1,4-dimethyl-4-vinylcyclohexene, 2,4-dimethyl-4-vinylcyclohexene and the like. Examples of 8-membered ring dimers include but not limited to 1,5-dimethylcycloocta-1,5-diene, 1,6-dimethylcycloocta-1,5-diene and the like. In one embodiment, neat biologically produced isoprene is dimerized by heating to a temperature of about 100 °C to about 300 °C, preferably about 150 °C to about 250 °C. The pressure is maintained at about 2 to 3 atm. In another embodiment, a catalyst suitable for catalyzing dimerization of conjugated dienes may be used. The proportions of various dimers in the product mixture may be controlled by the catalyst and other reactions conditions. For example, a nickel catalyst can promote formation of 8-membered ring dimers.

[0635] In some embodiments, a method is provided for producing a cyclic dimer of isoprene comprising photo-dimerization of bioisoprene. The cyclic dimer of isoprene produced may be one or more 4-membered ring dimer or a mixture thereof. Examples of Examples of 4-membered ring dimers include but not limited to 1,2-di(prop-1-en-2-yl)cyclobutane, 1,3-di(prop-1-en-2-yl)cyclobutane, 1-methyl-1-vinyl-3-(prop-1-en-2-yl)cyclobutane, 1-methyl-1-vinyl-2-(prop-1-en-2-yl)cyclobutane, 1,3-dimethyl-1,3-divinylcyclobutane, 1,2-dimethyl-1,2-divinylcyclobutane and the like. In one embodiment, neat biologically produced isoprene is dimerized by irradiating with UV light, preferably in presence of a photosensitizer such as benzophenone.

[0636] Isoprene dimers can be hydrogenated to form saturated C_{10} hydrocarbons that can serve as fuels or blended into fuels. In one embodiment, the unsaturated isoprene dimers are subjected to catalytic hydrogenation to produce partially hydrogenated and/or fully hydrogenated products. Examples of fully hydrogenated products include but not limited to 1-methyl-4-isopropylcyclohexane and 1-methyl-5-isopropylcyclohexane, 1,4-dimethyl-4-ethylcyclohexane, 2,4-dimethyl-4-ethylcyclohexane, 1,2-diisopropylcyclobutane and 1,3-diisopropylcyclobutane, 1-methyl-1-vinyl-3-(prop-1-en-2-yl)cyclobutane, 1-methyl-1-ethyl-2-(prop-2-yl)cyclobutane,

1,3-dimethyl-1,3-ethylcyclobutane, 1,2-dimethyl-1,2-diethylcyclobutane, 1,5-dimethylcyclooctane, 1,6-dimethylcyclooctane and the like.

[0637] In some embodiments, the method for producing a fuel constituent from isoprene comprises: (a) obtaining a commercially beneficial amount of any of the highly pure isoprene starting composition described herein; (b) chemically transforming at least a portion of the highly pure isoprene starting composition to a fuel constituent comprising: (i) heating the highly pure isoprene composition to over or about 100 °C under pressure; (ii) converting at least a portion of the starting isoprene composition to unsaturated cyclic isoprene dimers; and (iii) hydrogenating the unsaturated cyclic isoprene dimers to produce saturated cyclic isoprene dimers. In some embodiments, the starting isoprene composition is heated to over or about 100, 125, 150, 175, 200, 225 or 250 °C under pressure. In some embodiments, the starting isoprene composition is heated in the presence of an antioxidant (e.g. 2,6-di-tert-butyl-4-methylphenol) to prevent radical-mediated polymerization. In one embodiment, the thermal dimerization of isoprene is performed in presence of dinitrocresol as polymerization inhibitor. Suitable antioxidants may be used as polymerization inhibitors. In some embodiments, at least a portion of the starting isoprene composition to a mixture of unsaturated dimers that includes 6 and 8-membered rings, for examples, limonene (1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene), 1-methyl-5-(prop-1-en-2-yl)cyclohex-1-ene, 1,4-dimethyl-4-vinylcyclohexene, 2,4-dimethyl-4-vinylcyclohexene, 1,5-dimethylcycloocta-1,5-diene, 1,6-dimethylcycloocta-1,5-diene, 2,6-dimethyl-1,3-cyclooctadiene, 2,6-dimethyl-1,4-cyclooctadiene, 3,7-dimethyl-1,5-cyclooctadiene, 3,7-dimethyl-1,3-cyclooctadiene and 3,6-dimethyl-1,3-cyclooctadiene. In some embodiments, greater than or about 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, 99 or 100% of isoprene in starting isoprene composition is converted to unsaturated cyclic isoprene dimers. In some embodiments, hydrogenation of the unsaturated cyclic isoprene dimers is catalyzed by a hydrogenation catalyst such as a palladium based catalyst (e.g. Pd/C, e.g. 5% (wt.) Pd/C.) In some embodiments, the saturated cyclic isoprene dimers comprise one or more C10 hydrocarbons selected from the group consisting of 1-methyl-4-isopropylcyclohexane, 1-methyl-3-isopropylcyclohexane, 1,5-dimethylcyclooctane and 1,4-dimethylcyclooctane. In some preferred embodiments, the starting isoprene composition is a bioisoprene composition.

[0638] In some embodiments, the method for producing a fuel constituent from isoprene comprises: (a) obtaining a commercially beneficial amount of any of the highly pure isoprene starting composition described herein; (b) chemically transforming at least a portion of the highly pure isoprene starting composition to a fuel constituent comprising: (i) contacting the

highly pure isoprene composition with a catalyst system; (ii) converting at least a portion of the starting isoprene composition to unsaturated isoprene dimers and/or trimers; and (iii) hydrogenating the unsaturated dimers and/or trimers to produce saturated C10 and/or C15 hydrocarbons. In some embodiments, greater than or about 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, 99 or 100% of isoprene in starting isoprene composition is converted to unsaturated dimers and/or trimers of isoprene. In some embodiments, the starting isoprene composition is contacted with appropriate catalyst systems known in the art to yield one or more of unsaturated cyclic isoprene dimers selected from the group consisting of 1,2-di(prop-1-en-2-yl)cyclobutane, 1,3-di(prop-1-en-2-yl)cyclobutane, 1-methyl-1-vinyl-3-(prop-1-en-2-yl)cyclobutane, 1-methyl-1-vinyl-2-(prop-1-en-2-yl)cyclobutane, 1,3-dimethyl-1,3-divinylcyclobutane, 1,2-dimethyl-1,2-divinylcyclobutane, 1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene), 1-methyl-5-(prop-1-en-2-yl)cyclohex-1-ene, 1,4-dimethyl-4-vinylcyclohexene, 2,4-dimethyl-4-vinylcyclohexene, 1,5-dimethylcycloocta-1,5-diene, 1,6-dimethylcycloocta-1,5-diene, 1,4-dimethyl-4-vinyl-1-cyclohexene, 2,4-dimethyl-4-vinyl-1-cyclohexene, 2,7-dimethyl-1,3,7-octatriene, 2,7-dimethyl-2,4,6-octatriene, 2,6-dimethyl-1,3-cyclooctadiene, 2,6-dimethyl-1,4-cyclooctadiene, 3,7-dimethyl-1,5-cyclooctadiene, 3,7-dimethyl-1,3-cyclooctadiene and 3,6-dimethyl-1,3-cyclooctadiene. In some embodiments, the starting isoprene composition is contacted with appropriate catalyst systems known in the art to yield one or more of unsaturated cyclic isoprene trimers such as trimethylcyclododecatrienes and trimethyldodecatetraenes and the like. In some embodiments, the starting isoprene composition is contacted with appropriate catalyst systems known in the art to yield one or more of unsaturated linear dimers and/or trimers of isoprene such as dimethyloctatrienes (e.g. 2,7-dimethyl-1,3,7-octatriene and 2,7-dimethyl-2,4,6-octatriene) and trimethyldodecatetraenes (e.g. 2,6,10-trimethyl-1,5,9,11-dodecatetraene, a-farnesene and ß-farnesene) and the like. In some preferred embodiments, the starting isoprene composition is a bioisoprene composition.

[0639] In some embodiments, the method for producing a fuel constituent from isoprene comprises contacting the highly pure isoprene composition with a catalyst system to convert at least a portion of the starting isoprene composition to unsaturated isoprene dimers and/or trimers. In some embodiments, the catalyst system comprise a Ni, Fe or Co catalyst and the isoprene in the starting composition is converted an 8-membered ring isoprene dimer comprising dimethylcyclooctadienes such as 1,5-dimethylcycloocta-1,5-diene and 1,6-dimethylcycloocta-1,5-diene. In some embodiments, the catalyst system comprises a nickel compound. Ni-catalyzed dimerization of isoprene yields a dimethyl-1,5-cyclooctadiene mixture consisting of

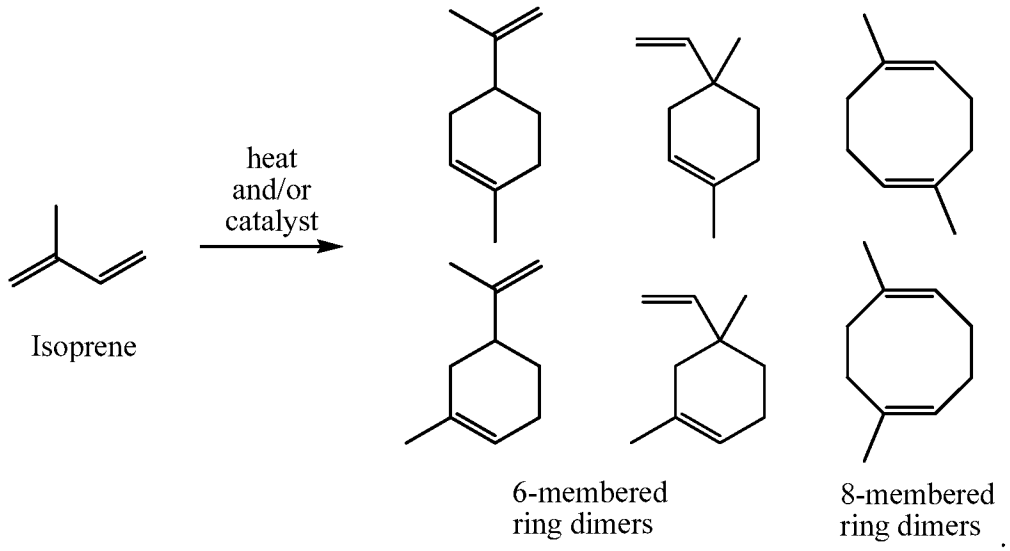
about 90 to 10, 80 to 20, 70 to 30, 60 to 40, 50 to 50, 40 to 60, 30 to 70, 20 to 80 and 10 to 90 ratio of 1,5-dimethyl-1,5-cyclooctadiene to 1,6-dimethyl-1,5-cyclooctadiene. In one embodiment, the catalyst system comprises a 3-component catalyst containing Ni carboxylates or ß-ketones, organoaluminum or organomagnesium compounds and substituted triphenylphosphite. For example, a degassed solution of a highly pure isoprene starting composition in anhydrous toluene is mixed with $Ni(acac)_2$, Et_3Al, and tris(3,4-bis(dimethylamino)phenyl)phosphite under nitrogen atmosphere and the mixture is heated at 95 °C to give dimethylcyclooctadiene. In another embodiment, the catalyst system comprises Fe carboxylates or ß-diketone compounds, organo-Al or Mg compounds, and 2,2'-dipyridyl derivatives having electron-donating groups. For example, a starting isoprene composition is heated with a mixture of Fe acetylacetonate, 4,4'-dimethyl-2,2'-dipyridyl, and Et_3Al in toluene to gave 1,6-dimethyl-1,5-cyclooctadiene in good yields. In some preferred embodiments, the starting isoprene composition is a bioisoprene composition.

[0640] In some embodiments, the method for producing a fuel constituent from isoprene comprises contacting the highly pure isoprene composition with a catalyst system to convert at least a portion of the starting isoprene composition to unsaturated isoprene dimers and/or trimers. In some embodiments, the catalyst system comprises a Ru, Fe, Ni or Pd catalyst or a combination thereof and the isoprene in the starting composition is converted to a mixture comprising 6-membered ring isoprene dimers. In one embodiment, the catalyst system is a $Fe(NO)_2Cl$-bis(1,5-cyclooctadiene)nickel catalyst system and the isoprene in the starting composition is cyclodimerized at low temperatures (e.g. from -5 to +20 °C) to give 1-methyl- and 2-methyl-4-isopropenyl-1-cyclohexene and 1,4- and 2,4-dimethyl-4-vinyl-1-cyclohexene. In another embodiment, contacting with a mixed catalyst of a tris(substituted hydrocarbyl) phosphite, arsenite, or antimonite and a Group VIII metal(0) compound (e.g. Ni acetylacetonate) converts the isoprene in the starting composition to 1,4-dimethyl-4-vinyl-1-cyclohexene and 1,5-dimethyl-1,5-cyclooctadiene. In another embodiment, contacting an isoprene starting composition with a ruthenium catalyst (e.g. see Itoh, Kenji; Masuda, Katsuyuki; Fukahori, Takahiko; Nakano, Katsumasa; Aoki, Katsuyuki; Nagashima, Hideo, *Organometallics* (**1994**), 13(3), 1020-9) converts isoprene to C10 cyclic dimers (e.g. a mixture of dimethyl-cyclooctadienes). The ruthenium catalyst can be synthesized in two steps from $RuCl_3$ and pentamethylcyclopentadiene (C_5Me_5H). The process may be performed in batch mode and the catalyst recovered and recycled. In some preferred embodiments, the starting

isoprene composition is a bioisoprene composition. One embodiment of the method is illustrated in Scheme XI.

[0641] In some embodiments, the method for producing a fuel constituent from isoprene comprises contacting the highly pure isoprene composition with a catalyst system to convert at least a portion of the starting isoprene composition to unsaturated isoprene dimers and/or trimers. In some embodiments, the catalyst system comprises a Ni, Ti, Al, or Mg catalyst or a mixture thereof and the isoprene in the starting composition is converted to a mixture comprising cyclic isoprene trimers such as trimethylcyclododecatrienes. In one embodiment, the catalyst system for the trimerization of isoprene comprising Ni and/or Ti, one or more organometallic compound, and a Group VA compound. The reaction may be conducted in a hydroxyl group-containing solvent. In another embodiment, oligomerization of isoprene catalyzed by nickel naphthenate and isoprenemagnesium in the presence of various phosphites as electron donors give cyclic dimers containing dimethylcyclooctadiene, in particular 1,1,1-tris(hydroxymethyl)propane phosphite gives trimethylcyclododecatriene selectively.

Scheme XI



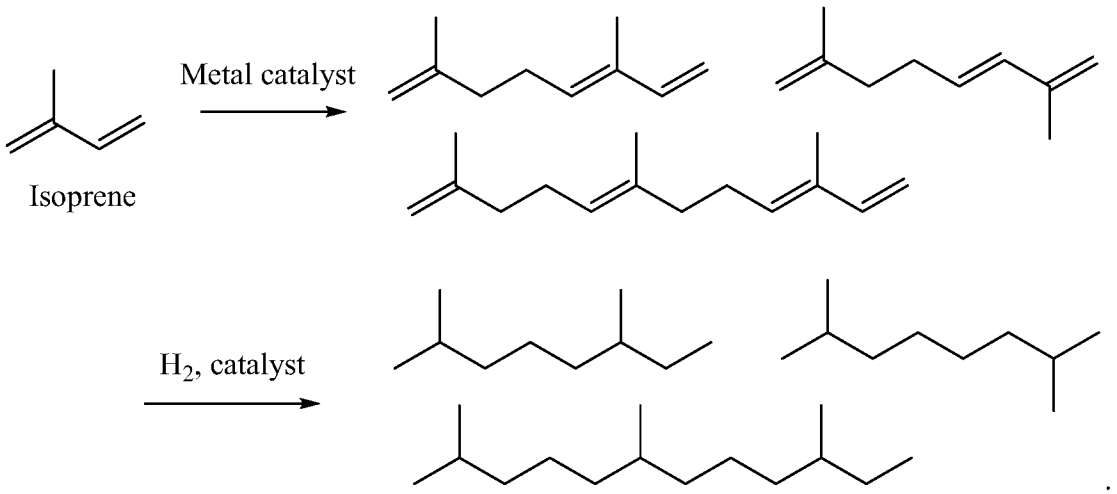
[0642] In some embodiments, the method for producing a fuel constituent from isoprene comprises contacting the highly pure isoprene composition with a catalyst system to convert at least a portion of the starting isoprene composition to unsaturated isoprene dimers and/or trimers. In some embodiments, the catalyst system comprises a Ni, Pd or Cr catalyst or a mixture thereof and the isoprene in the starting composition is converted telomers or linear dimers and/or trimers. In one embodiment, a catalyst system comprising Pd(0) complexes (e.g. $Pd(acac)_2$-Ph_3P and $Pd(OAc)_2$-Ph_3P) catalyzes dimerization and telomerization of isoprene to

give linear isoprene dimers (e.g. 2,7-dimethyl-1,3,7-octatriene). In one variation, the reaction is performed in methanol as a solvent and methyl ethers such as methoxydimethyloctadienes (e.g. 1-methoxy-2,7-dimethyl-2,7-octadiene and 3-methoxy-2,7-dimethyl-1,7-octadiene) are produced. In another embodiment, the catalyst system comprises divalent and trivalent transition metal-exchanged montmorillonites (e.g. Cr^{3+}-montmorillonite). In another embodiment, the catalyst system comprises a Ni(0)-aminophosphinite catalyst and the isoprene in the starting composition is converted to regioselective tail-to-tail linear dimers. In some variations, the linear dimer formation is accompanied by a competitive cyclodimerization reaction. In another embodiment, the catalyst system comprises a chromium *N,N*-bis(diarylphosphino)amine catalyst (e.g. see Bowen, L.; Charernsuk, M.; Wass, D.F. *Chem. Commun.* (**2007**) 2835-2837) and the isoprene is converted to linear and cyclic C15 trimers. In one variation, the chromium catalyst is made *in situ* from $CrCl_3(THF)_3$ and a PNP phosphine ligand.

[0643] In some embodiments, the method for producing a fuel constituent from a bioisoprene composition comprises chemically transforming a substantial portion of the isoprene in the bioisoprene composition by (i) contacting the bioisoprene composition with a catalyst for catalyzing trimerization of isoprene to produce an unsaturated isoprene trimer and (ii) hydrogenating the isoprene trimer to produce a fuel constituent. In some embodiments, at least about 95% of isoprene in the bioisoprene composition is converted to non-isoprene compounds during the chemical transformation.

[0644] In some embodiments, the method for producing a fuel constituent from isoprene comprises: (a) obtaining a commercially beneficial amount of any of the highly pure isoprene starting composition described herein; (b) converting at least a portion of the starting isoprene composition to unsaturated isoprene derivatives; and (c) hydrogenating the unsaturated isoprene derivatives to produce saturated compounds. In some embodiments, hydrogenation of unsaturated isoprene derivatives to saturated compounds is performed in batch mode. In some embodiments, hydrogenation of unsaturated isoprene derivatives to saturated compounds is performed in continuous mode. One embodiment of the method is illustrated in Scheme XII.

Scheme XII



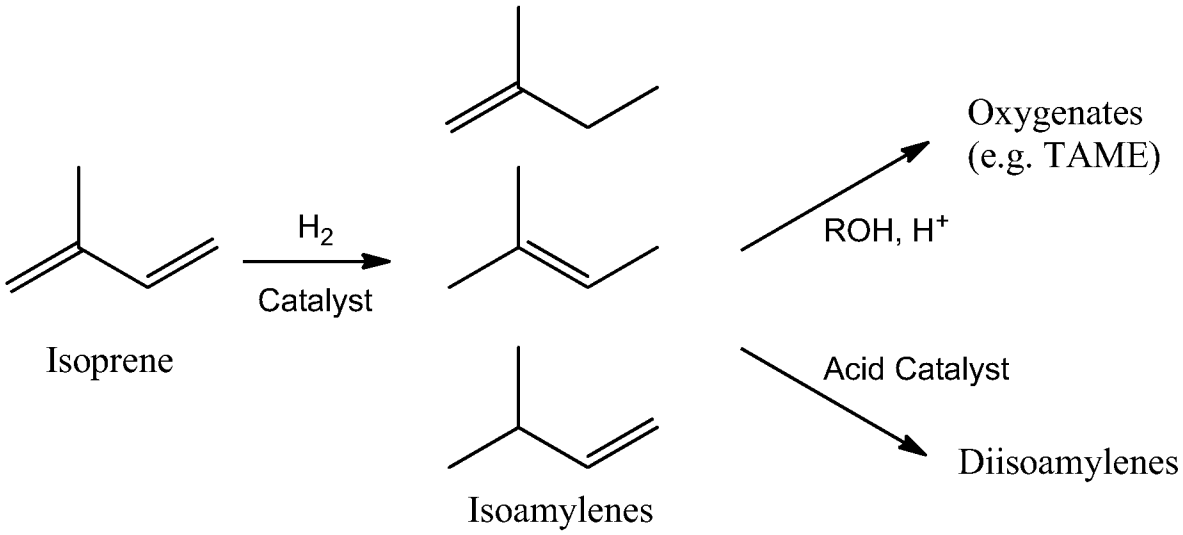
[0645] In some embodiments, the method for producing a fuel constituent from isoprene comprises: (a) obtaining a commercially beneficial amount of any of the highly pure isoprene starting composition described herein; (b) converting at least a portion of the starting isoprene composition to oxygenated isoprene derivatives; and optionally (c) hydrogenating any unsaturated oxygenated isoprene derivatives to produce saturated oxygenates. In some embodiments, the isoprene starting composition is contacted with a catalyst in the presence of alcohols (e.g. methanol or ethanol or mixtures thereof) to form oxygenated isoprene derivatives comprise one or more unsaturated or saturated ethers (e.g. methyl ethers or ethyl ethers or mixtures thereof). In some embodiments, the catalyst is an acid catalyst such as sulfuric acid, solid phase sulfuric acid (e.g., Dowex Marathon®), liquid and solid-phase fluorosulfonic acids (e.g. trifluoromethanesulfonic acid and Nafion-H (DuPont)). Zeolite catalysts can also been used, for example beta-zeolite, under conditions similar to those described by Hensel *et al.* [Hensen, K.; Mahaim, C.; Holderich, W.F., *Applied Catalysis A: General* (**1997**) 140(2), 311-329.] for the methoxylation of limonene and related monoterpenes. In some embodiments, the isoprene starting compositions or the unsaturated isoprene dimers or trimers undergo oxidation/hydrogenation to form the hydroxylated isoprene derivatives comprise one or more alcohols such as C5, C10 and C15 alcohols and diols. In some embodiments, the isoprene starting composition undergoes hydroxylation/esterification to form alcohols and esters. For example, bioisoprene or an unsaturated intermediate undergoes peroxidation to epoxides with peracids such as peracetic and 3-chloroperbenzoic acid; and hydration to give alcohols and diols with i) water and acid catalysts and ii) hydroboration methods. Such reactions are described in, for example, Michael B. Smith and Jerry March, *Advanced Organic Chemistry: Reactions,*

Mechanisms, and Structure, Sixth Edition, John Wiley & Sons, 2007. In some preferred embodiments, the starting isoprene composition is a bioisoprene composition.

[0646] In some embodiments, the method for producing a fuel constituent from isoprene comprises: (a) obtaining a commercially beneficial amount of any of the highly pure isoprene starting composition described herein; (b) partially hydrogenating the starting isoprene composition to mono-olefins (e.g. 2-methylbut-1-ene, 3-methyl-but-1-ene and 2-methylbut-2-ene); and (c) producing the fuel constituent from the mono-olefins. In one embodiment, the mono-olefin undergoes dimerization. In another embodiment, the mono-olefin is reacted with other olefins using traditional hydrocarbon cationic catalysis, such as that used to covert isobutylene to isooctane. The product of dimerization or reaction with other olefins are then undergoes hydrogenation to give saturated fuel constituents. In some preferred embodiments, the highly pure isoprene is a bioisoprene composition. In some embodiment, the starting isoprene composition partially hydrogenated using a palladium catalyst, such as $Pd/CaCO_3$, $Pd/BaSO_4$, Pd/C, Pd black, Pd/SiO_3, Pd/Al_2O_3, or Pd/SiO_2. In some embodiment, the starting isoprene composition partially hydrogenated using a silica-supported polyamidoamine (PAMAM) dendrimer-palladium complex. In some embodiment, the starting isoprene composition partially hydrogenated using a palladium-gold and palladium-silver catalysts (*e.g.*, $Pd\text{-}Au/SiO_2$ and $Pd\text{-}Ag/SiO_2$) have high selectivity for reducing isoprene to mono-olefins over fully reduced C5 alkanes. In some embodiment, the starting isoprene composition partially hydrogenated using a Group VIB metal on an inorganic support (*e.g.*, a metal zeolite) as a catalyst, e.g. a Mo/Al_2O_3 catalyst. In some embodiment, the starting isoprene composition partially hydrogenated using a monolithic catalyst bed, which may be in a honeycomb configuration. Catalyst support materials for the monolithic catalyst bed may include metals such as nickel, platinum, palladium, rhodium, ruthenium, silver, iron, copper, cobalt, chromium, iridium, tin, and alloys or mixtures thereof. In some embodiment, the starting isoprene composition partially hydrogenated using an eggshell $Pd/d\text{-}Al_2O_3$ catalyst, particularly if the reaction is free of water. In some embodiment, the starting isoprene composition partially hydrogenated using a Group VIII metal catalyst promoted by a metal from Group IB, VIB, VIIB, or zinc to reduce poisoning of the catalyst. Methods for converting isoprene into isoamylenes (mono-olefins) can be performed in continuous or batch mode using both homogeneous or heterogeneous catalysts. One embodiment of the method is illustrated in Scheme XIII.

[0647] In some embodiments, isoamylenes produce from partial hydrogenatin of isoprene undergos trimerization in liquid phase over ion exchange resins and zeolites. See, GranGranollers, Ind. Eng. Chem. Res., 49 (8), pp 3561–3570 (2001).

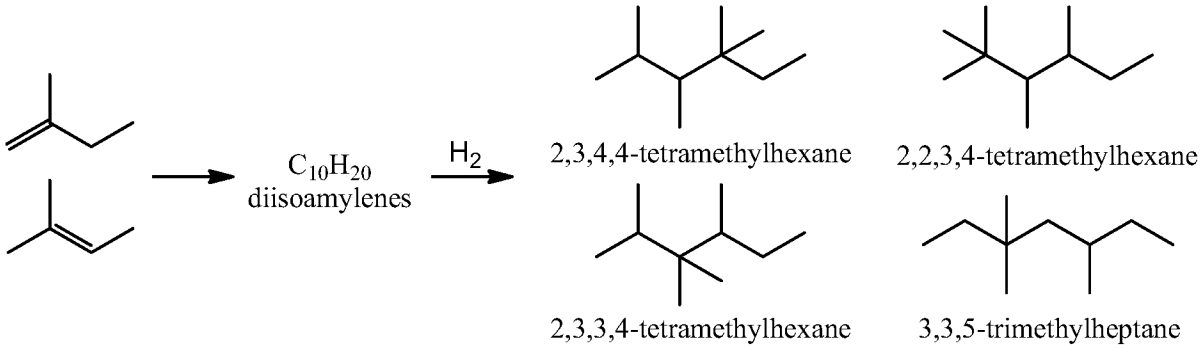
Scheme XIII



[0648] In some embodiments, the method for producing a fuel constituent from isoprene comprises: (a) obtaining a commercially beneficial amount of any of the highly pure isoprene starting composition described herein; (b) partially hydrogenating the starting isoprene composition to a mono-olefin (e.g. 2-methylbut-1-ene, 3-methyl-but-1-ene and 2-methylbut-2-ene); (c) contacting the mono-olefin with a catalyst for dimerization of mono-olefins to form a dimate with another mono-olefin; and (d) hydrogenating the product of dimerization (dimate) to produce saturated hydrocarbon fuel constituents. In some preferred embodiments, the highly pure isoprene is a bioisoprene composition. In some embodoments, the other mono-olefin is an isoamylene derived from the highly pure isoprene starting composition. In some embodoments, the other mono-olefin is an olefin from another source such as propylene, butane or isobutene. In some embodiements, the catalyst for dimerization is a resin catalyst (*e.g.*, Amberlyst, Amberlyst 35, Amberlyst 15, Amberlyst XN1010, Amberlyst XE586). In some embodiements, the catalyst for dimerization is a catalytic material containing an acidic mesoporous molecular sieve, such as a mesoporous sieve embedded in a zeolite structure, ZSM-5, ferrierite, ZSM-22, or ZSM-23. In some embodiements, the catalyst for dimerization is the catalytic material is thermally stable at high temperatures (*e.g.*, at least 900 °C). In some embodiements, the catalyst for dimerization is a catalytic material containing an acidic mesoporous molecular sieve. In some embodiements, the catalyst for dimerization is a solid acidic catalyst (*e.g.*, a solid phosphoric

acid catalyst, acidic ion exchange resins). One embodiment of the method is illustrated in Scheme XIV.

Scheme XIV



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[0649] In some embodiments, the method for producing a fuel constituent from isoprene comprises: (a) obtaining a commercially beneficial amount of any of the highly pure isoprene starting composition described herein; (b) partially hydrogenating the starting isoprene composition to an isoamylene (e.g. 2-methylbut-1-ene, 3-methyl-but-1-ene and 2-methylbut-2-ene); (c) contacting the isoamylene with an alcohol and a catalyst to form an oxygenate; and (c) producing producing the fuel constituent from the isoamylene oxygenate. In some preferred embodiments, the highly pure isoprene is a bioisoprene composition. In some embodiements, the isoamylene is contacted with ethanol and the fuel oxygenate formed is an ether such as TAME.

[0650] In some embodiments, any of the methods described herein further include purifying the starting isoprene composition before the chemical transformation. 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. In some embodiments, bioisoprene is recovered from fermentation off-gas by initial adsorption to activated carbon, followed by desorption and condensation to give a liquid isoprene composition for chemical transformation to a fuel constituent.

Continuous Process for Producing Fuels

[0651] The invention also provides a continuous process for producing a fuel constituent from isoprene comprising: (a) continuously producing a commercially beneficial amount of highly pure isoprene; and (b) continuously transforming chemically at least a portion of the commercially beneficial amount of highly pure isoprene to a fuel constituent. In a continuous process according to the invention, a stream of highly pure isoprene is produced continuously, for example, by a biological process (e.g. culturing cells producing isoprene) and is passed to a reactor for chemical transformation. In some embodiments, the isoprene from a fermentation off-gas is separated from water and other permanent gases (e.g., O_2, N_2 and CO_2) using extractive distillation, condensation, adsorption or membranes in a continuous purification unit, where the oxygen level is lowered to ppm range and other impurities of concern are removed; the isoprene vapor is catalytically converted to dimers (C10) and trimers (C15) using a heterogeneous catalyst; the desired product is separated and the unreacted isoprene is returned to the reactor for further conversion. In some embodiments, the catalytic dimerization is monitored by measuring the levels of desirable C10 hydrocarbons in the gas phase.

[0652] In some embodiments, a stream of biologically produced isoprene is passed through a chemical reactor (e.g., a reaction tube) maintained at a temperature of about 100 °C to about 300 °C, where the biologically produced isoprene undergoes dimerization reactions. In one variation, the isoprene dimers are separated from the product stream and at least a portion of the unreacted isoprene is recycled back into the chemical reactor. In one embodiment, the unsaturated isoprene dimers produced is contacted with a hydrogenation catalyst and a hydrogen source in a second chemical reactor producing partially hydrogenated and/or fully hydrogenated products. The process can further comprise a step of recovering isoprene from the off-gas of a bio-reactor producing isoprene.

[0653] In some embodiments, a stream of bioisoprene containing co-produced hydrogen is first separated from the hydrogen gas, the bioisoprene stream is passed through a chemical reactor (e.g., a reaction tube) maintained at a temperature of about 100 °C to about 300 °C, where the biologically produced isoprene undergoes dimerization reactions. In one variation, the

isoprene dimers are separated from the product stream and at least a portion of the unreacted isoprene is recycled back into the chemical reactor. In one embodiment, the unsaturated isoprene dimers produced is contacted with a hydrogenation catalyst and a hydrogen source in a second chemical reactor producing partially hydrogenated and/or fully hydrogenated products. In one variation, the hydrogen stream isolated from the isoprene-hydrogen co-production is used in the hydrogenation step. The process can further comprise a step of recovering isoprene from the off-gas of a bio-reactor producing isoprene. The process can further comprise a step of purifying the hydrogen stream isolated from the isoprene-hydrogen co-production using purification methods known in the art such as cryogenic condensation and solid matrix adsorption.

Removal of Dienes and Polymers from Fuel Products

[0654] Fuel compositions often contain unsaturated compounds (olefins, diolefins and polyolefins) that can form gums, resins, polymers and other undesirable byproducts over time (for example, see Pereira and Pasa (2006) *Fuel*, **85**, 1860–1865 and references therein). In general, as the degree of unsaturation increases of a given compound, the more likely that compound is to form such byproducts. Isoprene is a 1,3-diene that readily forms undesirable polymeric byproducts when present in fuel compositions. While there exist fuel additives (anti-oxidants, radical quenchers etc.) that can reduce the extent of byproduct formation, such byproducts can still form over time. Olefins can also contribute to the formation of ground-level ozone when released into the atmosphere upon evaporation from fuels, or as the result of incomplete combustion of olefin-containing fuels.

[0655] Accordingly, fuel compositions derived wholly or in part from isoprene should contain little to no free isoprene. A range of methods can be used to either remove isoprene from fuel compositions such as purification by distillation, reaction with alcohols to form ethers, or hydrogenation to convert isoprene to saturated derivatives. Alternately, isoprene can be treated with a dienophile such as malic anhydride producing inert adducts that do not contribute to the formation of undesirable byproducts.

[0656] Provided are fuel compositions comprising isoprene derivatives that are substantially free of isoprene. In some embodiements, the fuel composition contains less than 0.01%, 0.1%, 1%, 2%, 3%, 4%, 5%, 10% or 15% isoprene. Also provided are methods for chemically transforming a bioisoprene composition to fuel compositions comprising isoprene derivatives that are substantially free of isoprene. In some embodiments, a substantial portion of the

isoprene from the bioisoprene composition is converted to fuel constituents. In some embodiments, a substantial portion of the isoprene from the bioisoprene composition is converted to intermediates that can be further converted to produce fuel constituents. In some embodiments, a substantial portion of the isoprene from the bioisoprene composition is converted to compounds other than isoprene. A "substantial portion" is 99.99%, 99.9%, 99%, 98%, 97%, 96%, 95%, 90%, 85% or 80%.

[0657] In some instances, isoprene and other conjugated dienes can form polymeric products with gum-like consistencies that can reduce the yields of desired products and/or deactivate catalysts. (See, *e.g.*, R.C.C. Pereira and V.M.D. Pasa. *Fuel* 85 (2006) 1860-1865.) In some embodiments, methods are provided for determining the amount of conjugated diene present in a product mixture. D.F. Andrade et al. describe various methods for determining the amount of conjugated diene present (*Fuel* (2010), doi:10.106/j.fuel/2010.01.003), including the following: 1) UOP-326 method (maleic anhydride method), a semi-quantitative method in which the amount of maleic anhydride consumed via Diels-Alder reaction with the diene is measured; 2) polarography; 3) gas chromatography, in which the diene may be reacted with a derivatization agent, such as 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) or 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD); 4) HPLC; 5) supercritical fluid chromatography; 6) NMR; 7) UV and near IR spectroscopy; and 8) other spectroscopic methods, which may include first derivatizing the diene with p-nitrobenezenediazonium fluoroborate.

[0658] Method are provided herein for minimizing gum-formation and/or reducing the amount of gum that has been produced. In some embodiments, an anti-oxidant is used to minimize gum-formation. In other embodiments, polymeric by-products are recycled back to the process stream. In some embodiments, depolymerization of polymeric by-products is carried out via olefin metathesis. Olefin metathesis can be carried out using a molybdenum or tungsten catalyst, such as 2,6-diisopropylphenylimido neophylidene molybdenum bis(2-tertbutylphenoxide). (See US 5,446,102.)

[0659] In some embodiments, any of the methods described herein further include characterizing the products of these reactions and assessing the potential fuel value. For example, the products are characterized by standard methods known in the art, e.g. GC/MS, NMR, UV-Vis and IR spectroscopies, boiling temperature, density and other physical properties. The products can be further characterized by dual carbon-isotopic fingerprinting (see, US Patent Number 7,169,588). The potential fuel value of the products are assessed by parameters measuring fuel properties such as the energy density, heating value, water solubility,

octane/cetane number, density, viscosity, surface tension, enthalpy of vaporization, vapor diffusivity, flash point, autoignition point, flammability limits, cloud point and chemical stability.

[0660] As with commercial petroleum fuels, BioIsoFuel™ products can be tested for their acidity, density, trace mineral content, benzene, total aromatics content, water content, and corrosivity. To assure that minor impurities in BioIsoFuel™ products are not adversely affecting their material properties, samples can also be tested for their corrosivity and compatibility with fuel systems by standard ASTM tests such as Karl Fischer for water and copper strip for corrosivity.

Fuel Compositions

[0661] The invention provides a fuel composition comprising a fuel constituent produced by any of the methods and processes described herein. In some embodiments, the fuel constituent comprises one or more of the hydrocarbons selected from the group consisting of saturated C10 and C15 hydrocarbons derived from isoprene and oxygenated derivatives of isoprene. The hydrocarbon BioIsoFuel™ products are expected to be completely compatible with petroleum gasoline and diesel. Blends of BioIsoFuel™ with commercial petroleum fuels are expected to have more desirable properties, as they do not contain acids, sulfur, aromatics, or other undesirable impurities.

[0662] In some preferred embodiments, the fuel constituent comprises derivatives of bioisoprene. It is anticipated that there will be a significant demand for fuels derived from bioisoprene which is made from renewable, non-petrochemical based resources. It is believed that industrial customers and consumers would prefer to purchase fuel components derived from such environmentally friendly sources to those that are made with isoprene derived from a petrochemical process. It is further believed that customers would be willing to pay premium prices for such environmentally friendly products that are made with renewable resources. Fuel components derived from bioisoprene compositions described herein offer the benefit of being verifiable as to be derived from non-petrochemical based resources.

[0663] In some embodiments, the fuel constituent comprises one or more of the saturated cyclic C10 hydrocarbons such as 1,2-bis(isopropyl)cyclobutane, 1,2-bis(isopropyl)cyclobutane, 1-methyl-4-isopropylcyclohexane, 1-methyl-3-isopropylcyclohexane, 1-ethyl-1,4-dimethylcyclohexane, 1-ethyl-1,3-dimethylcyclohexane, 1,5-dimethylcyclooctane and 1,4-dimethylcyclooctane. In some embodiments, the fuel constituent comprises one or more of the

saturated cyclic C15 hydrocarbons such as 1,5,9-trimethylcyclododecane and 1,5,10-trimethylcyclododecane. In some embodiments, the fuel constituent comprises one or more of the saturated aliphatic C10 hydrocarbons such as 2,6-dimethyloctane, 2,7-dimethyloctane and 3,6-dimethyloctane. In some embodiments, the fuel constituent comprises one or more of the saturated aliphatic C15 hydrocarbons such as 2,6,10-trimethyldodecane, 2,7,10-trimethyldodecane and 3,7,10-trimethyldodecane. In some embodiments, the fuel constituent comprises 2-methylbutane. In some embodiments, the fuel constituent comprises one or more of the hydrocarbons selected from the group consisting of 1,2-bis(isopropyl)cyclobutane, 1,2-bis(isopropyl)cyclobutane, 1-methyl-4-isopropylcyclohexane, 1-methyl-3-isopropylcyclohexane, 1-ethyl-1,4-dimethylcyclohexane, 1-ethyl-1,3-dimethylcyclohexane, 1,5-dimethylcyclooctane, 1,4-dimethylcyclooctane, 1,5,9-trimethylcyclododecane, 1,5,10-trimethylcyclododecane, 2,6-dimethyloctane, 2,7-dimethyloctane, 3,6-dimethyloctane, 2,6,10-trimethyldodecane, 2,7,10-trimethyldodecane, 3,7,10-trimethyldodecane and 2-methylbutane.

[0664] In some embodiments, the fuel constituent comprises one or more of the saturated hydroxylates such as C5 alcohols and diols (e.g. 3-methyl-butan-1-ol, 2-methyl-butan-1-ol and 2-methyl-butan-2-ol, 3-methyl-butan-1,3-diol and 2-methyl-butan-2,3-diol), C-10 alcohols and diols (e.g. 3,7-dimethyloctan-1-ol, 2,7-dimethyloctan-1-ol, 2,7-dimethyloctan-2-ol and 2,7-dimethyloctan-2,7-diol) and cyclic C-10 alcohols (e.g. 2-(4-methylcyclohexyl)propan-2-ol, 2-(4-methylcyclohexyl)propan-1-ol, 2-(1,4-dimethylcyclohexyl)ethanol and 4-ethyl-1,4-dimethylcyclohexanol). In some embodiments, the fuel constituent comprises one or more of the saturated ethers such as 1,3-diethoxy-3-methylbutane, 1-ethoxy-3-methylbutane, 1-methoxy-2,7-dimethyloctane and 3-methoxy-2,7-dimethyloctane. In some embodiments, the fuel constituent comprises one or more of the isoprene derived oxygenates selected from the group consisting of 3-methyl-butan-1-ol, 2-methyl-butan-1-ol, 2-methyl-butan-2-ol, 3-methyl-butan-1,3-diol, 2-methyl-butan-2,3-diol, 3,7-dimethyloctan-1-ol, 2,7-dimethyloctan-1-ol, 2,7-dimethyloctan-2-ol, 2,7-dimethyloctan-2,7-diol, 2-(4-methylcyclohexyl)propan-2-ol, 2-(4-methylcyclohexyl)propan-1-ol, 2-(1,4-dimethylcyclohexyl)ethanol, 4-ethyl-1,4-dimethylcyclohexanol, 1,3-diethoxy-3-methylbutane, 1-ethoxy-3-methylbutane, 1-methoxy-2,7-dimethyloctane and 3-methoxy-2,7-dimethyloctane.

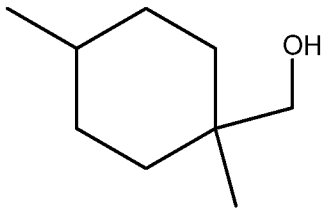
[0665] In some embodiments, the fuel constituent comprises less than or about 0.5 μg/L a product from a C5 hydrocarbon other than isoprene after undergoing the steps according to any of the methods and processes described herein. In one embodiment, he fuel constituent is substantially free of a product from a C5 hydrocarbon other than isoprene after undergoing the

steps according to any of the methods and processes described herein. In some embodiments, the fuel constituent comprises less than or about 0.2, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 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 starting composition. In some embodiments, the fuel constituent comprises C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) at a concentration of less than 100, 10, 1, 0.1 or 0.01 ppm.

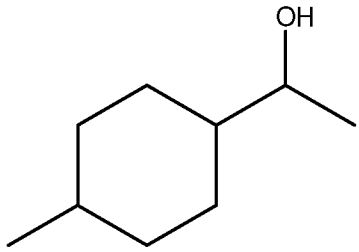
[0666] It is understood that components other than isoprene in the bioisoprene compositions described herein will be converted to different products after undergoing various processes described herein. The sensitivities of metal-based catalysts used in the methods/processes to the components other than isoprene are different depending on the nature and levels of these components. For the thermal dimerization process, a far wider range of compounds will be tolerated. In some cases, a component other than isoprene will react with isoprene to produce adducts, for example the Diels-Alder reactions of methacrolein and methyl vinyl ketone with isoprene to give 6-membered products. The unsaturated adducts can be further hydrogenated to saturated derivatives which are present in the fuel constituents and compositions, e.g. 1,4-dimethyl-1-(hydroxymethyl)cyclohexane and 1-(1-hydroxyethyl)-4-methylcyclohexane (see Scheme XV).

Scheme XV. Examples of trace impurities derived from Bioisoprene™

i) Hydrogenated Diels-Alder adducts

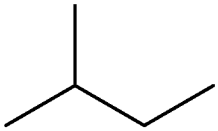


1,4-dimethyl-1-(hydroxymethyl)cyclohexane

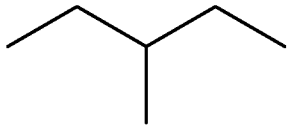


1-(1-hydroxyethyl)-4-methylcyclohexane

ii) Alkanes

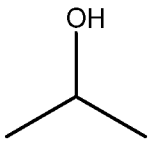


2-methylbutane

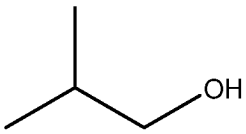


3-methylpentane

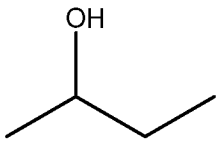
iii) Alcohols



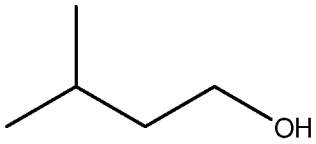
2-propanol



2-methyl-1-propanol

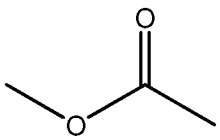


2-butanol

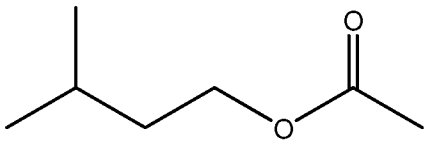


3-methyl-1-butanol

iv) Esters



ethyl acetate



3-methyl-1-butyl acetate

[0667] In some embodiments, the fuel constituent comprises a product from a compound selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms after undergoing the steps according to any of the methods and processes described herein. In some embodiments, the fuel constituent comprises one or more products from one or more compounds selected from the group consisting of ethanol, acetone, methanol, acetaldehyde, methacrolein, methyl vinyl ketone, 2-methyl-2-vinyloxirane, *cis*- and *trans*-3-methyl-1,3-pentadiene, a C5 prenyl alcohol (such as 3-methyl-3-

buten-1-ol or 3-methyl-2-buten-1-ol), 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine after undergoing the steps according to any of the methods and processes described herein. In some embodiments, the fuel constituent comprises one or more compounds selected from the group consisting of 1,4-dimethyl-1-(hydroxymethyl)cyclohexane, 1-(1-hydroxyethyl)-4-methylcyclohexane, 2-methylbutane, 3-methylpentane, 2-propanol, 2-methyl-1-propanol, 2-butanol, 3-methyl-1-butanol, ethyl acetate and 3-methyl-1-butyl acetate. In some embodiments, the fuel constituent comprises one or more compounds selected from the group consisting of 1,4-dimethyl-1-(hydroxymethyl)cyclohexane, 1-(1-hydroxyethyl)-4-methylcyclohexane, 2-methylbutane, 3-methylpentane, 2-propanol, 2-methyl-1-propanol, 2-butanol, 3-methyl-1-butanol, ethyl acetate and 3-methyl-1-butyl acetate at levels greater than or about 10 ppm, 1 ppm, 100 ppb, 10 ppb or 1 ppb.

[0668] In some embodiments, the fuel constituent comprises one or more compounds selected from the group consisting of ethanol, acetone, methanol, acetaldehyde, methacrolein, methyl vinyl ketone, 2-methyl-2-vinyloxirane, *cis*- and *trans*-3-methyl-1,3-pentadiene, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol), citronellol (3,7-dimethyl-6-octen-1-ol), (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine at levels greater than or about 10 ppm, 1 ppm, 100 ppb, 10 ppb or 1 ppb.

Carbon fingerprinting

[0669] BioIsoFuels derived from bioisoprene can be distinguished from fuels derived form petrochemical carbon on the basis of dual carbon-isotopic fingerprinting. Additionally, the specific source of biosourced carbon (e.g. glucose vs. glycerol) can be determined by dual carbon-isotopic fingerprinting (see, US Patent Number 7,169,588, which is herein incorporated by reference).

[0670] This method usefully distinguishes chemically-identical materials, and apportions carbon in products by source (and possibly year) of growth of the biospheric (plant) component. The isotopes, ^{14}C and ^{13}C, bring complementary information to this problem. The radiocarbon dating isotope (^{14}C), with its nuclear half life of 5730 years, clearly allows one to apportion specimen carbon between fossil ("dead") and biospheric ("alive") feedstocks [Currie, L. A. "Source Apportionment of Atmospheric Particles," Characterization of Environmental Particles, J. Buffle and H. P. van Leeuwen, Eds., 1 of Vol. I of the IUPAC Environmental Analytical Chemistry Series (Lewis Publishers, Inc) (1992) 3 74]. The basic assumption in radiocarbon dating is that the constancy of ^{14}C concentration in the atmosphere leads to the constancy of ^{14}C in living organisms. When dealing with an isolated sample, the age of a sample can be deduced approximately by the relationship $t = (-5730/0.693)\ln(A/A_O)$ (Equation 14) where t = age, 5730 years is the half-life of radiocarbon, and A and A_O are the specific ^{14}C activity of the sample and of the modem standard, respectively [Hsieh, Y., Soil Sci. Soc. Am J., 56, 460, (1992)]. However, because of atmospheric nuclear testing since 1950 and the burning of fossil fuel since 1850, ^{14}C has acquired a second, geochemical time characteristic. Its concentration in atmospheric CO_2 – and hence in the living biosphere – approximately doubled at the peak of nuclear testing, in the mid-1960s. It has since been gradually returning to the steady-state cosmogenic (atmospheric) baseline isotope rate ($^{14}C/^{12}C$) of ca. 1.2×10^{-12}, with an approximate relaxation "half-life" of 7-10 years. (This latter half-life must not be taken literally; rather, one must use the detailed atmospheric nuclear input/decay function to trace the variation of atmospheric and biospheric ^{14}C since the onset of the nuclear age.) It is this latter biospheric ^{14}C time characteristic that holds out the promise of annual dating of recent biospheric carbon. ^{14}C can be measured by accelerator mass spectrometry (AMS), with results given in units of "fraction of modem carbon" (f_M). f_M is defined by National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs) 4990B and 4990C, known as oxalic acids standards HOxI and HOxII, respectively. The fundamental definition relates to 0.95 times

the $^{14}C/^{12}C$ isotope ratio HOxI (referenced to AD 1950). This is roughly equivalent to decay-corrected pre-Industrial Revolution wood. For the current living biosphere (plant material), $f_M \sim 1.1$.

[0671] The stable carbon isotope ratio ($^{13}C/^{12}C$) provides a complementary route to source discrimination and apportionment. The $^{13}C/^{12}C$ ratio in a given biosourced material is a consequence of the $^{13}C/^{12}C$ ratio in atmospheric carbon dioxide at the time the carbon dioxide is fixed and also reflects the precise metabolic pathway. Regional variations also occur. Petroleum, C_3 plants (the broadleaf), C_4 plants (the grasses), and marine carbonates all show significant differences in $^{13}C/^{12}C$ and the corresponding $d^{13}C$ values. Furthermore, lipid matter of C_3 and C_4 plants analyze differently than materials derived from the carbohydrate components of the same plants as a consequence of the metabolic pathway. Within the precision of measurement, ^{13}C shows large variations due to isotopic fractionation effects, the most significant of which for the instant invention is the photosynthetic mechanism. The major cause of differences in the carbon isotope ratio in plants is closely associated with differences in the pathway of photosynthetic carbon metabolism in the plants, particularly the reaction occurring during the primary carboxylation, i. e., the initial fixation of atmospheric CO_2. Two large classes of vegetation are those that incorporate the "C_3" (or Calvin-Benson) photosynthetic cycle and those that incorporate the "C_4" (or Hatch-Slack) photosynthetic cycle. C_3 plants, such as hardwoods and conifers, are dominant in the temperate climate zones. In C_3 plants, the primary CO_2 fixation or carboxylation reaction involves the enzyme ribulose-1,5-diphosphate carboxylase and the first stable product is a 3-carbon compound. C_4 plants, on the other hand, include such plants as tropical grasses, corn and sugar cane. In C_4 plants, an additional carboxylation reaction involving another enzyme, phosphoenol-pyruvate carboxylase, is the primary carboxylation reaction. The first stable carbon compound is a 4-carbon acid which is subsequently decarboxylated. The CO_2 thus released is refixed by the C_3 cycle.

[0672] Both C_4 and C_3 plants exhibit a range of $^{13}C/^{12}C$ isotopic ratios, but typical values are ca. -10 to -14 per mil (C_4) and -21 to -26 per mil (C_3) [Weber et al., J. Agric. Food Chem., 45, 2942 (1997)]. Coal and petroleum fall generally in this latter range. The ^{13}C measurement scale was originally defined by a zero set by pee dee belemnite (PDB) limestone, where values are given in parts per thousand deviations from this material. The "$d^{13}C$", values are in parts per thousand (per mil), abbreviated ‰, and are calculated as follows (Equation 15):

$$\delta^{13}C \equiv \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 100\% \quad \text{(Equation 15)}$$

Since the PDB reference material (RM) has been exhausted, a series of alternative RMs have been developed in cooperation with the IAEA, USGS, NIST, and other selected international isotope laboratories. Notations for the per mil deviations from PDB is $d^{13}C$. Measurements are made on CO_2 by high precision stable ratio mass spectrometry (IRMS) on molecular ions of masses 44, 45 and 46.

[0673] For isoprene derived from extractive distillation of C_5 streams from petroleum refineries, $\delta^{13}C$ is about -22‰ to about -24‰. This range is typical for light, unsaturated hydrocarbons derived from petroleum, and products derived from petroleum-based isoprene typically contain isoprenic units with the same $\delta^{13}C$. Bioisoprene produced by fermentation of corn-derived glucose ($\delta^{13}C$ -10.73‰) with minimal amounts of other carbon-containing nutrients (e.g., yeast extract) produces isoprene which can be polymerized into polyisoprene with $\delta^{13}C$ -14.66‰ to -14.85‰. Products produced from such bioisoprene are expected to have $\delta^{13}C$ values that are less negative than those derived from petroleum-based isoprene. For isoprene derived from the reaction of isobutylene with formaldehyde, $\delta^{13}C$ values can be about -34.4‰ because formaldehyde is often derived from feedstocks with much more negative $\delta^{13}C$ values.

[0674] The fuels and fuel constituents of this invention which are made with isoprene from the cell cultures that utilize bio-renewable carbon sources can be identified as such by virtue of their $d^{13}C$ value and other fuel characteristics. In some embodiments, the fuel constituent derived from bioisoprene has $\delta^{13}C$ values of greater (less negative) than -22‰. In some embodiments, the fuel constituent derived from bioisoprene has $\delta^{13}C$ values of greater than -20, -18, -16, -14, -12, or -10 ‰. In some embodiments, the fuel constituent derived from bioisoprene has a $\delta^{13}C$ value which is within the range of -22 to -10, -21 to -12, or -20 to -14 ‰. In some embodiments, the fuel constituent derived from bioisoprene has a $\delta^{13}C$ value which is within the range of -34 to -24, -34 to -25, -33 to -25, -32 to -24, -32 to -25, -31 to -25, -30 to -29, -30.0 to -29.5, -29.5 to -28.5, or -29.0 to -28.5 ‰.

[0675] In some embodiments, the fuel constituent derived from bioisoprene comprises radioactive carbon-14. In some embodiments, the $^{14}C/^{12}C$ ratio is greater than or about 1.0×10^{-12}, 1.05×10^{-12}, 1.1×10^{-12}, 1.15×10^{-12},or 1.2×10^{-12}. In some embodiments, the fuel constituent derived from bioisoprene has an f_M value of greater than or about 0.9, 0.95, 1.0, 1.05 or 1.1. In

some embodiments, the fuel constituent derived from bioisoprene has an f_M value of greater than or about 0.9, 0.95, 1.0, 1.05 or 1.1 and $\delta^{13}C$ values of greater (less negative) than -22‰. In some embodiments, the fuel constituent derived from bioisoprene has an f_M value of greater than or about 0.9, 0.95, 1.0, 1.05 or 1.1 and a $\delta^{13}C$ value which is within the range of -22 to -10, -21 to -12, or -20 to -14 ‰. In some embodiments, the fuel constituent derived from bioisoprene has an f_M value of greater than or about 0.9, 0.95, 1.0, 1.05 or 1.1 and a $\delta^{13}C$ value which is within the range of -34 to -24, -34 to -25, -33 to -25, -32 to -24, -32 to -25, -31 to -25, -30 to -29, -30.0 to -29.5, -29.5 to -28.5, or -29.0 to -28.5 ‰.

[0676] The bioisoprene derivatives and the associated BioIsoFuels, intermediates, and mixtures may be completely distinguished from their petrochemical derived counterparts on the basis of ^{14}C (f_M) and dual carbon-isotopic fingerprinting, indicating new compositions of matter.

[0677] In some embodiments, the fuel constituent of the invention has an energy density higher than that of ethanol. In some embodiments, the fuel constituent boosts the cetane number of a fuel, e.g., a petroleum-based fuel. In some embodiments, the fuel constituent reduces emission of petroleum based fuels. In some embodiments, the fuel composition has an octane number in the range between about 80 to about 120. In some embodiments, the fuel composition has a cetane number in the range between about 30 to about 130.

[0678] In some embodiments, a fuel composition of the invention comprises one or more dimethylcyclooctane compounds. In some embodiments, a fuel composition of the invention comprises one or more C10 hydrocarbons such as substitued cyclohexanes. In some embodiments, a fuel composition of the invention comprises one or more isoprene derived oxygenates described herein. In some embodiments, any of the fuel compositions described herein further comprises a petroleum based fuel in the amount of from about 1% to about 95% by weight or volume, based on the total weight or volume of the total fuel composition.

[0679] The invention further provides methods for making a fuel composition comprising obtaining a petroleum distillate and adding a fuel constituent of the invention.

[0680] The invention can be further understood by reference to the following examples, which are provided by way of illustration and are not meant to be limiting.

EXAMPLES

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*

[0681] The protein sequence for the kudzu (*Pueraria montana*) isoprene synthase gene (IspS) was obtained from GenBank (AAQ84170). A kudzu isoprene synthase gene, optimized for *E. coli* codon usage, was purchased from DNA2.0 (SEQ ID NO:1). The isoprene synthase gene was removed from the supplied plasmid by restriction endonuclease digestion with *Bsp*LU11I /*Pst*I, gel-purified, and ligated into pTrcHis2B (Invitrogen) that had been digested with *Nco*I/*Pst*I. The construct was designed such that the stop codon in the isoprene synthase gene 5' to the *Pst*I site. As a result, when the construct was expressed the His-Tag is not attached to the isoprene synthase protein. The resulting plasmid, pTrcKudzu, was verified by sequencing (Figures 2 and 3; SEQ ID NO:2).

[0682] The isoprene synthase gene was also cloned into pET16b (Novagen). In this case, the isoprene synthase gene was inserted into pET16b such that the recombinant isoprene synthase protein contained the N-terminal His tag. The isoprene synthase gene was amplified from pTrcKudzu by PCR using the primer set pET-His-Kudzu-2F: 5'-CGTGAGATCATATGTGTGCGACCTCTTCTCAATTTAC (SEQ ID NO:49) and pET-His-Kudzu-R: 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:50). These primers added an *Nde*I site at the 5'-end and a *Bam*H1 site at the 3' end of the gene respectively. The plasmid pTrcKudzu, described above, was used as template DNA, Herculase polymerase (Stratagene) was used according to manufacture's directions, and primers were added at a concentration of 10 pMols. The PCR was carried out in a total volume of 25 µl. The PCR product was digested with *Nde*I/*Bam*H1 and cloned into pET16b digested with the same enzymes. The ligation mix was transformed into *E. coli* Top10 (Invitrogen) and the correct clone selected by sequencing. The resulting plasmid, in which the kudzu isoprene synthase gene was expressed from the T7 promoter, was designated pETNHisKudzu (Figures 4 and 5; SEQ ID NO:51).

[0683] The kudzu isoprene synthase gene was also cloned into the low copy number plasmid pCL1920. Primers were used to amplify the kudzu isoprene synthase gene from pTrcKudzu described above. The forward primer added a *Hin*dIII site and an *E. coli* consensus RBS to the 5' end. The *Pst*I cloning site was already present in pTrcKudzu just 3' of the stop codon so the reverse primer was constructed such that the final PCR product includes the *Pst*I site. The sequences of the primers were: HindIII-rbs-Kudzu F: 5'-CATATGAAAGCTTGTATCGATTAAATAAGGAGGAATAAACC (SEQ ID NO:51) and BamH1-Kudzu R:

[0684] 5'- CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:50). The PCR product was amplified using Herculase polymerase with primers at a concentration of 10 pmol and with 1 ng of template DNA (pTrcKudzu). The amplification protocol included 30 cycles of (95° C for 1 minute, 60° C for 1 minute, 72° C for 2 minutes). The product was digested with *Hin*dIII and *Pst*I and ligated into pCL1920 which had also been digested with *Hin*dIII and *Pst*I. The ligation mix was transformed into *E. coli* Top10. Several transformants were checked by sequencing. The resulting plasmid was designated pCL-lac-Kudzu (Figures 6 and 7; SEQ ID NO:4).

II. Determination of isoprene production

[0685] 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).

[0686] 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).

[0687] 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 70,000 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

[0688] 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

[0689] 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, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with potassium hydroxide (KOH) and q.s. to volume. The final product was filter sterilized with 0.22 μ filter (only, do not autoclave). The recipe for 1000X Modified Trace Metal Solution was as follows: Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 μ filter.

[0690] 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

[0691] The protein sequence for the poplar (*Populus alba* x *Populus tremula*) isoprene synthase (Schnitzler, J-P, *et al.* (2005) *Planta* 222:777-786) was obtained from GenBank (CAC35696). A gene, codon optimized for *E. coli*, was purchased from DNA2.0 (p9796-poplar, Figures 30 and 31; SEQ ID NO:14). The isoprene synthase gene was removed from the supplied plasmid by restriction endonuclease digestion with *Bsp*LU11I /*Pst*I, gel-purified, and ligated into pTrcHis2B that had been digested with *Nco*I/*Pst*I. The construct is cloned such that the stop codon in the insert is before the *Pst*I site, which results in a construct in which the His-Tag is not attached to the isoprene synthase protein. The resulting plasmid pTrcPoplar (Figures 32 and 33; SEQ ID NO:15), was verified by sequencing.

Example 2B: Demonstration of isoprene synthase activity from several *populus* isoprene synthases

[0692] The following isoprene synthases were examined; *Populus alba* (Accession number BAD98243; Figures 137A and B; SEQ ID NO:30), *Populus nigra* (Accession number CAL69918; Figures 137C and D; SEQ ID NO:31), *Populus tremuloides* (Accession number AAQ16588; Figure 137 E, F, and G; SEQ ID NOs:32-33), *Populus trichocarpa* (Accession number ACD70404; Figures 137H and I; SEQ ID NO:34), *Populus alba x Populus tremula* (Accession number CAJ29303; Figures 137J and K; SEQ ID NO:35), and MCM112-Kudzu.

[0693] pET24Kudzu (also referred to as MCM112) was constructed as follows: the kudzu isoprene synthase gene was subcloned into the pET24d vector (Novagen) from the pCR2.1 vector (Invitrogen). The kudzu IspS gene was amplified from pTrcKudzu template DNA using primers MCM50 5'-GATCATGCAT TCGCCCTTAG GAGGTAAAAAAACATGTGTGCGACCTCTTC TCAATTTACT (SEQ ID NO:52); and MCM53 5'-CGGTCGACGGATCCCTGCAG TTAGACATAC ATCAGCTG (SEQ ID NO:50). PCR reactions were carried out using Taq DNA Polymerase (Invitrogen), and the resulting PCR product was cloned into pCR2.1-TOPO TA cloning vector (Invitrogen), and transformed into *E. coli* Top10 chemically competent cells (Invitrogen). Transformants were plated on L-agar containing carbenicillin (50 µg/ml) and incubated overnight at 37°C. Five ml Luria Broth cultures containing carbenicillin 50 µg/ml were inoculated with single transformants and grown

overnight at 37°C. Five colonies were screened for the correct insert by sequencing of plasmid DNA isolated from 1 ml of liquid culture (Luria Broth) and purified using the QIAprep Spin Mini-prep Kit (Qiagen). The resulting plasmid, designated MCM93, contains the kudzu IspS coding sequence in a pCR2.1 backbone (Figure 137L). The sequence of MCM93 (SEQ ID NO:36) is shown in Figures 137M and N.

[0694] The kudzu coding sequence was removed by restriction endonuclease digestion with *Pci*I and *Bam*H1 (Roche) and gel purified using the QIAquick Gel Extraction kit (Qiagen). The pET24d vector DNA was digested with *Nco*I and *Bam*HI (Roche), treated with shrimp alkaline phosphatase (Roche), and purified using the QIAprep Spin Mini-prep Kit (Qiagen). The kudzu IspS fragment was ligated to the *Nco*I/*Bam*H1 digested pET24d using the Rapid DNA Ligation Kit (Roche) at a 5:1 fragment to vector ratio in a total volume of 20 µl. A portion of the ligation mixture (5 µl) was transformed into *E. coli* Top 10 chemically competent cells and plated on L agar containing kanamycin (50 µg/ml). The correct transformant was confirmed by sequencing and transformed into chemically competent BL21(λDE3)pLysS cells (Novagen). A single colony was selected after overnight growth at 37°C on L agar containing kanamycin (50 µg/ml). A map of the resulting plasmid designated as pET24D-Kudzu is shown in Figure 137O. The sequence of pET24D-Kudzu (SEQ ID NO:37) is shown in Figures 137P and Q.

[0695] *Escherichia coli* optimized isoprene synthase genes cloned into the pET24a expression vector (Novagen) were purchased from DNA2.0 (Menlo Park, CA) for *Populus tremuloides*, *Populus alba*, *Populus nigra* and *Populus trichocarpa*. Genes were synthesized with the chloroplast transit peptide sequence removed, resulting in expression of mature proteins.

[0696] The construct for the Kudzu isoprene synthase was used as control in this example. The plasmids were transformed into the *E. coli* expression host BL21(DE3)plysS and transformants were grown in 0.6 ml TM3 medium. The recipe for TM3 medium is as follows: K_2HPO_4 (13.6 g/l) KH_2PO_4 (13.6 g/l), $MgSO_4$ * $7H_2O$ (2 g/L) Citric Acid Monohydrate (2 g/L) Ferric Ammonium Citrate (0.3 g/L) $(NH_4)_2SO_4$ (3.2 g/L) yeast extract (0.2 g/L) 1 ml of 1000x Trace Elements solution, pH adjusted to 6.8 with ammonium hydroxide qs to volume with sterile DI H_2O and filter sterilized with a 0.22 micron filter. The recipe for 1000X Trace Elements solution is as follows: Citric Acids * H_2O (40 g/L), $MnSO_4$ * H_2O (30 g/L), NaCl (10 g/L), $FeSO_4$ * 7 H_2O (1 g/L), $CoCl_2$ * 6 H_2O (1 g/L), $ZnSO_4$ * 7 H_2O (1 g/L), $CuSO_4$ * 5 H_2O (100 mg/L), H_3BO_3 (100 mg/L), $NaMoO_4$ * 2 H_2O (100 mg/L). Each component was dissolved one at a time in DI H_2O, pH adjusted to 3.0 with HCl/NaOH, qs to volume and filter sterilized with a 0.22 micron filter.

[0697] The cultures were induced with 400 μM IPTG and growth was continued to OD_{600} of about 5. Aliquots of culture were transferred to a deep well glass plate and wells were sealed with aluminum plate sealer. The plate was incubated at 25°C for 30 minutes with shaking at 450 rpm. The reactions were heat inactivated by raising the temperature to 70°C for 5 minutes. Whole cell head space was measured by the GCMS method as described in Example 1, Part II.

[0698] K_m values were obtained from cultures grown in similar manner but cells were harvested and lysed by a freeze/thaw lysozyme protocol. A volume of 400 μL of culture was transferred into a new 96-well plate (Perkin Elmer, Catalog No. 6008290) and cells were harvested by centrifugation in a Beckman Coulter Allegra 6R centrifuge at 2500 x g. The pellet was resuspended in 200 mL of hypotonic buffer (5 mM $MgCL_2$, 5 mM Tris HCl, 5 mM DTT pH 8.0) and the plate was frozen at -80°C for a minimum time of 60 minutes. Cell lysate was prepared by thawing the plate and adding 32 mL of isoprene synthase DMAPP assay buffer (57 mM Tris HCl, 19 mM $MgCl_2$, 74 mg/mL DNase I (Sigma Catalog No. DN-25), $2.63x10^5$ U/mL of ReadyLyse lysozyme solution (Epicentre Catalog No. R1802M), and 5 mg/mL of molecular biology grade BSA. The plate was incubated with shaking at 25°C for 30 minutes and then placed on ice. DMAPP and lysate were added at desired concentration in a sealed deep well glass block for the whole cell head space assay described above. The reactions were allowed to proceed for 1 hour and then terminated by the heat step described above and head space activity was measured also as described.

[0699] In an alternate approach, the activity of the enzymes was measured from cells cultured in 25 mL volume and induced similarly as described above. Cells were harvested by centrifugation and the pellets were lysed by French pressing in buffer consisting of 50% glycerol mixed 1:1with 20 mM Tris/HCl pH 7.4, 20 mM $MgCl_2$, 200 mM KCl, 1 mM DTT. A lysate volume of 25 μL was assayed for isoprene synthase activity in 2 mL screw cap vials containing 75 μL of assay buffer (66.6 mM Tris/HCl pH 8, 6.66 mM DMAPP, 43 mM, $MgCl_2$). The reaction was incubated for 15 minutes at 30°C and was quenched by the addition of 100 μL of 250 mM EDTA through the septum of the vial. Isoprene was measured by GC/MS as described in Example 1, Part II.

[0700] All methods for the determination of activity showed that the poplar enzyme derived from the pure bred poplars were several-fold higher than the *Populus* [*alba x tremula*]. Figures 138 and 139 showed these results for the whole cell head space assay and the DMAPP assay, respectively, and surprisingly indicate that enzymes from *P. nigra, P. tremuloides, P. trichocarpa*, and *P. alba* all had significantly higher activity than hybrid [*P. alba x P.tremula*].

[0701] The DMAPP assay was performed as follows: a volume of 400 µL of culture was transferred into a new 96-well plate (Perkin Elmer, Catalog No. 6008290) and cells were harvested by centrifugation in a Beckman Coulter Allegra 6R centrifuge at 2500 x g. The pellet was resuspended in 200 mL of hypotonic buffer (5 mM $MgCL_2$, 5 mM Tris HCl, 5 mM DTT pH 8.0) and the plate was frozen at -80°C for a minimum time of 60 minutes. Cell lysate was prepared by thawing the plate and adding 32 mL of isoprene synthase DMAPP assay buffer (57 mM Tris HCl, 19 mM $MgCl_2$, 74 mg/mL DNase I (Sigma Catalog No. DN-25), $2.63x10^5$ U/mL of ReadyLyse lysozyme solution (Epicentre Catalog No. R1802M), and 5 mg/mL of molecular biology grade BSA. The plate was incubated with shaking at 25°C for 30 minutes and then placed on ice. For isoprene production an 80 mL aliquot of lysate was transferred to a 96-deep well glass plate (Zinsser Catalog No. 3600600) and 20 mL of a 10 mM DMAPP solution in 100 mM $KHPO_4$, pH 8.2 (Cayman Chemical Catalog No. 63180) was added. The plate was sealed with an aluminum plate seal (Beckman Coultor Catalog No. 538619) and incubated with shaking at 30°C for 60 minutes. The enzymatic reactions were terminated by heating the glass block (70°C for 5 minutes). The cell head space of each well was quantitatively analyzed as described in Example 1, Part II.

[0702] Notably, *P. alba, P.tremuloides, P. trichocarpa* had higher activity than the isoprene synthase from Kudzu. The enzyme from *P. alba* was expressed with the greatest activity of all enzymes tested. The higher activities observed with the cell lysate compared to the whole cell head space assay was likely due to limitations in DMAPP, the substrate for these enzymes, delivered by the endogenous deoxyxylulose 5-phosphate (DXP) pathway of the cell.

[0703] K_m kinetic parameter was measured to be about 2 to 3 mM for all enzymes for which the value was determined.

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

[0704] 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

[0705] 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

[0706] The *aprE* promoter was amplified from chromosomal DNA from *Bacillus subtilis* using the following primers:

CF 797 (+) Start *aprE* promoter MfeI

5'- GACATCAATTGCTCCATTTTCTTCTGCTATC (SEQ ID NO:53)

CF 07-43 (-) Fuse *aprE* promoter to Kudzu ispS

5'- ATTGAGAAGAGGTCGCACACACTCTTTACCCTCTCCTTTTA (SEQ ID NO:54)

b) Amplification of the isoprene synthase gene

[0707] The kudzu isoprene synthase gene was amplified from plasmid pTrcKudzu (SEQ ID NO:2). The gene had been codon optimized for *E. coli* and synthesized by DNA 2.0. The following primers were used:

CF 07-42 (+) Fuse the aprE promoter to kudzu isoprene synthase gene (GTG start codon)

5'- TAAAAGGAGAGGGTAAAGAGTGTGTGCGACCTCTTCTCAAT (SEQ ID NO:55)

CF 07-45 (-) Fuse the 3' end of kudzu isoprene synthase gene to the terminator

5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:56)

c) Amplification of the transcription terminator

[0708] The terminator from the alkaline serine protease of *Bacillus amyliquefaciens* was amplified from a previously sequenced plasmid pJHPms382 using the following primers:

CF 07-44 (+) Fuse the 3' end of kudzu isoprene synthase to the terminator

5'- GATTAACCAGCTGATGTATGTCTAAAAAAAACCGGCCTTGG (SEQ ID NO:57)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

[0709] The kudzu fragment was fused to the terminator fragment using PCR with the following primers:
CF 07-42 (+) Fuse the *aprE* promoter to kudzu isoprene synthase gene (GTG start codon)
5'- TAAAAGGAGAGGGTAAAGAGTGTGTGCGACCTCTTCTCAAT (SEQ ID NO:55)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

[0710] The kudzu-terminator fragment was fused to the promoter fragment using PCR with the following primers:
CF 797 (+) Start *aprE* promoter MfeI
5'- GACATCAATTGCTCCATTTTCTTCTGCTATC (SEQ ID NO:53)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

[0711] The fusion PCR fragment was purified using a Qiagen kit and digested with the restriction enzymes *Mfe*I and *Bam*HI. This digested DNA fragment was gel purified using a Qiagen kit and ligated to a vector known as pBS19, which had been digested with *Eco*RI and *Bam*HI and gel purified.

[0712] The ligation mix was transformed into *E. coli* Top 10 cells and colonies were selected on LA+50 carbenicillin plates. A total of six colonies were chosen and grown overnight in LB+50 carbenicillin and then plasmids were isolated using a Qiagen kit. The plasmids were digested with *Eco*RI and *Bam*HI to check for inserts and three of the correct plasmids were sent in for sequencing with the following primers:
CF 149 (+) EcoRI start of *aprE* promoter
5'- GACATGAATTCCTCCATTTTCTTCTGC (SEQ ID NO:59)

CF 847 (+) Sequence in pXX 049 (end of *aprE* promoter)
5'- AGGAGAGGGTAAAGAGTGAG (SEQ ID NO:60)

CF 07-45 (-) Fuse the 3' end of kudzu isoprene synthase to the terminator
5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:56)

CF 07-48 (+) Sequencing primer for kudzu isoprene synthase
5'- CTTTTCCATCACCCACCTGAAG (SEQ ID NO:61)

CF 07-49 (+) Sequencing in kudzu isoprene synthase
5'- GGCGAAATGGTCCAACAACAAAATTATC (SEQ ID NO:62)

[0713] The plasmid designated pBS Kudzu #2 (Figures 52 and 12; SEQ ID NO:5) was correct by sequencing and was transformed into BG 3594 comK, a *Bacillus subtilis* host strain. Selection was done on LA + 5 chloramphenicol plates. A transformant was chosen and struck to single colonies on LA + 5 chloramphenicol, then grown in LB+5 chloramphenicol until it reached an OD_{600} of 1.5. It was stored frozen in a vial at -80° C in the presence of glycerol. The resulting strain was designated CF 443.

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

[0714] 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 $FeSO_4*7H_2O$, 0.1 g $MnSO_4*H_2O$, 0.1 g $ZnSO_4*H_2O$, 0.05 g $CuCl_2*2H_2O$, 0.1 g $CoCl_2*6H_2O$, 0.1 g $Na_2MoO_4*2H_2O$, q.s. to 1 L with H_2O. Shake flasks were incubated at 37° C and samples were taken at 18, 24, and 44 hours. At 18 hours the headspaces of CF443 and the control strain were sampled. This represented 18 hours of accumulation of isoprene. The

amount of isoprene was determined by gas chromatography as described in Example 1. Production of isoprene was enhanced significantly by expressing recombinant isoprene synthase (Figure 11).

III. Production of isoprene by CF443 in 14 L fermentation

[0715] Large scale production of isoprene from *B. subtilis* containing the recombinant kudzu isoprene synthase gene on a replication plasmid was determined from a fed-batch culture. Bacillus strain CF 443, expressing a kudzu isoprene synthase gene, or control stain which does not express a kudzu isoprene synthase gene were cultivated by conventional fed-batch fermentation in a nutrient medium containing soy meal (Cargill), sodium and potassium phosphate, magnesium sulfate and a solution of citric acid, ferric chloride and manganese chloride. Prior to fermentation the media is macerated for 90 minutes using a mixture of enzymes including cellulases, hemicellulases and pectinases (see, WO95/04134). 14-L batch fermentations are fed with 60% wt/wt glucose (Cargill DE99 dextrose, ADM Versadex greens or Danisco invert sugar) and 99% wt/wt oil (Western Family soy oil, where the 99% wt/wt is the concentration of oil before it was added to the cell culture medium). Feed was started when glucose in the batch was non-detectable. The feed rate was ramped over several hours and was adjusted to add oil on an equal carbon basis. The pH was controlled at 6.8 – 7.4 using 28% w/v ammonium hydroxide. In case of foaming, antifoam agent was added to the media. The fermentation temperature was controlled at 37°C and the fermentation culture was agitated at 750 rpm. Various other parameters such as pH, DO%, airflow, and pressure were monitored throughout the entire process. The DO% is maintained above 20. Samples were taken over the time course of 36 hours and analyzed for cell growth (OD_{550}) and isoprene production. Results of these experiments are presented in Figures 53A and 53B.

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

[0716] 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*

[0717] The *Yarrowia lipolytica* codon-optimized kudzu IS gene was synthesized by DNA 2.0 (SEQ ID NO:6) (Figure 13). This plasmid served as the template for the following PCR amplification reaction: 1 µl plasmid template (20 ng/ul), 1 µl Primer EL-945 (10 µM) 5'-GCTTATGGATCCTCTAGACTATTACACGTACATCAATTGG (SEQ ID NO:63), 1 µl Primer EL-965 (10 µM) 5'-CACCATGTGTGCAACCTCCTCCCAGTTTAC (SEQ ID NO:64), 1 µl dNTP (10mM), 5 µl 10x PfuUltra II Fusion HS DNA Polymerase Buffer, 1 µl PfuUltra II Fusion HS DNA Polymerase, 40 µl water in a total reaction volume of 50 µl. The forward primer contained an additional 4 nucleotides at the 5'-end that did not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but was required for cloning into the pENTR/D-TOPO vector. The reverse primer contained an additional 21 nucleotides at the 5'-end that did not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but were inserted for cloning into other vector backbones. Using the MJ Research PTC-200 Thermocycler, the PCR reaction was performed as follows: 95° C for 2 minutes (first cycle only), 95° C for 30 seconds, 55° C for 30 seconds, 72° C for 30 seconds (repeat for 27 cycles), 72° C for 1 minute after the last cycle. The PCR product was analyzed on a 1.2% E-gel to confirm successful amplification of the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene.

[0718] 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.

[0719] 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.

[0720] 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*

[0721] 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.*

[0722] The starting point for the construction of vectors for the expression of the kudzu isoprene synthase gene in *Yarrowia lipolytica* was the vector pSPZ1(MAP29Spb). The complete sequence of this vector (SEQ ID NO:7) is shown in Figure 15.

[0723] The following fragments were amplified by PCR using chromosomal DNA of a *Y. lipolytica* strain GICC 120285 as the template: a promotorless form of the URA3 gene, a fragment of 18S ribosomal RNA gene, a transcription terminator of the *Y. lipolytica* XPR2 gene

and two DNA fragments containing the promoters of XPR2 and ICL1 genes. The following PCR primers were used:

ICL1 3

5'- GGTGAATTCAGTCTACTGGGGATTCCCAAATCTATATATACTGCAGGTGAC (SEQ ID NO:65)

ICL1 5

5'- GCAGGTGGGAAACTATGCACTCC (SEQ ID NO:66)

XPR 3

5'- CCTGAATTCTGTTGGATTGGAGGATTGGATAGTGGG (SEQ ID NO:67)

XPR 5

5'- GGTGTCGACGTACGGTCGAGCTTATTGACC (SEQ ID NO:68)

XPRT3

5'- GGTGGGCCCGCATTTTGCCACCTACAAGCCAG (SEQ ID NO:69)

XPRT 5

5'- GGTGAATTCTAGAGGATCCCAACGCTGTTGCCTACAACGG (SEQ ID NO:70)

Y18S3

5'- GGTGCGGCCGCTGTCTGGACCTGGTGAGTTTCCCCG (SEQ ID NO:71)

Y18S 5

5'- GGTGGGCCCATTAAATCAGTTATCGTTTATTTGATAG (SEQ ID NO:72)

YURA3

5'- GGTGACCAGCAAGTCCATGGGTGGTTTGATCATGG (SEQ ID NO:73)

YURA 50

5'- GGTGCGGCCGCCTTTGGAGTACGACTCCAACTATG (SEQ ID NO:74)

YURA 51

5'- GCGGCCGCAGACTAAATTTATTTCAGTCTCC (SEQ ID NO:75)

[0724] 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.

[0725] Synthetic DNA molecules encoding the kudzu isoprene synthase gene, codon-optimized for expression in *Yarrowia,* was obtained from DNA 2.0 (Figure 16; SEQ ID NO:8). Full detail of the construction scheme of the plasmids pYLA(KZ1) and pYLI(KZ1) carrying the synthetic kudzu isoprene synthase gene under control of XPR2 and ICL1 promoters respectively is presented in Figure 18. Control plasmids in which a mating factor gene (MAP29) is inserted in place of an isoprene synthase gene were also constructed (Figure 18E and 18F).

[0726] A similar cloning procedure can be used to express a poplar (*Populus alba* x *Populus tremula*) isoprene synthase gene. The sequence of the poplar isoprene is described in Miller B. *et al.* (2001) *Planta* 213, 483-487 and shown in Figure 17 (SEQ ID NO:9). A construction scheme for the generation the plasmids pYLA(POP1) and pYLI(POP1) carrying synthetic poplar isoprene synthase gene under control of XPR2 and ICL1 promoters respectively is presented in Figure 18A and B.

II. Production of isoprene by recombinant strains of *Y. lipolytica.*

[0727] 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.

[0728] 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

[0729] 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:76) and MCM23 5'-GATCCGATCGTCAGAAGAACTCGTCAAGAAGGC (SEQ ID NO:77), digested with *Hin*dIII and *Pvu*I, and end-filled. A transformant carrying a plasmid conferring kanamycin resistance (pTrcKudzuKan) was selected on LA containing kanamycin 50 μg/ml.

ii) Construction of pTrcKudzu yIDI Kan

[0730] pTrcKudzuKan was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *idi* from *S. cerevisiae* with a synthetic RBS. The primers for PCR were NsiI-YIDI 1 F 5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC (SEQ ID NO:78) and PstI-YIDI 1 R 5'- CCTTCTGCAGGACGCGTTGTTATAGC (SEQ ID NO:79); and the template

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

iii) Construction of pTrcKudzu DXS Kan

[0731] Plasmid pTrcKudzuKan was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *dxs* from *E. coli* with a synthetic RBS. The primers for PCR were MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAATACCCG (SEQ ID NO:80) and MCM14 5'- CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:81); and the template was *E. coli* genomic DNA. The PCR product was digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The resulting transformation reaction was transformed into TOP10 cells and selected on LA with kanamycin 50 μg/ml. Several transformants were isolated and sequenced and the resulting plasmid was called pTrcKudzu-DXS(kan) (Figures 36 and 37; SEQ ID NO:17).

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

[0732] pTrcKudzu-yIDI(kan) was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *E. coli dxs* with a synthetic RBS (primers MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAATACCCG (SEQ ID NO:80) and MCM14 5'- CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:81); template TOP10 cells) which had been digested with *Nsi*I and *Pst*I and gel purified. The final plasmid was called pTrcKudzu-yIDI-dxs (kan) (Figures 21 and 22; SEQ ID NO:10).

v) Construction of pCL PtrcKudzu

[0733] A fragment of DNA containing the promoter, structural gene and terminator from Example 1 above was digested from pTrcKudzu using *Ssp*I and gel purified. It was ligated to pCL1920 which had been digested with *Pvu*II, treated with SAP and heat killed. The resulting ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 μg/ml. Several clones were isolated and sequenced and two were selected. pCL PtrcKudzu

and pCL PtrcKudzu (A3) have the insert in opposite orientations (Figures 38-41; SEQ ID NOs:18-19).

vi) Construction of pCL PtrcKudzu yIDI

[0734] The *Nsi*I-*Pst*I digested, gel purified, IDI PCR amplicon from (ii) above was ligated into pCL PtrcKudzu which had been digested with *Pst*I, treated with SAP, and heat killed. The ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 μg/ml. Several clones were isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu yIDI (Figures 42 and 43; SEQ ID NO:20).

vii) Construction of pCL PtrcKudzu DXS

[0735] The *Nsi*I-*Pst*I digested, gel purified, DXS PCR amplicon from (iii) above was ligated into pCL PtrcKudzu (A3) which had been digested with *Pst*I, treated with SAP, and heat killed. The ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 μg/ml. Several clones were isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu DXS (Figures 44 and 45; SEQ ID NO:21).

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

[0736] 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.

[0737] Plasmid pTrcKudzu-yIDI-dxs (kan) was introduced into *E. coli* strain BL21 by transformation. The resulting strain BL21/pTrc Kudzu IDI DXS was grown overnight in LB containing kanamycin (50 μg/ml) at 20° C and used to inoculate shake flasks of TM3 (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_2O, and filter sterilized) containing 1% glucose. Flasks were incubated at 30° C until an OD_{600} of 0.8 was reached, and then induced with 400 μM IPTG.

Samples were taken at various times after induction and the amount of isoprene in the head space was measured as described in Example 1. Results are shown in Figure 23H.

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

[0738] The strain BL21 pTrcKudzuIDIDXS was tested for the ability to generate isoprene from three types of biomass; bagasse, corn stover and soft wood pulp with glucose as a control. Hydrolysates of the biomass were prepared by enzymatic hydrolysis (Brown, L and Torget, R., 1996, NREL standard assay method Lap-009 "Enzymatic Saccharification of Lignocellulosic Biomass") and used at a dilution based upon glucose equivalents. In this example, glucose equivalents were equal to 1% glucose. A single colony from a plate freshly transformed cells of BL21 (DE3) pTrcKudzu yIDI DXS (kan) was used to inoculate 5 ml of LB plus kanamycin (50 μg/ml). The culture was incubated overnight at 25° C with shaking. The following day the overnight culture was diluted to an OD_{600} of 0.05 in 25 ml of TM3 + 0.2% YE + 1% feedstock. The feedstock was corn stover, bagasse, or softwood pulp. Glucose was used as a positive control and no glucose was used as a negative control. Cultures were incubated at 30° C with shaking at 180 rpm. The culture was monitored for OD_{600} and when it reached an OD_{600} of ~0.8, cultures were analyzed at 1 and 3 hours for isoprene production as described in Example 1. Cultures are not induced. All cultures containing added feedstock produce isoprene equivalent to those of the glucose positive control. Experiments were done in duplicate and are shown in Figure 46.

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

[0739] A single colony from a plate freshly transformed cells of BL21 (λDE3)/pTrcKudzu yIDI DXS (kan) was used to inoculate 5 mL of LB + kanamycin (50 μg/ml). The culture was incubated overnight at 25° C with shaking. The following day the overnight culture was diluted to an OD_{600} of 0.05 in 25 ml of TM3 + 0.2% YE + 1% feedstock. Feedstock was glucose, inverted glucose or corn stover. The invert sugar feedstock (Danisco Invert Sugar) was prepared by enzymatically treating sucrose syrup. AFEX corn stover was prepared as described below (Part V). The cells were grown at 30° C and the first sample was measured when the cultures reached an OD_{600} ~0.8-1.0 (0 hour). The cultures were analyzed for growth as measured by OD_{600} and for isoprene production as in Example 1 at 0, 1 and 3 hours. Results are shown in Figure 47.

V. Preparation of hydrolysate from AFEX pretreated corn stover

[0740] 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

[0741] Fermentation was performed at the 14-L scale as previously described with *E. coli* cells containing the pTrcKudzu yIDI DXS plasmid described above. Yeast extract (Bio Springer, Montreal, Quebec, Canada) was fed at an exponential rate. The total amount of yeast extract delivered to the fermentor was varied between 70-830 g during the 40 hour fermentation. Optical density of the fermentation broth was measured at a wavelength of 550 nm. The final optical density within the fermentors was proportional to the amount of yeast extract added (Figure 48A). The isoprene level in the off-gas from the fermentor was determined as previously described. The isoprene titer increased over the course of the fermentation (Figure 48B). The amount of isoprene produced was linearly proportional to the amount of fed yeast extract (Figure 48C).

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

[0742] 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):

[0743] 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:

[0744] Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0745] 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.

[0746] Yeast extract (Bio Springer, Montreal, Quebec, Canada) and glucose were fed at exponential rates. The total amount of glucose and yeast extract delivered to the bioreactor during the 50 hour fermentation was 181.2 kg and 17.6 kg, respectively. The optical density within the bioreactor over time is shown in Figure 49A. The isoprene level in the off-gas from the bioreactor was determined as previously described. The isoprene titer increased over the course of the fermentation (Figure 49B). The total amount of isoprene produced during the 50 hour fermentation was 55.1 g and the time course of production is shown in Figure 49C.

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

I. Cloning the lower MVA pathway

[0747] The strategy for cloning the lower mevalonic pathway was as follows. Four genes of the mevalonic acid biosynthesis pathway; mevalonate kinase (MVK), phosphomevalonate kinase

(PMK), diphosphomevalonte decarboxylase (MVD) and isopentenyl diphosphate isomerase genes were amplified by PCR from *S. cerevisiae* chromosomal DNA and cloned individually into the pCR BluntII TOPO plasmid (Invitrogen). In some cases, the *idi* gene was amplified from *E. coli* chromosomal DNA. The primers were designed such that an *E. coli* consensus RBS (AGGAGGT (SEQ ID NO:82) or AAGGAGG (SEQ ID NO:83)) was inserted at the 5' end, 8 bp upstream of the start codon and a *Pst*I site was added at the 3' end. The genes were then cloned one by one into the pTrcHis2B vector until the entire pathway was assembled.

[0748] Chromosomal DNA from *S. cerevisiae* S288C was obtained from ATCC (ATCC 204508D). The MVK gene was amplified from the chromosome of *S. cerevisiae* using primers MVKF (5'-AGGAGGTAAAAAAACATGTCATTACCGTTCTTAACTTCTGC, SEQ ID NO:84) and MVK-Pst1-R (5'-ATGGCTGCAGGCCTATCGCAAATTAGCTTATGAAGTCCATGGTAAATTCGTG, SEQ ID NO:85) using PfuTurbo as per manufacturer's instructions. The correct sized PCR product (1370 bp) was identified by electrophoresis through a 1.2% E-gel (Invitrogen) and cloned into pZeroBLUNT TOPO. The resulting plasmid was designated pMVK1. The plasmid pMVK1 was digested with *Sac*I and *Taq*1 restriction endonucleases and the fragment was gel purified and ligated into pTrcHis2B digested with *Sac*I and *Bst*BI. The resulting plasmid was named pTrcMVK1.

[0749] The second gene in the mevalonic acid biosynthesis pathway, PMK, was amplified by PCR using primers: PstI-PMK1 R (5'-GAATTCGCCCTTCTGCAGCTACC, SEQ ID NO:86) and BsiHKA I-PMK1 F (5'-CGACTGGTGCACCCTTAAGGAGGAAAAAAACATGTCAG, SEQ ID NO:87). The PCR reaction was performed using Pfu Turbo polymerase (Stratagene) as per manufacturer's instructions. The correct sized product (1387 bp) was digested with *Pst*I and *Bsi*HKI and ligated into pTrcMVK1 digested with *Pst*I. The resulting plasmid was named pTrcKK. The MVD and the *idi* genes were cloned in the same manner. PCR was carried out using the primer pairs PstI-MVD 1 R (5'-GTGCTGGAATTCGCCCTTCTGCAGC, SEQ ID NO:88) and NsiI-MVD 1 F (5'-GTAGATGCATGCAGAATTCGCCCTTAAGGAGG, SEQ ID NO:89) to amplify the MVD gene and PstI-YIDI 1 R (5'-CCTTCTGCAGGACGCGTTGTTATAGC, SEQ ID NO:79) and NsiI-YIDI 1 F (5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC, SEQ ID NO:78) to amplify the yIDI gene. In some cases the IPP isomerase gene, *idi* from *E. coli* was used. To amplify *idi* from *E. coli* chromosomal DNA, the following primer set was used: PstI-CIDI 1 R (5'-GTGTGATGGATATCTGCAGAATTCG, SEQ ID NO:90) and NsiI-CIDI 1 F (5'-

CATCAATGCATCGCCCTTAGGAGGTAAAAAAACATG, SEQ ID NO:91). Template DNA was chromosomal DNA isolated by standard methods from *E. coli* FM5 (WO 96/35796 and WO 2004/033646, which are each hereby incorporated by reference in their entireties, particularly with respect to isolation of nucleic acids). The final plasmids were named pKKDIy for the construct encoding the yeast *idi* gene or pKKDIc for the construct encoding the *E. coli idi* gene. The plasmids were transformed into *E. coli* hosts BL21 for subsequent analysis. In some cases the isoprene synthase from kudzu was cloned into pKKDIy yielding plasmid pKKDIyIS.

[0750] The lower MVA pathway was also cloned into pTrc containing a kanamycin antibiotic resistance marker. The plasmid pTrcKKDIy was digested with restriction endonucleases *Apa*I and *Pst*I, the 5930 bp fragment was separated on a 1.2% agarose E-gel and purified using the Qiagen Gel Purification kit according to the manufacturer's instructions. The plasmid pTrcKudzuKan, described in Example 7, was digested with restriction endonucleases *Apa*I and *Pst*I, and the 3338 bp fragment containing the vector was purified from a 1.2% E-gel using the Qiagen Gel Purification kit. The 3338 bp vector fragment and the 5930 bp lower MVA pathway fragment were ligated using the Roche Quick Ligation kit. The ligation mix was transformed into *E. coli* TOP10 cells and tranformants were grown at 37° C overnight with selection on LA containing kanamycin (50 μg/ml). The transformants were verified by restriction enzyme digestion and one was frozen as a stock. The plasmid was designated pTrcKanKKDIy.

II. Cloning a kudzu isoprene synthase gene into pTrcKanKKDIy

[0751] The kudzu isoprene synthase gene was amplified by PCR from pTrcKudzu, described in Example 1, using primers MCM50 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGTGTGCGACCTCTTCTCAATTTACT (SEQ ID NO:52) and MCM53 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:50). The resulting PCR fragment was cloned into pCR2.1 and transformed into *E. coli* TOP10. This fragment contains the coding sequence for kudzu isoprene synthase and an upstream region containing a RBS from *E. coli*. Transformants were incubated overnight at 37° C with selection on LA containing carbenicillin (50 μg/ml). The correct insertion of the fragment was verified by sequencing and this strain was designated MCM93.

[0752] The plasmid from strain MCM93 was digested with restriction endonucleases *Nsi*I and *Pst*I to liberate a 1724 bp insert containing the RBS and kudzu isoprene synthase. The 1724 bp fragment was separated on a 1.2% agarose E-gel and purified using the Qiagen Gel Purification

kit according to the manufacturer's instructions. Plasmid pTrcKanKKDIy was digested with the restriction endonuclease *Pst*I, treated with SAP for 30 minutes at 37° C and purified using the Qiagen PCR cleanup kit. The plasmid and kudzu isoprene synthase encoding DNA fragment were ligated using the Roche Quick Ligation kit. The ligation mix was transformed into *E. coli* TOP10 cells and transformants were grown overnight at 37° C with selection on LA containing Kanamycin at 50 μg/ml. The correct transformant was verified by restriction digestion and the plasmid was designated pTrcKKDyIkISKan (Figures 24 and 25; SEQ ID NO:11). This plasmid was transformed into BL21(λDE3) cells (Invitrogen).

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

[0753] Strain BL21/pTrcKKDyIkISKan was cultured in MOPS medium (Neidhardt *et al.*, (1974) *J. Bacteriology* 119:736-747) adjusted to pH 7.1 and supplemented with 0.5% glucose and 0.5% mevalonic acid. A control culture was also set up using identical conditions but without the addition of 0.5% mevalonic acid. The culture was started from an overnight seed culture with a 1% inoculum and induced with 500 μM IPTG when the culture had reached an OD_{600} of 0.3 to 0.5. The cultures were grown at 30° C with shaking at 250 rpm. The production of isoprene was analyzed 3 hours after induction by using the head space assay described in Example 1. Maximum production of isoprene was 6.67 x 10^{-4} mol/L_{broth}/OD_{600}/hr where L_{broth} is the volume of broth and includes both the volume of the cell medium and the volume of the cells. The control culture not supplemented with mevalonic acid did not produce measurable isoprene.

IV. Cloning the upper MVA pathway

[0754] The upper mevalonate biosynthetic pathway, comprising two genes encoding three enzymatic activities, was cloned from *Enterococcus faecalis*. The *mvaE* gene encodes a protein with the enzymatic activities of both acetyl-CoA acetyltransferase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the first and third proteins in the pathway, and the *mvaS* gene encodes second enzyme in the pathway, HMG-CoA synthase. The *mvaE* gene was amplified from *E. faecalis* genomic DNA (ATCC 700802D-5) with an *E. coli* ribosome binding site and a spacer in front using the following primers:
CF 07-60 (+) Start of *mvaE* w/ RBS + ATG start codon *Sac*I

5' - GAGACATGAGCTCAGGAGGTAAAAAAACATGAAAACAGTAGTTATTATTG (SEQ ID NO:93)

CF 07-62 (-) Fuse *mvaE* to *mvaS* with RBS in between
5' - TTTATCAATCCCAATTGTCATGTTTTTTTACCTCCTTTATTGTTTTCTTAAATC (SEQ ID NO:94)

[0755] The *mvaS* gene was amplified from *E. faecalis* genomic DNA (ATCC 700802D-5) with a RBS and spacer from *E. coli* in front using the following primers:
CF 07-61 (+) Fuse *mvaE* to *mvaS* with RBS in between
5' -
GATTTAAGAAAACAATAAAGGAGGTAAAAAAACATGACAATTGGGATTGATAAA (SEQ ID NO:95)

CF 07-102 (-) End of *mvaS* gene *Bgl*II
5' -GACATGACATAGATCTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:96)

[0756] The PCR fragments were fused together with PCR using the following primers:
CF 07-60 (+) Start of *mvaE* w/ RBS + ATG start codon *Sac*I
5' -GAGACATGAGCTCAGGAGGTAAAAAAACATGAAAACAGTAGTTATTATTG (SEQ ID NO:93)

CF 07-102 (-) End of *mvaS* gene *Bgl*II
5'-GACATGACATAGATCTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:96)

[0757] The fusion PCR fragment was purified using a Qiagen kit and digested with the restriction enzymes *Sac*I and *Bgl*II. This digested DNA fragment was gel purified using a Qiagen kit and ligated into the commercially available vector pTrcHis2A, which had been digested with *Sac*I and *Bgl*II and gel purified.

[0758] The ligation mix was transformed into *E. coli* Top 10 cells and colonies were selected on LA+50 μg/ml carbenicillin plates. A total of six colonies were chosen and grown overnight in LB+50 μg/ml carbenicillin and plasmids were isolated using a Qiagen kit. The plasmids were

digested with *Sac*I and *Bgl*II to check for inserts and one correct plasmid was sequenced with the following primers:

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

5' – ATGAAAACAGTAGTTATTATTGATGC (SEQ ID NO:97)

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

5' – ATGTTATTGTTTTCTTAAATCATTTAAAATAGC (SEQ ID NO:98)

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

5' – ATGACAATTGGGATTGATAAAATTAG (SEQ ID NO:99)

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

5' – TTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:100)

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

5' – GAAATAGCCCCATTAGAAGTATC (SEQ ID NO:101)

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

5' – TTGCCAATCATATGATTGAAAATC (SEQ ID NO:102)

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

5' – GCTATGCTTCATTAGATCCTTATCG (SEQ ID NO:103)

CF 07-89 (+) Sequence *mvaS*

5' – GAAACCTACATCCAATCTTTTGCCC (SEQ ID NO:104)

[0759] The plasmid called pTrcHis2AUpperPathway#1 was correct by sequencing and was transformed into the commercially available *E. coli* strain BL21. Selection was done on LA+ 50 µg/ml carbenicillin. Two transformants were chosen and grown in LB+ 50 µg/ml carbenicillin until they reached an OD_{600} of 1.5. Both strains were frozen in a vial at -80° C in the presence of glycerol. Strains were designated CF 449 for pTrcHis2AUpperPathway#1 in BL21, isolate #1 and CF 450 for pTrcHis2AUpperPathway#1 in BL21, isolate #2. Both clones were found to behave identically when analyzed.

V. Cloning of UpperMVA Pathway into pCL1920

[0760] The plasmid pTrcHis2AUpperPathway was digested with the restriction endonuclease *Ssp*I to release a fragment containing pTrc-*mvaE-mvaS*-(His tag)-terminator. In this fragment, the his-tag was not translated. This blunt ended 4.5 kbp fragment was purified from a 1.2% E-gel using the Qiagen Gel Purification kit. A dephosphorylated, blunt ended 4.2 kbp fragment from pCL1920 was prepared by digesting the vector with the restriction endonuclease *Pvu*II, treating with SAP and gel purifying from a 1.2% E-gel using the Qiagen Gel Purification kit. The two fragments were ligated using the Roche Quick Ligation Kit and transformed into TOP10 chemically competent cells. Transformants were selected on LA containing spectinomycin (50 μg/ml). A correct colony was identified by screening for the presence of the insert by PCR. The plasmid was designated pCL PtrcUpperPathway (Figures 26 and 27A-27D; SEQ ID NO:12).

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

[0761] To obtain a strain with a complete mevalonic acid pathway plus kudzu isoprene synthase, plasmids pTrcKKDyIkISkan and pCLpTrcUpperPathway were both transformed into BL21(λDE3) competent cells (Invitrogen) and transformants were selected on LA containing kanamycin (50 μg/ml) and Spectinomycin (50 μg/ml). The transformants were checked by plasmid prep to ensure that both plasmids were retained in the host. The strain was designated MCM127.

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

[0762] Single colonies of the BL21/pTrcHis2A-*mvaE/mvaS* or FM5/p pTrcHis2A-*mvaE/mvaS* are inoculated into LB + carbenicillin (100 μg/ml) and are grown overnight at 37° C with shaking at 200 rpm. These cultures were diluted into 50 ml medium in 250 ml baffled flasks to an OD_{600} of 0.1. The medium was TM3 + 1 or 2% glucose + carbenicillin (100 μg/ml) or TM3 + 1% glucose + hydrolyzed soy oil + carbenicillin (100 μg/ml) or TM3 + biomass (prepared bagasse, corn stover or switchgrass). Cultures were grown at 30° C with shaking at 200 rpm for approximately 2-3 hours until an OD_{600} of 0.4 was reached. At this point the expression from the *mvaE mvaS* construct was induced by the addition of IPTG (400 μM). Cultures were incubated for a further 20 or 40 hours with samples taken at 2 hour intervals to 6 hour post induction and then at 24, 36 and 48 hours as needed. Sampling was done by removing 1 ml of culture, measuring the OD_{600}, pelleting the cells in a microfuge, removing the supernatant and analyzing it for mevalonic acid.

[0763] A 14 liter fermentation of *E. coli* cells with nucleic acids encoding *Enterococcus faecalis* AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase polypeptides produced 22 grams of mevalonic acid with TM3 medium and 2% glucose as the cell medium. A shake flask of these cells produced 2-4 grams of mevalonic acid per liter with LB medium and 1% glucose as the cell culture medium. The production of mevalonic acid in these strains indicated that the MVA pathway was functional in *E. coli*.

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

[0764] The following strains were created by transforming in various combinations of plasmids containing the upper and lower MVA pathway and the kudzu isoprene synthase gene as described above and the plasmids containing the idi, dxs, and dxr and isoprene synthase genes described in Example 7. The host cells used were chemically competent BL21(λDE3) and the transformations were done by standard methods. Transformants were selected on L agar containing kanamycin (50 μg/ml) or kanamycin plus spectinomycin (both at a concentration of 50 μg/ml). Plates were grown at 37° C. The resulting strains were designated as follows:

Grown on Kanamycin plus Spectinomycin (50 μg/ml each)
MCM127 - pCL Upper MVA + pTrcKKDyIkIS (kan) in BL21(λDE3)
MCM131 - pCL1920 + pTrcKKDyIkIS (kan) in BL21(λDE3)
MCM125 - pCL Upper MVA + pTrcHis2B (kan) in BL21(λDE3)

Grown on Kanamycin (50 μg/ml)
MCM64 - pTrcKudzu yIDI DXS (kan) in BL21(λDE3)
MCM50 - pTrcKudzu (kan) in BL21(λDE3)
MCM123 - pTrcKudzu yIDI DXS DXR (kan) in BL21(λDE3)

[0765] The above strains were streaked from freezer stocks to LA + appropriate antibiotic and grown overnight at 37° C. A single colony from each plate was used to inoculate shake flasks (25 ml LB + the appropriate antibiotic). The flasks were incubated at 22° C overnight with shaking at 200 rpm. The next morning the flasks were transferred to a 37° C incubator and grown for a further 4.5 hours with shaking at 200 rpm. The 25 ml cultures were centrifuged to pellet the cells and the cells were resuspended in 5 ml LB + the appropriate antibiotic. The

cultures were then diluted into 25 ml LB+1% glucose + the appropriate antibiotic to an OD_{600} of 0.1. Two flasks for each strain were set up, one set for induction with IPTG (800 μM) the second set was not induced. The cultures were incubated at 37° C with shaking at 250 rpm. One set of the cultures were induced after 1.50 hours (immediately following sampling time point 1). At each sampling time point, the OD_{600} was measured and the amount of isoprene determined as described in Example 1. Results are presented in Table 3. The amount of isoprene made is presented as the amount at the peak production for the particular strain.

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

Strain	Isoprene (μg/liter/OD/hr)
MCM50	23.8
MCM64	289
MCM125	ND
MCM131	Trace
MCM127	874

ND: not detected

Trace: peak present but not integratable.

IX. Analysis of mevalonic acid

[0766] Mevalonolactone (1.0 g, 7.7 mmol) (CAS# 503-48-0) was supplied from Sigma-Aldrich (WI, USA) as a syrup that was dissolved in water (7.7 mL) and was treated with potassium hydroxide (7.7 mmol) in order to generate the potassium salt of mevalonic acid. The conversion to mevalonic acid was confirmed by ^1H NMR analysis. Samples for HPLC analysis were prepared by centrifugation at 14,000 rpm for 5 minutes to remove cells, followed by the addition of a 300 μl aliquot of supernatant to 900 μl of H_2O. Perchloric acid (36 μl of a 70% solution) was then added followed by mixing and cooling on ice for 5 minutes. The samples were then centrifuged again (14,000 rpm for 5 min) and the supernatant transferred to HPLC. Mevalonic acid standards (20, 10, 5, 1 and 0.5 g/L) were prepared in the same fashion. Analysis of mevalonic acid (20 μL injection volume) was performed by HPLC using a BioRad Aminex 87-H+ column (300 mm by 7.0 mm) eluted with 5 mM sulfuric acid at 0.6 mL/min with refractive index (RI) detection. Under these conditions mevalonic acid eluted as the lactone form at 18.5 minutes.

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

[0767] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase was used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions resulted in the production of 2.2 g/L of isoprene.

Medium Recipe (per liter fermentation medium):

[0768] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0769] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0770] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pCL PtrcUpperPathway (Figure 26) and pTrcKKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into soytone-yeast extract-glucose medium. After the inoculum grew to OD 1.0 when measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0771] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 54 hour fermentation was 3.7 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 25 μM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 μM when OD_{550} reached 190. IPTG concentration was raised to 100 μM at 38 hours of fermentation. The OD_{550} profile within the bioreactor over time is shown in Figure 54. The isoprene level in the off gas from the bioreactor was determined as

described herein. The isoprene titer increased over the course of the fermentation to a final value of 2.2 g/L (Figure 55). The total amount of isoprene produced during the 54 hour fermentation was 15.9 g, and the time course of production is shown in Figure 56.

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

[0772] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase was used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions resulted in the production of 3.0 g/L of isoprene.

Medium Recipe (per liter fermentation medium):

[0773] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0774] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0775] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0776] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time, the glucose feed was decreased to meet metabolic demands. The total amount of

glucose delivered to the bioreactor during the 59 hour fermentation was 2.2 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 μM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 μM when OD_{550} reached 190. The OD_{550} profile within the bioreactor over time is shown in Figure 93. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 3.0 g/L (Figure 94). The total amount of isoprene produced during the 59 hour fermentation was 22.8 g, and the time course of production is shown in Figure 95. The molar yield of utilized carbon that went into producing isoprene during fermentation was 2.2%. The weight percent yield of isoprene from glucose was 1.0%.

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

[0777] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides, *Pueraria lobata* isoprene synthase, and Kudzu isoprene synthase was used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions resulted in the production of 3.3 g/L of isoprene.

i) Construction of pCLPtrcUpperPathwayHGS2

[0778] The gene encoding isoprene synthase from *Pueraria lobata* was PCR-amplified using primers NsiI-RBS-HGS F (CTTGATGCATCCTGCATTCGCCCTTAGGAGG, SEQ ID NO:105) and pTrcR (CCAGGCAAATTCTGTTTTATCAG, SEQ ID NO:106), and pTrcKKDyIkIS as a template. The PCR product thus obtained was restriction-digested with *Nsi*I and *Pst*I and gel-purified. The plasmid pCL PtrcUpperPathway was restriction-digested with *Pst*I and dephosphorylated using rAPid alkaline phosphatase (Roche) according to manufacturer's instructions.

[0779] These DNA fragments were ligated together and the ligation reaction was transformed into *E. coli* Top10 chemcially competent cells (Invitrogen), plated on L agar containing spectinomycin (50 μg/ml) and incubated overnight at 37 °C. Plasmid DNA was prepared from 6 clones using the Qiaquick Spin Mini-prep kit. The plasmid DNA was digested with restriction enzymes *Eco*RV and *Mlu*I to identify a clone in which the insert had the right orientation (*i.e.*, the gene oriented in the same way as the pTrc promoter).

[0780] The resulting correct plasmid was designated pCLPtrcUpperPathwayHGS2. This plasmid was assayed using the headspace assay described herein and found to produce isoprene in *E. coli* Top10, thus validating the functionality of the gene. The plasmid was transformed into BL21(LDE3) containing pTrcKKDyIkIS to yield the strain BL21/pCLPtrcUpperPathwayHGS2-pTrcKKDyIkIS. This strain has an extra copy of the isoprene synthase compared to the BL21/pCL PtrcUpperMVA and pTrc KKDyIkIS strain (Example 8, part XI). This strain also had increased expression and activity of HMGS compared to the BL21/pCL PtrcUpperMVA and pTrc KKDyIkIS strain used in Example 8, part XI.

ii) Isoprene fermentation from *E. coli* expressing pCLPtrcUpperPathwayHGS2-pTrcKKDyIkIS and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium):

[0781] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0782] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0783] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pCLPtrcUpperPathwayHGS2 and pTrc KKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0 measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0784] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 58 hour fermentation was 2.1 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 μM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 50 μM when OD_{550} reached 170. The OD_{550} profile within the bioreactor over time is shown in Figure 104. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 3.3 g/L (Figure 105). The total amount of isoprene produced during the 58 hour fermentation was 24.5 g and the time course of production is shown in Figure 106. The molar yield of utilized carbon that went into producing isoprene during fermentation was 2.5%. The weight percent yield of isoprene from glucose was 1.2%. Analysis showed that the activity of the isoprene synthase was increased by approximately 3-4 times that compared to BL21 expressing CL PtrcUpperMVA and pTrc KKDyIkIS plasmids (data not shown).

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

[0785] A synthetic operon containing mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and the IPP isomerase was integrated into the chromosome of *E. coli*. If desired, expression may be altered by integrating different promoters 5' of the operon.

[0786] Table 4 lists primers used for this experiment.

Table 4. Primers

MCM78	attTn7 up rev for integration construct	gcatgctcgagcggccgcTTTTAATCAAACATCCTGCCAA CTC (SEQ ID NO:107)
MCM79	attTn7 down rev for integration construct	gatcgaagggcgatcgTGTCACAGTCTGGCGAAACCG (SEQ ID NO:108)
MCM88	attTn7 up forw for integration construct	ctgaattctgcagatatcTGTTTTTCCACTCTTCGTTCACTT T (SEQ ID NO:109)
MCM89	attTn7 down forw for integration construct	tctagagggcccAAGAAAAATGCCCCGCTTACG (SEQ ID NO:110)
MCM104	GI1.2 promoter – MVK	Gatcgcggccgcgcccttgacgatgccacatcctgagcaaataattcaaccac taattgtgagcggataacacaaggaggaaacagctatgtcattaccgttcttaact tc (SEQ ID NO:111)
MCM105	aspA terminator – yIDI	Gatcgggccccaagaaaaaaggcacgtcatctgacgtgccttttttatttgtaga cgcgttgttatagcattcta (SEQ ID NO:112)
MCM120	Forward of attTn7: attTn7 homology, GB	aaagtagccgaagatgacggtttgtcacatggagttggcaggatgtttgattaaa agcAATTAACCCTCACTAAAGGGCGG (SEQ ID

	marker homology	NO:113)
MCM127	Rev complement of 1.2 GI: GB marker homology(extra long), promoter, RBS, ATG	AGAGTGTTCACCAAAAATAATAACCTTTCCCGG TGCAgaagttaagaacggtaatgacatagctgtttcctccttgtgttatccgct cacaattagtggttgaattatttgctcaggatgtggcatcgtcaagggcTAAT ACGACTCACTATAGGGCTCG (SEQ ID NO:114)

i) Target vector construction

[0787] The attTn7 site was selected for integration. Regions of homology upstream (attTn7 up) (primers MCM78 and MCM79) and downstream (attTn7 down) (primers MCM88 and MCM89) were amplified by PCR from MG1655 cells. A 50 μL reaction with 1μL 10μM primers, 3μL ddH_2O, 45μL Invitrogen Platinum PCR Supermix High Fidelity, and a scraped colony of MG1655 was denatured for 2:00 at 94 °C, cycled 25 times (2:00 at 94 °C, 0:30 at 50 °C, and 1:00 at 68 °C), extended for 7:00 at 72 °C, and cooled to 4 °C. This resulting DNA was cloned into pCR2.1 (Invitrogen) according to the manufacturer's instructions, resulting in plasmids MCM278 (attTn7 up) and MCM252 (attTn7 down). The 832bp *Apa*I-*Pvu*I fragment digested and gel purified from MCM252 was cloned into *Apa*I-*Pvu*I digested and gel purified plasmid pR6K, creating plasmid MCM276. The 825bp *Pst*I-*Not*I fragment digested and gel purified from MCM278 was cloned into *Pst*I-*Not*I digested and gel purified MCM276, creating plasmid MCM281.

ii) Cloning of lower pathway and promoter

[0788] MVK-PMK-MVD-IDI genes were amplified from pTrcKKDyIkIS with primers MCM104 and MCM105 using Roche Expand Long PCR System according to the manufacturer's instructions. This product was digested with *Not*I and *Apa*I and cloned into MCM281 which had been digested with *Not*I and *Apa*I and gel purified. Primers MCM120 and MCM127 were used to amplify CMR cassette from the GeneBridges FRT-gb2-Cm-FRT template DNA using Stratagene Pfu Ultra II. A PCR program of denaturing at 95 °C for 4:00, 5 cycles of 95 °C for 0:20, 55 °C for 0:20, 72 °C for 2:00, 25 cycles of 95 °C for 0:20, 58 °C for 0:20, 72 °C for 2:00, 72 °C for 10:00, and then cooling to 4 °C was used with four 50μL PCR reactions containing 1μL ~10ng/μL template, 1μL each primer, 1.25 μL 10mM dNTPs, 5μL 10x buffer, 1μL enzyme, and 39.75μL ddH_2O. Reactions were pooled, purified on a Qiagen PCR cleanup column, and used to electroporate water-washed Pir1 cells containing plasmid MCM296. Electroporation was carried out in 2mM cuvettes at 2.5V and 200 ohms.

Electroporation reactions were recovered in LB for 3hr at 30 °C. Transformant MCM330 was selected on LA with CMP5, Kan50 (Figures 107 and 108A-108C; SEQ ID NO:25).

iii) Integration into *E. coli* chromosome

[0789] Miniprepped DNA (Qiaquick Spin kit) from MCM330 was digested with *Sna*BI and used to electroporate BL21(DE3) (Novagen) or MG1655 containing GeneBridges plasmid pRedET Carb. Cells were grown at 30 °C to ~OD1 then induced with 0.4% L-arabinose at 37 °C for 1.5 hours. These cells were washed three times in 4 °C ddH2O before electroporation with 2μL of DNA. Integrants were selected on L agar with containing chloramphenicol (5 μg/ml) and subsequently confirmed to not grow on L agar + Kanamycin (50 μg/ml). BL21 integrant MCM331 and MG1655 integrant MCM333 were frozen.

iv) Construction of pET24D-Kudzu encoding Kudzu isoprene synthase

[0790] The kudzu isoprene synthase gene was subcloned into the pET24d vector (Novagen) from the pCR2.1 vector (Invitrogen). In particular, the kudzu isoprene synthase gene was amplified from the pTrcKudzu template DNA using primers MCM50 5'-GATCATGCAT TCGCCCTTAG GAGGTAAAAA AACATGTGTG CGACCTCTTC TCAATTTACT (SEQ ID NO:52) and MCM53 5'-CGGTCGACGG ATCCCTGCAG TTAGACATAC ATCAGCTG (SEQ ID NO:50). PCR reactions were carried out using Taq DNA Polymerase (Invitrogen), and the resulting PCR product was cloned into pCR2.1-TOPO TA cloning vector (Invitrogen), and transformed into *E. coli* Top10 chemically competent cells (Invitrogen). Transformants were plated on L agar containing carbenicillin (50 μg/ml) and incubated overnight at 37°C. Five ml Luria Broth cultures containing carbenicillin 50 μg/ml were inoculated with single transformants and grown overnight at 37°C. Five colonies were screened for the correct insert by sequencing of plasmid DNA isolated from 1 ml of liquid culture (Luria Broth) and purified using the QIAprep Spin Mini-prep Kit (Qiagen). The resulting plasmid, designated MCM93, contains the kudzu isoprene synthase coding sequence in a pCR2.1 backbone.

[0791] The kudzu coding sequence was removed by restriction endonuclease digestion with *Pci*I and *Bam*H1 (Roche) and gel purified using the QIAquick Gel Extraction kit (Qiagen). The pET24d vector DNA was digested with *Nco*I and *Bam*HI (Roche), treated with shrimp alkaline phosphatase (Roche), and purified using the QIAprep Spin Mini-prep Kit (Qiagen). The kudzu isoprene synthase fragment was ligated to the *Nco*I/*Bam*H1 digested pET24d using the Rapid DNA Ligation Kit (Roche) at a 5:1 fragment to vector ratio in a total volume of 20 μl. A portion

of the ligation mixture (5 µl) was transformed into *E. coli* Top 10 chemically competent cells and plated on L agar containing kanamycin (50 µg/ml). The correct transformant was confirmed by sequencing and transformed into chemically competent BL21(λDE3)pLysS cells (Novagen). A single colony was selected after overnight growth at 37°C on L agar containing kanamycin (50 µg/ml). A map of the resulting plasmid designated as pET24D-Kudzu is shown in Figure 109. The sequence of pET24D-Kudzu (SEQ ID NO:26) is shown in Figures 110A and 110B. Isoprene synthase activity was confirmed using a headspace assay.

v) Production strains

[0792] Strains MCM331 and MCM333 were cotransformed with plasmids pCLPtrcupperpathway and either pTrcKudzu or pETKudzu, resulting in the strains shown in Table 5.

Table 5. Production Strains

Background	Integrated Lower	Upper MVA plasmid	Isoprene synthase plasmid	Production Stain
BL21(DE3)	MCM331	pCLPtrcUpper Pathway	pTrcKudzu	MCM343
BL21(DE3)	MCM331	pCLPtrcUpper Pathway	pET24D-Kudzu	MCM335
MG1655	MCM333	pCLPtrcUpper Pathway	pTrcKudzu	MCM345

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

Medium Recipe (per liter fermentation medium):

[0793] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0794] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0795] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the gi1.2 integrated lower MVA pathway described above and the pCL PtrcUpperMVA and pTrcKudzu plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0796] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time, the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 57 hour fermentation was 3.9 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 100 μM when the carbon dioxide evolution rate reached 100 mmol/L/hr. The OD_{550} profile within the bioreactor over time is shown in Figure 111A. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 1.6 g/L (Figure 111B). The specific productivity of isoprene over the course of the fermentation is shown in Figure 111C and peaked at 1.2 mg/OD/hr. The total amount of isoprene produced during the 57 hour fermentation was 16.2 g. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.9%. The weight percent yield of isoprene from glucose was 0.4%.

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

[0797] A 15-L scale fermentation of *E. coli* expressing Kudzu isoprene synthase was used to produce isoprene from cells fed glycerol in fed-batch culture. This experiment demonstrates that

growing cells in the presence of glycerol (without glucose) resulted in the production of 2.2 mg/L of isoprene.

Medium Recipe (per liter fermentation medium):

[0798] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glycerol 5.1 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0799] The medium was generated using the following components per liter fermentation medium: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0800] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pTrcKudzu plasmid. This experiment was carried out to monitor isoprene formation from glycerol at the desired fermentation pH 7.0 and temperature 35°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LA broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into soytone-yeast extract-glucose medium and grown at 35°C. After the inoculum grew to OD 1.0, measured at 550 nm, 600 mL was used to inoculate a 7.5-L bioreactor.

[0801] Glycerol was fed at an exponential rate until cells reached an optical density at 550 nm (OD_{550}) of 153. The total amount of glycerol delivered to the bioreactor during the 36 hour fermentation was 1.7 kg. Other than the glucose in the inoculum, no glucose was added to the bioreactor. Induction was achieved by adding IPTG. The IPTG concentration was brought to 20 μM when the OD_{550} reached a value of 50. The OD_{550} profile within the bioreactor over time is shown in Figure 57. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value

of 2.2 mg/L (Figure 58). The total amount of isoprene produced during the 54 hour fermentation was 20.9 mg, and the time course of production is shown in Figure 59.

XV. Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale using invert sugar as a carbon source

[0802] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase was used to produce isoprene from cells fed invert sugar in fed-batch culture. This experiment demonstrates that growing cells in the presence of invert sugar resulted in the production of 2.4 g/L of isoprene.

Medium Recipe (per liter fermentation medium):

[0803] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Invert sugar 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0804] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in Di H2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0805] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from invert sugar at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0806] Invert sugar was fed at an exponential rate until cells reached the stationary phase. After this time the invert sugar feed was decreased to meet metabolic demands. The total amount of invert sugar delivered to the bioreactor during the 44 hour fermentation was 2.4 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 μM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 50 μM when OD_{550} reached 200. The OD_{550} profile within the bioreactor over time is shown in Figure 96. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 2.4 g/L (Figure 97). The total amount of isoprene produced during the 44 hour fermentation was 18.4 g and the time course of production is shown in Figure 98. The molar yield of utilized carbon that went into producing isoprene during fermentation was 1.7%. The weight percent yield of isoprene from glucose was 0.8%.

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

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

[0807] The upper pathway from *Enterococcus faecalis* is integrated into *B. subtilis* under control of the *aprE* promoter. The upper pathway consists of two genes; *mvaE*, which encodes for AACT and HMGR, and *mvaS*, which encodes for HMGS. The two genes are fused together with a stop codon in between, an RBS site in front of *mvaS*, and are under the control of the *aprE* promoter. A terminator is situated after the *mvaE* gene. The chloramphenicol resistance marker is cloned after the *mvaE* gene and the construct is integrated at the *aprE* locus by double cross over using flanking regions of homology.

[0808] Four DNA fragments are amplified by PCR such that they contain overhangs that will allowed them to be fused together by a PCR reaction. PCR amplifications are carried out using Herculase polymerase according to manufacturer's instructions.

1. PaprE

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:115)

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

5'- CAATAATAACTACTGTTTTCACTCTTTACCCTCTCCTTTTAA (SEQ ID NO:116)

Template: *Bacillus subtilis* chromosomal DNA

2. *mvaE*

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

5'- TTAAAAGGAGAGGGTAAAGAGTGAAAACAGTAGTTATTATTG (SEQ ID NO:117)

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

5'- TTTATCAATCCCAATTGTCATGTTTTTTTACCTCCTTTATTGTTTTCTTAAATC (SEQ ID NO:94)

Template: *Enterococcus faecalis* chromosomal DNA (from ATCC)

3. *mvaS*

CF 07-61 (+) Fuse *mvaE* to *mvaS* with RBS in between

5'-

GATTTAAGAAAACAATAAAGGAGGTAAAAAAACATGACAATTGGGATTGATAAA (SEQ ID NO:95)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator

5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:118)

Template: *Enterococcus faecalis* chromosomal DNA

4. *B. amyliquefaciens* alkaline serine protease terminator

CF 07-123 (+) Fuse the end of mvaS to the terminator

5'- ACCGTTCGTTCTTATCGAAACTAAAAAAAACCGGCCTTGGCCCCG (SEQ ID NO:119)

CF 07-46 (-) End of *B. amyliquefaciens* terminator BamHI

5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

Template: *Bacillus amyliquefaciens* chromosomal DNA

PCR Fusion Reactions

5. Fuse *mvaE* to *mvaS*

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

5'- TTAAAAGGAGAGGGTAAAGAGTGAAAACAGTAGTTATTATTG (SEQ ID NO:117)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator

5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:118)

Template: #2 and 3 from above

6. Fuse *mvaE-mvaS* to *aprE* promoter

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:115)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator

5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:118)

Template #1 and #4 from above

7. Fuse PaprE-*mvaE-mvaS* to terminator

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:115)

CF 07-46 (-) End of *B. amyliquefaciens* terminator BamHI

5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

Template: #4 and #6

[0809] The product is digested with restriction endonucleases *Pst*I/*Bam*HI and ligated to pJM102 (Perego, M. 1993. Integrational vectors for genetic manipulation in *Bacillus subtilis*, p. 615–624. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington,D.C.) which is digested with *Pst*I/*Bam*HI. The ligation is transformed into *E.coli* TOP 10 chemically competent cells and transformants are selected on LA containing carbenicillin (50 μg/ml). The correct plasmid is identified by sequencing and is designated pJMUpperpathway2 (Figures 50 and 51). Purified plasmid DNA is transformed into *Bacillus subtilis aprEnprE* Pxyl-*comK* and transformants are selected on L agar containing chloramphenicol (5 μg/ml). A correct colony is selected and is plated sequentially on L agar containing chloramphenicol 10, 15 and 25 μg/ml to amplify the number of copies of the cassette containing the upper pathway.

[0810] The resulting strain is tested for mevalonic acid production by growing in LB containing 1% glucose and 1%. Cultures are analyzed by GC for the production of mevalonic acid.

[0811] This strain is used subsequently as a host for the integration of the lower mevalonic acid pathway.

[0812] The following primers are used to sequence the various constructs above.

Sequencing primers:

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:115)

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

5'- ATGAAAACAGTAGTTATTATTGATGC (SEQ ID NO:97)

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

5'- ATGTTATTGTTTTCTTAAATCATTTAAAATAGC (SEQ ID NO:98)

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

5'- ATGACAATTGGGATTGATAAAATTAG (SEQ ID NO:99)

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

5'- TTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:100)

CF 07-86 (+) Sequence in *mvaE*
5'- GAAATAGCCCCATTAGAAGTATC (SEQ ID NO:101)

CF 07-87 (+) Sequence in *mvaE*
5'- TTGCCAATCATATGATTGAAAATC (SEQ ID NO:102)

CF 07-88 (+) Sequence in *mvaE*
5'- GCTATGCTTCATTAGATCCTTATCG (SEQ ID NO:103)

CF 07-89 (+) Sequence *mvaS*
5'- GAAACCTACATCCAATCTTTTGCCC (SEQ ID NO:104)

[0813] Transformants are selected on LA containing chloramphenicol at a concentration of 5 μg/ml. One colony is confirmed to have the correct integration by sequencing and is plated on LA containing increasing concentrations of chloramphenicol over several days, to a final level of 25 μg/ml. This results in amplification of the cassette containing the genes of interest. The resulting strain is designated CF 455: pJMupperpathway#1 X *Bacillus subtilis aprEnprE* Pxyl *comK* (amplified to grow on LA containing chloramphenicol 25 μg/ml).

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

[0814] The lower MVA pathway, consisting of the genes mvk1, pmk, mpd and idi are combined in a cassette consisting of flanking DNA regions from the nprE region of the *B. subtilis* chromosome (site of integration), the *aprE* promoter, and the spectinomycin resistance marker (see Figures 28 and 29; SEQ ID NO:13). This cassette is synthesized by DNA2.0 and is integrated into the chromosome of *B. subtilis* containing the upper MVA pathway integrated at the aprE locus. The kudzu isoprene synthase gene is expressed from the replicating plasmid described in Example 4 and is transformed into the strain with both upper and lower pathways integrated.

Example 10: Exemplary isoprene compositions and methods of making them

I. Compositional analysis of fermentation off-gas containing isoprene

[0815] A 14 L scale fermentation was performed with a recombinant *E. coli* BL21 (DE3) strain containing two plasmids (pCL upperMev; pTrcKKDyIkIS encoding the full mevalonate pathway for isoprenoid precursor biosynthesis, an isoprenyl pyrophosphate isomerase from yeast, and an isoprene synthase from Kudzu. Fermentation off-gas from the 14 L tank was collected into 20 mL headspace vials at around the time of peak isoprene productivity (27.9 hours elapsed fermentation time, "EFT") and analyzed by headspace GC/MS for volatile components.

[0816] Headspace analysis was performed with an Agilent 6890 GC/MS system fitted with an Agilent HP-5MS GC/MS column (30 m x 250 μm; 0.25 μm film thickness). A combiPAL autoinjector was used for sampling 500 μL aliquots from 20 mL headspace vials. The GC/MS method utilized helium as the carrier gas at a flow of 1 mL/min. The injection port was held at 250 ^{o}C with a split ratio of 50:1. The oven temperature was held at 37 ^{o}C for an initial 2 minute period, followed an increase to 237 ^{o}C at a rate of 25 ^{o}C/min for a total method time of 10 minutes. The Agilent 5793N mass selective detector scanned from m/z 29 to m/z 300. The limit of detection of this system is approximately 0.1 $\mu g/L_{gas}$ or approximately 0.1 ppm. If desired, more sensitive equipment with a lower limit of detection may be used.

[0817] The off-gas consisted of 99.925 % (v/v) permanent gases (N_2, CO_2 and O_2), approximately 0.075% isoprene (2-methyl-1,3-butadiene) (~750 ppmv, 2100 μg/L) and minor amounts (<50 ppmv) of ethanol, acetone, and two C5 prenyl alcohols. The amount of water vapor was not determined but was estimated to be equal to the equilibrium vapor pressure at 0 ^{o}C. The composition of the volatile organic fraction was determined by integration of the area under the peaks in the GC/MS chromatogram (Figs. 86A and 86B) and is listed in Table 6. Calibration curves for ethanol and acetone standards enabled the conversion of GC area to gas phase concentration in units of μg/L using standard methods.

Table 6. Composition of volatile organic components in fermentation off-gas. The off-gas was analyzed at the 27.9 hour time point of a fermentation using an E. coli BL21 (DE3) strain expressing a heterologous mevalonate pathway, an isoprenyl pyrophosphate isomerase from yeast, and an isoprene synthase from Kudzu.

Compound	RT (min)	GC area	Area %	Conc. (μg/L)
Ethanol	1.669	239005	0.84	62 +/- 6
Acetone	1.703	288352	1.02	42 +/- 4
Isoprene (2-methyl-1,3-butadiene)	1.829	27764544	97.81	2000 +/- 200
3-methyl-3-buten-1-ol	3.493	35060	0.12	<10
3-methyl-2-buten-1-ol	4.116	58153	0.20	<10

II. Measurement of trace volatile organic compounds (VOCs) co-produced with isoprene during fermentation of a recombinant E. coli strain

[0818] A 14 L scale fermentation was performed with a recombinant *E. coli* BL21 (DE3) strain containing two plasmids (pCL upperMev; pTrcKKDyIkIS) encoding the full mevalonate pathway for isoprenoid precursor biosynthesis, an isoprenyl pyrophosphate isomerase from yeast, and an isoprene synthase from Kudzu.

[0819] Fermentation off-gas was passed through cooled headspace vials in order to concentrate and identify trace volatile organic components. The off-gas from this fermentation was sampled at a rate of 1 L/min for 10 minutes through a 20 mL headspace vial packed with quartz wool (2g) and cooled to –78 ^{o}C with dry ice. The vial was recapped with a fresh vial cap and analyzed by headspace GC/MS for trapped VOCs using the conditions described in Example 10, part I. The ratios of compounds observed in Figs. 87A-87D are a combination of overall level in the fermentation off-gas, the relative vapor pressure at -78 ^{o}C, and the detector response of the mass spectrometer. For example, the low level of isoprene relative to oxygenated volatiles (*e.g.*, acetone and ethanol) is a function of the high volatility of this material such that it does not accumulate in the headspace vial at -78 ^{o}C.

[0820] The presence of many of these compounds is unique to isoprene compositions derived from biological sources. The results are depicted in Figs. 87A-87D and summarized in Tables 7A and 7B.

Table 7A: Trace volatiles present in off-gas produced by E. coli BL21 (DE3) (pCL upperMev; pTrcKKDylkIS) following cryo-trapping at -78oC.

Compound	RT (min)	GC Area1	Area%2	Ratio%3
Acetaldehyde	1.542	4019861	4.841	40.14
Ethanol	1.634	10553620	12.708	105.39
Acetone	1.727	7236323	8.714	72.26
2-methyl-1,3-butadiene	1.777	10013714	12.058	100.00
1-propanol	1.987	163574	0.197	1.63
Diacetyl	2.156	221078	0.266	2.21
2-methyl-3-buten-2-ol	2.316	902735	1.087	9.01
2-methyl-1-propanol	2.451	446387	0.538	4.46
3-methyl-1-butanal	2.7	165162	0.199	1.65
1-butanol	2.791	231738	0.279	2.31
3-methyl-3-buten-1-ol	3.514	14851860	17.884	148.32
3-methyl-1-butanol	3.557	8458483	10.185	84.47
3-methyl-2-buten-1-ol	4.042	18201341	21.917	181.76
3-methyl-2-butenal	4.153	1837273	2.212	18.35
3-methylbutyl acetate	5.197	196136	0.236	1.96
3-methyl-3-buten-1-yl acetate	5.284	652132	0.785	6.51
2-heptanone	5.348	67224	0.081	0.67
2,5-dimethylpyrazine	5.591	58029	0.070	0.58
3-methyl-2-buten-1-yl acetate	5.676	1686507	2.031	16.84
6-methyl-5-hepten-2-one	6.307	101797	0.123	1.02
2,4,5-trimethylpyridine	6.39	68477	0.082	0.68
2,3,5-trimethylpyrazine	6.485	30420	0.037	0.30
(E)-3,7-dimethyl-1,3,6-octatriene	6.766	848928	1.022	8.48
(Z)-3,7-dimethyl-1,3,6-octatriene	6.864	448810	0.540	4.48
3-methyl-2-buten-1-yl butyrate	7.294	105356	0.127	1.05
Citronellal	7.756	208092	0.251	2.08
2,3-cycloheptenolpyridine	8.98	1119947	1.349	11.18

1 GC area is the uncorrected area under the peak corresponding to the listed compound.
2 Area % is the peak area expressed as a % relative to the total peak area of all compounds.
3 Ratio % is the peak area expressed as a % relative to the peak area of 2-methyl-1,3-butadiene.

Table 7B. Trace volatiles present in off-gas produced by E. coli BL21 (DE3) (pCL upperMev; pTrcKKDylkIS) following cryo-trapping at -196oC.

Compound	RT (min)	GC Area1	Area%2	Ratio%3

Compound	RT (min)	GC Area1	Area%2	Ratio%3
Acetaldehyde	1.54	1655710	0.276	0.33
Methanethiol	1.584	173620	0.029	0.03
Ethanol	1.631	10259680	1.707	2.03
Acetone	1.722	73089100	12.164	14.43
2-methyl-1,3-butadiene	1.771	506349429	84.269	100.00
methyl acetate	1.852	320112	0.053	0.06
1-propanol	1.983	156752	0.026	0.03
Diacetyl	2.148	67635	0.011	0.01
2-butanone	2.216	254364	0.042	0.05
2-methyl-3-buten-2-ol	2.312	684708	0.114	0.14
ethyl acetate	2.345	2226391	0.371	0.44
2-methyl-1-propanol	2.451	187719	0.031	0.04
3-methyl-1-butanal	2.696	115723	0.019	0.02
3-methyl-2-butanone	2.751	116861	0.019	0.02
1-butanol	2.792	54555	0.009	0.01
2-pentanone	3.034	66520	0.011	0.01
3-methyl-3-buten-1-ol	3.516	1123520	0.187	0.22
3-methyl-1-butanol	3.561	572836	0.095	0.11
ethyl isobutyrate	3.861	142056	0.024	0.03
3-methyl-2-buten-1-ol	4.048	302558	0.050	0.06
3-methyl-2-butenal	4.152	585690	0.097	0.12
butyl acetate	4.502	29665	0.005	0.01
3-methylbutyl acetate	5.194	271797	0.045	0.05
3-methyl-3-buten-1-yl acetate	5.281	705366	0.117	0.14
3-methyl-2-buten-1-yl acetate	5.675	815186	0.136	0.16
(E)-3,7-dimethyl-1,3,6-octatriene	6.766	207061	0.034	0.04
(Z)-3,7-dimethyl-1,3,6-octatriene	6.863	94294	0.016	0.02
2,3-cycloheptenolpyridine	8.983	135104	0.022	0.03

1 GC area is the uncorrected area under the peak corresponding to the listed compound.
2 Area % is the peak area expressed as a % relative to the total peak area of all compounds.
3 Ratio % is the peak area expressed as a % relative to the peak area of 2-methyl-1,3-butadiene.

III. Absence of C5 hydrocarbon isomers in isoprene derived from fermentation.

[0821] Cryo-trapping of isoprene present in fermentation off-gas was performed using a 2 mL headspace vial cooled in liquid nitrogen. The off-gas (1 L/min) was first passed through a 20 mL vial containing sodium hydroxide pellets in order to minimize the accumulation of ice and solid CO_2 in the 2 mL vial (-196 ^oC). Approximately 10L of off-gas was passed through the vial, after which it was allowed to warm to -78 ^oC with venting, followed by resealing with a fresh vial cap and analysis by GC/MS.

[0822] GC/MS headspace analysis was performed with an Agilent 6890 GC/MS system using a 100μL gas tight syringe in headspace mode. A Zebron ZB-624 GC/MS column (30 m x 250 μm; 1.40 μm film thickness) was used for separation of analytes. The GC autoinjector was fitted with a gas-tight 100 μL syringe, and the needle height was adjusted to allow the injection of a 50 μL headspace sample from a 2 mL GC vial. The GC/MS method utilized helium as the carrier gas at a flow of 1 mL/min. The injection port was held at 200 °C with a split ratio of 20:1. The oven temperature was held at 37 °C for the 5 minute duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 55, 66, 67 and 70. Under these conditions, isoprene was observed to elute at 2.966 minutes (Figure 88B). A standard of petroleum derived isoprene (Sigma-Aldrich) was also analyzed using this method and was found to contain additional C5 hydrocarbon isomers, which eluted shortly before or after the main peak and were quantified based on corrected GC area (Figure 88A).

Table 8A: GC/MS analysis of petroleum-derived isoprene

Compound	RT (min)	GC area	Area % of total C5 hydrocarbons
2-methyl-1-butene	2.689	18.2 x 103	0.017%
(Z)-2-pentene	2.835	10.6x 104	0.101%
Isoprene	2.966	10.4x 107	99.869%
1,3-cyclopentadiene (CPD)	3.297	12.8 x 103	0.012%

Table 8B: GC/MS analysis of fermentation-derived isoprene (% total C5 hydrocarbons)

Compound	RT (min)	Corrected GC Area	% of total C5 hydrocarbons
Isoprene	2.966	8.1 x 107	100%

[0823] In a separate experiment, a standard mixture of C5 hydrocarbons was analyzed to determine if the detector response was the same for each of the compounds. The compounds were 2-methyl-1-butene, 2-methyl-1,3-butadiene, (E)-2-pentene, (Z)-2-pentene and (E)-1,3-pentadiene. In this case, the analysis was performed on an Agilent DB-Petro column (100 m x 0.25 mm, 0.50 μm film thickness) held at 50 °C for 15 minutes. The GC/MS method utilized helium as the carrier gas at a flow of 1 mL/min. The injection port was held at 200 °C with a split ratio of 50:1. The Agilent 5793N mass selective detector was run in full scan mode from

m/z 19 to m/z 250. Under these conditions, a 100 μg/L concentration of each standard produced the same detector response within experimental error.

IV. Compositions comprising isoprene adsorbed to a solid phase.

[0824] Biologically-produced isoprene was adsorbed to activated carbon resulting in a solid phase containing 50 to 99.9% carbon, 0.1% to 50% isoprene, 0.01% to 5% water, and minor amounts (<0.1%) of other volatile organic components.

[0825] Fermentation off-gas was run through a copper condensation coil held at 0 ^{o}C, followed by a granulated silica desiccant filter in order to remove water vapor. The dehumidified off-gas was then run through carbon containing filters (Koby Jr, Koby Filters, MA) to the point at which breakthrough of isoprene was detected in the filter exhaust by GC/MS. The amount of isoprene adsorbed to the cartridge can be determined indirectly by calculating the concentration in the off-gas, the overall flow rate and the percent breakthrough over the collection period. Alternately the adsorbed isoprene can be recovered from the filters by thermal, vacuum, or solvent-mediated desorption.

V. Collection and analysis of condensed isoprene.

[0826] Fermentation off-gas is dehumidified, and the CO_2 removed by filtration through a suitable adsorbent (*e.g.*, ascarite). The resulting off-gas stream is then run through a liquid nitrogen-cooled condenser in order to condense the VOCs in the stream. The collection vessel contains t-butyl catechol to inhibit the resulting isoprene condensate. The condensate is analyzed by GC/MS and NMR in order to determine purity using standard methods, such as those described herein.

VI. Production of prenyl alcohols by fermentation

[0827] Analysis of off-gas from an *E. coli* BL21 (DE3) strain expressing a Kudzu isoprene synthase revealed the presence of both isoprene and 3-methyl-3-buten-1-ol (isoprenol). The levels of the two compounds in the fermentation off-gas over the fermentation are shown in Figure 89 as determined by headspace GC/MS. Levels of isoprenol (3-methyl-3-buten-1-ol, 3-MBA) attained was nearly 10 $\mu g/L_{offgas}$ in this experiment. Additional experiments produced levels of approximately 20 $\mu g/L_{offgas}$ in the fermentation off-gas.

Example 11: The de-coupling of growth and production of isoprene in *E. coli* expressing genes from the mevalonic acid pathway and fermented in a fed-batch culture

[0828] Example 11 illustrates the de-coupling of cell growth from mevalonic acid and isoprene production.

I. Fermentation Conditions

Medium Recipe (per liter fermentation medium):

[0829] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0830] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, CoCl2 * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0831] Fermentation was performed with *E. coli* cells containing the pTrcHis2AUpperPathway (also called pTrcUpperMVA, Figures 91 and 92A-92C; SEQ ID NO:23) (50 μg/ml carbenicillin) or the pCL PtrcUpperMVA (also called pCL PtrcUpperPathway (Figure 26)) (50 μg/ml spectinomycin) plasmids. For experiments in which isoprene was produced, the *E. coli* cells also contained the pTrc KKDyIkIS (50 μg/ml kanamycin) plasmid. These experiments were carried out to monitor mevalonic acid or isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of an *E. coli* strain taken from a frozen vial was streaked onto an LA broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to optical density 1.0 when measured at 550 nm, it was used to inoculate the bioreactor.

[0832] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. Induction was achieved by adding IPTG. The mevalonic acid concentration in fermentation broth was determined by

applying perchloric acid (Sigma-Aldrich # 244252) treated samples (0.3 M incubated at 4°C for 5 minutes) to an organic acids HPLC column (BioRad # 125-0140). The concentration was determined by comparing the broth mevalonic acid peak size to a calibration curve generated from mevalonolacetone (Sigma-Aldrich # M4667) treated with perchloric acid to form D,L-mevalonate. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer is defined as the amount of isoprene produced per liter of fermentation broth.

II. Mevalonic acid production from *E. coli* BL21 (DE3) cells expressing the pTrcUpperMVA plasmid at a 150-L scale

[0833] BL21 (DE3) cells that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 45 mL of tryptone-yeast extract medium and incubated at 30°C with shaking at 170 rpm for 5 hours. This solution was transferred to a 5-L bioreactor of tryptone-yeast extract medium, and the cells were grown at 30 °C and 27.5 rpm until the culture reached an OD_{550} of 1.0. The 5 L of inoculum was seeded into a 150-L bioreactor containing 45-kg of medium. The IPTG concentration was brought to 1.1 mM when the OD_{550} reached a value of 10. The OD_{550} profile within the bioreactor over time is shown in Figure 60A. The mevalonic acid titer increased over the course of the fermentation to a final value of 61.3 g/L (Figure 60B). The specific productivity profile throughout the fermentation is shown in Figure 60C and a comparison to Figure 60A illustrates the de-coupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 52.5 hour fermentation was 4.0 kg from 14.1 kg of utilized glucose. The molar yield of utilized carbon that went into producing mevalonic acid during fermentation was 34.2%.

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

[0834] BL21 (DE3) cells that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 1.0 mM when the OD_{550} reached a value of 10. The OD_{550} profile within the bioreactor over time is shown in Figure 61A. The mevalonic acid titer increased over the course of the fermentation to a final value of 53.9 g/L (Figure 61B). The specific productivity profile throughout the fermentation is shown in Figure 61C and a

comparison to Figure 61A illustrates the de-coupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 46.6 hour fermentation was 491 g from 2.1 kg of utilized glucose. The molar yield of utilized carbon that went into producing mevalonic acid during fermentation was 28.8%.

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

[0835] FM5 cells that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 1.0 mM when the OD_{550} reached a value of 30. The OD_{550} profile within the bioreactor over time is shown in Figure 62A. The mevalonic acid titer increased over the course of the fermentation to a final value of 23.7 g/L (Figure 62B). The specific productivity profile throughout the fermentation is shown in Figure 62C and a comparison to Figure 62A illustrates the de-coupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 51.2 hour fermentation was 140 g from 1.1 kg of utilized glucose. The molar yield of utilized carbon that went into producing mevalonic acid during fermentation was 15.2%.

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

[0836] BL21 (DE3) cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 25 μM when the OD_{550} reached a value of 10. The IPTG concentration was raised to 50 μM when OD_{550} reached 190. The IPTG concentration was raised to 100 μM at 38 hours of fermentation. The OD_{550} profile within the bioreactor over time is shown in Figure 63A. The isoprene titer increased over the course of the fermentation to a final value of 2.2 g/L broth (Figure 63B). The specific productivity profile throughout the fermentation is shown in Figure 63C and a comparison to Figure 63A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 54.4 hour

fermentation was 15.9 g from 2.3 kg of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 1.53%.

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

[0837] BL21 (DE3) tuner cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 26 μM when the OD_{550} reached a value of 10. The IPTG concentration was raised to 50 μM when OD_{550} reached 175. The OD_{550} profile within the bioreactor over time is shown in Figure 64A. The isoprene titer increased over the course of the fermentation to a final value of 1.3 g/L broth (Figure 64B). The specific productivity profile throughout the fermentation is shown in Figure 64C and a comparison to Figure 64A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 48.6 hour fermentation was 9.9 g from 1.6 kg of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 1.34%.

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

[0838] MG1655 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 24 μM when the OD_{550} reached a value of 45. The OD_{550} profile within the bioreactor over time is shown in Figure 65A. The isoprene titer increased over the course of the fermentation to a final value of 393 mg/L broth (Figure 65B). The specific productivity profile throughout the fermentation is shown in Figure 65C and a comparison to Figure 65A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 67.4 hour fermentation was 2.2 g from 520 g of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.92%.

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

[0839] MG1655ack-pta cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 30 μM when the OD_{550} reached a value of 10. The OD_{550} profile within the bioreactor over time is shown in Figure 66A. The isoprene titer increased over the course of the fermentation to a final value of 368 mg/L broth (Figure 66B). The specific productivity profile throughout the fermentation is shown in Figure 66C and a comparison to Figure 66A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 56.7 hour fermentation was 1.8 g from 531 g of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.73%.

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

[0840] FM5 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 27 μM when the OD_{550} reached a value of 15. The OD_{550} profile within the bioreactor over time is shown in Figure 67A. The isoprene titer increased over the course of the fermentation to a final value of 235 mg/L broth (Figure 67B). The specific productivity profile throughout the fermentation is shown in Figure 67C and a comparison to Figure 67A illustrates the de-coupling of growth and isoprene production. The total amount of isoprene produced during the 52.3 hour fermentation was 1.4 g from 948 g of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.32%.

Example 12: Production of isoprene during the exponential growth phase of *E. coli* expressing genes from the mevalonic acid pathway and fermented in a fed-batch culture

[0841] Example 12 illustrates the production of isoprene during the exponential growth phase of cells.

Medium Recipe (per liter fermentation medium):

[0842] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0843] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in Di H2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0844] Fermentation was performed in a 15-L bioreactor with ATCC11303 *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0845] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 50 hour fermentation was 2.0 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 μM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 μM when OD_{550} reached 190. The OD_{550} profile within the bioreactor over time is shown in Figure 99. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 1.4 g/L (Figure

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

Example 13: Flammability modeling and testing of isoprene

I. Summary of flammability modeling and testing of isoprene

[0846] Flammability modeling and experiments were performed for various hydrocarbon/oxygen/nitrogen/water/carbon dioxide mixtures. This modeling and experimental tested was aimed at defining isoprene and oxygen/nitrogen flammability curves under specified steam and carbon monoxide concentrations at a fixed pressure and temperature. A matrix of the model conditions is shown in Table 9, and a matrix of the experiments performed is shown in Table 5.

Table 9. Summary of Modeled Isoprene Flammability

Series	Temperature (°C)	Pressure (psig)	Steam Concentration (wt%)	Carbon Dioxide Concentration (wt. %)	Isoprene Concentration (vol. %)	Oxygen Concentration (vol. %)
A	40	0	0	0	Varying	Varying
B	40	0	4	0	Varying	Varying
C	40	0	0	5	Varying	Varying
D	40	0	0	10	Varying	Varying
E	40	0	0	15	Varying	Varying
F	40	0	0	20	Varying	Varying
G	40	0	0	30	Varying	Varying

Table 10. Summary of Isoprene Flammability Tests

Series Number	Temperature (°C)	Pressure (psig)	Steam Concentration (vol. %)	Isoprene Concentration (vol. %)	Oxygen Concentration (vol. %)
1	40	0	0	Varying	Varying
2	40	0	4	Varying	Varying

II. Description of calculated adiabatic flame temperature (CAFT) model

[0847] Calculated adiabatic flame temperatures (CAFT) along with a selected limit flame temperature for combustion propagation were used to determine the flammability envelope for isoprene. The computer program used in this study to calculate the flame temperatures is the NASA Glenn Research Center CEA (Chemical Equilibrium with Applications) software.

[0848] There are five steps involved in determining the flammability envelope using an adiabatic flame temperature model for a homogeneous combustion mechanism (where both the fuel and oxidant are in the gaseous state): selection of the desired reactants, selection of the test condition, selection of the limit flame temperature, modification of the reactants, and construction of a flammability envelope from calculations.

[0849] In this first step, selection of desired reactants, a decision must be made as to the reactant species that will be present in the system and the quantities of each. In many cases the computer programs used for the calculations have a list of reactant and product species. If any of the data for the species to be studied are not found in the program, they may be obtained from other sources such as the JANAF tables or from the internet. In this current model data for water, nitrogen, oxygen and carbon dioxide were present in the program database. The program database did not have isoprene as a species; therefore the thermodynamic properties were incorporated manually.

[0850] The next step is to decide whether the initial pressure and temperature conditions that the combustion process is taking place in. In this model the pressure was 1 atmosphere (absolute) and the temperature was 40°C, the boiling point of isoprene.

[0851] The limit flame temperature for combustion can be either selected based on theoretical principles or determined experimentally. Each method has its own limitations.

[0852] Based on prior studies, the limit flame temperatures of hydrocarbons fall in the range of 1000 K to 1500 K. For this model, the value of 1500 K was selected. This is the temperature at which the reaction of carbon monoxide to carbon dioxide (a highly exothermic reaction and constitutes a significant proportion of the flame energy) becomes self sustaining.

[0853] Once the limit flame temperature has been decided upon, model calculations are performed on the given reactant mixture (species concentrations) and the adiabatic flame temperature is determined. Flame propagation is considered to have occurred only if the temperature is greater than the limit flame temperature. The reactant mixture composition is then modified to create data sets for propagation and non-propagation mixtures.

[0854] This type of model shows good agreement with the experimentally determined flammability limits. Regions outside the derived envelope are nonflammable and regions within it are flammable. The shape of the envelope forms a nose. The nose of the envelope is related to the limiting oxygen concentration (LOC) for gaseous fuels.

III. Results from calculated adiabatic flame temperature (CAFT) model

[0855] Plotted in Figs. 68 through 74 are the CAFT model results for Series A to G, respectively. The figures plot the calculated adiabatic flame temperature (using the NASA CEA program) as a function of fuel concentration (by weight) for several oxygen/nitrogen ratios (by weight). The parts of the curve that are above 1500 K, the selected limit flame temperature, contain fuel levels sufficient for flame propagation. The results may be difficult to interpret in the form presented in Figs. 68 through 74. Additionally, the current form is not conducive to comparison with experimental data which is generally presented in terms of volume percent.

[0856] Using Series A as an example the data in Figure 68 can be plotted in the form of a traditional flammability envelope. Using Figure 68 and reading across the 1500 K temperature line on the ordinate one can determine the fuel concentration for this limit flame temperature by dropping a tangent to the abscissa for each curve (oxygen to nitrogen ratio) that it intersects. These values can then be tabulated as weight percent of fuel for a given weight percent of oxidizer (Figure 75A). Then knowing the composition of the fuel (100 wt.% isoprene) and the composition of the oxidizer (relative content of water, oxygen and nitrogen) molar quantities can be established.

[0857] From these molar quantities percentage volume concentrations can be calculated. The concentrations in terms of volume percent can then be plotted to generate a flammability envelope (Figure 75B). The area bounded by the envelope is the explosible range and the area excluded is the non-explosible range. The "nose" of the envelope is the limiting oxygen concentration. Figs. 76A and 76B contain the calculated volume concentrations for the flammability envelope for Series B generated from data presented in Figure 69. A similar approach can be used on data presented in Figs. 70-74.

IV. Flammability testing experimental equipment and procedure

[0858] Flammability testing was conducted in a 4 liter high pressure vessel. The vessel was cylindrical in shape with an inner diameter of 6" and an internal height of 8.625". The temperature of the vessel (and the gases inside) was maintained using external heaters that were

controlled by a PID controller. To prevent heat losses, ceramic wool and reflective insulation were wrapped around the pressure vessel. Type K thermocouples were used the measure the temperature of the gas space as well as the temperature of the vessel itself. Figure 77 illustrates the test vessel.

[0859] Before a test was ran, the vessel was evacuated and purged with nitrogen to ensure that any gases from previous tests were removed. A vacuum was then pulled on the vessel. The pressure after this had been done was typically around 0.06 bar(a). Due to the nitrogen purging, the gas responsible for this initial pressure was assumed to be nitrogen. Using partial pressures, water, isoprene, nitrogen, and oxygen were then added in the appropriate amounts to achieve the test conditions in question. A magnetically driven mixing fan within the vessel ensured mixing of the gaseous contents. The gases were allowed to mix for about 2 minutes with the fan being turned off approximately 1 minute prior to ignition.

[0860] The igniter was comprised of a 1.5 ohm nicrome coil and an AC voltage source on a timer circuit. Using an oscilloscope, it was determined that 34.4 VAC were delivered to the igniter for 3.2 seconds. A maximum current of 3.8 amps occurred approximately halfway into the ignition cycle. Thus, the maximum power was 131 W and the total energy provided over the ignition cycle was approximately 210 J.

[0861] Deflagration data was acquired using a variable reluctance Validyne DP215 pressure transducer connected to a data acquisition system. A gas mixture was considered to have deflagrated if the pressure rise was greater than or equal to 5%.

V. Results of flammability testing

[0862] The first experimental series (Series 1) was run at 40°C and 0 psig with no steam. Running tests at varying concentrations of isoprene and oxygen produced the flammability curve shown in Figure 78A. The data points shown in this curve are only those that border the curve. A detailed list of all the data points taken for this series is shown in Figs. 80A and 80B.

[0863] Figure 78B summarizes the explosibility data points shown in Figure 78A. Figure 78C is a comparison of the experimental data with the CAFT model predicted flammability envelope. The model agrees very well with the experimental data. Discrepancies may be due to the non-adiabatic nature of the test chamber and limitations of the model. The model looks at an infinite time horizon for the oxidation reaction and does not take into consideration any reaction kinetic limitation.

[0864] Additionally, the model is limited by the number of equilibrium chemical species that are in its database and thus may not properly predict pyrolytic species. Also, the flammability envelope developed by the model uses one value for a limit flame temperature (1500K). The limit flame temperature can be a range of values from 1,000K to 1,500K depending on the reacting chemical species. The complex nature of pyrolytic chemical species formed at fuel concentrations above the stoichiometric fuel/oxidizer level is one reason why the model may not accurately predict the upper flammable limit for this system.

[0865] The second experimental series (Series 2) was run at 40°C and 0 psig with a fixed steam concentration of 4%. Running tests at varying concentrations of isoprene and oxygen produced the flammability curve shown in Figure 79A. The data points shown in this curve are only those that border the curve. A detailed list of all the data points taken for this series is shown in Figure 81. Due to the similarity between the data in Series 1 only the key points of lower flammable limit, limiting oxygen concentration, and upper flammable limits were tested. The addition of 4% steam to the test mixture did not significantly change the key limits of the flammability envelope. It should be noted that higher concentrations of steam/water and or other inertants may influence the flammability envelope.

[0866] Figure 79B summarizes the explosibility data points shown in Figure 79A. Figure 79C is a comparison of the experimental data with the CAFT model predicted flammability envelope. The model agrees very well with the experimental data. Discrepancies may be due to the same factors described in Series 1

VI. Calculation of Flammability Limits of Isoprene in Air at 3 Atmospheres of Pressure

[0867] The methods described in Example 13, parts I to IV were also used to calculate the flammability limits of isoprene at an absolute system pressure of 3 atmospheres and 40°C. These results were compared to those of Example 13, parts I to IV at an absolute system pressure of 1 atmosphere and 40°C. This higher pressure was tested because the flammability envelope expands or grows larger as the initial system pressure is increased. The upper flammability limit is affected the most, followed by the limiting oxygen composition. The lower flammability limit is the least affected (*see*, for example, "Bulletin 627 – Flammability Characteristics of Combustible Gases and Vapors" written by Michael G. Zabetakis and published by the former US Bureau of Mines (1965), which is hereby incorporated by reference in its entirety, particular with respect to the calculation of flammability limits).

[0868] In Figure 82, the calculated adiabatic flame temperature is plotted as a function of isoprene (fuel) concentration, expressed in weight percent of the total fuel/nitrogen/oxygen, where the system pressure was initially 3 atmospheres. The calculated flame temperatures are very similar to those determined initially in the 1 atmosphere system (Figure 83). As a result, when flammability envelopes are generated using the calculated adiabatic flammability data, the curves are very similar (see Figs. 84 and 85). Therefore, based on these theoretical calculations, a system pressure increase from 1 atmosphere to 3 atmosphere does not result in a significant increase/broadening of the flammability envelope. If desired, these model results may be validated using experimental testing (such as the experimental testing described herein at a pressure of 1 atmosphere).

VII. Summary of flammability studies

[0869] A calculated adiabatic temperature model was developed for the flammability envelope of the isoprene/oxygen/nitrogen/water/ carbon dioxide system at 40°C and 0 psig. The CAFT model that was developed agreed well with the experimental data generated by the tests conducted in this work. The experimental results from Series 1 and 2 validated the model results from Series A and B.

Example 14: Expression Constructs and Strains

I. Construction of plasmids encoding mevalonate kinase.

[0870] A construct encoding the *Methanosarcina mazei* lower MVA pathway (Accession numbers NC_003901.1, NC_003901.1, NC_003901.1, and NC_003901.1, which are each hereby incorporated by reference in their entireties) was synthesized with codon optimization for expression in *E. coli*. This construct is named *M. mazei* archeal Lower Pathway operon (Figures 112A-112C; SEQ ID NO:27) and encodes *M. mazei* MVK, a putative decarboxylase, IPK, and IDI enzymes. The gene encoding MVK (Accession number NC_003901.1) was PCR amplified using primers MCM165 and MCM177 (Table 11) using the Strategene Herculase II Fusion kit according to the manufacturer's protocol using 30 cycles with an annealing temperature of 55 °C and extension time of 60 seconds. This amplicon was purified using a Qiagen PCR column and then digested at 37 °C in a 10 μL reaction with PmeI (in the presence of NEB buffer 4 and BSA). After one hour, NsiI and Roche buffer H were added for an additional hour at 37 °C. The digested DNA was purified over a Qiagen PCR column and ligated to a similarly digested and

purified plasmid MCM29 (MCM29 is *E. coli* TOP10 (Invitrogen) transformed with pTrcKudzu encoding Kudzu isoprene synthase) in an 11 μL reaction 5μL Roche Quick Ligase buffer 1, 1 μL buffer 2, 1 μL plasmid, 3 μL amplicon, and 1 μL ligase (1 hour at room temperature). MCM 29 is pTrcKudzuKan. The ligation reaction was introduced into Invitrogen TOP10 cells and transformants selected on LA/kan50 plates incubated at 37 °C overnight. The MVK insert in the resulting plasmid MCM382 was sequenced (Figures 113A-113C; SEQ ID NO: 28).

Table 11. Oligonucleotides.

MCM161	*M.mazei* MVK for	CACCATGGTATCCTGTTCTGCG (SEQ ID NO:120)
MCM162	*M.mazei* MVK rev	TTAATCTACTTTCAGACCTTGC (SEQ ID NO:121)
MCM165	*M.mazei* MVK for w/ RBS	gcgaacgATGCATaaaggaggtaaaaaaacATGGTATCCTGTTCTG CGCCGGGTAAGATTTACCTG (SEQ ID NO:122)
MCM177	*M.mazei* MVK rev Pst	gggcccgtttaaactttaactagactTTAATCTACTTTCAGACCTTGC (SEQ ID NO:123)

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

[0871] Plasmid MCM382 was transformed into MCM331 cells (which contains chromosomal construct gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase) that had been grown to midlog in LB medium and washed three times in iced, sterile water. One μL of DNA was added to 50 μL of cell suspension, and this mixture was electroporated in a 2 mm cuvette at 2.5 volts, 25 uFd followed immediately by recovery in 500 μL LB medium for one hour at 37 °C. Transformant was selected on LA/kan50 and named MCM391. Plasmid MCM82 was introduced into this strain by the same electroporation protocol followed by selection on LA/kan50/spec50. The resulting strain MCM401 contains a cmp-marked chromosomal construct gi1.2KKDyI, kan-marked plasmid MCM382, and spec-marked plasmid MCM82 (which is pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS). *See* Table 12.

Table 12. Strains overexpressing mevalonate kinase and isoprene synthase.

MCM382	E. coli BL21 (lambdaDE3) pTrcKudzuMVK(M. mazei)GI1.2KKDyI
MCM391	MCM331 pTrcKudzuMVK(M. mazei)
MCM401	MCM331pTrcKudzuMVK(M.mazei)pCLPtrcUpperpathway
MCM396	MCM333pTrcKudzuMVK(M. mazei)
MCM406	MCM333pTrcKudzuMVK(M.mazei)pCLPtrcUpperpathway

III. Construction of plasmid MCM376 - MVK from *M. mazei* archeal Lower in pET200D.

[0872] The MVK ORF from the *M. mazei* archeal Lower Pathway operon (Figures 112A-112C; SEQ ID NO:27) was PCR amplified using primers MCM161 and MCM162 (Table 11) using the Invitrogen Platinum HiFi PCR mix. 45 μL of PCR mix was combined with 1 μL template, 1 μL of each primer at 10 μM, and 2 μL water. The reaction was cycled as follows: 94 °C for 2:00; 30 cycles of 94 °C for 0:30, 55 °C for 0:30, and 68 °C for 1:15; and then 72 °C for 7:00, and 4 °C until cool. 3 μL of this PCR reaction was ligated to Invitrogen pET200D plasmid according to the manufacturer's protocol. 3 μL of this ligation was introduced into Invitrogen TOP10 cells, and transformants were selected on LA/kan50. A plasmid from a transformant was isolated and the insert sequenced, resulting in MCM376 (Figures 114A-114C; SEQ ID NO:29).

IV. Creation of expression strain MCM378.

[0873] Plasmid MCM376 was transformed into Invitrogen BL21(DE3) pLysS cells according to the manufacturer's protocol. Transformant MCM378 was selected on LA/kan50.

Example 15: Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from *M. mazei*, and isoprene synthase from Kudzu and grown in fed-batch culture at the 20 mL batch scale

Medium Recipe (per liter fermentation medium):

[0874] Each liter of fermentation medium contained K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 1 g, and 1000X Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media was filter sterilized with a 0.22 micron filter. Glucose (2.5 g) and antibiotics were added after sterilization and pH adjustment.

1000X Trace Metal Solution:

[0875] 1000X Trace Metal Solution contained citric Acids $* H_2O$ 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in di

H_2O, pH to 3.0 with HCl/NaOH, then brought to volume and filter sterilized with a 0.22 micron filter.

Strains:

[0876] MCM343 cells are BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL Upper), the integrated lower MVA pathway (gi1.2KKDyI), and isoprene synthase from Kudzu (pTrcKudzu).

[0877] MCM401 cells are BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway), the integrated lower MVA pathway (gi1.2KKDyI), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(M.mazei)).

[0878] Isoprene production was analyzed by growing the strains in 100 mL bioreactors with a 20 mL working volume at a temperature of 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 30°C. A single colony was inoculated into media and grown overnight. The bacteria were diluted into 20 mL of media to reach an optical density of 0.05 measured at 550 nm. The 100 mL bioreactors were sealed, and air was pumped through at a rate of 8 mL/min. Adequate agitation of the media was obtained by stirring at 600 rpm using magnetic stir bars. The off-gas from the bioreactors was analyzed using an on-line Hiden HPR-20 mass spectrometer. Masses corresponding to isoprene, CO_2, and other gasses naturally occurring in air were monitored. Accumulated isoprene and CO_2 production were calculated by summing the concentration (in percent) of the respective gasses over time. Atmospheric CO_2 was subtracted from the total in order to estimate the CO_2 released due to metabolic activity.

[0879] Isoprene production from a strain expressing the full mevalonic acid pathway and Kudzu isoprene synthase (MCM343) was compared to a strain that in addition over-expressed MVK from *M. mazei* and Kudzu isoprene synthase (MCM401) in 100 mL bioreactors. The bacteria were grown under identical conditions in defined media with glucose as carbon source. Induction of isoprene production was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG) to a final concentration of either 100 μM or 200 μM. Off-gas measurements revealed that the strain over-expressing both MVK and isoprene synthase (MCM401) produced significantly more isoprene compared to the strain expressing only the mevalonic acid pathway and Kudzu isoprene synthase (MCM343) as shown in Figures 115A-115D. At 100 μM induction, the MCM401 strain produced 2-fold more isoprene compared to

the MCM343 strain. At 200 μM IPTG induction, the MCM401 strain produced 3.4-fold more isoprene when compared to the MCM343 strain. Analysis of CO_2 in the off-gas from the bioreactors, which is a measure of metabolic activity, indicates that metabolic activity was independent from IPTG induction and isoprene production.

Example 16: Production of isoprene by E. coli expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from M. mazei, and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium):

[0880] Each liter of fermentation medium contained K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in DI H_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0881] 1000X Modified Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0882] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS), the integrated lower MVA pathway (gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(M.mazei)). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated

into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate 5-L of medium in a 15-L bioreactor.

[0883] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 68 hour fermentation was 3.8 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 51 μM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 88 μM when OD_{550} reached 149. Additional IPTG additions raised the concentration to 119 μM at OD_{550} = 195 and 152 μM at OD_{550} = 210. The OD_{550} profile within the bioreactor over time is shown in Figure 116. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a final value of 23.8 g/L (Figure 117). The total amount of isoprene produced during the 68 hour fermentation was 227.2 g and the time course of production is shown in Figure 118. The metabolic activity profile, as measured by TCER, is shown in Figure 119. The total viable count (total colony forming units) decreased by two orders of magnitude between 10 and 39 hours of fermentation (Figure 120). The molar yield of utilized carbon that went into producing isoprene during fermentation was 13.0%. The weight percent yield of isoprene from glucose was 6.3%.

Example 17: Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from *M. mazei*, and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale (2x 100μM IPTG induction)

Medium Recipe (per liter fermentation medium):

[0884] Each liter of fermentation medium contained K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in DI H_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0885] 1000X Modified Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, $ZnSO_4$ * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0886] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS), the integrated lower MVA pathway (gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(*M.mazei*)). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate 5-L medium in a 15-L bioreactor.

[0887] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 55 hour fermentation was 1.9 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 111 μM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 193 μM when OD_{550} reached 155. The OD_{550} profile within the bioreactor over time is shown in Figure 121. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a final value of 19.5 g/L (Figure 122). The total amount of isoprene produced during the 55 hour fermentation was 133.8 g and the time course of production is shown in Figure 123. Instantaneous volumetric productivity levels reached values as high as 1.5 g isoprene/L broth/hr (Figure 124). Instantaneous yield levels reached as high as 17.7% w/w (Figure 125). The metabolic activity profile, as measured by TCER, is shown in Figure 126. The total viable count (total colony forming units) decreased by two orders of magnitude between 8 and 36 hours of fermentation (Figure 127). The molar yield of utilized carbon that went into producing isoprene during fermentation was 15.8%. The weight percent yield of isoprene from glucose over the entire fermentation was 7.4%.

[0888] In addition, as a control, fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS), the integrated lower MVA pathway (gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(*M.mazei*)). This experiment was carried out to monitor uninduced cell metabolic activity as measured by CER from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain (MCM401 described above) taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate 5-L medium in a 15-L bioreactor. Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands.

[0889] Figure 148 compares the CER profiles for the uninduced cells described above and the cells induced by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG) in Examples 16 and 17.

Example 18: Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from *M. mazei,* and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale (1x 50μM IPTG + 150μM IPTG fed induction)

Medium Recipe (per liter fermentation medium):

[0890] Each liter of fermentation medium contained K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0891] 1000X Modified Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, $ZnSO_4$ * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0892] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS), the integrated lower MVA pathway (gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(M.mazei)). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate 5-L medium in a 15-L bioreactor.

[0893] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 55 hour fermentation was 2.2 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 51 μM when the optical density at 550 nm (OD_{550}) reached a value of 10. In addition to the IPTG spike, at OD_{550} = 10 a constant feed began and delivered 164 mg of IPTG over 18 hours. The OD_{550} profile within the bioreactor over time is shown in Figure 128. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a final value of 22.0 g/L (Figure 129). The total amount of isoprene produced during the 55 hour fermentation was 170.5 g and the time course of production is shown in Figure 130. The metabolic activity profile, as measured by TCER, is shown in Figure 131. When the airflow to the bioreactor was decreased from 8 slpm to 4 slpm for a period of about 1.7 hours, the concentration of isoprene in the offgas increased from 0.51 to 0.92 w/w % (Figure 132). These elevated levels of isoprene did not appear to have any negative impact on cell metabolic activity as measured by the total carbon dioxide evolution rate (TCER), since TCER declined only 7% between 37.2 and 39.3 hours (Figure 132). The total viable count (total colony forming units) decreased by two orders

of magnitude between 7 and 36 hours of fermentation (Figure 133). The molar yield of utilized carbon that went into producing isoprene during fermentation was 16.6%. The weight percent yield of isoprene from glucose over the entire fermentation was 7.7%.

Example 19: The effect of externally applied isoprene on a wild-type *E. coli* grown in fed-batch culture at the 1-L scale

Medium Recipe (per liter fermentation medium):

[0894] Each liter of fermentation medium contained K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0895] 1000X Modified Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0896] Fermentation was performed in a 1-L bioreactor with BL21 (DE3) *E. coli* cells. This experiment was carried out to monitor the effects of isoprene on cell viability and metabolic activity in a glucose fed-batch bioreactor at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain from a frozen vial was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 50 mL was used to inoculate 0.5-L medium in a 1-L bioreactor.

[0897] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was fed to meet metabolic demands. Isoprene was fed into the bioreactor using nitrogen gas as a carrier. The rate of isoprene feeding was 1 g/L/hr during mid-growth phase (OD_{550} = 31-44) and lasted for a total of 75 minutes (13.2 to 14.4 hours). The OD_{550} profile within the bioreactor over time is shown in Figure 134. The metabolic activity profile, as measured by TCER, is shown in Figure 135. The total viable count (total colony

forming units) increased by 14-fold during the period when isoprene was introduced into the bioreactor (Figure 136).

Example 20: Production of isoprene and expression of isoprene synthase by *Saccharomyces cerevisiae*

[0898] The Kudzu isoprene synthase enzyme was optimized for expression according to a hybrid *Saccharomyces cerevisiae/Pichia pastoris* codon usage table, synthesized, and cloned into pDONR221:19430 (by DNA 2.0, Figure 140 for map and Figure 141 for sequence (SEQ ID NO:38)). A Gateway® Cloning (Invitrogen) reaction was performed according to the manufacturer's protocol: Since pDONR221:19430 was an "entry" vector, the LR Clonase II enzyme (the LR Reaction) was used to introduce the codon-optimized isoprene synthase into the "destination" vector pYES-DEST52 (Invitrogen).

[0899] The LR Reaction was then transformed into Top10 chemically competent cells (Invitrogen) according to the manufacturer's protocol, and bacteria harboring pYES-DEST52 plasmids with the isoprene synthase ORF were selected for on LA plates containing 50 μg/ml carbenicillin. Individual positive transformants were tested by colony PCR (see below for primer concentrations and thermocycling parameters) using illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare) with the T7 forward primer and the Yeast isoprene synthase -Rev2 primer (*See* Table 13).

Table 13. Primer sequences for amplifying isoprene synthase.

Primer Name	Sequence (5′ to 3′)	Purpose
Yeast HGS - For2	CACCAAAGACTTCATAGACT (SEQ ID NO:124)	Forward primer for yeast optimized isoprene synthase
Yeast HGS - Rev2	AGAGATATCTTCCTGCTGCT (SEQ ID NO:125)	Reverse primer for yeast optimized isoprene synthase
T7 Forward	TAATACGACTCACTATAGGG (SEQ ID NO:126)	PCR and sequencing primer

[0900] Plasmids that yielded a PCR fragment of the correct size (1354 bp) were purified by miniprep (Qiagen) and sent for sequencing (Quintara Biosciences, Berkeley, CA) with the T7 Forward and Yeast isoprene synthase-For2 primers (*See* Table 13). Results from sequencing runs were compared to the known sequence of pDONR221:19430 (using Vector NTI software, Invitrogen), and a single plasmid, pDW14, was selected for further study (Figure 142A for map and Figures 142B and C for the complete sequence (SEQ ID NO:39)). The sequence of pDW14

diverged from that of pDONR221:19430 by a single nucleotide (marked in bold in Figure 142B). The single nucleotide change (G to A) did not result in a change in the ORF, since it was in the third position of a lysine-encoding codon.

[0901] Purified pDW14 was transformed into *Saccharomyces cerevisiae* strain INVSc-1 using the protocol described in the S. c. EasyComp Transformation kit (Invitrogen). INVSc-1 strains harboring pDW14 or pYES-DEST52 (which contains an intact URA3 gene) were selected for and maintained on SC Minimal Medium with 2% glucose without uracil, as described in the pYES-DEST52 Gateway Vector manual (Invitrogen). Two independent isolates of INVSc-1 containing pDW14 and a single control strain with pYES-DEST52 were chosen for further analysis.

[0902] To induce isoprene synthase expression, cultures were grown overnight in liquid SC Minimal Medium. The cultures were then diluted to an OD_{600} of approximately 0.2 and grown for 2-3 hours. Cultures were spun by centrifugation, washed once, resuspended in an equal volume (10 ml) of SC minimal medium with 1% raffinose, 2% galactose without uracil, and grown overnight to induce the expression of isoprene synthase. The OD_{600} of the strains was determined (Figure 144A), and strains were harvested by centrifugation and resuspended in 2 ml of lysis buffer (a 1:1 mix of 50% glycerol and PEB pH 7.4: Tris Base 2.423 g/L, $MgCl_2$ (Anhydrous) 1.904 g/L, KCl 14.910 g/L, DTT 0.154 g/L,Glycerol 50 mL/L).

[0903] The lysis mixtures were passed through a french press three times, and lysates were analyzed by SDS-PAGE. For Coomassie gel analysis (Figure 143A), samples were diluted 1:1 with 2X SDS loading buffer with reducing agent, loaded (20 µl total volume) onto a 4-12% bis-tris gel , run in MES buffer, and stained using SimplyBlue SafeStain according to the manufacturer's protocol (the Invitrogen Novex system).

[0904] The WesternBreeze kit (Invitrogen) was used for transfer and chromogenic detection of isoprene synthase on a nitrocellulose membrane. The primary antibody was 1799A 10 week diluted 1:1000 in Invitrogen antibody diluent. Primary antibody binding was followed by development with a secondary antibody labeled with Alexa Fluor 488 (Invitrogen Catalog No. A-11008) to permit quantitative signal determination. The western blot procedure was carried out as described by Invitrogen. The fluorescence signal was recorded with a Molecular Dynamics Storm instrument using the blue filter setting and quantitatively analyzed with the Molecular Dynamics ImageQuant image analysis software package. Specific activity of the library members was calculated from the ratio of the amount of isoprene produced divided by either the A600 of the induction cultures or the isoprene synthase protein concentration

determined by western blot. Figure 143B shows that isoprene synthase was present in the induced INVSc-1 strains harboring pDW14 (lanes 2 and 3) in comparison to the control harboring pYES-DEST52 (lane 1).

[0905] The DMAPP assay for isoprene synthase headspace was performed on 25 μL of the lysate from each strain for which 5 μL 1 M MgCl2, 5 μL 100 mM DMAPP, and 65 μL 50 mM Tris pH 8 were added. The reaction was performed at 30°C for 15 minutes in a gas tight 1.8 mL GC tube. Reactions were terminated by addition of 100 μL 250 mM EDTA pH 8. Figure 144B showed the specific activity values (in μg HG/L/OD) of the induced strains harboring pDW14 in comparison to the control. Induced strains harboring pDW14 displayed approximately 20X higher activity than the control lacking isoprene synthase.

PCR Cycling Parameters

[0906] Illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare) were used with oligonucleotide primer pairs at a concentration of 0.4 μM each in 25 μl total volume/reaction. For analysis of plasmids resulting from the LR Clonase reaction (Invitrogen), a small amount of bacteria from individual colonies on a selective plate was added to each tube containing the PCR mix described above. The reaction cycle was as follows: 1) 95°C for 4 minutes; 2) 95°C for 20 seconds; 3) 52°C for 20 seconds; 4) 72°C for 30 seconds; 5 cycles of steps 2 through 4; 5) 95°C for 20 seconds; 6) 55°C for 20 seconds; 7) 72°C for 30 seconds; 25 cycles of steps 5 through 7, 72°C for 10 minutes, and 4°C until cool.

Example 21: Production of isoprene in *Pseudomonas* and other Gram negative bacteria

Construction of pBBR5HGSOpt2_2, conjugation in *Pseudomonas* and measurement of isoprene synthase activity

[0907] A gene encoding isoprene synthase from *Pueraria lobata* (Kudzu plant) was codon-optimized for different microbial species of interest (Table 14; fluo-opt2v2 was the sequence chosen) and was synthesized by DNA2.0, Menlo Park, CA. The map and sequence of fluo-opt2v2 can be found in Figures 145A and 145B (SEQ ID NO:40). HindIII and BamHI restriction sites were added to the synthesized sequence for easier cloning, and a RBS was added in front of the ATG to enhance transcription.

[0908] Number of rare codons, as a function of the microbial species, in different versions of codon-optimized isoprene synthase from *Pueraria lobata*. Several rounds of optimization led to a gene with no rare codons in the all the species of interest.

Table 14. Number of rare codons.

Organism	fluo-opt1 (quote)	fluo-opt2	fluo-opt3	E. coli opt	fluo-opt2v2
Pseudomonas fluorescens Pf-5	19	X	X	57	0
Phodopseudomonas palustris CGA009	37	13	3	74	0
Pseudomonas putida F1	0	0	0	29	0
Corynebacterium glutamicum (ATCC)	4 (Ser)	0	0	0	0
Pseudomonas fluorescens PfO-1	1 (Val)	0	0	57	0

[0909] The gene was provided by DNA2.0 in a cloning vector. The vector was digested with HindIII/BamHI, the band corresponding to the insert of interest was gel-purified, and relegated with HindIII/BamHI-digested pBBR1MCS5 (Kovach et al, *Gene* 166:175-176, 1995, which is incorporated by reference in its entirety, particularly with respect to pBBR1MCS5), Figure 146A for map and Figures 146B and C for sequence (SEQ ID NO:41). This resulted in plasmid pBBR5HGSOpt2_2 (Figure 147A for map and Figures 147B and C for sequence (SEQ ID NO:42)) in which isoprene synthase was expressed from the lac promoter presented in pBBR1MCS5.

[0910] The vector was transformed in *E. coli* S17-1 and mated with *Pseudomonas putida* F1 ATCC700007 and *Pseudomonas fluorescens* ATCC 13525. After conjugation on LB, selection for plasmid-harboring Pseudomonas strains was on M9 + 16 mM sodium citrate +Gentamicin 50 μg/ml. Presence of the plasmid in the strains thus generated was checked by plasmid preparation using the Qiagen kit (Valencia, CA).

[0911] Isoprene synthase activities of the recombinant strains *P. putida*, pBBR5HGSOpt2_2 and *P. fluorescens*, pBBR5HGSOpt2_2 were assayed by growing the strains in TM3 medium (as described in Example 1 Part II) + 10 g/L glucose, harvesting the biomass in mid-log phase, breaking the cells by French Press and proceeding with the DMAPP assay. Results of the assay were presented in Table 15. The presence of activity measured by the DMAPP assay confirmed that isoprene synthase was expressed in *Pseudomonas*.

[0912] Isoprene synthase activity was examined in *Pseudomonas putida* and *Pseudomonas fluorescens* expressing isoprene synthase from the lac promoter, using plasmid pBBR5HGSOpt2_2

Table 15. Isoprene synthase activity in *Pseudomonas putida* and *Pseudomonas fluorescens.*

Strain	OD	Isoprene synthase activity mg isoprene/(L.h.OD)
P. fluorescens, pBBR5HGSOpt2_2	1.46	0.96
P. putida, pBBR5HGSOpt2_2	3.44	0.65
Control (*P. putida* w/o plasmid)	8.32	To be determined

Example 22: Growth of *E. coli* and *Pseudomonas* strains on sugar cane compared to glucose, and expression of isoprene synthase using both substrates

I. Preparation of liquid sugar cane

[0913] Crystallized raw cane sugar was dissolved in water in the following way: 750 g H_2O was added to 250 g sugar. The solution was stirred and gently heated until dissolution. Some material was not soluble. The weight of the solution was adjusted to 1 kg after dissolution to replenish the evaporated water. The volume of the solution was measured to be 940 mL. Hence the concentration of the solution was 265 g/L. The product label claimed 14 g of carbohydrate for 15 g of raw sugar cane. Hence the carbohydrate concentration of the solution was 248 g/L. Dry solids were measured to be 24.03 %, close enough of the expected 250 g/kg. pH of the solution was 5.49. Glucose concentration was measured using an enzymatic/spectrophotometric assay, with glucose oxidase. The glucose concentration was 17.4 g/L.

[0914] As a majority of microorganisms do not use sucrose, but can use glucose and fructose, the solution was split in two. One half was autoclaved once for 30 minutes (sugar cane as is). Some inversion resulted, as the glucose content increased to 29.75 g/L (*See* Figure 149). The other half of the solution was adjusted to pH 4.0 using phosphoric acid, then the solution was inverted by autoclaving (inverted sugar cane). Three cycles of 30 min were sufficient to obtain complete inversion, as shown on Figure 149. Both solutions were used for the growth curves described below.

II. Growth curves of different strains of *E. coli* and *Pseudomonas* on sugar cane compared to glucose

[0915] One colony of each of the strains presented in Table 16 was inoculated in 25 ml TM3 + 10 g/L glucose, and was grown overnight at 30°C and 200 rpm. TM3 is described in Example 7, Section II. The morning after, 1 ml of each culture was used to inoculate flasks containing 25

mL TM3 and 10 g/L glucose, 10 g/L sugar cane as is, or 10 g/L inverted sugar cane (sugar cane solutions described above). The flasks were incubated at 30°C and 200 rpm and samples were taken regularly to measure OD600. Figures 150 and 151 show that growth rate and biomass yield were comparable for glucose and inverted sugar cane, both for *Pseudomonas* and *E. coli* strains. *P. fluorescens* showed some signs of being able to use sugar cane which has not been inverted too.

Table 16. Strains used in this study.

	Strain
Escherichia coli	BL21
	MG1655
	ATCC11303
	B REL 606
Pseudomonas	*putida* F1 (ATCC700007)
	Fluorescens (ATCC13525)

III. Comparison of isoprene production from *E. coli* expressing isoprene synthase when grown on glucose or sugar cane

[0916] *E. coli* MCM401 (BL21(DE3)) containing the full MVA pathway, mevalonate kinase from *M. mazei* and isoprene synthase from *Pueraria lobata*, as described in Example 14, Section II was grown in TM3 + either 10 g/L glucose or 10 g/L inverted sugar cane (based on carbohydrate concentration of the syrup). Flasks were inoculated from an overnight culture on TM3 + 10 g/L glucose at an OD_{600} = 0.2. Antibiotics were added where needed. After two hours, the *E. coli* cultures were induced with 400 μM IPTG. After 6 hours of growth, isoprene production and isoprene synthase activities, using the DMAPP assay as described in Example 2B, were measured. Results are presented in Table 17 and illustrate clearly that inverted sugar cane is equivalent to glucose in terms of isoprene and isoprene synthase production on a per cell basis.

Table 17.

Strain	Carbon Source	OD	Isoprene synthase activity mg isoprene/(L.h.OD)	Isoprene production mg isoprene/(L.h.OD)
MCM401	Glucose	2.20	21.06	8.98
MCM401	Sugar cane inverted	2.32	20.20	9.23

Example 23: Construction of *E. coli* strains expressing the *S. cerevisiae* gi1.2KKDyI operon, *P. alba* isoprene synthase, *M. mazei* mevalonate kinase, pCL Upper MVA (*E. faecalis* mvaE and mvaS) and ybhE (pgl)

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

[0917] *E. coli* BL21 (Novagen brand, EMD Biosciences, Inc.) was a recipient strain, transduced with MCM331 P1 lysate (lysate prepared according to the method described in Ausubel, *et al., Current Protocols in Molecular Biology*. John Wiley and Sons, Inc.). MCM331 cells contain chromosomal construct gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase (*i.e.*, the gi1.2-KKDyI operon from *S. cerevisiae*). Transductants were selected for by spreading cells onto L Agar and 20 μg/μl chloramphenicol. The plates were incubated overnight at 30°C. Analysis of transductants showed no colonies on control plates (water + cells control plate for reversion and water and P1 lysate control plate for lysate contamination.

[0918] Four transductants were picked and used to inoculate 5 mL L Broth and 20 μg/μl chloramphenicol. The cultures were grown overnight at 30°C with shaking at 200 rpm. To make genomic DNA preps of each transductant for PCR analysis, 1.5mL of overnight cell culture were centrifuged. The cell pellet was resuspended with 400μl Resuspension Buffer (20mM Tris, 1mM EDTA, 50mM NaCl, pH 7.5) and 4μl RNase, DNase-free (Roche) was added. The tubes were incubated at 37°C for 30 minutes followed by the addition of 4μl 10% SDS and 4μl of 10mg/ml Proteinase K stock solution (Sigma-Aldrich). The tubes were incubated at 37°C for 1 hour. The cell lysate was transferred into 2 ml Phase Lock Light Gel tubes (Eppendorf) and 200μl each of saturated phenol pH7.9 (Ambion Inc.) and chloroform were added. The tubes were mixed well and microcentrifuged for 5 minutes. A second extraction was done with 400μl chloroform and the aqueous layer was transferred to a new eppendorf tube. The genomic DNA was precipitated by the addition of 1ml of 100% ethanol and centrifugation for 5 minutes. The genomic DNA pellet was washed with 1ml 70% ethanol. The ethanol was removed and the genomic DNA pellet was allowed to air dry briefly. The genomic DNA pellet was resuspended with 200μl TE.

[0919] Using Pfu Ultra II DNA polymerase (Stratagene) and 200ng/μl of genomic DNA as template, 2 different sets of PCR reaction tubes were prepared according to manufacturer's protocol. For set 1, primers MCM130 and GB Cm-Rev (Table 18) were used to ensure

transductants were successfully integrated into the attTn7 locus. PCR parameters for set 1 were 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 25 seconds, 72°C for 25 seconds (repeat steps 2-4 for 28 cycles), 72°C for 1 minute. For set 2, primers MVD For and MVD Rev (Table 18) were used to ensure that the gi1.2-KKDyI operon integrated properly. PCR parameters for set 2 were 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 25 seconds, 72°C for 10 seconds (repeat steps 2-4 for 28 cycles), 72°C for 1 minute. Analysis of PCR amplicons on a 1.2% E-gel (Invitrogen Corp.) showed that all 4 transductant clones were correct. One was picked and designated as strain EWL201.

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

[0920] The chloramphenicol marker was looped out of strain EWL201 using plasmid pCP20 as described by Datsenko and Wanner (2000) (Datsenko *et al.*, *Proc Natl. Acad. Sci* USA 97:6640-6645, 2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. (Datsenko *et al.*, *PNAS,* 97: 6640-6645, 2000). EWL201 cells were grown in L Broth to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50μl of cell suspension was mixed with 1μl of pCP20 and the cell suspension mixture was electroporated in a 2mm cuvette (Invitrogen Corp.) at 2.5 Volts and 25uFd using a Gene Pulser Electroporator (Bio-Rad Inc.). 1ml of LB was immediately added to the cells, then transferred to a 14ml polypropylene tube (Sarstedt) with a metal cap. Cells were allowed to recover by growing for 1 hour at 30°C. Transformants were selected on L Agar and 20μg/μl chloramphenicol and 50μg/μl carbenicillin and incubated at 30°C overnight. The next day, a single clone was grown in 10ml L Broth and 50μg/μl carbenicillin at 30°C until early log phase. The temperature of the growing culture was then shifted to 42°C for 2 hours. Serial dilutions were made, the cells were then spread onto LA plates (no antibiotic selection), and incubated overnight at 30°C. The next day, 20 colonies were picked and patched onto L Agar (no antibiotics) and LA and 20μg/μl chloramphenicol plates. Plates were then incubated overnight at 30°C. Cells able to grow on LA plates, but not LA and 20μg/μl chloramphenicol plates, were deemed to have the chloramphenicol marker looped out (picked one and designated as strain EWL204).

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

[0921] Generation of a synthetic gene encoding *Populus alba* isoprene synthase (*P. alba* HGS) was outsourced to DNA2.0 Inc. (Menlo Park, CA) based on their codon optimization method for *E. coli* expression. The synthetic gene was custom cloned into plasmid pET24a (Novagen brand, EMD Biosciences, Inc.) and delivered lyophilized (Figures 152, 153A-B; SEQ ID NO:43).

[0922] A PCR reaction was performed to amplify the *P. alba* isoprene synthase (*P. alba* HGS) gene using pET24 P. alba HGS as the template, primers MCM182 and MCM192, and Herculase II Fusion DNA polymerase (Stratagene) according to manufacturer's protocol. PCR conditions were as follows: 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 20 seconds, 72°C for 1 minute, repeat for 25 cycles, with final extension at 72°C for 3 minutes. The *P. alba* isoprene synthase PCR product was purified using QIAquick PCR Purification Kit (Qiagen Inc.).

[0923] *P. alba* isoprene synthase PCR product was then digested in a 20µl reaction containing 1µl *Bsp*HI endonuclease (New England Biolabs) with 2µl 10X NEB Buffer 4. The reaction was incubated for 2 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 20µl reaction containing 1µl *Pst*I endonuclease (Roche) with 2µl 10X Buffer H. The reaction was incubated for 2 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. Plasmid pTrcHis2B (Invitrogen Corp.) was digested in a 20µl reaction containing 1µl *Nco*I endonuclease (Roche), 1µl *Pst*I endonuclease, and 2µl 10X Buffer H. The reaction was incubated for 2 hours at 37°C. The digested pTrcHis2B vector was gel purified using a 1.2% E-gel (Invitrogen Corp.) and extracted using the QIAquick Gel Extraction Kit (Qiagen) (Figure 154). Using the compatible cohesive ends of *Bsp*HI and *Nco*I sites, a 20µl ligation reaction was prepared containing 5µl *P. alba* isoprene synthase insert, 2µl pTrc vector, 1µl T4 DNA ligase (New England Biolabs), 2µl 10X ligase buffer, and 10µl ddH_2O. The ligation mixture was incubated at room temperature for 40 minutes. The ligation mixture was desalted by floating a 0.025µm nitrocellulose membrane filter (Millipore) in a petri dish of ddH_2O and applying the ligation mixture gently on top of the nitrocellulose membrane filter for 30 minutes at room temperature. MCM446 cells (*see* Section II) were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50µl of cell suspension was mixed with 5µl of desalted pTrc P.alba HGS ligation mix. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser

Electroporator. 1ml of LB is immediately added to the cells, then transferred to a 14ml polypropylene tube (Sarstedt) with a metal cap. Cells were allowed to recover by growing for 2 hour at 30°C. Transformants were selected on L Agar and 50μg/μl carbenicillin and 10mM mevalonic acid and incubated at 30°C. The next day, 6 transformants were picked and grown in 5ml L Broth and 50μg/μl carbenicillin tubes overnight at 30°C. Plasmid preps were performed on the overnight cultures using QIAquick Spin Miniprep Kit (Qiagen). Due to the use of BL21 cells for propagating plasmids, a modification of washing the spin columns with PB Buffer 5X and PE Buffer 3X was incorporated to the standard manufacturer's protocol for achieving high quality plasmid DNA. Plasmids were digested with *Pst*I in a 20μl reaction to ensure the correct sized linear fragment. All 6 plasmids were the correct size and shipped to Quintara Biosciences (Berkeley, CA) for sequencing with primers MCM65, MCM66, EL1000 (Table 18). DNA sequencing results showed all 6 plasmids were correct. One plasmid was picked designated as plasmid EWL230 (Figures 155, 156A-B; SEQ ID NO:44).

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

[0924] A PCR reaction was performed to amplify the *Methanosarcina mazei* (*M. mazei*) MVK gene using MCM376 as the template (see section (v) below), primers MCM165 and MCM177 (see Table 18), and Pfu Ultra II Fusion DNA polymerase (Stratagene) according to manufacturer's protocol. PCR conditions were as follows: 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 25 seconds, 72°C for 18 seconds, repeat for 28 cycles, with final extension at 72°C for 1 minute. The *M. mazei* MVK PCR product was purified using QIAquick PCR Purification Kit (Qiagen Inc.).

[0925] The *M. mazei* MVK PCR product was then digested in a 40μl reaction containing 8μl PCR product, 2μl *Pme*I endonuclease (New England Biolabs), 4μl 10X NEB Buffer 4, 4μl 10X NEB BSA, and 22μl of ddH_2O. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 47μl reaction containing 2μl *Nsi*I endonuclease (Roche), 4.7μl 10X Buffer H, and 40μl of PmeI digested *M. mazei* MVK fragment. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then gel purified using a 1.2% E-gel and extracted using the QIAquick Gel Extraction Kit. Plasmid EWL230 was digested in a 40μl reaction containing 10μl plasmid, 2μl PmeI endonuclease, 4μl 10X NEB Buffer 4, 4μl 10X NEB BSA, and 20μl of ddH_2O. The reaction was incubated for 3 hours at 37°C. The

digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 47µl reaction containing 2µl *Pst*I endonuclease, 4.7µl 10X Buffer H, and 40µl of *Pme*I digested EWL230 linear fragment. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then gel purified using a 1.2% E-gel and extracted using the QIAquick Gel Extraction Kit (Figure 157). Using the compatible cohesive ends of *Nsi*I and *Pst*I sites, a 20µl ligation reaction was prepared containing 8µl *M. mazei* MVK insert, 3µl EWL230 plasmid, 1µl T4 DNA ligase, 2µl 10X ligase buffer, and 6µl ddH_2O. The ligation mixture was incubated overnight at 16°C. The next day, the ligation mixture was desalted by floating a 0.025µm nitrocellulose membrane filter in a petri dish of ddH_2O and applying the ligation mixture gently on top of the nitrocellulose membrane filter for 30 minutes at room temperature. MCM446 cells were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50µl of cell suspension was mixed with 5µl of desalted pTrc P.alba-mMVK ligation mix. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB is immediately added to the cells, then the cells are transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hour at 30°C. Transformants were selected on LA and 50µg/µl carbenicillin and 5mM mevalonic acid plates and incubated at 30°C. The next day, 6 transformants were picked and grown in 5ml LB and 50µg/µl carbenicillin tubes overnight at 30°C. Plasmid preps were performed on the overnight cultures using QIAquick Spin Miniprep Kit. Due to the use of BL21 cells for propagating plasmids, a modification of washing the spin columns with PB Buffer 5X and PE Buffer 3X was incorporated to the standard manufacturer's protocol for achieving high quality plasmid DNA. Plasmids were digested with PstI in a 20µl reaction to ensure the correct sized linear fragment. Three of the 6 plasmids were the correct size and shipped to Quintara Biosciences for sequencing with primers MCM65, MCM66, EL1000, EL1003, and EL1006 (Table 18). DNA sequencing results showed all 3 plasmids were correct. One was picked and designated as plasmid EWL244 (Figures 158 and 159A-B; SEQ ID NO:45).

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

[0926] The MVK ORF from the *M. mazei* archaeal Lower Pathway operon (Figures 160A-C; SEQ ID NO:46) was PCR amplified using primers MCM161 and MCM162 (Table 18) using the Invitrogen Platinum HiFi PCR mix. 45 µL of PCR mix was combined with 1 µL template, 1 µL

of each primer at 10 μM, and 2 μL water. The reaction was cycled as follows: 94 °C for 2:00 minutes; 30 cycles of 94 °C for 0:30 minutes, 55 °C for 0:30 minutes and 68 °C for 1:15 minutes; and then 72 °C for 7:00 minutes, and 4 °C until cool. 3 μL of this PCR reaction was ligated to Invitrogen pET200D plasmid according to the manufacturer's protocol. 3 μL of this ligation was introduced into Invitrogen TOP10 cells, and transformants were selected on LA/kan50. A plasmid from a transformant was isolated and the insert sequenced, resulting in MCM376 (Figures 161A-C).

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

[0927] MCM331 cells (which contain chromosomal construct gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase) were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. Mixed 50μl of cell suspension with 1μl of plasmid EWL244. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB is immediately added to the cells, and then the cells were transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hours at 30°C. Transformants were selected on LA and 50μg/μl carbenicillin and 5mM mevalonic acid plates and incubated at 37°C. One colony was selected and designated as strain EWL251.

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

[0928] EWL251 cells were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. Mixed 50μl of cell suspension with 1μl of plasmid MCM82 (comprising pCL PtrcUpperPathway (also known as "pCL Upper MVA"), encoding *E. faecalis* mvaE and mvaS). Plasmid pCL Ptrc Upper Pathway was constructed as described in Example 8 above. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25 μFd using a Gene Pulser Electroporator. 1ml of LB was immediately added to the cells. Cells were then transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hours at 30°C. Transformants were selected on LA and 50μg/μl carbenicillin and 50μg/μl spectinomycin plates and incubated at 37°C. One colony was picked and designated as strain EWL256.

Table 18: Primer Sequences

Primer name	Primer sequence
MCM130	ACCAATTGCACCCGGCAGA (SEQ ID NO:127)
GB Cm Rev	GCTAAAGCGCATGCTCCAGAC (SEQ ID NO:128)
MVD For	GACTGGCCTCAGATGAAAGC (SEQ ID NO:129)
MVD Rev	CAAACATGTGGCATGGAAAG (SEQ ID NO:130)
MCM182	GGGCCCGTTTAAACTTTAACTAGACTCTGCAGTTAGCGTTCAAACGGCAGAA (SEQ ID NO:131)
MCM192	CGCATGCATGTCATGAGATGTAGCGTGTCCACCGAAAA (SEQ ID NO:132)
MCM65	ACAATTTCACACAGGAAACAGC (SEQ ID NO:133)
MCM66	CCAGGCAAATTCTGTTTTATCAG (SEQ ID NO:106)
EL1000	GCACTGTCTTTCCGTCTGCTGC (SEQ ID NO:134)
MCM165	GCGAACGATGCATAAAGGAGGTAAAAAAACATGGTATCCTGTTCTGCGCCGGG TAAGATTTACCTG (SEQ ID NO:122)
MCM177	GGGCCCGTTTAAACTTTAACTAGACTTTAATCTACTTTCAGACCTTGC (SEQ ID NO:123)
EL1003	GATAGTAACGGCTGCGCTGCTACC (SEQ ID NO:137)
EL1006	GACAGCTTATCATCGACTGCACG (SEQ ID NO:138)
MCM161	CACCATGGTATCCTGTTCTGCG (SEQ ID NO:120)
MCM162	TTAATCTACTTTCAGACCTTGC (SEQ ID NO:121)

viii) Construction of strain RM111608-2 (Cm-GI1.2-KKDyI, pTrc P.alba-mMVK, pCL Upper MVA, pBBRCMPGI1.5-pgl)

[0929] The BL21 strain of *E. coli* producing isoprene (EWL256) was constructed with constitutive expression of the *ybhE* gene (encoding *E. coli* 6-phosphogluconolactonase) on a replicating plasmid pBBR1MCS5(Gentamycin) (obtained from Dr. K. Peterson, Louisiana State University).

[0930] FRT-based recombination cassettes, and plasmids for Red/ET-mediated integration and antibiotic marker loopout were obtained from Gene Bridges GmbH (Germany). Procedures using these materials were carried out according to Gene Bridges protocols. Primers Pgl-F (SEQ ID NO:139) and PglGI1.5-R (SEQ ID NO:140) were used to amplify the resistance cassette

from the FRT-gb2-Cm-FRT template using Stratagene Herculase II Fusion kit according to the manufacturer's protocol. The PCR reaction (50 μL final volume) contained: 5 μL buffer, 1 μL template DNA (FRT-gb2-Cm-F from Gene Bridges), 10 pmols of each primer, and 1.5 μL 25mM dNTP mix, made to 50 μL with dH_2O. The reaction was cycled as follows: 1 x 2 minutes, 95°C then 30 cycles of (30 seconds at 95°C; 30 seconds at 63°C; 3 minutes at 72°C).

[0931] The resulting PCR product was purified using the QiaQick PCR purification kit (Qiagen) and electroporated into electrocompetent MG1655 cells harboring the pRed-ET recombinase-containing plasmid as follows. Cells were prepared by growing in 5 mLs of L broth to and OD600~0.6 at 30°C. The cells were induced for recombinase expression by the addition of 4% arabinose and allowed to grow for 30 minutes at 30°C followed by 30 minutes of growth at 37°C. An aliquot of 1.5 mLs of the cells was washed 3-4 times in ice cold dH_2O. The final cell pellet was resuspended in 40 μL of ice cold dH_2O and 2-5 μL of the PCR product was added. The electroporation was carried out in 1-mm gap cuvettes, at 1.3 kV in a Gene Pulser Electroporator (Bio-Rad Inc.). Cells were recovered for 1-2 hours at 30°C and plated on L agar containing chloramphenicol (5 μg/mL). Five transformants were analyzed by PCR and sequencing using primers flanking the integration site (2 primer sets: pgl and 49 rev and 3' EcoRV-pglstop; Bottom Pgb2 and Top GB's CMP (946)). A correct transformant was selected and this strain was designated MG1655 GI1.5-pgl::CMP.

[0932] The chromosomal DNA of MG1655 GI1.5-pgl::CMP was used as template to generate a PCR fragment containing the FRT-CMP-FRT-GI1.5 - ybhE construct. This construct was cloned into pBBR1MCS5(Gentamycin) as follows. The fragment, here on referred to as CMP-GI1.5-pgl, was amplified using the 5' primer Pglconfirm-F (SEQ ID NO:141) and 3' primer 3' EcoRV-pglstop (SEQ ID NO:142). The resulting fragment was cloned using the Invitrogen TOPO-Blunt cloning kit into the plasmid vector pCR-Blunt II-TOPO as suggested from the manufacturer. The *Nsi*I fragment harboring the CMP-GI1.5-pgl fragment was cloned into the *Pst*I site of pBBR1MCS5 (Gentamycin). A 20μl ligation reaction was prepared containing 5μl CMP-GI1.5-pgl insert, 2μl pBBR1MCS5 (Gentamycin) vector, 1μl T4 DNA ligase (New England Biolabs), 2μl 10X ligase buffer, and 10μl ddH_2O. The ligation mixture was incubated at room temperature for 40 minutes then 2-4 μL were electroporated into electrocompetent Top10 cells (Invitrogen) using the parameters disclosed above. Transformants were selected on L agar containing 10 μg/ml chloramphenicol and 5 μg/ml Gentamycin. The sequence of the selected clone was determined using a number of the primers described above as well as with the

in-house T3 and Reverse primers provided by Sequetech, CA. This plasmid was designated pBBRCMPGI1.5-pgl (Figures 162, 163A-B and SEQ ID NO:48).

[0933] Plasmid pBBRCMPGI1.5-pgl was electroporated into EWL256, as described herein and transformants were plated on L agar containing Chloramphenicol (10 μg/mL), Gentamycin (5 μg/mL), spectinomycin (50 μg/mL), and carbenicillin (50 μg/mL). One transformant was selected and designated strain RM111608-2.

Primers:

Pgl-F

5'-
ACCGCCAAAAGCGACTAATTTTAGCTGTTACAGTCAGTTGAATTAACCCTCACTAAA
GGGCGGCCGC-3' (SEQ ID NO:139)

PglGI1.5-R
5'-
GCTGGCGATATAAACTGTTTGCTTCATGAATGCTCCTTTGGGTTACCTCCGGGAAAC
GCGGTTGATTTGTTTAGTGGTTGAATTATTTGCTCAGGATGTGGCATAGTCAAGGGC
GTGACGGCTCGCTAATACGACTCACTATAGGGCTCGAG-3' (SEQ ID NO:140)

3' EcoRV-pglstop:

5'-CTT GAT ATC TTA GTG TGC GTT AAC CAC CAC (SEQ ID NO:142)

pgl +49 rev: CGTGAATTTGCTGGCTCTCAG (SEQ ID NO:136)

Bottom Pgb2: GGTTTAGTTCCTCACCTTGTC (SEQ ID NO:135)

Top GB's CMP (946): ACTGAAACGTTTTCATCGCTC (SEQ ID NO:92)

Pglconfirm-F

5'-ACCGCCAAAAGCGACTAATTTTAGCT-3' (SEQ ID NO:141)

Example 24: Improvement of isoprene production by constitutive expression of *ybhE* (pgl) in *E. coli.*

[0934] This example shows production of isoprene in a strain constitutively expressing *E. coli ybhE* (pgl) compared to a control strain expressing *ybhE* at wild-type levels (*i.e.*, EWL256). The gene *ybhE* (pgl) encodes *E. coli* 6-phosphogluconolactonase that suppresses posttranslational gluconylation of heterologously expressed proteins and improves product solubility and yield

while also improving biomass yield and flux through the pentose phosphate pathway (Aon *et al.*, *Applied and Environmental Microbiology*, 74(4): 950-958, 2008).

i) Small scale analysis

[0935] Media Recipe (per liter fermentation media): K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 1 g, 1000X Trace Metals Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media was filter-sterilized with a 0.22 micron filter. Glucose 5.0 g and antibiotics were added after sterilization and pH adjustment.

[0936] 1000X Trace Metal Solution (per liter fermentation media): Citric Acid * H_2O 40g, $MnSO_4$ * H_2O 30g, NaCl 10g, $FeSO_4$ * $7H_2O$ 1g, $CoCl_2$ * $6H_2O$ 1g, $ZnSO_4$ * $7H_2O$ 1g, $CuSO_4$ * $5H_2O$ 100mg, H_3BO_3 100mg, $NaMoO_4$ * $2H_2O$ 100mg. Each component is dissolved one at a time in diH_2O. The pH is adjusted to 3.0 with HCl/NaOH, and then the solution is brought to volume and filter-sterilized with a 0.22 micron filter.

(a) Experimental procedure

[0937] Isoprene production was analyzed by growing the strains in a Cellerator™ from MicroReactor Technologies, Inc. The working volume in each of the 24 wells was 4.5 mL. The temperature was maintained at 30°C, the pH setpoint was 7.0, the oxygen flow setpoint was 20 sccm and the agitation rate was 800 rpm. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 30°C. A single colony was inoculated into media with antibiotics and grown overnight. The bacteria were diluted into 4.5 mL of media with antibiotics to reach an optical density of 0.05 measured at 550 nm.

[0938] Off-gas analysis of isoprene was performed using a gas chromatograph-mass spectrometer (GC-MS) (Agilent) headspace assay. Sample preparation was as follows: 100 µL of whole broth was placed in a sealed GC vial and incubated at 30°C for a fixed time of 30 minutes. Following a heat kill step, consisting of incubation at 70°C for 5 minutes, the sample was loaded on the GC.

[0939] Optical density (OD) at a wavelength of 550 nm was obtained using a microplate reader (Spectramax) during the course of the run. Specific productivity was obtained by dividing the isoprene concentration (μg/L) by the OD reading and the time (hour).

[0940] The two strains EWL256 and RM11608-2 were assessed at 200 and 400 μM IPTG induction levels. Samples were analyzed for isoprene production and cell growth (OD550) at 1, 2.5, 4.75, and 8 hours post-induction. Samples were done in duplicate.

(b) Results

[0941] The experiment demonstrated that at 2 different concentrations of IPTG the strain expressing the *ybhE* (pgl) had a dramatic 2-3 fold increase in specific productivity of isoprene compared to the control strain.

ii) Isoprene fermentation from *E. coli* expressing Cm-GI1.2-KKDyI, *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, and *ybhE* (pgl) (RM111608-2) and grown in fed-batch culture at the 15-L scale

[0942] Medium Recipe (per liter fermentation medium): K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

[0943] 1000X Modified trace Metal Solution: Citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter

[0944] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL Upper), the integrated lower MVA pathway (gi1.2KKDyI), high expression of mevalonate kinase from *M. mazei* and isoprene synthase from *P. alba* (pTrcAlba-mMVK), and high expression of *E. coli pgl* (pBBR-pgl). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 34°C. A frozen vial of the *E. coli* strain was thawed and

inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 15-L bioreactor bringing the initial volume to 5-L.

[0945] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 40 hour (59 hour) fermentation was 3.1 kg (4.2 kg at 59 hour). Induction was achieved by adding IPTG. The IPTG concentration was brought to 110 μM when the optical density at 550 nm (OD_{550}) reached a value of 4. The IPTG concentration was raised to 192 μM when OD_{550} reached 150. The OD_{550} profile within the bioreactor over time is shown in Figure 164A. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 33.2 g/L at 40 hours (48.6 g/L at 59 hours) (Figure 164B). The isoprene titer increased over the course of the fermentation to a maximum value of 40.0 g/L at 40 hours (60.5 g/L at 59 hours) (Figure 164C). The total amount of isoprene produced during the 40-hour (59-hour) fermentation was 281.3 g (451.0 g at 59 hours) and the time course of production is shown in Figure 164D. The time course of volumetric productivity is shown in Figure 164E and shows that an average rate of 1.0 g/L/hr was maintained between 0 and 40 hours (1.4 g/L/hour between 19 and 59 hour). The metabolic activity profile, as measured by CER, is shown in Figure 164F. The molar yield of utilized carbon that went into producing isoprene during fermentation was 19.6% at 40 hours (23.6% at 59 hours). The weight percent yield of isoprene from glucose was 8.9% at 40 hours (10.7% at 59 hours).

Example 25: Co-production of isoprene and hydrogen in *E. coli* strains expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, pCL Upper MVA (*E. faecalis* mvaE and mvaS) and *ybhE* (pgl)

Collection and analysis of fermentation off-gas for hydrogen and isoprene levels

[0946] Fermentations were performed using strains RM111608-2 (*E. coli* BL21 (DE3), pCL Upper MVA, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK, pBBR cmR-gi1.5-pgl) and EWL 256 (*E. coli* BL21 (DE3), pCL Upper MVA, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK). Construction of bacterial strains is described in Example 23 above.

[0947] Large scale production of isoprene from *E. coli* was determined from a fed-batch culture of *E. coli* strains EWL256 and RM111608-2 expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, pCL Upper MVA (*E. faecalis mvaE* and *mvaS*) and either constitutively expressing *ybhE* (pgl) (RM111608-2) or normally expressing *ybhE* (pgl) (EWL256). This

experiment demonstrates that growing cells in the presence of glucose resulted in the co-production of isoprene and hydrogen.

[0948] The recipe for the fermentation medium (TM2) per liter of TM2 fermentation medium was as follows: K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 5 g, 1000X Modified Trace Metal Solution 1 ml. 1000X Modified Trace Metal Solution: Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, $ZnSO_4$ * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. For the 1000X Modified Trace Metal Solution, each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then brought to final volume in distilled water and filter sterilized with a 0.22 micron (μm) filter (this solution is not autoclaved). For the TM2 fermentation medium, all of the components were added together, dissolved in diH_2O, the pH was adjusted to 6.8 with potassium hydroxide (KOH), q.s. to volume, and the medium was filter sterilized with a 0.22 micron (μm) filter. Glucose was sourced from Cargill as 99DE (dextrose equivalent), 71% DS (dry solids) syrup.

[0949] Fermentations were performed in 15-L bioreactors with *E. coli* strains EWL256 or RM111608-2, containing the upper mevalonic acid (MVA) pathway (pCL Upper MVA), the integrated lower MVA pathway (cmR-gi1.2-yKKDyI), mevalonate kinase from *M. mazei* and isoprene synthase from *P. alba* (pTrcAlba-mMVK), and constitutively expressing *ybhE* (pgl) (RM111608-2) or normally expressing *ybhE* (pgl) (EWL256). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation conditions (pH 7.0 and temperature 34°C).

[0950] An inoculum of the appropriate *E. coli* strain taken from a frozen vial was prepared in peptone-yeast extract medium. After the inoculum grew to OD_{550} = 0.6, 600 mL was used to inoculate a 15-L bioreactor containing TM2 medium. Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 67 hour fermentation was 3.9 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 102 μM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 192 μM when OD_{550} reached 140. At various times after inoculation, samples were removed and the amount of isoprene produced was determined as described below. Levels of hydrogen, nitrogen, oxygen, carbon dioxide, and isoprene in the off gas from the bioreactor were determined using a Hiden HPR-20 mass spectrometer as discussed below.

[0951] Samples of fermentation off-gas from 15-L bioreactors were collected into 20 mL glass headspace vials by sparging the vials at 1 L_{offgas}/min for 10 seconds and sealed with metal screw caps fitted with teflon-coated septa (Agilent, CA). The vials were analyzed within 30 minutes of collection.

[0952] Analysis of the two samples was performed by infusion into a Hiden HPR-20 mass spectrometer (Hiden Analytics, U.K.) at a rate of 4 scc/min (4 mL/min) by placing the inlet tube of the mass spectrometer into the uncapped headspace vials for 1-2 minutes. The HPR-20 instrument was configured to scan masses corresponding to hydrogen (*m/z* 2), nitrogen (*m/z* 28), oxygen (*m/z* 32), carbon dioxide (*m/z* 44) and isoprene (*m/z* 67). The Faraday detector was used for masses 28, 32, 44 and 67. The SEM detector was used for hydrogen (*m/z* 2). Detector response was measured in arbitrary units of pressure (Torr). Absolute hydrogen levels were estimated by comparison to an authentic hydrogen gas standard. Results were recorded using MASsoft V 6.21.0.51 software (Hiden Analytics, United Kingdom).

Results

[0953] Off-gas samples were taken from two fermentation runs and analyzed as described above:

[0954] A) Strain RM111608-2 (*E. coli* BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK, pBBR cmR-gi1.5-pgl). Sample was taken at 64.8 hours into the run during which time the fermentation was being run anaerobically with a nitrogen sparge at 1 vvm.

[0955] B) Strain EWL256 (*E. coli* BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK). Sample was taken at 34.5 hours into the run during which time the fermentation was being run aerobically with an air sparge at 1 vvm.

[0956] The results are depicted in Figures 165A-B. In both cases low levels of hydrogen were detected, in addition to isoprene, oxygen and carbon dioxide. The baseline reading for hydrogen was 0.95 x 10^{-8} Torr. Both Sample A and B gave reading of around 1.3 x 10^{-8} Torr. Based on a comparison to a hydrogen standard, the amount of hydrogen present in the off-gas for samples A and B was estimated to be less than 10 ppmv (parts per million volume) but above the baseline. As shown in Figures 165A-B, both samples A and B also contained significant amounts of isoprene and carbon dioxide.

Example 26: Co-production of isoprene and hydrogen in *E. coli* strains expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, pCL Upper MVA (*E. faecalis* mvaE and mvaS) and *ybhE* (pgl)

Collection and analysis of fermentation off-gas for hydrogen and isoprene levels

[0957] The objective of this experiment is co-produce hydrogen and isoprene in an engineered strain of *E. coli*. For this purpose, a portion of the *hyc* operon encoding *E. coli* hydrogenase-3 will be expressed in strain EWL256 [BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK], prepared as described herein, although any of the bacterial strains described herein, such as RM111608-2, can be similarly modified. An expression construct comprising *hyc* operon genes *hycB* (gi|16130631), *hycC* (gi|16130630), *hycD* (gi|16130629), *hycE* (gi|16130628), *hycF* (gi|16130627), and *hycG* (gi|16130626) is prepared by standard cloning methods known in the art based upon publicly available gene sequences, and introduced into strain EWL256 to produce new strain EWL256+Hyd-3.

[0958] The impact of additional mutations on co-production of hydrogen and isoprene is assessed alone or in combination in EWL256+Hyd-3, by introducing genes involved in the maturation or regulation of hydrogenase-3 (*e.g.*, hycH (gi|16130625) and hycI (gi|16130624)), by inactivating or deleting genes involved in hydrogen uptake or transport (*e.g.*, *E. coli* hydrogenase-1 (*hya* operon) and hydrogenase-2 (*hyb* operon)) or related proteins (*e.g.*, formate dehydrogenase (*fdhF* (gi|16130624)), repressor of formate lyase (*hycA* (gi|16130632)), formate dehydrogenase N, alpha subunit (*fdnG* (gi|16129433)), formate dehydrogenase O, large subunit (*fdoG* (gi|16131734)), nitrate reductase (*narG* (gi|16129187)), fumarate reductase regulator (*fnr* (gi|16129295)), and acetyl-coenzyme A synthetase (*acs* (gi|16131895))), by activating genes involved in upregulation of hydrogenases (*e.g.*, activator of formate hydrogen lyase (*fhlA* (gi|16130638)), by inactivating or deleting genes involved in the production of fermentation side products (*e.g.*, lactate dehydrogenase (*ldhA* (gi|16129341)), fumarate reductase membrane protein (*frdC* (gi|16131977)), alcohol dehydrogenase (*adhE* (gi|16129202)), pyruvate oxidase (*poxB* (gi|16128839)), pyruvate dehydrogenase E1 component *ackA/pta* (*aceE* (gi|16128107)), formate dehydrogenase regulatory protein (*hycA* (gi|16130632)), and formate transporters A and B (*FocA* (gi|16128871) and *FocB* (gi|16130417)), or by expression of heterologous genes involved in hydrogen metabolism (*e.g.*, glyceraldehyde-3-phosphate dehydrogenase from *Clostridium acetobutylicum* (*gapC* (gi|15893997)).

[0959] Fermentations are performed using engineered variants of strain EWL 256+Hyd-3 (BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK and *hycB-F*), modified to comprise one or more additional mutations as described herein, either alone or in combination, essentially as described in Example 25 above. Co-production of hydrogen and isoprene is

assessed by analysis of off-gas samples essentially as described above. Strains are selected for further analysis based upon the rate of isoprene and hydrogen co-production.

[0960] 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.

Example 27: Production of isoprene or mevalonate from fatty acid or palm oil in *E. coli fadR atoC* LS5218 containing the upper or upper and lower Mevalonic Acid pathway plus kudzu isoprene synthase.

[0961] *Escherichia coli fadR atoC* strain LS5218 (#6966) was obtained from the Coli Genetic Stock Center. FadR encodes a transcription repressor that negatively regulates expression of the genes encoding fatty acid degradation enzymes (Campbell *et al.*, *J. Bacteriol.* 183: 5982-5990, 2001). AtoC is a response regulator in a two-component regulatory system wherein AtoS regulates acetolactate metabolism. The fadR atoC strain allows constitutive expression of the fatty acid degradation genes and incorporates long chain fatty acids into long-chain-length polyhydroxyalkanoates. When palm oil is used as a carbon source for either mevalonate or isoprene production, the palm oil was converted to glycerol plus fatty acid. Methods for this are well known in the art, and it can be done either enzymatically by incubation with a lipase (for example Porcine pancreatic lipase, *Candida rugosa* lipase, or other similar lipases) or chemically by saponification with a base such as sodium hydroxide.

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

[0962] Strain WW4 was created by electroporating pCLPtrcUpperPathway into LS5218 using standard methods (Sambrooke *et al.,* Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989). Incorporation of the plasmid was demonstrated by the production of mevalonic acid (MVA) when cells were cultured in TM3 medium supplemented with either C12 fatty acid (FA) or palm oil as the carbon source. To demonstrate production of MVA by WW4

from fatty acid, cells from an overnight culture were diluted 1 to 100 into 5 mL of modified TM3 medium (TM3 without yeast extract) supplemented with 0.25% C12 FA (Sigma cat # L9755). The first sign of MVA production (24 mg/L) was apparent after overnight incubation at 30°C of the IPTG induced culture. Production increased over three days with the final level of 194 mg/L of MVA produced. To demonstrate production of MVA by WW4 from oil, cells from an overnight culture were diluted 1 to 100 into modified TM3 medium supplemented with 200 mg of digested palm oil per 5 mL of TM3 medium. The first sign of MVA production (50 mg/L) was apparent after overnight incubation of the IPTG induced culture at 30°C. Production increased over three days with a final level of 500 mg/L of MVA produced.

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

[0963] *Escherichia coli* strain WW4 (LS5218 fadR atoC pCLPtrcUpperPathway) was transformed with pMCM118 [pTrcKKDyIkIS] to yield WW10. The incorporation of the plasmid was demonstrated by evidence of production of isoprene when the strain was cultured in TM3 and glucose and induced with IPTG (100, 300, or 900 μM). The strain was relatively sensitive to IPTG and showed a significant growth defect even at 100 μM IPTG. These results are shown in Figure 70A.

[0964] To test isoprene production from dodecanoic acid, WW10 was cultured overnight in L broth containing spectinomycin (50 μg/ml), and kanamycin (50 μg/ml) at 37C with shaking at 200 rpm. The cells were washed with modified TM3 medium by centrifugation and resuspension in their original culture volume with this medium. The washed and resuspended cells from this starter culture were diluted 1 to100 and 1 to 10 into 5 mL of modified TM3 medium containing 0.125% C12 Fatty Acid (Sigma cat # L9755).

[0965] To demonstrate production of mevalonate from palm oil, the oil was predigested with lipase at 37°C and 250 rpm for several days to release the fatty acids (evidence of hydrolysis was judged by the foam formed when tubes were shaken).

[0966] In addition, a culture was set up by diluting the washed cells at 1 to 10 into modified TM3 medium contained in test tubes with palm oil. A further tube was set up by the addition of 0.125% C12FA to the remainder (2.5 mL) of the washed cells without further dilution (bioconversion). After 3.75 hours of growth at 30°C with shaking at 250 rpm all of the cultures were induced by the addition of 50 μM IPTG. Incubation was continued for 4 hours after which time 200 μL of each of the cultures was assayed for isoprene accumulation with a modified head

space assay (1 hour accumulation at 30°C with shaking at 500 rpm). An additional isoprene assay was conducted by a 12 hour incubation of the assay glass block prior to GCMS analysis. Incubation of the induced cultures was continued overnight and 200 µL aliquots were again assayed for isoprene production (1 hour, 30 deg, 500 rpm Shel-Lab shaker) the following morning. Analysis of these cultures showed the production of significant levels of isoprene. The highest levels of isoprene were observed in the culture which was seeded at 1/10 dilution from the overnight starter culture after it had been incubated and induced overnight. This result suggests that this culture continued to grow and increase in cell density. These results are shown in Figure 70B. Cell density could not be measured directly because the fatty acid suspension had a turbid appearance. Cell density of this culture was therefore determined by plating an aliquot of the culture and showed 8 $x10^7$ colony forming units. This corresponds approximately to an OD_{600} of 0.1. Nevertheless, this culture provided significant isoprene production; no isoprene is observed for similar strains without the pathway described in this example.

Example 28: Expression of isoprene-synthase from plant in *Streptomyces sp.*

[0967] The gene for isoprene synthase Kudzu was obtained from plasmid pJ201:19813. Plasmid pJ201:19813 encodes isoprene synthase from *Pueraia lobata* (Kudzu plant) and was codon-optimized for *Pseudomonas fluorescens*, *Pseudomonas putida, Rhodopseudomonas palustris* and *Corynebacterium* (Figures 79A-79C (SEQ ID NO:123)). Digestion of plasmid pJ201:19813 with restriction enzymes NdeI and BamHI liberated gene iso19813 that was ligated into the *Streptomyces-E.coli* shuttle vector pUWL201PW (Doumith *et al.*, *Mol. Gen. Genet.* 264: 477-485, 2000; Figure 71) to generate pUWL201_iso. Successful cloning was verified by restriction analysis of pUWL201_iso. Expression of isoprene synthase iso19813 was under control of the *erm*-promoter which allows for constitutive expression in *Streptomycetes* species, but not for expression in *E. coli.*

[0968] PUWL201PW (no insert) and pUWL201_iso were introduced in *Streptomyces albus* J1074 (Sanchez *et al.*, *Chem. Biol.* 9:519-531, 2002) by transformation of protoplasts as described by Hopwood *et al.*, *The John innes foundation, Norwich*, 1985.

[0969] A 200 µl aliquot of protoplast suspensions was transformed with 1.9 µg pUWL201PW or 2.9 µg pUWL201_iso. After incubation overnight at 28°C on non-selective R5-agarplates, positive transformants were selected by further incubation for 4 days in R3-overlay agar containing thiostrepton (250 µg/ml). Thiostrepton resistant transformants were examined for presence of the pUWL-plasmids by plasmid preparation using Plasmid Mini Kit (Qiagen).

Prepared plasmid DNA was reintroduced in *E. coli* DH5α to generate sufficient amounts of plasmid DNA to be analyzed by restriction analysis. Positive transformants were selected on ampicillin-containing L-agar plates and insert analysis was done by digestion of plasmid DNA with *Nde*I and *Bam*HI endonucleases. Isoprene synthase was identified as a 1.7 kb fragment in positive pUWL201 iso clones while in the control strains (pUWL201PW) no such fragment was observed.

[0970] Wild type strain and transformants of *S. albus* containing control plasmid pUWL201PW or isoprene synthase encoding pUWL201_iso were analyzed for isoprene formation. Strains were cultivated in duplicate on solid media (tryptic soy broth agar, TSB; 2.5 ml) in presence or absence of thiostrepton (200 µg/ml) and incubated for 4 days at 28°C in sealed head-space vials (total volume 20 ml). 500 µl head-space samples (end point measurements) were analyzed by GC-MS in SIM-mode and isoprene was identified according to reference retention times and molecular masses (67 m/z). Isoprene present in head-space samples was quantified by previously generated calibration curves. While wild-type *S. albus* and control strains harboring pUWL201PW produced isoprene in concentrations slightly higher than the detection limit (0.04 – 0.07 ppm), *S. albus* harboring pUWL201_iso produced isoprene in at least tenfold excess compared to controls (0.75 ppm; Figure 72). The results demonstrate successful expression of plant-derived isoprene synthase in a prokaryotic organism of the *Actinomycetes* group.

Example 29: Recovery of Bioisoprene™

[0971] Bioisoprene™ was recovered from a set of four 14-L scale fermentations in a two-step operation involving stripping of isoprene from the fermentation off-gas stream by adsorption to activated carbon, followed by off-line steam desorption and condensation to give liquid Bioisoprene™ (Figures 166A and 166B). The total amount of Bioisoprene™ produced by the four fermentors was 1150 g (16.9 mol), of which 953 g (14 mol, 83%) was adsorbed by the carbon filters. Following the steam desorption/condensation step, the amount of liquid Bioisoprene™ recovered was 810 g, corresponding to an overall recovery yield of 70%. The recovered Bioisoprene™ was analyzed for the presence of impurities.

Analysis and impurity profile of Bioisoprene™ liquid

[0972] Recovered Bioisoprene™ liquid was analyzed by GC/MS and gas chromatography/flame ionization detection (GC/FID) to determine the nature and levels of impurities. The product was determined to be >99.5% pure and contained several dominant impurities in addition to many minor components. The GC/FID chromatogram is depicted in Figure 167, and the typical levels of impurities are shown in Table 19. The impurity profile was similar to other Bioisoprene™ batches produced on this scale.

Table 19. Summary of the nature and levels of impurities seen in several batches of Bioisoprene™.

Compound	Retention Time (min)		Conc. Range
	GC/MS	GC/FID	
Ethanol	1.59	11.89	<50 ppm
Acetone	1.624	12.673	<100 ppm
Methacrolein	1.851	15.369	<200 ppm
Methyl vinyl ketone	1.923	16.333	<20 ppm
Ethyl acetate	2.037	17.145	100 to 800 ppm
3-Methyl-1,3-pentadiene	2.27	18.875	50 to 500 ppm
Methyl vinyl oxirane	2.548	19.931	<100 ppm
Isoprenol	2.962	21.583	<500 ppm
3-methyl-1-butanol	2.99	21.783	<50 ppm
3-hexen-1-ol	4.019	24.819	<100 ppm
Isopentenyl acetate	4.466	25.733	200 to 1000 ppm
3-hexen-1-yl acetate	5.339	27.223	<400 ppm
limonene	5.715	27.971	< 500 ppm
Other cyclics	5.50 – 6.50	27.5 – 28.0	<200 ppm

Purification of Bioisoprene™ by treatment with adsorbents

[0973] Adsorbents are widely used by industry for the removal of trace impurities from hydrocarbon feedstocks. Suitable adsorbents include zeolite, alumina and silica-based materials. Bioisoprene™ can be substantially purified by passage over silica gel, and to a lesser extent with alumina. Figure 168 shows the GC/FID chromatograms of a Bioisoprene™ sample before (A) and after treatment with alumina (B) or silica (C). The Selexsorb™ adsorbent products from

BASF is one of the adsorbents of choice for the removal of polar impurities from Bioisoprene™. Specifically, the Selexsorb CD and CDX products are preferred given their proven utility for removal of polar impurities from isoprene and butadiene feedstocks.

Example 30: Chemical transformations of Bioisoprene™

[0974] Chemicals and solvents were used as received from Sigma Aldrich Corp (WI, USA). BioIsoprene™ was produced by fermentation of *E. coli* BL21 strains expressing Isoprene synthase and a heterologous mevalonic acid (MVA) isoprene precursor biosynthetic pathway. Bioisoprene was recovered from fermentation off-gas by adsorption to activated carbon, followed by steam desorption and condensation to obtain crude, liquid Bioisoprene. Bioisoprene was purified by fractional distillation immediately before use.

^{1}H NMR analysis

[0975] Proton (^{1}H) nuclear magnetic resonance (NMR) spectra were recorded on a Varian VNMRS 500 MHz NMR system. All NMR spectra are referenced to tetramethylsilane (TMS, 0 ppm) or chloroform ($CHCl_3$, 7,26 ppm)and peak frequencies are recorded in ppm unless otherwise specified. Samples were run in either deuterated chloroform ($CDCl_3$) or methanol (CD_3OD).

GC/MS analysis

[0976] 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 autosampler was set up to inject 1 μL of a liquid sample from a 10 μL liquids syringe. 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 100:1. The oven program began at 50° C for 2 minutes, increasing to 225°C at a rate of 25°C/min. followed by a 1 minute hold for a total run time of 10 minutes. The Agilent 5793N mass selective detector was run in scan mode from m/z 29 to 500. A solvent delay of 1.5 minutes was employed. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at 0.675 minutes.

GC/FID analysis

[0977] The analysis was performed using an Agilent 6890 GC/FID system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in liquids mode. An Agilent DB-Petro GC column (100 m x 0.25 mm; 0.50 μm film thickness) was used for separation of analytes. The autosampler was set up to inject 1 μL of a liquid sample from a 10 μL liquids syringe. The GC/FID method utilized helium as the carrier gas at a flow of 1 mL/minute. The injection port was held at 200° C with a split ratio of 50:1. The oven program began at 50° C for 15 minutes, increasing to 250°C at a rate of 25°C/min. followed by a 10 minute hold for a total run time of 33 minutes. The FID detector was held at 280°C in Constant makeup mode with a hydrogen flow of 35 mL/min and air flow of 250 mL/min. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at 13.54 minutes.

I. Preparation of cyclic dimers of isoprene through thermal Diels-Alder cycloaddition

[0978] Isoprene (1.02 g, 0.015 mole) was heated at 150°C for 24 hours in the presence of 2,6-di-tert-butyl-4-methylphenol (0.165 g, 0.05 mole) acting as an inhibitor of thermal polymerization. The reaction was carried out in a sealed thick-walled glass reaction vessel that was dried in the vacuum oven for 24 hours prior to the reaction. The reaction mixture was stirred with a magnetic stirrer. The progress of the reaction was monitored by gas chromatography with mass-spectrometer detector (GC-MS). After the reaction was finished, the resulting mixture of isomers was purified using silica gel chromatography with hexane as the eluent. Following concentration on a rotary vacuum evaporator the product was characterized by GC-MS and ^{1}H-NMR. GC-MS: product A: 5.17, 5.19 min; product B: 5.72, 5.74 min; product C: 6.11 min. ^{1}H-NMR ($CDCl_3$) d: 5.8 (m); 5.4 (m); 4.95 (m); 2.4-1.2 (m).

II. Preparation of isoprene oligomers through Pd-catalyzed oligomerization

[0979] Isoprene (2.03 g, 0.03 mole) was mixed with isopropanol (1.79 g ,0.03 mole) in a sealed thick-walled glass reaction vessel. Prior to the reaction the glass chamber was dried in a vacuum oven for 24 hours. Transfer of all reagents was done under inert nitrogen atmosphere. Palladium acetylacetonate (0.55 mg, 0.06 mmole) and triphenylphosphine (1.49 mg, 0.19 mmol) were added to the reaction mixture. The reaction chamber was then heated to 100°C for 24 hours with mixing provided via magnetic stirrer. The course of the reaction was analyzed by GC-MS. Once the reaction was finished the products were isolated by silica gel column chromatography using hexane as the eluent. The final products were characterized by GC-MS and ^{1}H-NMR spectroscopy. GC-MS: product A: 5.54 min; product B: 6.6 min; product C: 6.85 min.

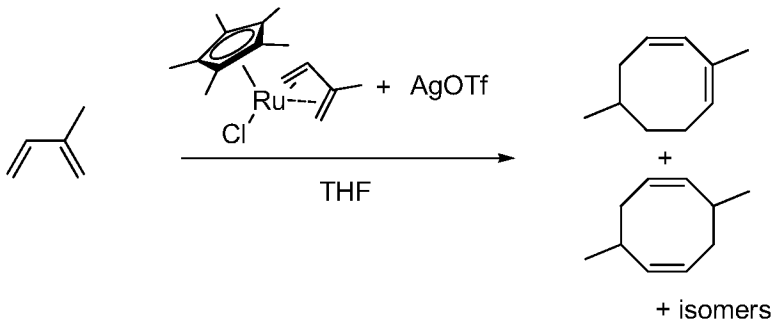
III. Hydrogenation of unsaturated compounds

[0980] The mixture of isomers obtained in Example 2 (1.36 g, 0.01 mole) is put in a glass chamber equipped with magnetic stirrer and containing a hydrogenation catalyst (Pd/C, 5 wt%) (0.21 g, 0.1 mmol). All glassware is vacuum dried prior to carrying out the experiments. Hydrogen gas is introduced into the system and the pressure is kept at 3 atm. After several hours the reaction mixture Pd/C is filtered from the reaction mixture and the products are separated using silica gel chromatography. Final analysis is done using GC-MS and NMR.

IV. Preparation of ethoxylated derivatives of isoprene

[0981] Isoprene (0.982 g, 0.014 mole) was mixed with absolute ethanol (0.665 g, 0.014 mol) in a thick glass wall chamber equipped with magnetic stirrer. A catalytic amount of concentrated sulfuric acid was added to the reaction mixture, followed by stirring overnight at 85°C. The progress of the reaction was monitored by GC-MS. After 16 hours of heating the GC-MS trace revealed the presence of a mixture of isomers with following retention times: product A: 2.66 min $m/z^+ = 99$; product B: 3.88 $m/z^+ = 99, 114$; product C: 5.41min, $m/z^+ = 87, 99, 114$.

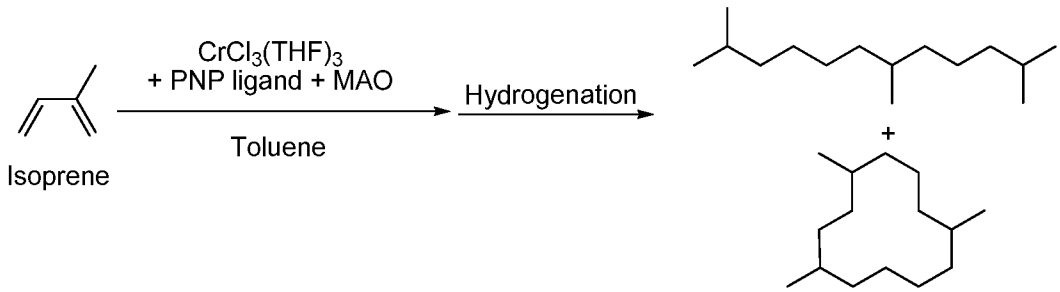
V. Conversion of isoprene to C10 cyclic dimers using a ruthenium catalyst



[0982] The conversion of isoprene into C10 cyclic dimers has been achieved in excellent yield using a ruthenium catalyst to a mixture of dimethyl-cyclooctadienes (Itoh, Kenji; Masuda, Katsuyuki; Fukahori, Takahiko; Nakano, Katsumasa; Aoki, Katsuyuki; Nagashima, Hideo, *Organometallics* (**1994**), 13(3), 1020-9.) Conversions higher than 95% are reported at very moderate temperatures (as low as 60°C). The catalyst, a pentamethylcyclopentadienyl-based ruthenium organometallic compound, can be prepared in two easy steps using ruthenium chloride ($RuCl_3$) and pentamethylcyclopentadiene (C_5Me_5H) as starting materials. For the

dimerization reaction the catalyst is activated with silver triflate (AgOTf) to produce the active species, but other activators could be used as well.

VI. Conversion of isoprene to C15 trimers using a chromium catalyst



3:1 mixture (linear:cyclic)

[0983] Conversion of isoprene into the C15 trimers can be carried out using a chromium catalyst with a P-N-P ligand, *N,N*-bis(diarylphosphino)amine. The catalyst is prepared *in situ* and it is activated using MAO, as described in Bowen, L.; Charernsuk, M.; Wass, D.F. *Chem. Commun.* (**2007**) 2835-2837. The reported conversion of isoprene into the C15 trimers, consisting in a mixture (3:1) of linear and cyclic products, is as high as 95% at moderate temperatures (70°C). Tetramers are identified as the major other product in every case.

VII. Hydrogenation of isoprene

[0984] Isoprene (10 mL of a 10% solution in absolute ethanol (v/v)) was hydrogenated to 2-methylbutane (isopentane) in a continuous manner using an H-cube hydrogenation instrument (ThalesNano, Princeton, NJ, U.S.A.). The isoprene solution was pumped at 0.5 mL/min through a 10% Pd/C catalyst cartridge held at 70°C. Hydrogen gas was introduced using "full mode" at 1 atm pressure. The product was collected and analyzed by ^{1}H NMR and GC/FID which confirmed the conversion of isoprene to 2-methylbutane in over 90% yield, in addition to minor amounts of partially hydrogenated mono-olefins. ^{1}H NMR (500 MHz, CDCl3): δ0.8 (m, 9H, CH_3); 1.12 (m, 2H, CH_2); 1.37 (m, 1H, CH). GC/FID: 2-methylbutane; retention time = 12.69 minutes.

VIII. Partial hydrogenation of isoprene

[0985] BioIsoprene™ product (50 mL, 0.5 mol) was mixed with toluene (200 mL) and partially hydrogenated over a 5% Pd/C catalyst on an Midi-Cube hydrogenation instrument (ThalesNano, Budapest, Hungary) at 40°C and 5 bar hydrogen pressure. Substrate flow rate was 10 mL/min and hydrogen was delivered at 125 mL/min (5 mmol/min). The product stream was recycled through the instrument for a period of 2 hours after which time an aliquot of the product was analyzed by GC/MS and GC/FID which showed that the majority of the starting material had been converted to a mixture of isoamylenes (2-methyl-1-butene, 2-methyl-2-butene and 3-methyl-1-butene), in addition to isopentane and some unreacted isoprene (Figure 170).

IX. Selective hydrogenation of BioIsoprene™ product

[0986] BioIsoprene™ product is selectively hydrogenated under the conditions cited in the above example using an eggshell Pd/d-Al_2O_3 catalyst giving a mixture of isoamylenes where 2-methyl-2-butene is the dominant product accounting for >50% of the total isoamylenes and 3-methyl-1-butene is the minor product accounting for <25% of the total isoamylene products as determined by GC/MS analysis. The amount of isopentane and residual isoprene account for <10% of the total product stream. A similar result is obtained when a sulfided palladium on carbon catalyst is used to perform the reaction.

X. Partial hydrogenation of BioIsoprene™ product in the gas phase

[0987] A dry gas stream containing BioIsoprene™ product is mixed with a slight excess of hydrogen gas (mol/mol) and the gaseous mixture passed over a heterogenous hydrogenation catalyst, such as a Group IB-promoted palladium catalyst with high pore volume as described in US Pat. Appl. 20090203520, to produce a mixture of isoamylenes and one or more impurities derived from the fermentation process from which the BioIsoprene™ product was originally derived. The conversion is carried out at pressures ranging from 0.5 to 200 bar, and temperatures from 0°C to 200°C.

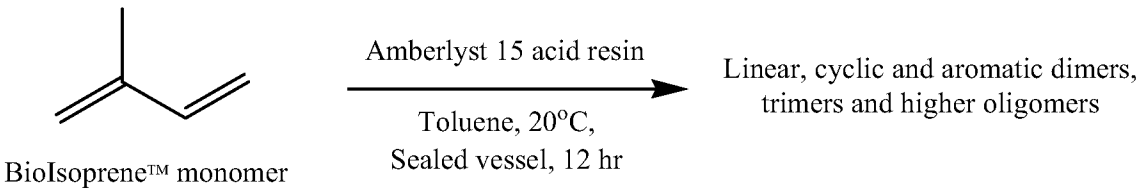
XI. Dimerization of Isoamylenes with a solid acid catalyst

[0988] 2-Methyl-2-butene (1.5 mL) and toluene (4 mL) were stirred at room temperature with Amberlyst 15 acid resin (186 mg) for 12 h at room temperature. An aliquot (500 uL) was removed from the reaction mixture and transferred to a GC vial. Analysis of the mixture was

performed by GC/MS (Figure 171) and revealed the partial conversion to C10 dimers (diisoamlylenes) suitable as BioIsofuel™ components.

[0989] The products of the dimerization reaction (diisoamylenes, C10 dimate) are optionally fully hydrogenated under conditions described in Example 30, part VII, or through conditions known in the art for the hydrogenation of olefins to isoparaffins [for example, see Marichonna (2001)].

XII. Oligomerization of BioIsoprene™ product with a solid acid catalyst



[0990] A mixture of BioIsoprene™ monomer, 2-methyl-2-butene (1.5 mL) and toluene (4 mL) was stirred at room temperature with Amberlyst 15 acid resin (186 mg) for 12 h at room temperature. An aliquot (500 uL) was removed from the reaction mixture and transferred to a GC vial. Analysis of the mixture was performed by GC/MS (Figure 172) and revealed a complex mixture of products consisting of isoprene, linear, cyclic and aromatic C10, C15 and higher oligomers.

XIII. Continuous oligomerization of BioIsoprene™ product with a solid acid catalyst

[0991] BioIsoprene™ monomer is continuously converted into C10 dimers and C15 trimers in a dimerization reactor containing Amberlyst 15 ion exchange resin or an equivalent catalyst. The BioIsoprene™ feed stream comprises BioIsoprene™ monomer and optionally C5 derivatives of BioIsoprene™ and a co-solvent. The process is conducted at temperatures ranging from 20 to 200°C and pressures from 0.5 to 200 bar. The products of the dimerization step are fractionated in a first fractionation column to separate unreacted isoprene from higher (>C5) oligomers. The C5 fraction is returned to the dimerization reactor and the heavy >C5 fraction is introduced into a second fractionation column in which the desired C10/C15 fraction is collected from the overhead stream. The bottom fraction consisting of >C15 oligomers is fed into a heavy recycle reactor containing a metathesis catalyst such as the Grubbs 2nd generation catalyst. The metathesis catalyst converts a portion of the higher oligomer fraction into lighter

components by olefin cross-metathesis reactions that are subsequently fed into fractionation column #1 as depicted in Figure 173.

[0992] Overall, the process results in conversion of BioIsoprene monomer into C10 dimer and C15 trimer BioIsofuel™ precursors which are then subjected to partial or complete hydrogenation under conditions described in example 30, section VII. The resulting partially or fully saturated compounds are suitable as BioIsofuel™ compositions and as BioIsofuel™ blendstocks.

Example 31 $^{13}C/^{12}C$ isotope analysis

[0993] ^{13}C analysis can be done by loading 0.5 to 1.0 mg samples into tin cups for carbon isotopic analysis using a Costech ECS4010 Elemental Analyzer as an inlet for a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer. Samples are dropped into a cobaltous/cobaltic oxide combustion reactor at 1020°C with combustion gases being passed in a helium stream at 85mL/min through a copper reactor (650°C) to convert NO_x to N_2. CO_2 and N_2 are separated using a 3-m 5Å molecular sieve column. Then, $^{13}C/^{12}C$ ratios are calibrated to the VPDB scale using two laboratory standards (Acetanilide B, -29.52 ± 0.02‰m and cornstarch A, -11.01 ±0.02‰) which have been carefully calibrated to the VPDB scale by off-line combustion and dual-inlet analysis using the 2-standard approach of T. B. Coplen et al, New Guidelines for $d^{13}C$ Measurements, Anal. Chem., 78, 2439-2441 (2006). The teachings of Coplen are incorporated herein by reference for the purpose of teaching the technique for determining $d^{13}C$ values.

[0994] U.S. Provisional Patent Application No. 61/133,521 filed on June 30, 2008 and WO 2010/05525 A1 list $\delta^{13}C$ values for feedstock and polymers of isoprene derived from various sources, including ones listed in Table 20.

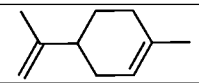
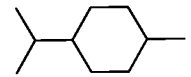
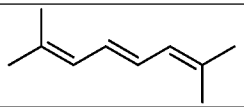
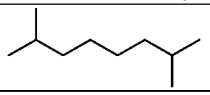
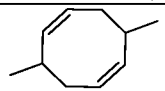
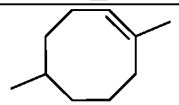
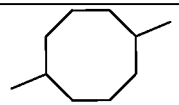
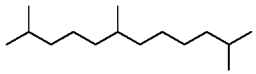
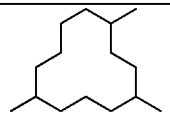
Table 20

Sample	$\delta^{13}C$
Palm oil	-30.00
Yeast extract	-25.70
Commercial polyisoprene from extractive distillation	-23.83
Sugar from softwood pulp	-23.00
Polyisoprene from Isoprene Sample B (emulsion polymerization)	-19.67
Invert Sugar	-15.37
Polyisoprene from Isoprene Sample A (Neodymium catalyst)	-14.85
Glucose from bagasse	-13.00
Glucose from corn stover	-11.20
Cornstarch	-11.10
Glucose	-10.73

Example 32 – Exemplary Fuel Properties

[0995] Table 21 lists fuel properties of certain compounds that can be made from isoprene using methods described herein.

Table 21

	Formula		MW (g/mol)	Boiling point (°C)	Density (g/cm^3)	Vapor pressure (Torr, 25°C)*	ΔHc (kcal/ mol)	Lower Heating Value (kBtu/gal)	Higher Heating Value (kBtu/gal)	Octane (Cetane)
a-Limonene	$C_{10}H_{16}$		136.23	177	0.8477	1.54	1474	130.6	137.9	88
1-Methyl-4-isopropylcyclohexane	$C_{10}H_{20}$		140.27	170.2	0.8060	2.16	1561	126.5	134.9	75
2,7-dimethyl-,(4E)- 2,4,6-Octatriene	$C_{10}H_{16}$		136.23	186.4*	0.782	0.915	1481	121.1	127.8	110
2,7-dimethyl-octane	$C_{10}H_{22}$		142.28	160	0.728	3.35	1620	116.5	124.7	97
3,7-dimethyl-1,5-cyclooctadiene	$C_{10}H_{16}$		136.23	182.7*	0.860	1.09	1490	134.0	141.4	95
1,5-Dimethylcyclooctene (BIF-10)	$C_{10}H_{18}$		138.25	178.5*	0.830	1.33	1545	131.6	139.4	90
1,5-Dimethylcyclooctane	$C_{10}H_{20}$		140.27	158.5	0.800	1.39	1560	125.4	133.8	85
2,6,11-Trimethyldodecane	$C_{15}H_{32}$		212.41	247.8*	0.766	0.0396	2400	121.7	130.1	(65)
Cyclododecane, 1,4,8-trimethyl	$C_{15}H_{30}$		210.40	278.0*	0.850	7.38E-3	2380	135.7	144.6	(40)
BIF-15	$C_{15}H_{31.5}$	3:1 mixture of linear and cyclic C15	211.9	255.4	0.787	0.0315	2395	125.2	133.7	(58.8)

[0996] 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.

[0997] 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.

Appendix 1

Exemplary 1-deoxy-D-xylulose-5-phosphate synthase nucleic acids and polypeptides

ATH: AT3G21500(DXPS1) AT4G15560(CLA1) AT5G11380(DXPS3)
OSA: 4338768 4340090 4342614
CME: CMF089C
PFA: MAL13P1.186
TAN: TA20470
TPV: TP01_0516
ECO: b0420(dxs)
ECJ: JW0410(dxs)
ECE: Z0523(dxs)
ECS: ECs0474
ECC: c0531(dxs)
ECI: UTI89_C0443(dxs)
ECP: ECP_0479
ECV: APECO1_1590(dxs)
ECW: EcE24377A_0451(dxs)
ECX: EcHS_A0491
STY: STY0461(dxs)
STT: t2441(dxs)
SPT: SPA2301(dxs)
SEC: SC0463(dxs)
STM: STM0422(dxs)
YPE: YPO3177(dxs)
YPK: y1008(dxs)
YPM: YP_0754(dxs)
YPA: YPA_2671
YPN: YPN_0911
YPP: YPDSF_2812
YPS: YPTB0939(dxs)
YPI: YpsIP31758_3112(dxs)
SFL: SF0357(dxs)
SFX: S0365(dxs)
SFV: SFV_0385(dxs)
SSN: SSON_0397(dxs)
SBO: SBO_0314(dxs)
SDY: SDY_0310(dxs)
ECA: ECA1131(dxs)
PLU: plu3887(dxs)
BUC: BU464(dxs)
BAS: BUsg448(dxs)
WBR: WGLp144(dxs)
SGL: SG0656
KPN: KPN_00372(dxs)
BFL: Bfl238(dxs)
BPN: BPEN_244(dxs)
HIN: HI1439(dxs)
HIT: NTHI1691(dxs)
HIP: CGSHiEE_04795
HIQ: CGSHiGG_01080
HDU: HD0441(dxs)
HSO: HS_0905(dxs)
PMU: PM0532(dxs)
MSU: MS1059(dxs)
APL: APL_0207(dxs)
XFA: XF2249
XFT: PD1293(dxs)
XCC: XCC2434(dxs)
XCB: XC_1678
XCV: XCV2764(dxs)
XAC: XAC2565(dxs)
XOO: XOO2017(dxs)
XOM: XOO_1900(XOO1900)
VCH: VC0889
VVU: VV1_0315
VVY: VV0868
VPA: VP0686
VFI: VF0711
PPR: PBPRA0805
PAE: PA4044(dxs)
PAU: PA14_11550(dxs)
PAP: PSPA7_1057(dxs)
PPU: PP_0527(dxs)
PST: PSPTO_0698(dxs)
PSB: Psyr_0604
PSP: PSPPH_0599(dxs)
PFL: PFL_5510(dxs)
PFO: Pfl_5007
PEN: PSEEN0600(dxs)
PMY: Pmen_3844
PAR: Psyc_0221(dxs)
PCR: Pcryo_0245
ACI: ACIAD3247(dxs)
SON: SO_1525(dxs)
SDN: Sden_2571
SFR: Sfri_2790
SAZ: Sama_2436
SBL: Sbal_1357
SLO: Shew_2771
SHE: Shewmr4_2731
SHM: Shewmr7_2804
SHN: Shewana3_2901
SHW: Sputw3181_2831
ILO: IL2138(dxs)
CPS: CPS_1088(dxs)
PHA: PSHAa2366(dxs)
PAT: Patl_1319
SDE: Sde_3381
PIN: Ping_2240
MAQ: Maqu_2438
MCA: MCA0817(dxs)
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FTW: FTW_0925(dxs)
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FTH: FTH_1047(dxs)
FTA: FTA_1131(dxs)
FTN: FTN_0896(dxs)
NOC: Noc_1743
AEH: Mlg_1381
HCH: HCH_05866(dxs)
CSA: Csal_0099
ABO: ABO_2166(dxs)
AHA: AHA_3321(dxs)
BCI: BCI_0275(dxs)
RMA: Rmag_0386
VOK: COSY_0360(dxs)
NME: NMB1867
NMA: NMA0589(dxs)
NMC: NMC0352(dxs)
NGO: NGO0036
CVI: CV_2692(dxs)
RSO: RSc2221(dxs)
REU: Reut_A0882
REH: H16_A2732(dxs)
RME: Rmet_2615
BMA: BMAA0330(dxs)
BMV: BMASAVP1_1512(dxs)
BML: BMA10299_1706(dxs)
BMN: BMA10247_A0364(dxs)
BXE: Bxe_B2827
BUR: Bcep18194_B2211
BCN: Bcen_4486
BCH: Bcen2424_3879
BAM: Bamb_3250
BPS: BPSS1762(dxs)
BPM: BURPS1710b_A0842(dxs)
BPL: BURPS1106A_A2392(dxs)
BPD: BURPS668_A2534(dxs)
BTE: BTH_II0614(dxs)
BPE: BP2798(dxs)
BPA: BPP2464(dxs)
BBR: BB1912(dxs)
RFR: Rfer_2875
POL: Bpro_1747
PNA: Pnap_1501
AJS: Ajs_1038
MPT: Mpe_A2631
HAR: HEAR0279(dxs)
MMS: mma_0331
NEU: NE1161(dxs)
NET: Neut_1501
NMU: Nmul_A0236
EBA: ebA4439(dxs)
AZO: azo1198(dxs)
DAR: Daro_3061
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HHE: HH0608(dxs)
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WSU: WS1996
TDN: Tmden_0475
CJE: Cj0321(dxs)
CJR: CJE0366(dxs)
CJJ: CJJ81176_0343(dxs)
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CJD: JJD26997_1642(dxs)
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CHA: CHAB381_1297(dxs)
CCO: CCC13826_1594(dxs)
ABU: Abu_2139(dxs)
NIS: NIS_0391(dxs)
SUN: SUN_2055(dxs)
GSU: GSU0686(dxs-1) GSU1764(dxs-2)
GME: Gmet_1934 Gmet_2822
PCA: Pcar_1667
PPD: Ppro_1191 Ppro_2403
DVU: DVU1350(dxs)
DVL: Dvul_1718
DDE: Dde_2200
LIP: LI0408(dsx)
DPS: DP2700
ADE: Adeh_1097
MXA: MXAN_4643(dxs)
SAT: SYN_02456
SFU: Sfum_1418
PUB: SAR11_0611(dxs)
MLO: mlr7474
MES: Meso_0735
SME: SMc00972(dxs)
ATU: Atu0745(dxs)
ATC: AGR_C_1351
RET: RHE_CH00913(dxs)
RLE: RL0973(dxs)
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BBT: BBta_2479(dxs)
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RPD: RPD_4305
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BBK: BARBAKC583_0400(dxs)
CCR: CC_2068
SIL: SPO0247(dxs)
SIT: TM1040_2920
RSP: RSP_0254(dxsA) RSP_1134(dxs)
JAN: Jann_0088 Jann_0170
RDE: RD1_0101(dxs) RD1_0548(dxs)
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MGM: Mmc1_1048
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BSU: BG11715(dxs)
BHA: BH2779
BAN: BA4400(dxs)
BAR: GBAA4400(dxs)
BAA: BA_4853
BAT: BAS4081
BCE: BC4176(dxs)
BCA: BCE_4249(dxs)
BCZ: BCZK3930(dxs)
BTK: BT9727_3919(dxs)
BTL: BALH_3785(dxs)
BLI: BL01523(dxs)
BLD: BLi02598(dxs)
BCL: ABC2462(dxs)
BAY: RBAM_022600
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LMF: LMOf2365_1382(dxs)
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LWE: lwe1380(tktB)
LLA: L108911(dxsA) L123365(dxsB)
LLC: LACR_1572 LACR_1843
LLM: llmg_0749(dxsB)
SAK: SAK_0263
LPL: lp_2610(dxs)
LJO: LJ0406
LAC: LBA0356
LSL: LSL_0209(dxs)
LGA: LGAS_0350
STH: STH1842
CAC: CAC2077 CA_P0106(dxs)
CPE: CPE1819
CPF: CPF_2073(dxs)
CPR: CPR_1787(dxs)
CTC: CTC01575
CNO: NT01CX_1983
CTH: Cthe_0828
CDF: CD1207(dxs)
CBO: CBO1881(dxs)
CBA: CLB_1818(dxs)
CBH: CLC_1825(dxs)
CBF: CLI_1945(dxs)
CKL: CKL_1231(dxs)
CHY: CHY_1985(dxs)
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DRM: Dred_1078
PTH: PTH_1196(dxs)
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CSC: Csac_1853
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MTC: MT2756(dxs)
MBO: Mb2701c(dxs1) Mb3413c(dxs2)
MLE: ML1038(dxs)
MPA: MAP2803c(dxs)
MAV: MAV_3577(dxs)
MSM: MSMEG_2776(dxs)
MMC: Mmcs_2208
CGL: NCgl1827(cgl1902)
CGB: cg2083(dxs)
CEF: CE1796
CDI: DIP1397(dxs)
CJK: jk1078(dxs)
NFA: nfa37410(dxs)
RHA: RHA1_ro06843
SCO: SCO6013(SC1C3.01) SCO6768(SC6A5.17)
SMA: SAV1646(dxs1) SAV2244(dxs2)
TWH: TWT484

TWS: TW280(Dxs)
LXX: Lxx10450(dxs)
CMI: CMM_1660(dxsA)
AAU: AAur_1790(dxs)
PAC: PPA1062
TFU: Tfu_1917
FRA: Francci3_1326
FAL: FRAAL2088(dxs)
ACE: Acel_1393
SEN: SACE_1815(dxs) SACE_4351
BLO: BL1132(dxs)
BAD: BAD_0513(dxs)
FNU: FN1208 FN1464
RBA: RB2143(dxs)
CTR: CT331(dxs)
CTA: CTA_0359(dxs)
CMU: TC0608
CPN: CPn1060(tktB_2)
CPA: CP0790
CPJ: CPj1060(tktB_2)
CPT: CpB1102
CCA: CCA00304(dxs)
CAB: CAB301(dxs)
CFE: CF0699(dxs)
PCU: pc0619(dxs)
TPA: TP0824
TDE: TDE1910(dxs)
LIL: LA3285(dxs)
LIC: LIC10863(dxs)
LBJ: LBJ_0917(dxs)
LBL: LBL_0932(dxs)
SYN: sll1945(dxs)
SYW: SYNW1292(Dxs)
SYC: syc1087_c(dxs)
SYF: Synpcc7942_0430
SYD: Syncc9605_1430
SYE: Syncc9902_1069
SYG: sync_1410(dxs)
SYR: SynRCC307_1390(dxs)
SYX: SynWH7803_1223(dxs)
CYA: CYA_1701(dxs)
CYB: CYB_1983(dxs)
TEL: tll0623
GVI: gll0194
ANA: alr0599
AVA: Ava_4532
PMA: Pro0928(dxs)
PMM: PMM0907(Dxs)
PMT: PMT0685(dxs)
PMN: PMN2A_0300
PMI: PMT9312_0893
PMB: A9601_09541(dxs)
PMC: P9515_09901(dxs)
PMF: P9303_15371(dxs)
PMG: P9301_09521(dxs)
PMH: P9215_09851
PMJ: P9211_08521
PME: NATL1_09721(dxs)
TER: Tery_3042
BTH: BT_1403 BT_4099
BFR: BF0873 BF4306
BFS: BF0796(dxs) BF4114
PGI: PG2217(dxs)
CHU: CHU_3643(dxs)
GFO: GFO_3470(dxs)
FPS: FP0279(dxs)
CTE: CT0337(dxs)
CPH: Cpha266_0671
PVI: Cvib_0498
PLT: Plut_0450
DET: DET0745(dxs)
DEH: cbdb_A720(dxs)
DRA: DR_1475
DGE: Dgeo_0994
TTH: TTC1614
TTJ: TTHA0006
AAE: aq_881
TMA: TM1770
PMO: Pmob_1001

Exemplary acetyl-CoA-acetyltransferase nucleic acids and polypeptides

HSA: 38(ACAT1) 39(ACAT2)
PTR: 451528(ACAT1)
MCC: 707653(ACAT1) 708750(ACAT2)
MMU: 110446(Acat1) 110460(Acat2)
RNO: 25014(Acat1)
CFA: 484063(ACAT2) 489421(ACAT1)
GGA: 418968(ACAT1) 421587(RCJMB04_34i5)
XLA: 379569(MGC69098) 414622(MGC81403)
414639(MGC81256)
444457(MGC83664)
XTR: 394562(acat2)
DRE: 30643(acat2)
SPU: 759502(LOC759502)
DME: Dmel_CG10932 Dmel_CG9149
CEL: T02G5.4 T02G5.7 T02G5.8(kat-1)
ATH: AT5G48230(ACAT2/EMB1276)
OSA: 4326136 4346520
CME: CMA042C CME087C
SCE: YPL028W(ERG10)
AGO: AGOS_ADR165C
PIC: PICST_31707(ERG10)
CAL: CaO19.1591(erg10)
CGR: CAGL0L12364g
SPO: SPBC215.09c
MGR: MGG_01755 MGG_13499
ANI: AN1409.2
AFM: AFUA_6G14200 AFUA_8G04000
AOR: AO090103000012 AO090103000406
CNE: CNC05280
UMA: UM03571.1
DDI: DDB_0231621
PFA: PF14_0484
TET: TTHERM_00091590 TTHERM_00277470
TTHERM_00926980
TCR: 511003.60
ECO: b2224(atoB)
ECJ: JW2218(atoB) JW5453(yqeF)
ECE: Z4164(yqeF)
ECS: ECs3701
ECC: c2767(atoB) c3441(yqeF)
ECI: UTI89_C2506(atoB) UTI89_C3247(yqeF)
ECP: ECP_2268 ECP_2857
ECV: APECO1_3662(yqeF) APECO1_4335(atoB)
APECO1_43352(atoB)
ECX: EcHS_A2365
STY: STY3164(yqeF)
STT: t2929(yqeF)
SPT: SPA2886(yqeF)
SEC: SC2958(yqeF)
STM: STM3019(yqeF)
SFL: SF2854(yqeF)
SFX: S3052(yqeF)
SFV: SFV_2922(yqeF)
SSN: SSON_2283(atoB) SSON_3004(yqeF)
SBO: SBO_2736(yqeF)
ECA: ECA1282(atoB)
ENT: Ent638_3299
SPE: Spro_0592
HIT: NTHI0932(atoB)
XCC: XCC1297(atoB)
XCB: XC_2943
XCV: XCV1401(thlA)
XAC: XAC1348(atoB)
XOO: XOO1881(atoB)
XOM: XOO_1778(XOO1778)
VCH: VCA0690
VCO: VC0395_0630
VVU: VV2_0494 VV2_0741
VVY: VVA1043 VVA1210
VPA: VPA0620 VPA1123 VPA1204
PPR: PBPRB1112 PBPRB1840
PAE: PA2001(atoB) PA2553 PA3454 PA3589
PA3925
PAU: PA14_38630(atoB)
PPU: PP_2051(atoB) PP_2215(fadAx) PP_3754
PP_4636
PPF: Pput_2009 Pput_2403 Pput_3523 Pput_4498
PST: PSPTO_0957(phbA-1) PSPTO_3164(phbA-2)
PSB: Psyr_0824 Psyr_3031
PSP: PSPPH_0850(phbA1) PSPPH_2209(phbA2)
PFL: PFL_1478(atoB-2) PFL_2321 PFL_3066
PFL_4330(atoB-2) PFL_5283
PFO: Pfl_1269 Pfl_1739 Pfl_2074 Pfl_2868
PEN: PSEEN3197 PSEEN3547(fadAx)
PSEEN4635(phbA)
PMY: Pmen_1138 Pmen_2036 Pmen_3597
Pmen_3662 Pmen_3820
PAR: Psyc_0252 Psyc_1169
PCR: Pcryo_0278 Pcryo_1236 Pcryo_1260
PRW: PsycPRwf_2011
ACI: ACIAD0694 ACIAD1612 ACIAD2516(atoB)
SON: SO_1677(atoB)
SDN: Sden_1943
SFR: Sfri_1338 Sfri_2063
SAZ: Sama_1375
SBL: Sbal_1495
SBM: Shew185_1489
SBN: Sbal195_1525

SLO: Shew_1667 Shew_2858
SPC: Sputcn32_1397
SSE: Ssed_1473 Ssed_3533
SPL: Spea_2783
SHE: Shewmr4_2597
SHM: Shewmr7_2664
SHN: Shewana3_2771
SHW: Sputw3181_2704
ILO: IL0872
CPS: CPS_1605 CPS_2626
PHA: PSHAa0908 PSHAa1454(atoB)
PSHAa1586(atoB)
PAT: Patl_2923
SDE: Sde_3149
PIN: Ping_0659 Ping_2401
MAQ: Maqu_2117 Maqu_2489 Maqu_2696
Maqu_3162
CBU: CBU_0974
LPN: lpg1825(atoB)
LPF: lpl1789
LPP: lpp1788
NOC: Noc_1891
AEH: Mlg_0688 Mlg_2706
HHA: Hhal_1685
HCH: HCH_05299
CSA: Csal_0301 Csal_3068
ABO: ABO_0648(fadAx)
MMW: Mmwyl1_0073 Mmwyl1_3021 Mmwyl1_3053
Mmwyl1_3097 Mmwyl1_4182
AHA: AHA_2143(atoB)
CVI: CV_2088(atoB) CV_2790(phaA)
RSO: RSc0276(atoB) RSc1632(phbA) RSc1637(bktB)
RSc1761(RS02948)
REU: Reut_A0138 Reut_A1348 Reut_A1353
Reut_B4561 Reut_B4738
Reut_B5587 Reut_C5943 Reut_C6062
REH: H16_A0170 H16_A0867 H16_A0868
H16_A0872 H16_A1297
H16_A1438(phaA) H16_A1445(bktB) H16_A1528
H16_A1713 H16_A1720
H16_A1887 H16_A2148 H16_B0380 H16_B0381
H16_B0406 H16_B0662
H16_B0668 H16_B0759 H16_B1369 H16_B1771
RME: Rmet_0106 Rmet_1357 Rmet_1362 Rmet_5156
BMA: BMA1316 BMA1321(phbA) BMA1436
BMV: BMASAVP1_A1805(bktB)
BMASAVP1_A1810(phbA)
BML: BMA10299_A0086(phbA) BMA10299_A0091
BMN: BMA10247_1076(bktB)
BMA10247_1081(phbA)
BXE: Bxe_A2273 Bxe_A2335 Bxe_A2342
Bxe_A4255 Bxe_B0377 Bxe_B0739
Bxe_C0332 Bxe_C0574 Bxe_C0915
BVI: Bcep1808_0519 Bcep1808_1717
Bcep1808_2877 Bcep1808_3594
Bcep1808_4015 Bcep1808_5507 Bcep1808_5644
BUR: Bcep18194_A3629 Bcep18194_A5080
Bcep18194_A5091
Bcep18194_A6102 Bcep18194_B0263
Bcep18194_B1439
Bcep18194_C6652 Bcep18194_C6802
Bcep18194_C6874
Bcep18194_C7118 Bcep18194_C7151
Bcep18194_C7332
BCN: Bcen_1553 Bcen_1599 Bcen_2158 Bcen_2563
Bcen_2998 Bcen_6289
BCH: Bcen2424_0542 Bcen2424_1790
Bcen2424_2772 Bcen2424_5368
Bcen2424_6232 Bcen2424_6276
BAM: Bamb_0447 Bamb_1728 Bamb_2824
Bamb_4717 Bamb_5771 Bamb_5969
BPS: BPSL1426 BPSL1535(phbA) BPSL1540
BPM: BURPS1710b_2325(bktB)
BURPS1710b_2330(phbA)
BURPS1710b_2453(atoB-2)
BPL: BURPS1106A_2197(bktB)
BURPS1106A_2202(phbA)
BPD: BURPS668_2160(bktB) BURPS668_2165(phbA)
BTE: BTH_I2144 BTH_I2256 BTH_I2261
PNU: Pnuc_0927
BPE: BP0447 BP0668 BP2059
BPA: BPP0608 BPP1744 BPP3805 BPP4216
BPP4361
BBR: BB0614 BB3364 BB4250 BB4804 BB4947
RFR: Rfer_0272 Rfer_1000 Rfer_1871 Rfer_2273
Rfer_2561 Rfer_2594
Rfer_3839
POL: Bpro_1577 Bpro_2140 Bpro_3113 Bpro_4187
PNA: Pnap_0060 Pnap_0458 Pnap_0867 Pnap_1159
Pnap_2136 Pnap_2804
AAV: Aave_0031 Aave_2478 Aave_3944 Aave_4368
AJS: Ajs_0014 Ajs_0124 Ajs_1931 Ajs_2073
Ajs_2317 Ajs_3548
Ajs_3738 Ajs_3776
VEI: Veis_1331 Veis_3818 Veis_4193
DAC: Daci_0025 Daci_0192 Daci_3601 Daci_5988
MPT: Mpe_A1536 Mpe_A1776 Mpe_A1869
Mpe_A3367
HAR: HEAR0577(phbA)
MMS: mma_0555
NEU: NE2262(bktB)

NET: Neut_0610
EBA: ebA5202 p2A409(tioL)
AZO: azo0464(fadA1) azo0469(fadA2) azo2172(thlA)
DAR: Daro_0098 Daro_3022
HPA: HPAG1_0675
HAC: Hac_0958(atoB)
GME: Gmet_1719 Gmet_2074 Gmet_2213
Gmet_2268 Gmet_3302
GUR: Gura_3043
BBA: Bd0404(atoB) Bd2095
DOL: Dole_0671 Dole_1778 Dole_2160 Dole_2187
ADE: Adeh_0062 Adeh_2365
AFW: Anae109_0064 Anae109_1504
MXA: MXAN_3791
SAT: SYN_02642
SFU: Sfum_2280 Sfum_3582
RPR: RP737
RCO: RC1134 RC1135
RFE: RF_0163(paaJ)
RBE: RBE_0139(paaJ)
RAK: A1C_05820
RBO: A1I_07215
RCM: A1E_04760
PUB: SAR11_0428(thlA)
MLO: mlr3847
MES: Meso_3374
PLA: Plav_1573 Plav_2783
SME: SMa1450 SMc03879(phbA)
SMD: Smed_0499 Smed_3117 Smed_5094
Smed_5096
ATU: Atu2769(atoB) Atu3475
ATC: AGR_C_5022(phbA) AGR_L_2713
RET: RHE_CH04018(phbAch)
RHE_PC00068(ypc00040) RHE_PF00014(phbAf)
RLE: RL4621(phaA) pRL100301 pRL120369
BME: BMEI0274 BMEII0817
BMF: BAB1_1783(phbA-1) BAB2_0790(phbA-2)
BMS: BR1772(phbA-1) BRA0448(phbA-2)
BMB: BruAb1_1756(phbA-1) BruAb2_0774(phbA-2)
BOV: BOV_1707(phbA-1)
OAN: Oant_1130 Oant_3107 Oant_3718 Oant_4020
BJA: bll0226(atoB) bll3949 bll7400 bll7819
blr3724(phbA)
BRA: BRADO0562(phbA) BRADO0983(pimB)
BRADO3110 BRADO3134(atoB)
BBT: BBta_3558 BBta_3575(atoB) BBta_5147(pimB)
BBta_7072(pimB)
BBta_7614(phbA)
RPA: RPA0513(pcaF) RPA0531 RPA3715(pimB)
RPB: RPB_0509 RPB_0525 RPB_1748
RPC: RPC_0504 RPC_0636 RPC_0641 RPC_0832
RPC_1050 RPC_2005
RPC_2194 RPC_2228
RPD: RPD_0306 RPD_0320 RPD_3105 RPD_3306
RPE: RPE_0168 RPE_0248 RPE_3827
NWI: Nwi_3060
XAU: Xaut_3108 Xaut_4665
CCR: CC_0510 CC_0894 CC_3462
SIL: SPO0142(bktB) SPO0326(phbA) SPO0773
SPO3408
SIT: TM1040_0067 TM1040_2790 TM1040_3026
TM1040_3735
RSP: RSP_0745 RSP_1354 RSP_3184
RSH: Rsph17029_0022 Rsph17029_2401
Rsph17029_3179 Rsph17029_3921
RSQ: Rsph17025_0012 Rsph17025_2466
Rsph17025_2833
JAN: Jann_0262 Jann_0493 Jann_4050
RDE: RD1_0025 RD1_0201(bktB) RD1_3394(phbA)
PDE: Pden_2026 Pden_2663 Pden_2870 Pden_2907
Pden_4811 Pden_5022
DSH: Dshi_0074 Dshi_3066 Dshi_3331
MMR: Mmar10_0697
HNE: HNE_2706 HNE_3065 HNE_3133
NAR: Saro_0809 Saro_1069 Saro_1222 Saro_2306
Saro_2349
SAL: Sala_0781 Sala_1244 Sala_2896 Sala_3158
SWI: Swit_0632 Swit_0752 Swit_2893 Swit_3602
Swit_4887 Swit_5019
Swit_5309
ELI: ELI_01475 ELI_06705 ELI_12035
GBE: GbCGDNIH1_0447
ACR: Acry_1847 Acry_2256
RRU: Rru_A0274 Rru_A1380 Rru_A1469 Rru_A1946
Rru_A3387
MAG: amb0842
MGM: Mmc1_1165
ABA: Acid345_3239
BSU: BG11319(mmgA) BG13063(yhfS)
BHA: BH1997 BH2029 BH3801(mmgA)
BAN: BA3687 BA4240 BA5589
BAR: GBAA3687 GBAA4240 GBAA5589
BAA: BA_0445 BA_4172 BA_4700
BAT: BAS3418 BAS3932 BAS5193
BCE: BC3627 BC4023 BC5344
BCA: BCE_3646 BCE_4076 BCE_5475
BCZ: BCZK3329(mmgA) BCZK3780(thl)
BCZK5044(atoB)
BCY: Bcer98_2722 Bcer98_3865
BTK: BT9727_3379(mmgA) BT9727_3765(thl)
BT9727_5028(atoB)

BTL: BALH_3262(mmgA) BALH_3642(fadA) BALH_4843(atoB)
BLI: BL03925(mmgA)
BLD: BLi03968(mmgA)
BCL: ABC0345 ABC2989 ABC3617 ABC3891(mmgA)
BAY: RBAM_022450
BPU: BPUM_2374(yhfS) BPUM_2941 BPUM_3373
OIH: OB0676 OB0689 OB2632 OB3013
GKA: GK1658 GK3397
SAU: SA0342 SA0534(vraB)
SAV: SAV0354 SAV0576(vraB)
SAM: MW0330 MW0531(vraB)
SAR: SAR0351(thl) SAR0581
SAS: SAS0330 SAS0534
SAC: SACOL0426 SACOL0622(atoB)
SAB: SAB0304(th1) SAB0526
SAA: SAUSA300_0355 SAUSA300_0560(vraB)
SAO: SAOUHSC_00336 SAOUHSC_00558
SAJ: SaurJH9_0402
SAH: SaurJH1_0412
SEP: SE0346 SE2384
SER: SERP0032 SERP0220
SHA: SH0510(mvaC) SH2417
SSP: SSP0325 SSP2145
LMO: lmo1414
LMF: LMOf2365_1433
LIN: lin1453
LWE: lwe1431
LLA: L11745(thiL) L25946(fadA)
LLC: LACR_1665 LACR_1956
LLM: llmg_0930(thiL)
SPY: SPy_0140 SPy_1637(atoB)
SPZ: M5005_Spy_0119 M5005_Spy_0432 M5005_Spy_1344(atoB)
SPM: spyM18_0136 spyM18_1645(atoB)
SPG: SpyM3_0108 SpyM3_1378(atoB)
SPS: SPs0110 SPs0484
SPH: MGAS10270_Spy0121 MGAS10270_Spy0433 MGAS10270_Spy1461(atoB)
SPI: MGAS10750_Spy0124 MGAS10750_Spy0452 MGAS10750_Spy1453(atoB)
SPJ: MGAS2096_Spy0123 MGAS2096_Spy0451 MGAS2096_Spy1365(atoB)
SPK: MGAS9429_Spy0121 MGAS9429_Spy0431 MGAS9429_Spy1339(atoB)
SPF: SpyM50447(atoB2)
SPA: M6_Spy0166 M6_Spy0466 M6_Spy1390
SPB: M28_Spy0117 M28_Spy0420 M28_Spy1385(atoB)
SAK: SAK_0568
LJO: LJ1609
LAC: LBA0626(thiL)
LSA: LSA1486
LDB: Ldb0879
LBU: LBUL_0804
LBR: LVIS_2218
LCA: LSEI_1787
LGA: LGAS_1374
LRE: Lreu_0052
EFA: EF1364
OOE: OEOE_0529
STH: STH2913 STH725 STH804
CAC: CAC2873 CA_P0078(thiL)
CPE: CPE2195(atoB)
CPF: CPF_2460
CPR: CPR_2170
CTC: CTC00312
CNO: NT01CX_0538 NT01CX_0603
CDF: CD1059(thlA1) CD2676(thlA2)
CBO: CBO3200(thl)
CBE: Cbei_0411 Cbei_3630
CKL: CKL_3696(thlA1) CKL_3697(thlA2) CKL_3698(thlA3)
AMT: Amet_4630
AOE: Clos_0084 Clos_0258
CHY: CHY_1288 CHY_1355(atoB) CHY_1604 CHY_1738
DSY: DSY0632 DSY0639 DSY1567 DSY1710 DSY2402 DSY3302
DRM: Dred_0400 Dred_1491 Dred_1784 Dred_1892
SWO: Swol_0308 Swol_0675 Swol_0789 Swol_1486 Swol_1934 Swol_2051
TTE: TTE0549(paaJ)
MTA: Moth_1260
MTU: Rv1135A Rv1323(fadA4) Rv3546(fadA5)
MTC: MT1365(phbA)
MBO: Mb1167 Mb1358(fadA4) Mb3576(fadA5) Mb3586c(fadA6)
MBB: BCG_1197 BCG_1385(fadA4) BCG_3610(fadA5) BCG_3620c(fadA6)
MLE: ML1158(fadA4)
MPA: MAP2407c(fadA3) MAP2436c(fadA4)
MAV: MAV_1544 MAV_1573 MAV_1863 MAV_5081
MSM: MSMEG_2224 MSMEG_4920
MUL: MUL_0357
MVA: Mvan_1976 Mvan_1988 Mvan_4305 Mvan_4677 Mvan_4891
MGI: Mflv_1347 Mflv_1484 Mflv_2040 Mflv_2340 Mflv_4356 Mflv_4368

MMC: Mmcs_1758 Mmcs_1769 Mmcs_3796
Mmcs_3864
MKM: Mkms_0251 Mkms_1540 Mkms_1805
Mkms_1816 Mkms_2836 Mkms_3159
Mkms_3286 Mkms_3869 Mkms_3938 Mkms_4227
Mkms_4411 Mkms_4580
Mkms_4724 Mkms_4764 Mkms_4776
MJL: Mjls_0231 Mjls_1739 Mjls_1750 Mjls_2819
Mjls_3119 Mjls_3235
Mjls_3800 Mjls_3850 Mjls_4110 Mjls_4383
Mjls_4705 Mjls_4876
Mjls_5018 Mjls_5063 Mjls_5075
CGL: NCgl2309(cgl2392)
CGB: cg2625(pcaF)
CEF: CE0731 CE2295
CJK: jk1543(fadA3)
NFA: nfa10750(fadA4)
RHA: RHA1_ro01455 RHA1_ro01623
RHA1_ro01876 RHA1_ro02517(catF)
RHA1_ro03022 RHA1_ro03024 RHA1_ro03391
RHA1_ro03892
RHA1_ro04599 RHA1_ro05257 RHA1_ro08871
SCO: SCO5399(SC8F4.03)
SMA: SAV1384(fadA5) SAV2856(fadA1)
ART: Arth_1160 Arth_2986 Arth_3268 Arth_4073
NCA: Noca_1371 Noca_1797 Noca_1828 Noca_2764
Noca_4142
TFU: Tfu_1520 Tfu_2394
FRA: Francci3_3687
FRE: Franean1_1044 Franean1_2711 Franean1_2726
Franean1_3929
Franean1_4037 Franean1_4577
FAL: FRAAL2514 FRAAL2618 FRAAL5910(atoB)
ACE: Acel_0626 Acel_0672
SEN: SACE_1192(mmgA) SACE_2736(fadA6)
SACE_4011(catF)
SACE_6236(fadA4)
STP: Strop_3610
SAQ: Sare_1316 Sare_3991
RXY: Rxyl_1582 Rxyl_1842 Rxyl_2389 Rxyl_2530
FNU: FN0495
BGA: BG0110(fadA)
BAF: BAPKO_0110(fadA)
LIL: LA0457(thiL1) LA0828(thiL2) LA4139(fadA)
LIC: LIC10396(phbA)
LBJ: LBJ_2862(paaJ-4)
LBL: LBL_0209(paaJ-4)
SYN: slr1993(phaA)
SRU: SRU_1211(atoB) SRU_1547
CHU: CHU_1910(atoB)
GFO: GFO_1507(atoB)
FJO: Fjoh_4612
FPS: FP0770 FP1586 FP1725
RRS: RoseRS_3911 RoseRS_4348
RCA: Rcas_0702 Rcas_3206
HAU: Haur_0522
DRA: DR_1072 DR_1428 DR_1960 DR_2480
DR_A0053
DGE: Dgeo_0755 Dgeo_1305 Dgeo_1441 Dgeo_1883
TTH: TTC0191 TTC0330
TTJ: TTHA0559
TME: Tmel_1134
FNO: Fnod_0314
PMO: Pmob_0515
HMA: rrnAC0896(acaB3) rrnAC2815(aca2)
rrnAC3497(yqeF)
rrnB0240(aca1) rrnB0242(acaB2) rrnB0309(acaB1)
TAC: Ta0582
TVO: TVN0649
PTO: PTO1505
APE: APE_2108
SSO: SSO2377(acaB-4)
STO: ST0514
SAI: Saci_0963 Saci_1361(acaB1)
MSE: Msed_0656
PAI: PAE1220
PIS: Pisl_0029 Pisl_1301
PCL: Pcal_0781
PAS: Pars_0309 Pars_1071
CMA: Cmaq_1941

Exemplary HMG-CoA synthase nucleic acids and polypeptides

HSA: 3157(HMGCS1) 3158(HMGCS2)

PTR: 457169(HMGCS2) 461892(HMGCS1)
MCC: 702553(HMGCS1) 713541(HMGCS2)
MMU: 15360(Hmgcs2) 208715(Hmgcs1)
RNO: 24450(Hmgcs2) 29637(Hmgcs1)
CFA: 479344(HMGCS1) 607923(HMGCS2)
BTA: 407767(HMGCS1)
SSC: 397673(CH242-38B5.1)
GGA: 396379(HMGCS1)
XLA: 380091(hmgcs1) 447204(MGC80816)
DRE: 394060(hmgcs1)
SPU: 578259(LOC578259)
DME: Dmel_CG4311(Hmgs)
CEL: F25B4.6
ATH: AT4G11820(BAP1)
OSA: 4331418 4347614
CME: CMM189C
SCE: YML126C(ERG13)
AGO: AGOS_ADL356C
PIC: PICST_83020
CAL: CaO19_7312(CaO19.7312)
CGR: CAGL0H04081g
SPO: SPAC4F8.14c(hcs)
MGR: MGG_01026
ANI: AN4923.2
AFM: AFUA_3G10660 AFUA_8G07210
AOR: AO090003000611 AO090010000487
CNE: CNC05080 CNG02670
UMA: UM05362.1
ECU: ECU10_0510
DDI: DDBDRAFT_0217522 DDB_0219924(hgsA)
TET: TTHERM_00691190
TBR: Tb927.8.6110
YPE: YPO1457
YPK: y2712(pksG)
YPM: YP_1349(pksG)
YPA: YPA_0750
YPN: YPN_2521
YPP: YPDSF_1517
YPS: YPTB1475
CBD: COXBU7E912_1931
TCX: Tcr_1719
DNO: DNO_0799
BMA: BMAA1212
BPS: BPSS1002
BPM: BURPS1710b_A2613
BPL: BURPS1106A_A1384
BPD: BURPS668_A1470
BTE: BTH_II1670

MXA: MXAN_3948(tac) MXAN_4267(mvaS)
BSU: BG10926(pksG)
OIH: OB2248
SAU: SA2334(mvaS)
SAV: SAV2546(mvaS)
SAM: MW2467(mvaS)
SAR: SAR2626(mvaS)
SAS: SAS2432
SAC: SACOL2561
SAB: SAB2420(mvaS)
SAA: SAUSA300_2484
SAO: SAOUHSC_02860
SAJ: SaurJH9_2569
SAH: SaurJH1_2622
SEP: SE2110
SER: SERP2122
SHA: SH0508(mvaS)
SSP: SSP0324
LMO: lmo1415
LMF: LMOf2365_1434(mvaS)
LIN: lin1454
LWE: lwe1432(mvaS)
LLA: L13187(hmcM)
LLC: LACR_1666
LLM: llmg_0929(hmcM)
SPY: SPy_0881(mvaS.2)
SPZ: M5005_Spy_0687(mvaS.1)
SPM: spyM18_0942(mvaS2)
SPG: SpyM3_0600(mvaS.2)
SPS: SPs1253
SPH: MGAS10270_Spy0745(mvaS1)
SPI: MGAS10750_Spy0779(mvaS1)
SPJ: MGAS2096_Spy0759(mvaS1)
SPK: MGAS9429_Spy0743(mvaS1)
SPF: SpyM51121(mvaS)
SPA: M6_Spy0704
SPB: M28_Spy0667(mvaS.1)
SPN: SP_1727
SPR: spr1571(mvaS)
SPD: SPD_1537(mvaS)
SAG: SAG1316
SAN: gbs1386
SAK: SAK_1347
SMU: SMU.943c
STC: str0577(mvaS)
STL: stu0577(mvaS)
STE: STER_0621
SSA: SSA_0338(mvaS)
SSU: SSU05_1641

SSV: SSU98_1652
SGO: SGO_0244
LPL: lp_2067(mvaS)
LJO: LJ1607
LAC: LBA0628(hmcS)
LSA: LSA1484(mvaS)
LSL: LSL_0526
LDB: Ldb0881(mvaS)
LBU: LBUL_0806
LBR: LVIS_1363
LCA: LSEI_1785
LGA: LGAS_1372
LRE: Lreu_0676
PPE: PEPE_0868
EFA: EF1363

OOE: OEOE_0968
LME: LEUM_1184
NFA: nfa22120
SEN: SACE_4570(pksG)
BBU: BB0683
BGA: BG0706
BAF: BAPKO_0727
FJO: Fjoh_0678
HAL: VNG1615G(mvaB)
HMA: rrnAC1740(mvaS)
HWA: HQ2868A(mvaB)
NPH: NP2608A(mvaB_1) NP4836A(mvaB_2)

Exemplary hydroxymethylglutaryl-CoA reductase nucleic acids and polypeptides

HSA: 3156(HMGCR)
PTR: 471516(HMGCR)
MCC: 705479(HMGCR)
MMU: 15357(Hmgcr)
RNO: 25675(Hmgcr)
CFA: 479182(HMGCR)
BTA: 407159(HMGCR)
GGA: 395145(RCJMB04_14m24)
SPU: 373355(LOC373355)
DME: Dmel_CG10367(Hmgcr)
CEL: F08F8.2
OSA: 4347443
SCE: YLR450W(HMG2) YML075C(HMG1)
AGO: AGOS_AER152W
CGR: CAGL0L11506g
SPO: SPCC162.09c(hmg1)
ANI: AN3817.2
AFM: AFUA_1G11230 AFUA_2G03700
AOR: AO090103000311 AO090120000217
CNE: CNF04830
UMA: UM03014.1
ECU: ECU10_1720
DDI: DDB_0191125(hmgA) DDB_0215357(hmgB)
TBR: Tb927.6.4540
TCR: 506831.40 509167.20
LMA: LmjF30.3190
VCH: VCA0723
VCO: VC0395_0662
VVU: VV2_0117
VVY: VVA0625
VPA: VPA0968
VFI: VFA0841

PAT: Patl_0427
CBU: CBU_0030 CBU_0610
CBD: COXBU7E912_0151
COXBU7E912_0622(hmgA)
TCX: Tcr_1717
DNO: DNO_0797
CVI: CV_1806
SUS: Acid_5728 Acid_6132
SAU: SA2333(mvaA)
SAV: SAV2545(mvaA)
SAM: MW2466(mvaA)
SAB: SAB2419c(mvaA)
SEP: SE2109
LWE: lwe0819(mvaA)
LLA: L10433(mvaA)
LLC: LACR_1664
LLM: llmg_0931(mvaA)
SPY: SPy_0880(mvaS.1)
SPM: spyM18_0941(mvaS1)
SPG: SpyM3_0599(mvaS.1)
SPS: SPs1254
SPH: MGAS10270_Spy0744
SPI: MGAS10750_Spy0778
SPJ: MGAS2096_Spy0758
SPK: MGAS9429_Spy0742
SPA: M6_Spy0703
SPN: SP_1726
SAG: SAG1317
SAN: gbs1387
STC: str0576(mvaA)
STL: stu0576(mvaA)
STE: STER_0620

SSA: SSA_0337(mvaA)
LPL: lp_0447(mvaA)
LJO: LJ1608
LSL: LSL_0224
LBR: LVIS_0450
LGA: LGAS_1373
EFA: EF1364
NFA: nfa22110
BGA: BG0708(mvaA)
SRU: SRU_2422
FPS: FP2341
MMP: MMP0087(hmgA)
MMQ: MmarC5_1589
MAC: MA3073(hmgA)
MBA: Mbar_A1972
MMA: MM_0335
MBU: Mbur_1098
MHU: Mhun_3004
MEM: Memar_2365
MBN: Mboo_0137
MTH: MTH562
MST: Msp_0584(hmgA)
MSI: Msm_0227
MKA: MK0355(HMG1)
AFU: AF1736(mvaA)
HAL: VNG1875G(mvaA)
HMA: rrnAC3412(mvaA)
HWA: HQ3215A(hmgR)
NPH: NP0368A(mvaA_2) NP2422A(mvaA_1)
TAC: Ta0406m
TVO: TVN1168
PTO: PTO1143
PAB: PAB2106(mvaA)
PFU: PF1848
TKO: TK0914
RCI: RCIX1027(hmgA) RCIX376(hmgA)
APE: APE_1869
IHO: Igni_0476
HBU: Hbut_1531
SSO: SSO0531
STO: ST1352
SAI: Saci_1359
PAI: PAE2182
PIS: Pisl_0814
PCL: Pcal_1085
PAS: Pars_0796

Exemplary mevalonate kinase nucleic acids and polypeptides

HSA: 4598(MVK)
MCC: 707645(MVK)
MMU: 17855(Mvk)
RNO: 81727(Mvk)
CFA: 486309(MVK)
BTA: 505792(MVK)
GGA: 768555(MVK)
DRE: 492477(zgc:103473)
SPU: 585785(LOC585785)
DME: Dmel_CG33671
OSA: 4348331
SCE: YMR208W(ERG12)
AGO: AGOS_AER335W
PIC: PICST_40742(ERG12)
CGR: CAGL0F03861g
SPO: SPAC13G6.11c
MGR: MGG_06946
ANI: AN3869.2
AFM: AFUA_4G07780
AOR: AO090023000793
CNE: CNK01740
ECU: ECU09_1780
DDI: DDBDRAFT_0168621
TET: TTHERM_00637680
TBR: Tb927.4.4070
TCR: 436521.9 509237.10
LMA: LmjF31.0560
CBU: CBU_0608 CBU_0609
CBD: COXBU7E912_0620(mvk)
LPN: lpg2039
LPF: lpl2017
LPP: lpp2022
BBA: Bd1027(lmbP) Bd1630(mvk)
MXA: MXAN_5019(mvk)
OIH: OB0225
SAU: SA0547(mvaK1)
SAV: SAV0590(mvaK1)
SAM: MW0545(mvaK1)
SAR: SAR0596(mvaK1)
SAS: SAS0549
SAC: SACOL0636(mvk)
SAB: SAB0540(mvaK1)
SAA: SAUSA300_0572(mvk)
SAO: SAOUHSC_00577
SEP: SE0361
SER: SERP0238(mvk)

SHA: SH2402(mvaK1)
SSP: SSP2122
LMO: lmo0010
LMF: LMOf2365_0011
LIN: lin0010
LWE: lwe0011(mvk)
LLA: L7866(yeaG)
LLC: LACR_0454
LLM: llmg_0425(mvk)
SPY: SPy_0876(mvaK1)
SPZ: M5005_Spy_0682(mvaK1)
SPM: spyM18_0937(mvaK1)
SPG: SpyM3_0595(mvaK1)
SPS: SPs1258
SPH: MGAS10270_Spy0740(mvaK1)
SPI: MGAS10750_Spy0774(mvaK1)
SPJ: MGAS2096_Spy0753(mvaK1)
SPK: MGAS9429_Spy0737(mvaK1)
SPF: SpyM51126(mvaK1)
SPA: M6_Spy0699
SPB: M28_Spy0662(mvaK1)
SPN: SP_0381
SPR: spr0338(mvk)
SPD: SPD_0346(mvk)
SAG: SAG1326
SAN: gbs1396
SAK: SAK_1357(mvk)
SMU: SMU.181
STC: str0559(mvaK1)
STL: stu0559(mvaK1)
STE: STER_0598
SSA: SSA_0333(mvaK1)
SSU: SSU05_0289
SSV: SSU98_0285
SGO: SGO_0239(mvk)
LPL: lp_1735(mvaK1)
LJO: LJ1205
LAC: LBA1167(mvaK)
LSA: LSA0908(mvaK1)
LSL: LSL_0685(eRG)
LDB: Ldb0999(mvk)
LBU: LBUL_0906
LBR: LVIS_0858
LCA: LSEI_1491
LGA: LGAS_1033
LRE: Lreu_0915
PPE: PEPE_0927
EFA: EF0904(mvk)
OOE: OEOE_1100
LME: LEUM_1385
NFA: nfa22070
BGA: BG0711
BAF: BAPKO_0732
FPS: FP0313
MMP: MMP1335
MAE: Maeo_0775
MAC: MA0602(mvk)
MBA: Mbar_A1421
MMA: MM_1762
MBU: Mbur_2395
MHU: Mhun_2890
MEM: Memar_1812
MBN: Mboo_2213
MST: Msp_0858(mvk)
MSI: Msm_1439
MKA: MK0993(ERG12)
HAL: VNG1145G(mvk)
HMA: rrnAC0077(mvk)
HWA: HQ2925A(mvk)
NPH: NP2850A(mvk)
PTO: PTO1352
PHO: PH1625
PAB: PAB0372(mvk)
PFU: PF1637(mvk)
TKO: TK1474
RCI: LRC399(mvk)
APE: APE_2439
HBU: Hbut_0877
SSO: SSO0383
STO: ST2185
SAI: Saci_2365(mvk)
MSE: Msed_1602
PAI: PAE3108
PIS: Pisl_0467
PCL: Pcal_1835

Exemplary phosphomevalonate kinase nucleic acids and polypeptides

HSA: 10654(PMVK)
PTR: 457350(PMVK)
MCC: 717014(PMVK)
MMU: 68603(Pmvk)
CFA: 612251(PMVK)
BTA: 513533(PMVK)
DME: Dmel_CG10268
ATH: AT1G31910

OSA: 4332275
SCE: YMR220W(ERG8)
AGO: AGOS_AER354W
PIC: PICST_52257(ERG8)
CGR: CAGL0F03993g
SPO: SPAC343.01c
MGR: MGG_05812
ANI: AN2311.2
AFM: AFUA_5G10680
AOR: AO090010000471
CNE: CNM00100
UMA: UM00760.1
DDI: DDBDRAFT_0184512
TBR: Tb09.160.3690
TCR: 507913.20 508277.140
LMA: LmjF15.1460
MXA: MXAN_5017
OIH: OB0227
SAU: SA0549(mvaK2)
SAV: SAV0592(mvaK2)
SAM: MW0547(mvaK2)
SAR: SAR0598(mvaK2)
SAS: SAS0551
SAC: SACOL0638
SAB: SAB0542(mvaK2)
SAA: SAUSA300_0574
SAO: SAOUHSC_00579
SAJ: SaurJH9_0615
SEP: SE0363
SER: SERP0240
SHA: SH2400(mvaK2)
SSP: SSP2120
LMO: lmo0012
LMF: LMOf2365_0013
LIN: lin0012
LWE: lwe0013
LLA: L10014(yebA)
LLC: LACR_0456
LLM: llmg_0427
SPY: SPy_0878(mvaK2)
SPZ: M5005_Spy_0684(mvaK2)
SPM: spyM18_0939
SPG: SpyM3_0597(mvaK2)
SPS: SPs1256
SPH: MGAS10270_Spy0742(mvaK2)
SPI: MGAS10750_Spy0776(mvaK2)
SPJ: MGAS2096_Spy0755(mvaK2)
SPK: MGAS9429_Spy0739(mvaK2)
SPF: SpyM51124(mvaK2)
SPA: M6_Spy0701
SPB: M28_Spy0664(mvaK2)
SPN: SP_0383
SPR: spr0340(mvaK2)
SPD: SPD_0348(mvaK2)
SAG: SAG1324
SAN: gbs1394
SAK: SAK_1355
SMU: SMU.938
STC: str0561(mvaK2)
STL: stu0561(mvaK2)
STE: STER_0600
SSA: SSA_0335(mvaK2)
SSU: SSU05_0291
SSV: SSU98_0287
SGO: SGO_0241
LPL: lp_1733(mvaK2)
LJO: LJ1207
LAC: LBA1169
LSA: LSA0906(mvaK2)
LSL: LSL_0683
LDB: Ldb0997(mvaK)
LBU: LBUL_0904
LBR: LVIS_0860
LCA: LSEI_1092
LGA: LGAS_1035
LRE: Lreu_0913
PPE: PEPE_0925
EFA: EF0902
NFA: nfa22090
BGA: BG0710
BAF: BAPKO_0731
NPH: NP2852A
SSO: SSO2988
STO: ST0978
SAI: Saci_1244

Exemplary diphosphomevalonate decarboxylase nucleic acids and polypeptides

HSA: 4597(MVD)
PTR: 468069(MVD)
MCC: 696865(MVD)
MMU: 192156(Mvd)
RNO: 81726(Mvd)
CFA: 489663(MVD)
GGA: 425359(MVD)
DME: Dmel_CG8239
SCE: YNR043W(MVD1)
AGO: AGOS_AGL232C

PIC: PICST_90752
CGR: CAGL0C03630g
SPO: SPAC24C9.03
MGR: MGG_09750
ANI: AN4414.2
AFM: AFUA_4G07130
AOR: AO090023000862
CNE: CNL04950
UMA: UM05179.1
DDI: DDBDRAFT_0218058
TET: TTHERM_00849200
TBR: Tb10.05.0010 Tb10.61.2745
TCR: 507993.330 511281.40
LMA: LmjF18.0020
CBU: CBU_0607(mvaD)
CBD: COXBU7E912_0619(mvaD)
LPN: lpg2040
LPF: lpl2018
LPP: lpp2023
TCX: Tcr_1734
DNO: DNO_0504(mvaD)
BBA: Bd1629
MXA: MXAN_5018(mvaD)
OIH: OB0226
SAU: SA0548(mvaD)
SAV: SAV0591(mvaD)
SAM: MW0546(mvaD)
SAR: SAR0597(mvaD)
SAS: SAS0550
SAC: SACOL0637(mvaD)
SAB: SAB0541(mvaD)
SAA: SAUSA300_0573(mvaD)
SAO: SAOUHSC_00578
SAJ: SaurJH9_0614
SAH: SaurJH1_0629
SEP: SE0362
SER: SERP0239(mvaD)
SHA: SH2401(mvaD)
SSP: SSP2121
LMO: lmo0011
LMF: LMOf2365_0012(mvaD)
LIN: lin0011
LWE: lwe0012(mvaD)
LLA: L9089(yeaH)
LLC: LACR_0455
LLM: llmg_0426(mvaD)
SPY: SPy_0877(mvaD)
SPZ: M5005_Spy_0683(mvaD)
SPM: spyM18_0938(mvd)
SPG: SpyM3_0596(mvaD)
SPS: SPs1257
SPH: MGAS10270_Spy0741(mvaD)
SPI: MGAS10750_Spy0775(mvaD)
SPJ: MGAS2096_Spy0754(mvaD)
SPK: MGAS9429_Spy0738(mvaD)
SPF: SpyM51125(mvaD)
SPA: M6_Spy0700
SPB: M28_Spy0663(mvaD)
SPN: SP_0382
SPR: spr0339(mvd1)
SPD: SPD_0347(mvaD)
SAG: SAG1325(mvaD)
SAN: gbs1395
SAK: SAK_1356(mvaD)
SMU: SMU.937
STC: str0560(mvaD)
STL: stu0560(mvaD)
STE: STER_0599
SSA: SSA_0334(mvaD)
SSU: SSU05_0290
SSV: SSU98_0286
SGO: SGO_0240(mvaD)
LPL: lp_1734(mvaD)
LJO: LJ1206
LAC: LBA1168(mvaD)
LSA: LSA0907(mvaD)
LSL: LSL_0684
LDB: Ldb0998(mvaD)
LBU: LBUL_0905
LBR: LVIS_0859
LCA: LSEI_1492
LGA: LGAS_1034
LRE: Lreu_0914
PPE: PEPE_0926
EFA: EF0903(mvaD)
LME: LEUM_1386
NFA: nfa22080
BBU: BB0686
BGA: BG0709
BAF: BAPKO_0730
GFO: GFO_3632
FPS: FP0310(mvaD)
HAU: Haur_1612
HAL: VNG0593G(dmd)
HMA: rrnAC1489(dmd)
HWA: HQ1525A(mvaD)
NPH: NP1580A(mvaD)
PTO: PTO0478 PTO1356
SSO: SSO2989
STO: ST0977
SAI: Saci_1245(mvd)
MSE: Msed_1576

Exemplary isopentenyl phosphate kinases (IPK) nucleic acids and polypeptides

Methanobacterium thermoautotrophicum gi|2621082
Methanococcus jannaschii DSM 2661 gi|1590842 ; *Methanocaldococcus jannaschii* gi|1590842
Methanothermobacter thermautotrophicus gi|2621082
Picrophilus torridus DSM9790 (IG-57) gi|48477569
Pyrococcus abyssi gi|14520758
Pyrococcus horikoshii OT3 gi|3258052
Archaeoglobus fulgidus DSM4304 gi|2648231

Exemplary isopentenyl-diphosphate Delta-isomerase (IDI) nucleic acids and polypeptides

HSA: 3422(IDI1) 91734(IDI2)
PTR: 450262(IDI2) 450263(IDI1)
MCC: 710052(LOC710052) 721730(LOC721730)
MMU: 319554(Idi1)
RNO: 89784(Idi1)
GGA: 420459(IDI1)
XLA: 494671(LOC494671)
XTR: 496783(idi2)
SPU: 586184(LOC586184)
CEL: K06H7.9(idi-1)
ATH: AT3G02780(IPP2)
OSA: 4338791 4343523
CME: CMB062C
SCE: YPL117C(IDI1)
AGO: AGOS_ADL268C
PIC: PICST_68990(IDI1)
CGR: CAGL0J06952g
SPO: SPBC106.15(idi1)
ANI: AN0579.2
AFM: AFUA_6G11160
AOR: AO090023000500
CNE: CNA02550
UMA: UM04838.1
ECU: ECU02_0230
DDI: DDB_0191342(ipi)
TET: TTHERM_00237280 TTHERM_00438860
TBR: Tb09.211.0700
TCR: 408799.19 510431.10
LMA: LmjF35.5330
EHI: 46.t00025
ECO: b2889(idi)
ECJ: JW2857(idi)
ECE: Z4227
ECS: ECs3761
ECC: c3467
ECI: UTI89_C3274
ECP: ECP_2882
ECV: APECO1_3638
ECW: EcE24377A_3215(idi)
ECX: EcHS_A3048
STY: STY3195
STT: t2957
SPT: SPA2907(idi)
SEC: SC2979(idi)
STM: STM3039(idi)
SFL: SF2875(idi)
SFX: S3074
SFV: SFV_2937
SSN: SSON_3042 SSON_3489(yhfK)
SBO: SBO_3103
SDY: SDY_3193
ECA: ECA2789
PLU: plu3987
ENT: Ent638_3307
SPE: Spro_2201
VPA: VPA0278
VFI: VF0403
PPR: PBPRA0469(mvaD)
PEN: PSEEN4850
CBU: CBU_0607(mvaD)
CBD: COXBU7E912_0619(mvaD)
LPN: lpg2051
LPF: lpl2029
LPP: lpp2034
TCX: Tcr_1718
HHA: Hhal_1623
DNO: DNO_0798
EBA: ebA5678 p2A143
DVU: DVU1679(idi)
DDE: Dde_1991
LIP: LI1134
BBA: Bd1626
AFW: Anae109_4082
MXA: MXAN_5021(fni)
RPR: RP452
RTY: RT0439(idi)
RCO: RC0744
RFE: RF_0785(fni)
RBE: RBE_0731(fni)
RAK: A1C_04190

RBO: A1I_04755
RCM: A1E_02555
RRI: A1G_04195
MLO: mlr6371
RET: RHE_PD00245(ypd00046)
XAU: Xaut_4134
SIL: SPO0131
SIT: TM1040_3442
RSP: RSP_0276
RSH: Rsph17029_1919
RSQ: Rsph17025_1019
JAN: Jann_0168
RDE: RD1_0147(idi)
DSH: Dshi_3527
BSU: BG11440(ypgA)
BAN: BA1520
BAR: GBAA1520
BAA: BA_2041
BAT: BAS1409
BCE: BC1499
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SAS: SAS2237
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Exemplary isoprene synthase nucleic acids and polypeptides

Genbank Accession Nos.
AY341431
AY316691
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CLAIMS

1. A method for producing a fuel constituent from a bioisoprene composition comprising chemically transforming a substantial portion of the isoprene in the bioisoprene composition to non-isoprene compounds by:

(a) subjecting the bioisoprene composition to heat or catalytic conditions suitable for isoprene dimerization to produce an isoprene dimer and then catalytically hydrogenating the isoprene dimer to form a saturated C10 fuel constituent; or

(b) (i) partially hydrogenating the bioisoprene composition to produce an isoamylene, (ii) dimerizing the isoamylene with a mono-olefin selected from the group consisting of isoamylene, propylene and isobutene to form a dimate and (iii) completely hydrogenating the dimate to produce a fuel constituent.

2. The method of claim 1, wherein at least about 95% of isoprene in the bioisoprene composition is converted to non-isoprene compounds.

3. The method of claim 1, wherein the bioisoprene composition is heated to about 150 °C to about 250 °C to produce an unsaturated cyclic isoprene dimer and the unsaturated cyclic isoprene dimer is hydrogenated catalytically to produce a saturated cyclic isoprene dimer fuel constituent.

4. The method of claim 1, wherein the method comprises: (i) contacting the bioisoprene composition with a catalyst for catalyzing cyclo-dimerization of isoprene to produce an unsaturated cyclic isoprene dimer and the unsaturated cyclic isoprene dimer is hydrogenated catalytically to produce a saturated cyclic isoprene dimer fuel constituent.

5. The method of claim 4, wherein the catalyst for catalyzing cyclo-dimerization of isoprene comprising a catalyst selected from the group consisting of a nickel catalyst, iron catalysts and chromium catalysts.

6. The method of claim 1, wherein the step of partially hydrogenating the bioisoprene composition comprises contacting the bioisoprene composition with hydrogen gas and a catalyst for catalyzing partial hydrogenation of isoprene.

7. The method of claim 6, wherein the catalyst for catalyzing partial hydrogenation of isoprene comprises a palladium catalyst.

8. The method of claim 1, wherein the step of dimerizing the isoamylene with a mono-olefin comprises contacting the isoamylene with the mono-olefin in the presence of a catalyst for catalyzing dimerization of mono-olefin.

9. The method of claim 8, wherein the catalyst for catalyzing dimerization of mono-olefin comprises an acid catalyst.

10. The method of claim 1, further comprising purifying the isoprene from the bioisoprene composition prior to chemically transforming the bioisoprene composition to a fuel constituent.

11. A system for producing a fuel constituent from a bioisoprene composition, wherein a substantial portion of the isoprene in the bioisoprene composition is chemically converted to non-isoprene compounds, the system comprising a bioisoprene composition and :

(a) (i) one or more chemicals capable of dimerizing isoprene in the bioisoprene composition or a source of heat capable of dimerizing isoprene in the bioisoprene composition; and (ii) a catalyst capable of hydrogenating the isoprene dimer to form a saturated C10 fuel constituent; or

(b) (i) a chemical capable of partially hydrogenating isoprene in the bioisoprene composition to produce an isoamylene, (ii) a chemical capable of dimerizing the isoamylene with mono-olefins selected from the group consisting of isoamylene, propylene and isobutene to form a dimate and (iii) a chemical capable of completely hydrogenating the dimate to produce a fuel constituent.

12. The system of claim 11, wherein the bioisoprene composition comprising greater than about 2 mg of isoprene and comprising greater than or about 99.94% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition.

13. The system of claim 11, wherein the one or more chemicals capable of dimerizing isoprene comprises catalyst for catalyzing cyclo-dimerization of isoprene comprising a catalyst selected from the group consisting of ruthenium catalysts, nickel catalysts, iron catalysts and chromium catalysts.

14. The system of claim 11, wherein the catalyst for hydrogenating the unsaturated isoprene dimers comprises a catalyst selected from the group consisting of palladium catalysts, nickel catalysts, ruthenium catalysts and rhodium catalysts.

15. The system of claim 11, wherein the chemical capable of partially hydrogenating isoprene comprises a palladium catalyst.

16. The system of claim 11, wherein the chemical capable of dimerizing the isoamylene with mono-olefins comprises an acid catalyst.

17. A fuel composition comprising a fuel constituent produced by the method of claim 1.

18. The fuel composition of claim 17, wherein the fuel composition is substantially free of isoprene.

19. The fuel composition of claim 17, wherein the fuel composition has $\delta^{13}C$ value which is greater than -22‰ or within the range of -32‰ to -24‰.

Figure 1

1-
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Figure 2

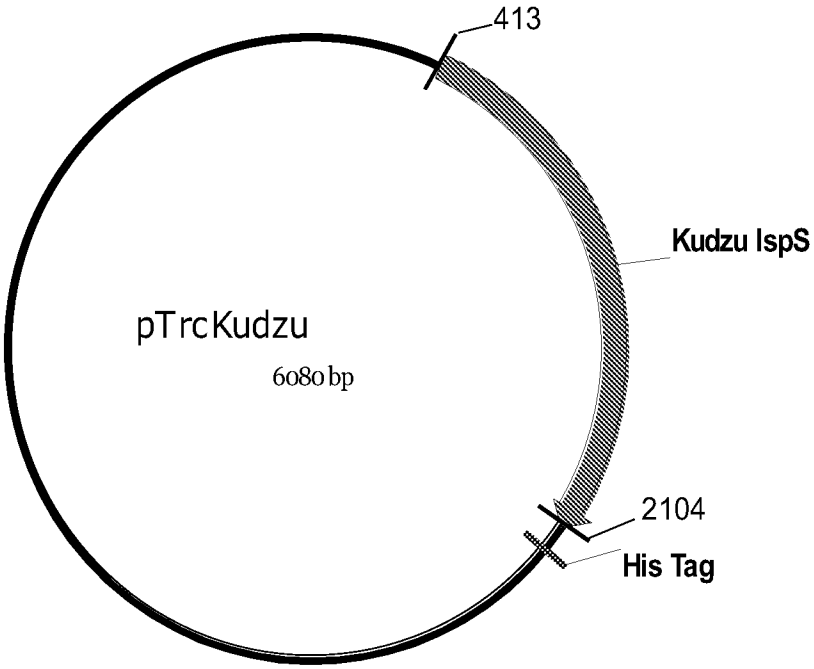

Figure 3A

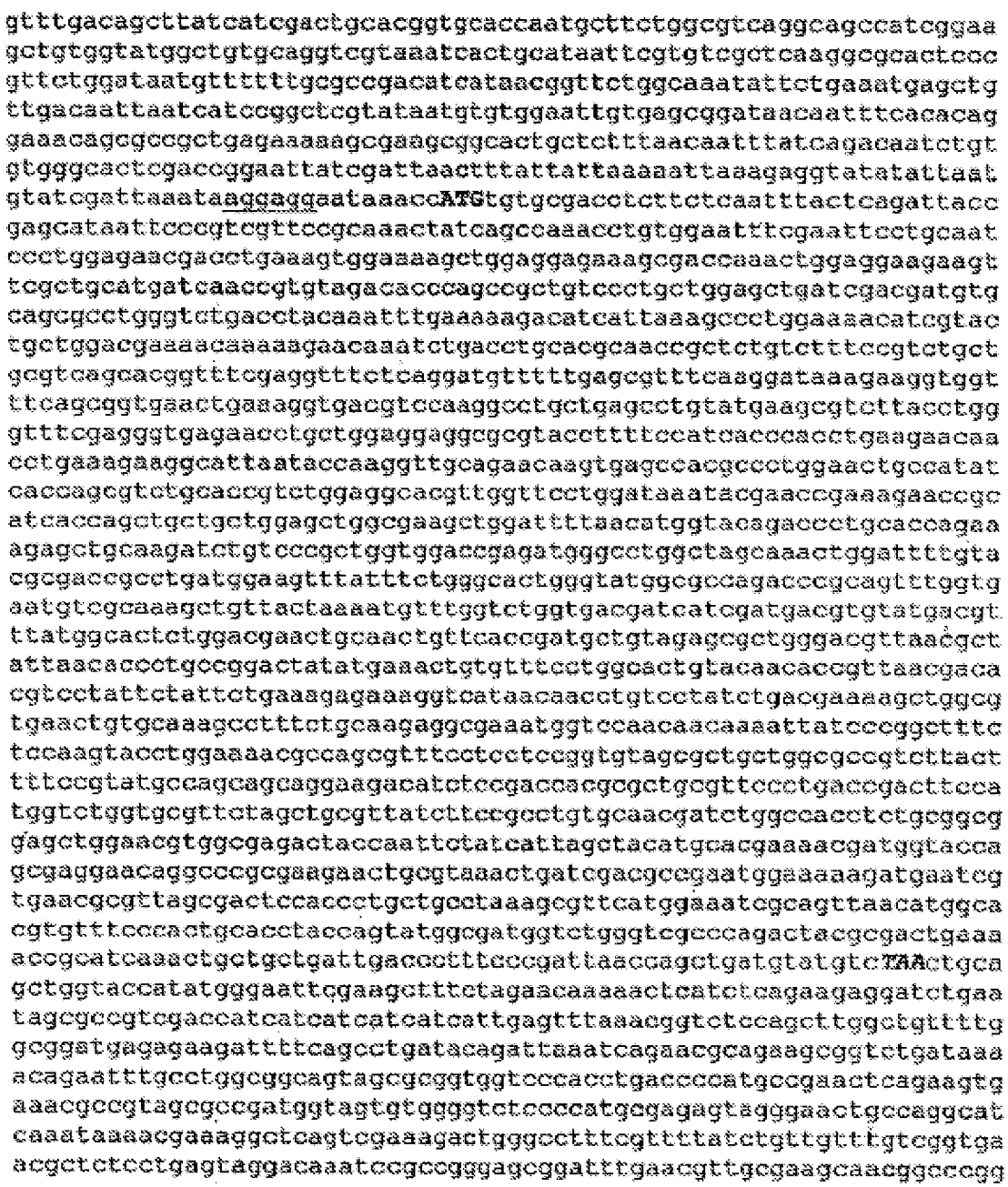
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Figure 3B

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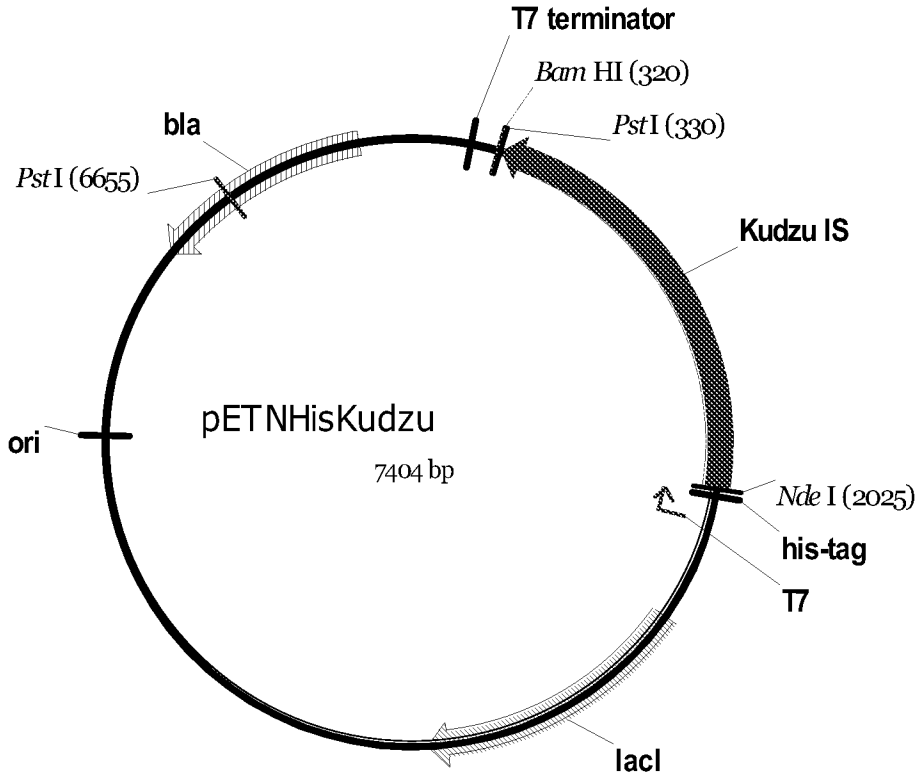
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Figure 5A

1-

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Figure 5B

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Figure 5C

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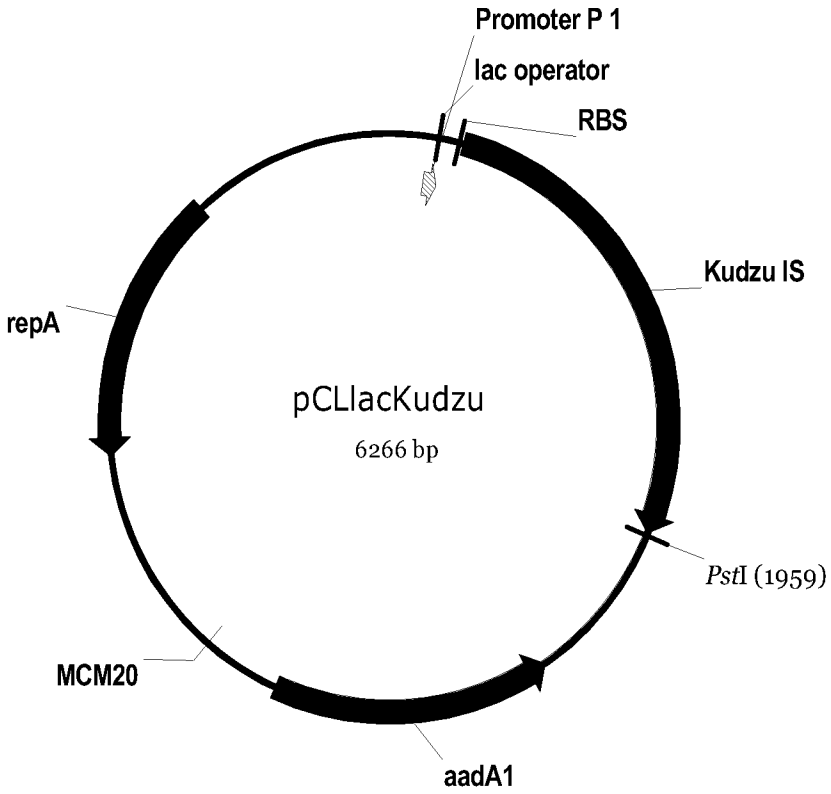
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Figure 7A

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Figure 7B

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Figure 7C

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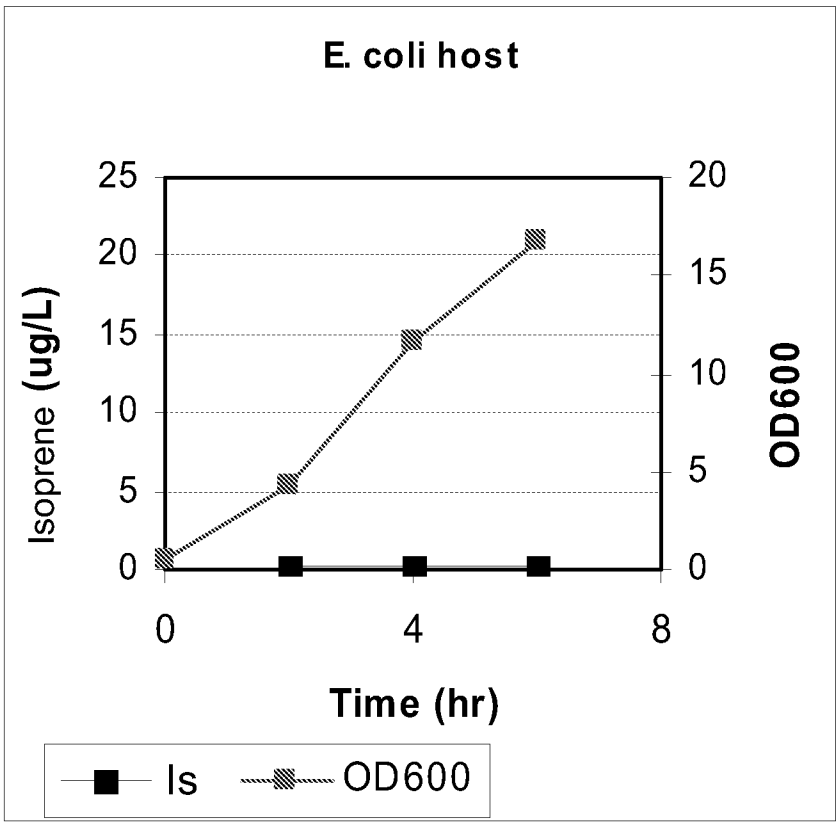
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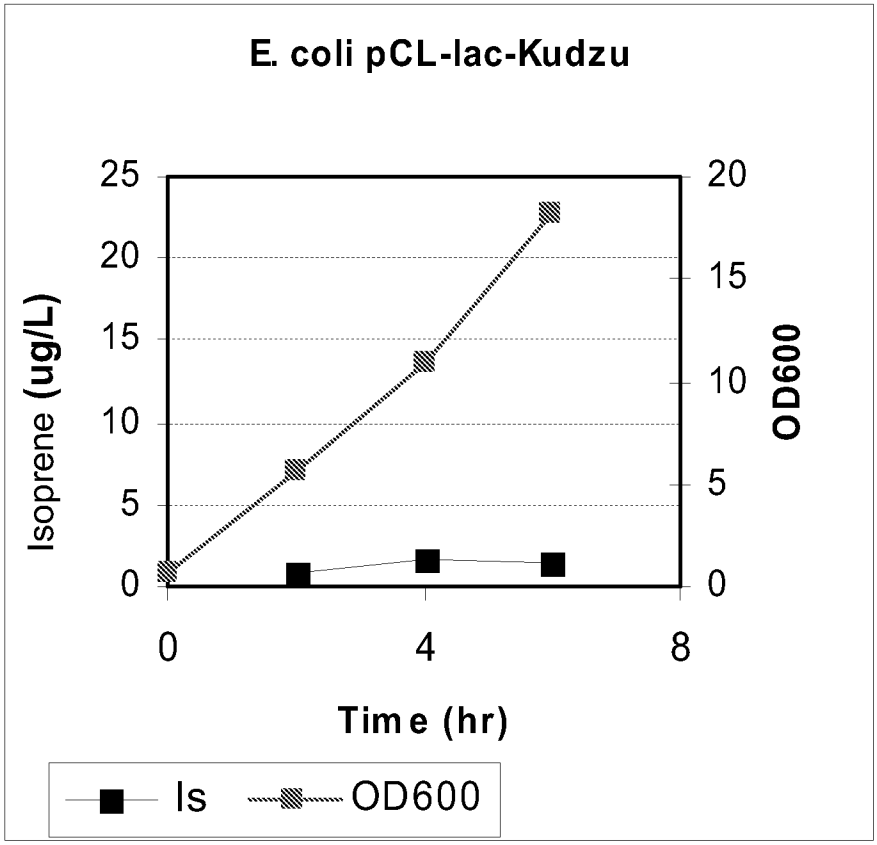
Figure 8B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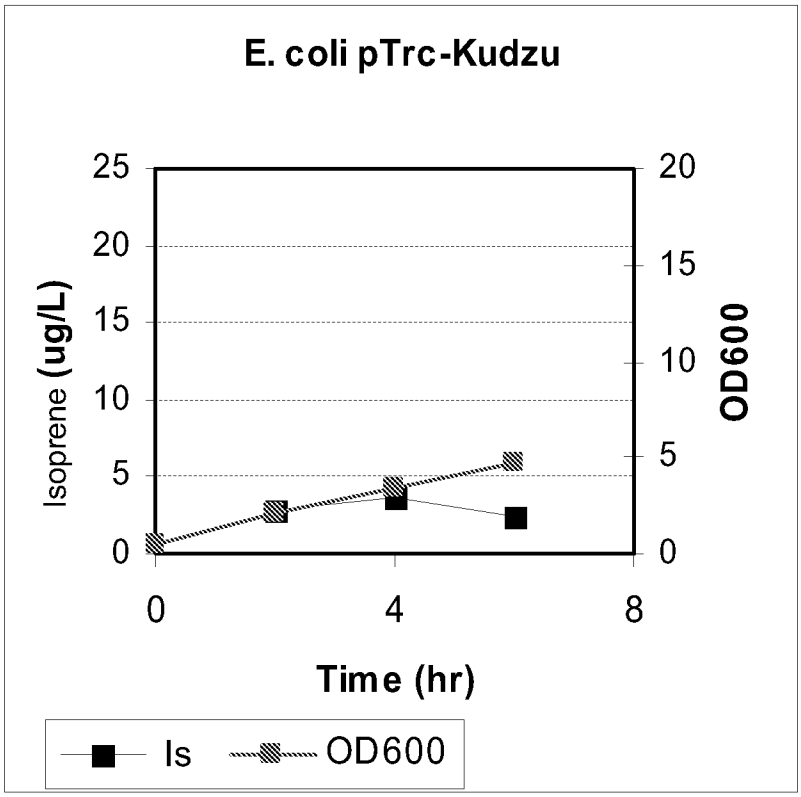
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Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 8D

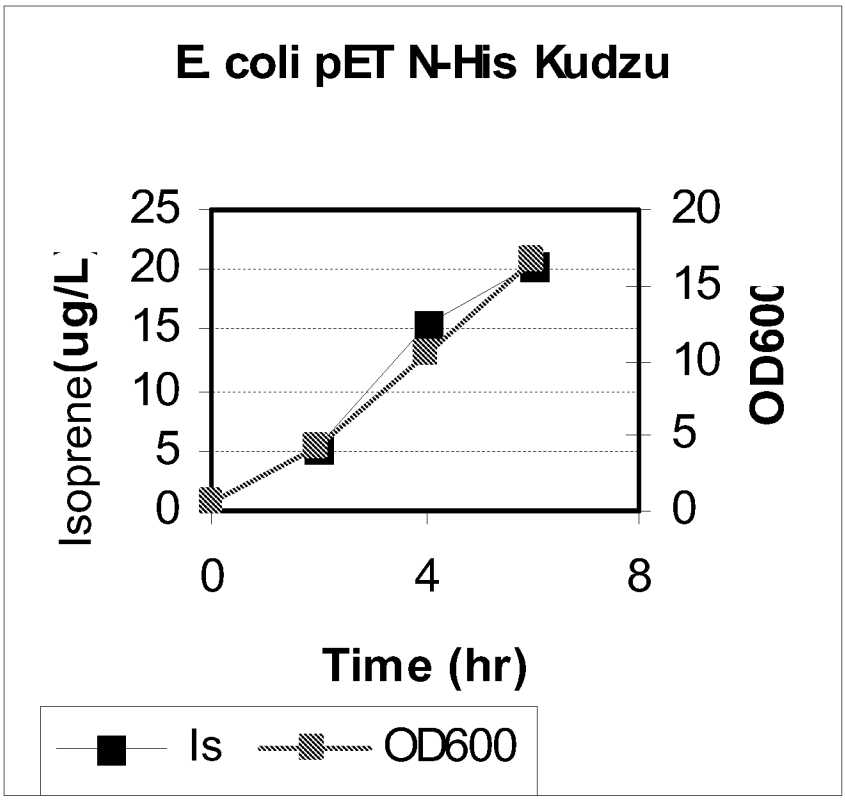
E. coli pET N-His Kudzu
Isoprene(ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 9A

A.

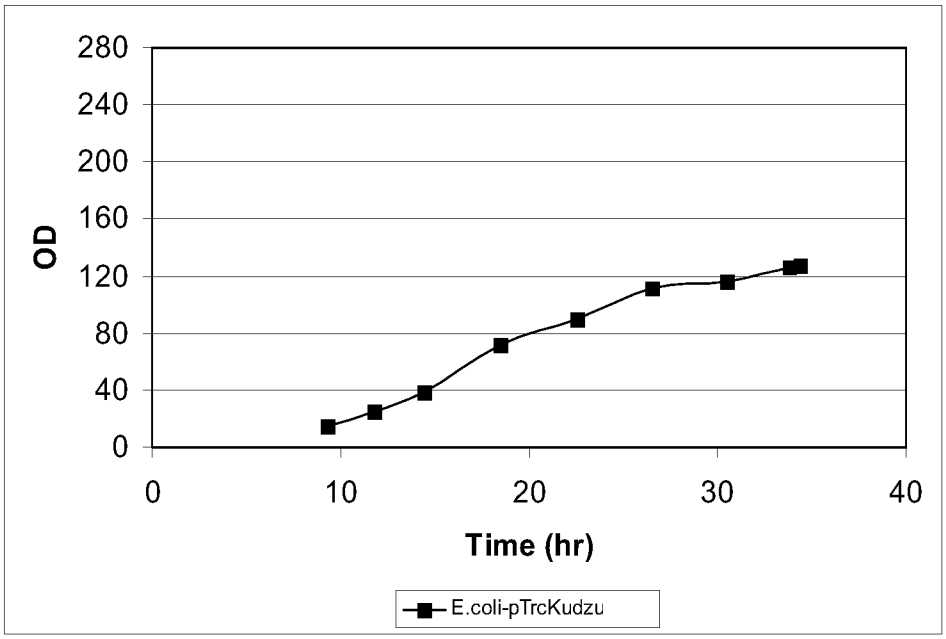
280
240
200
160
120
80
40
0
OD
0
10
20
30
40
Time (hr)
E.coli-pTrcKudzu

Figure 9B

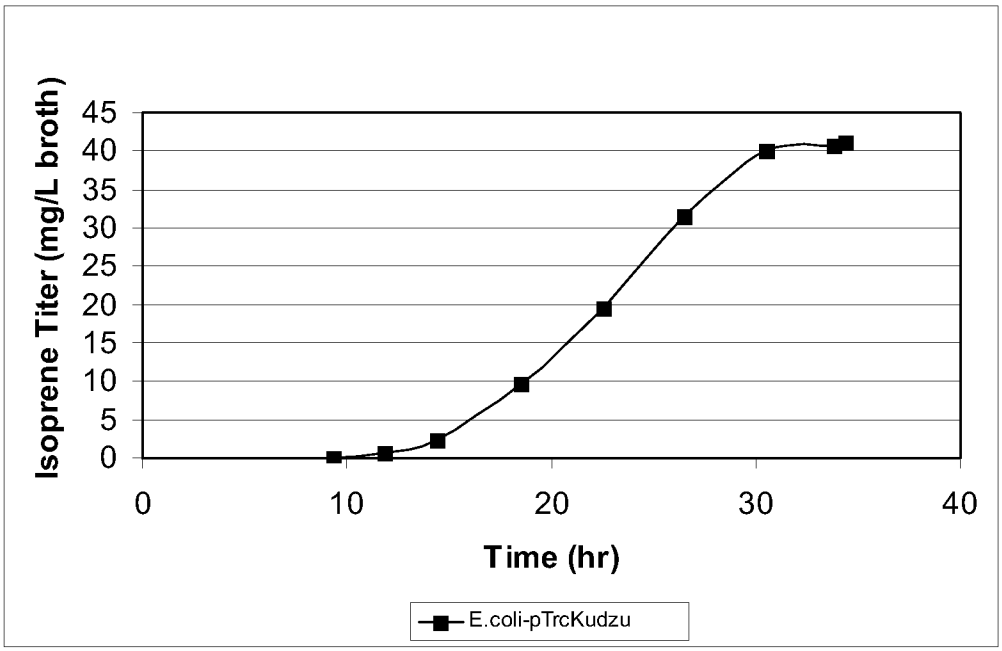
Isoprene Titer (mg/L broth)
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
Time (hr)
E.coli-pTrcKudzu

Figure 10A

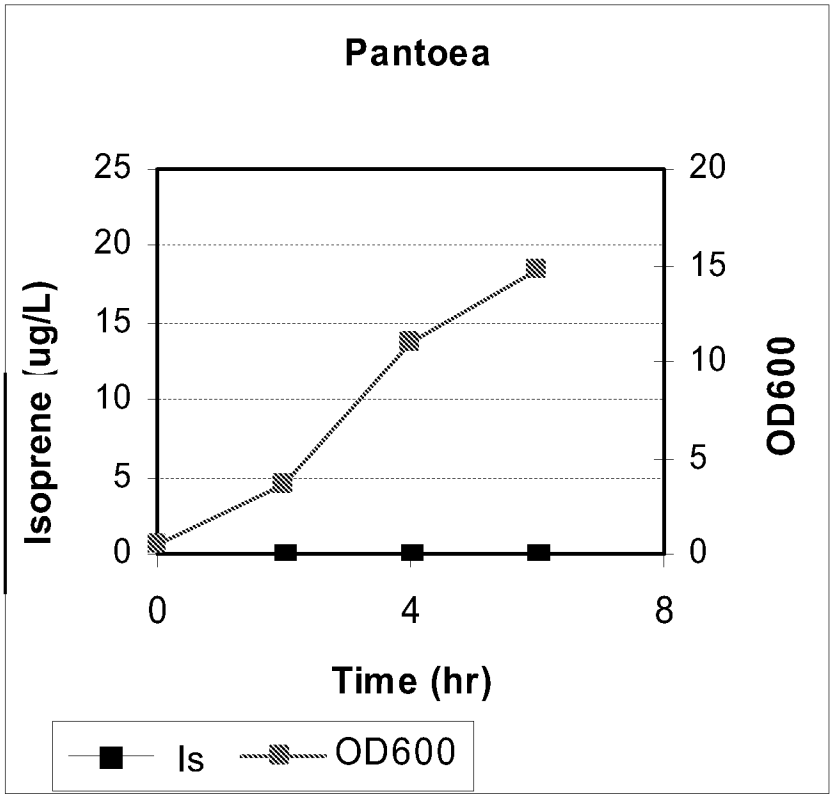
Pantoea
Isoprene (ug/L)
OD600
Time (hr)
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Is
OD600

Figure 10B

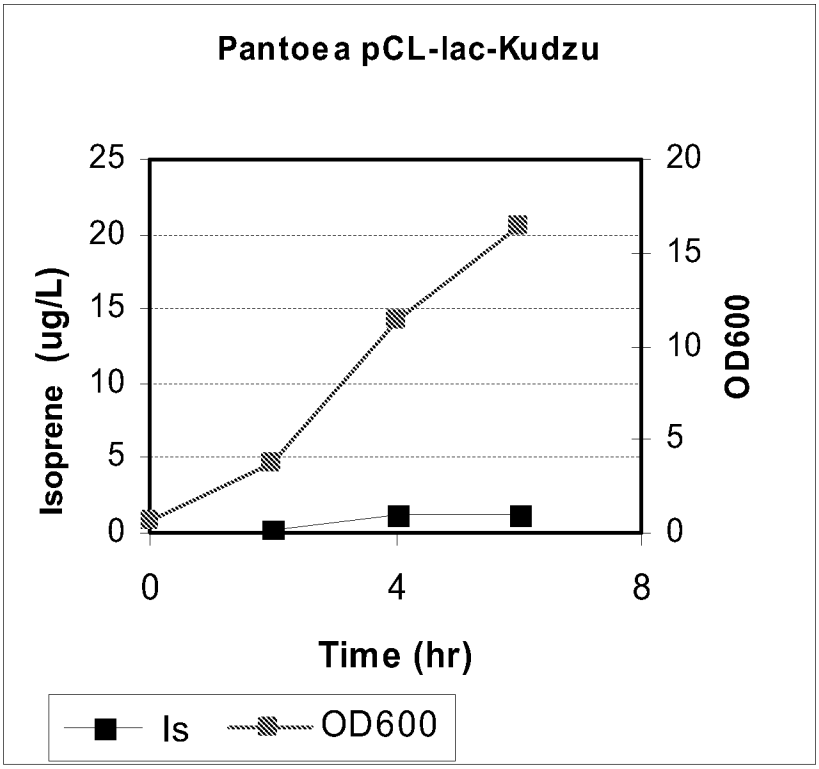
Pantoea pCL-lac-Kudzu
Isoprene (ug/L)
OD600
Time (hr)
Is
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8

Figure 10C

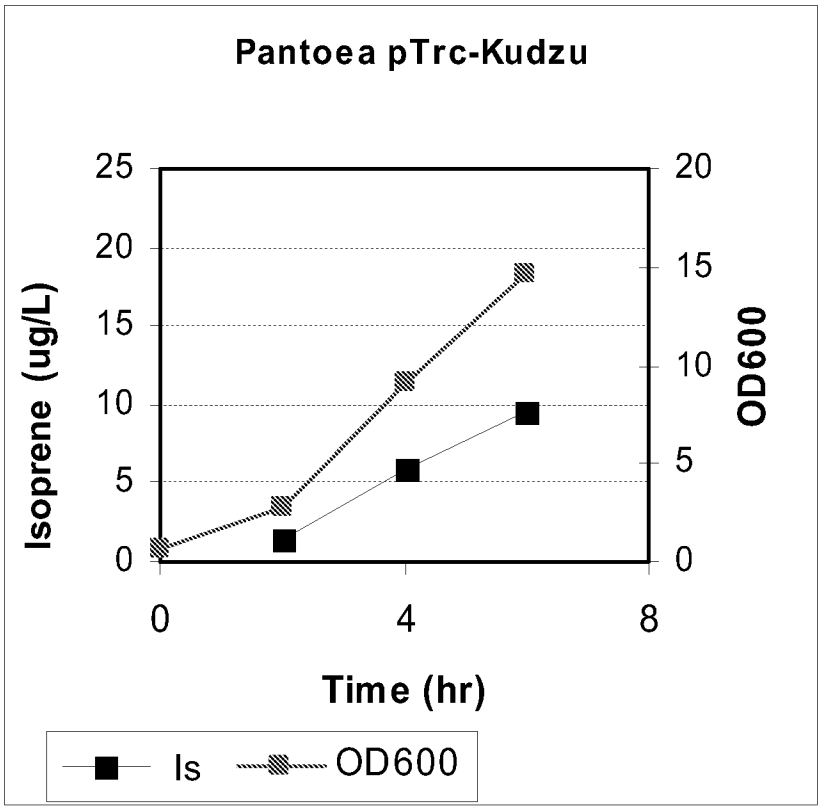
Pantoea pTrc-Kudzu
Isoprene (ug/L)
OD600
Time (hr)
0
5
10
15
20
25
0
4
8
Is
OD600

Figure 11

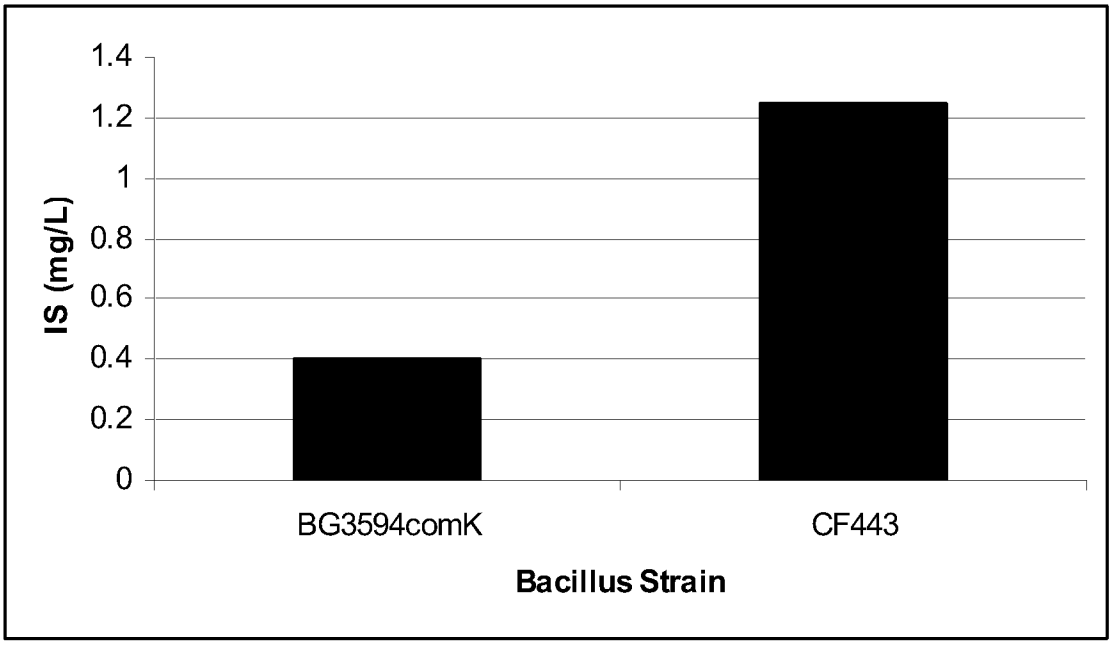

Figure 12A

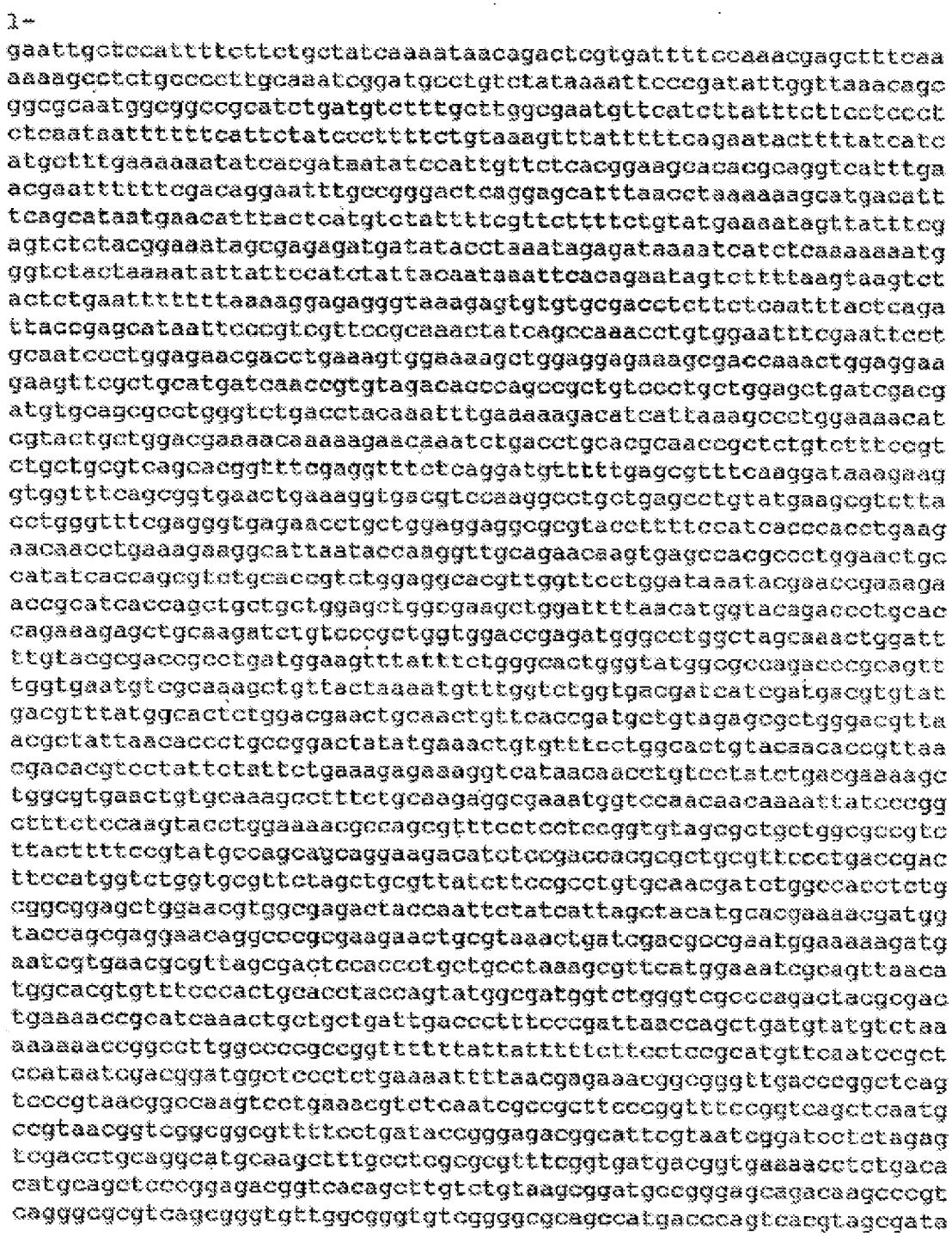
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Figure 12B

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Figure 12C

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SEQ ID NO:5

Figure 13

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CAACCCCTGTCTTTGCTGGAGCTGATCGACGATGTGCAGCGGTTGGGTTTGACTTATAAATTCG
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GACGTCTTCGAGAGATTTAAGGACAAGGAGGGAGGATTTAGCGGCGAGCTGAAGGGAGACGTTC
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TTCACTGACGCCGTCGAGCGATGGGATGTGAACGCCATTAATACTCTCCCTGACTATATGAAGC
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SEQ ID NO:6

Figure 14

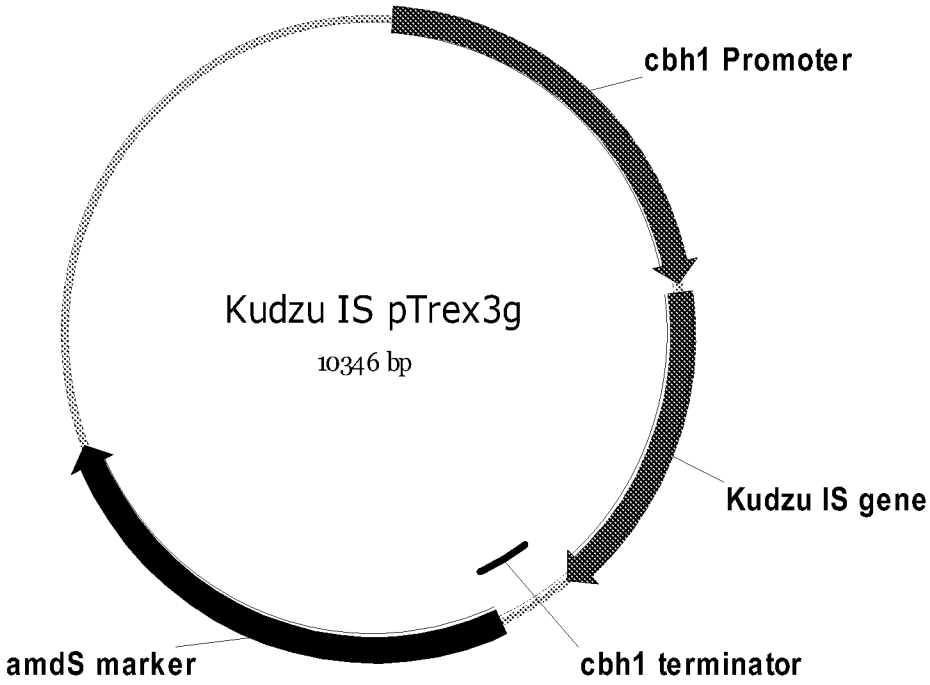
cbh1 Promoter
Kudzu IS pTrex3g
10346 bp
Kudzu IS gene
amdS marker
cbh1 terminator

Figure 15A

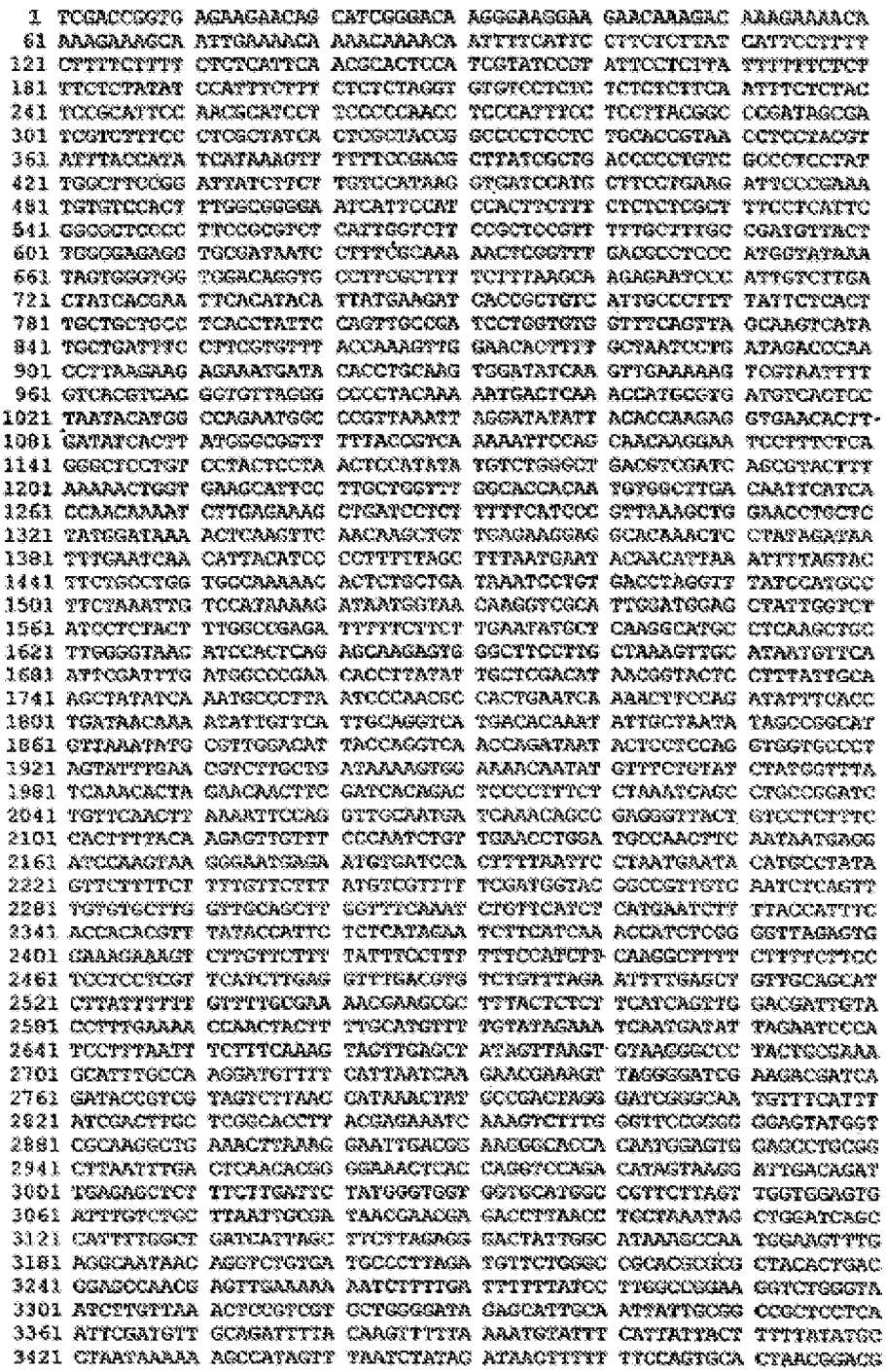

Figure 15B

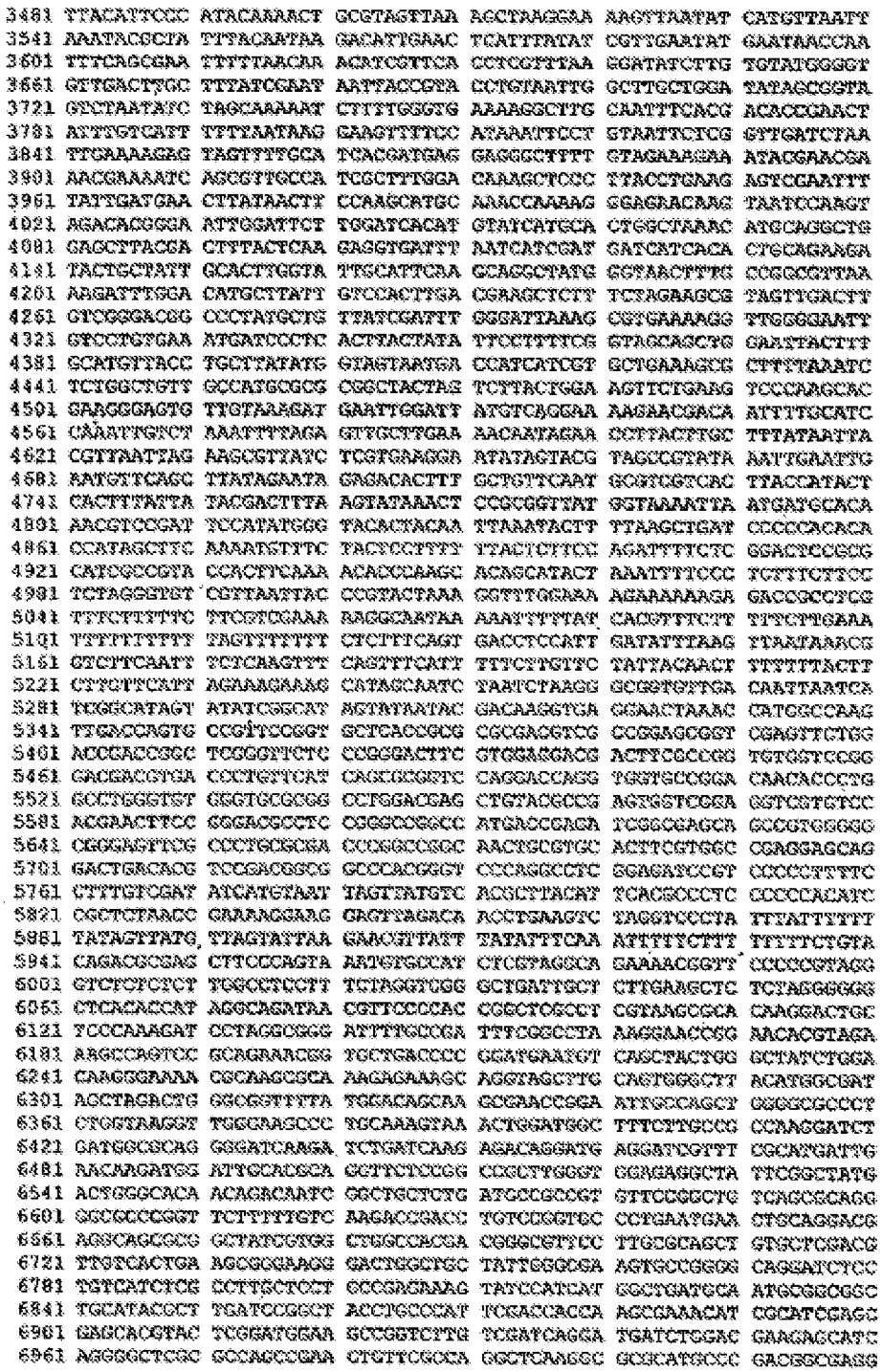

Figure 15C

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7141 TGGCTACCCG 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
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8041 CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT TTTGCTCACA TGTTCTTTCC
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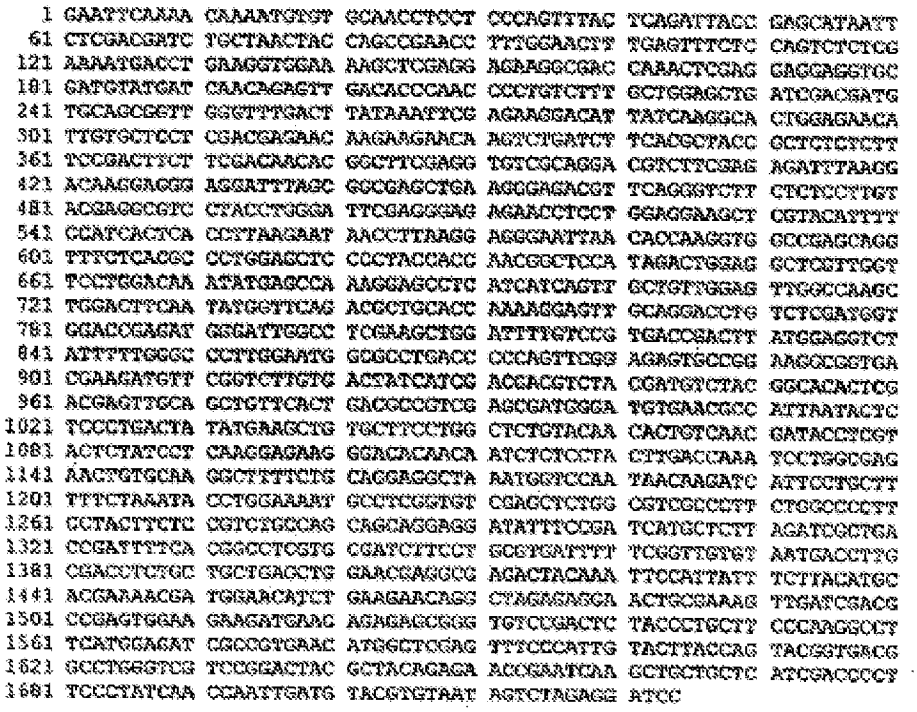
SEQ ID NO:7

Figure 16



SEQ ID NO:8

Figure 17

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 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
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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
```

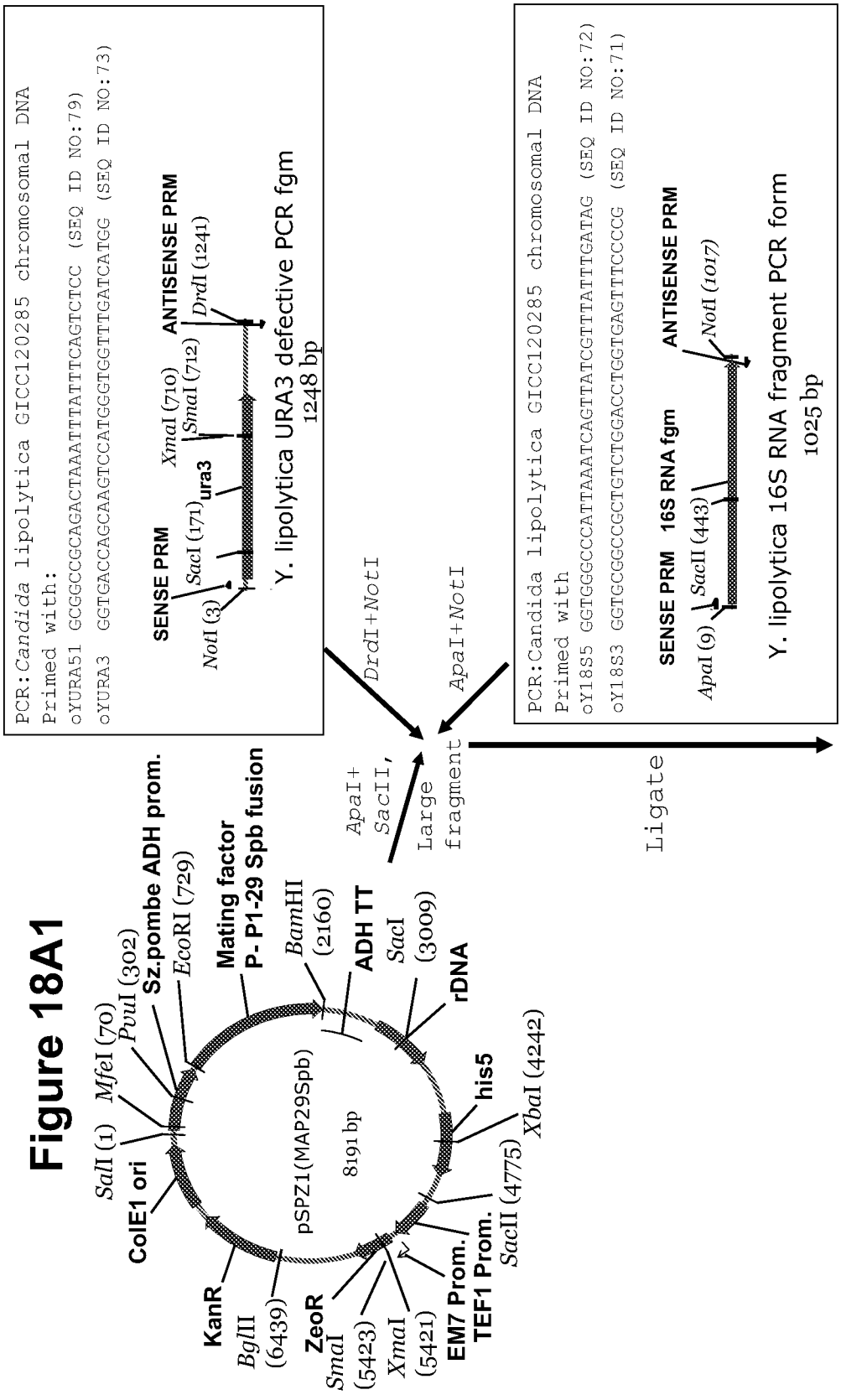
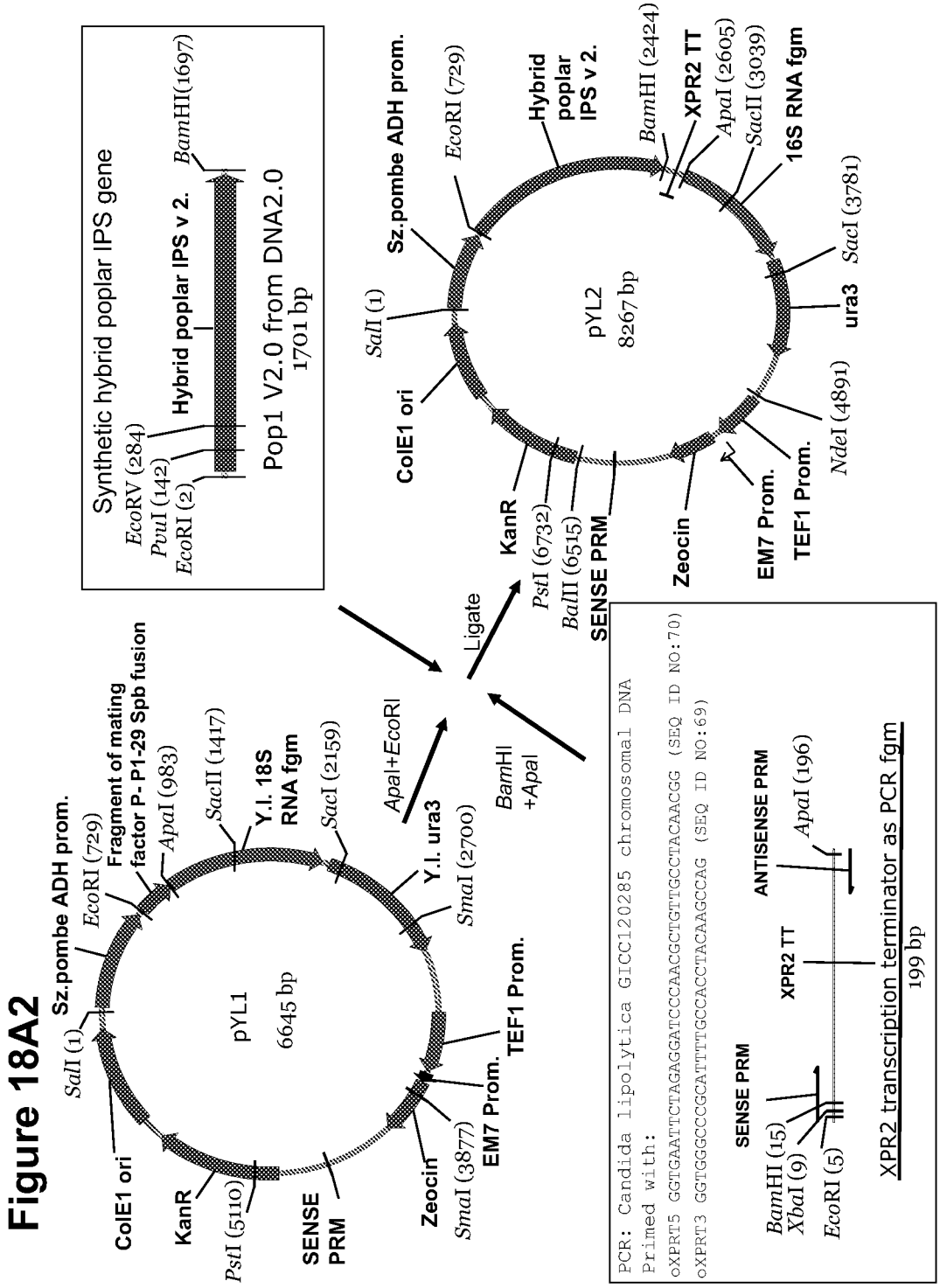


Figure 18A1
PCR:Candida lipolytica GICC120285 chromosomal DNA
Primed with:
oYURA51 GCGGCCGCAGACTAAATTTATTTCAGTCTCC (SEQ ID NO:79)
oYURA3 GGTGACCAGCAAGTCCATGGGTGGTTTGATCATGG (SEQ ID NO:73)
SENSE PRM
NotI (3)
SacI (171)
ura3
XmaI (710)
SmaI (712)
ANTISENSE PRM
DrdI (1241)
Y. lipolytica URA3 defective PCR fgm
1248 bp
DrdI+NotI
ApaI+NotI
PCR:Candida lipolytica GICC120285 chromosomal DNA
Primed with
oY18S5 GGTGGGCCCATTAAATCAGTTATCGTTTATTTGATAG (SEQ ID NO:72)
oY18S3 GGTGCGGCCGCTGTCTGGACCTGGTGAGTTTCCCCG (SEQ ID NO:71)
SENSE PRM 16S RNA fgm
ApaI (9)
SacII (443)
ANTISENSE PRM
NotI (1017)
Y. lipolytica 16S RNA fragment PCR form
1025 bp
ApaI+
SacII,
Large
fragment
Ligate
SalI (1)
MfeI (70)
PvuI (302)
Sz.pombe ADH prom.
EcoRI (729)
Mating factor
P- P1-29 Spb fusion
BamHI
(2160)
ADH TT
SacI
(3009)
rDNA
his5
XbaI (4242)
SacII (4775)
TEF1 Prom.
EM7 Prom.
XmaI
(5421)
SmaI
(5423)
ZeoR
BglII
(6439)
KanR
ColE1 ori
pSPZ1(MAP29Spb)
8191 bp

Figure 18A2


Synthetic hybrid poplar IPS gene
EcoRV (284)
PvuI (142)
EcoRI (2)
Hybrid poplar IPS v 2.
BamHI(1697)
Pop1 V2.0 from DNA2.0
1701 bp
pYL1
6645 bp
Sz.pombe ADH prom.
SalI (1)
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)
TEF1 Prom.
EM7 Prom.
Zeocin
SmaI (3877)
SENSE PRM
PstI (5110)
KanR
ColE1 ori
ApaI+EcoRI
BamHI +ApaI
Ligate
pYL2
8267 bp
Sz.pombe ADH prom.
EcoRI (729)
Hybrid poplar IPS v 2.
BamHI (2424)
XPR2 TT
ApaI (2605)
SacII (3039)
16S RNA fgm
SacI (3781)
ura3
NdeI (4891)
TEF1 Prom.
EM7 Prom.
Zeocin
SENSE PRM
PstI (6732)
BalII (6515)
KanR
ColE1 ori
SalI (1)
PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with:
oXPRT5 GGTGAATTCTAGAGGATCCCAACGCTGTTGCCTACAACGG (SEQ ID NO:70)
oXPRT3 GGTGGGCCCGCATTTTGCCACCTACAAGCCAG (SEQ ID NO:69)
SENSE PRM
ANTISENSE PRM
BamHI (15)
XbaI (9)
EcoRI (5)
XPR2 TT
ApaI (196)
XPR2 transcription terminator as PCR fgm
199 bp

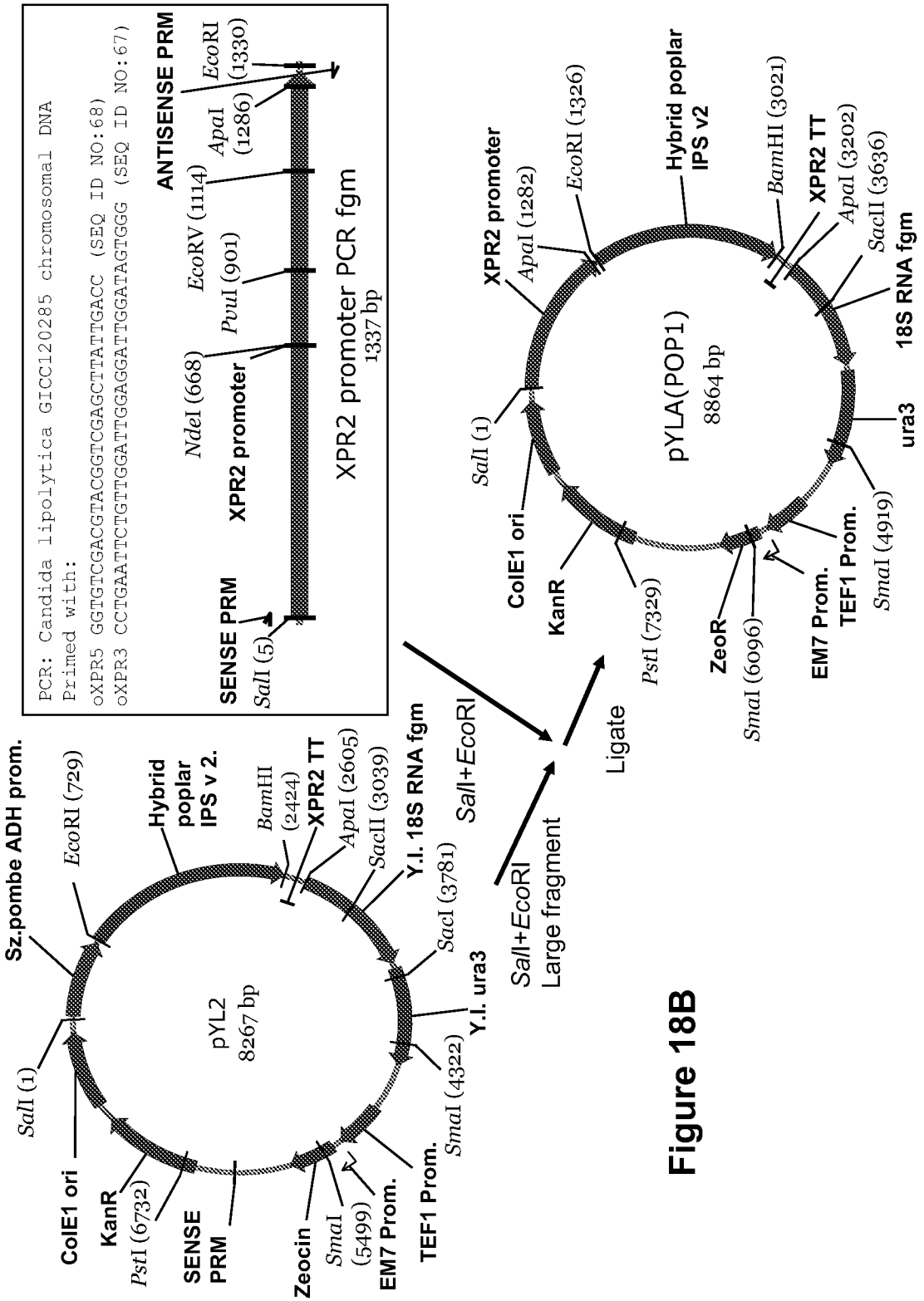

PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with:
oXPR5 GGTGTCGACGTACGGTCGAGCTTATTGACC (SEQ ID NO:68)
oXPR3 CCTGAATTCTGTTGGATTGGAGGATTGGATAGTGGG (SEQ ID NO:67)
SENSE PRM
XPR2 promoter
ANTISENSE PRM
SalI (5)
NdeI (668)
PvuI (901)
EcoRV (1114)
ApaI (1286)
EcoRI (1330)
XPR2 promoter PCR fgm
1337 bp
pYL2
8267 bp
Sz.pombe ADH prom.
EcoRI (729)
Hybrid poplar IPS v 2.
BamHI (2424)
XPR2 TT
ApaI (2605)
SacII (3039)
Y.I. 18S RNA fgm
SacI (3781)
Y.I. ura3
SmaI (4322)
TEF1 Prom.
EM7 Prom.
SmaI (5499)
Zeocin
SENSE PRM
PstI (6732)
KanR
ColE1 ori
SalI (1)
SalI+EcoRI
SalI+EcoRI
Large fragment
Ligate
pYLA(POP1)
8864 bp
SalI (1)
ColE1 ori
KanR
PstI (7329)
XPR2 promoter
ApaI (1282)
EcoRI (1326)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
ura3
SmaI (4919)
TEF1 Prom.
EM7 Prom.
SmaI (6096)
ZeoR

Figure 18B

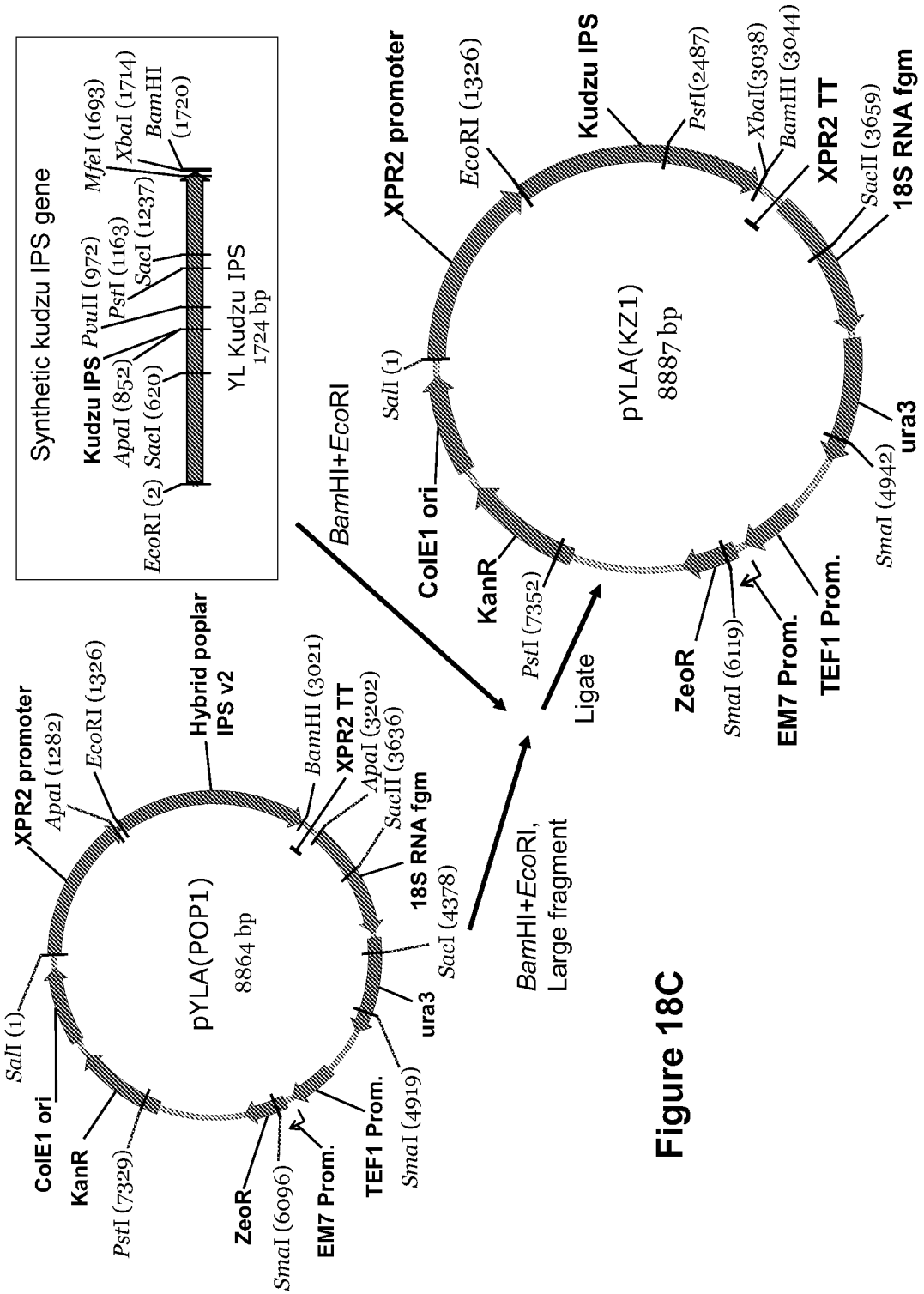

Synthetic kudzu IPS gene
Kudzu IPS
EcoRI (2)
ApaI (852)
SacI (620)
PvuII (972)
PstI (1163)
SacI (1237)
MfeI (1693)
XbaI (1714)
BamHI (1720)
YL Kudzu IPS
1724 bp
pYLA(POP1)
8864 bp
SalI (1)
ColE1 ori
KanR
PstI (7329)
ZeoR
SmaI (6096)
EM7 Prom.
TEF1 Prom.
SmaI (4919)
ura3
SacI (4378)
18S RNA fgm
SacII (3636)
XPR2 TT
ApaI (3202)
BamHI (3021)
Hybrid poplar IPS v2
EcoRI (1326)
ApaI (1282)
XPR2 promoter
BamHI+EcoRI
BamHI+EcoRI, Large fragment
Ligate
pYLA(KZ1)
8887 bp
SalI (1)
ColE1 ori
KanR
PstI (7352)
ZeoR
SmaI (6119)
EM7 Prom.
TEF1 Prom.
SmaI (4942)
ura3
18S RNA fgm
SacII (3659)
XPR2 TT
BamHI (3044)
XbaI(3038)
PstI(2487)
Kudzu IPS
EcoRI (1326)
XPR2 promoter

Figure 18C

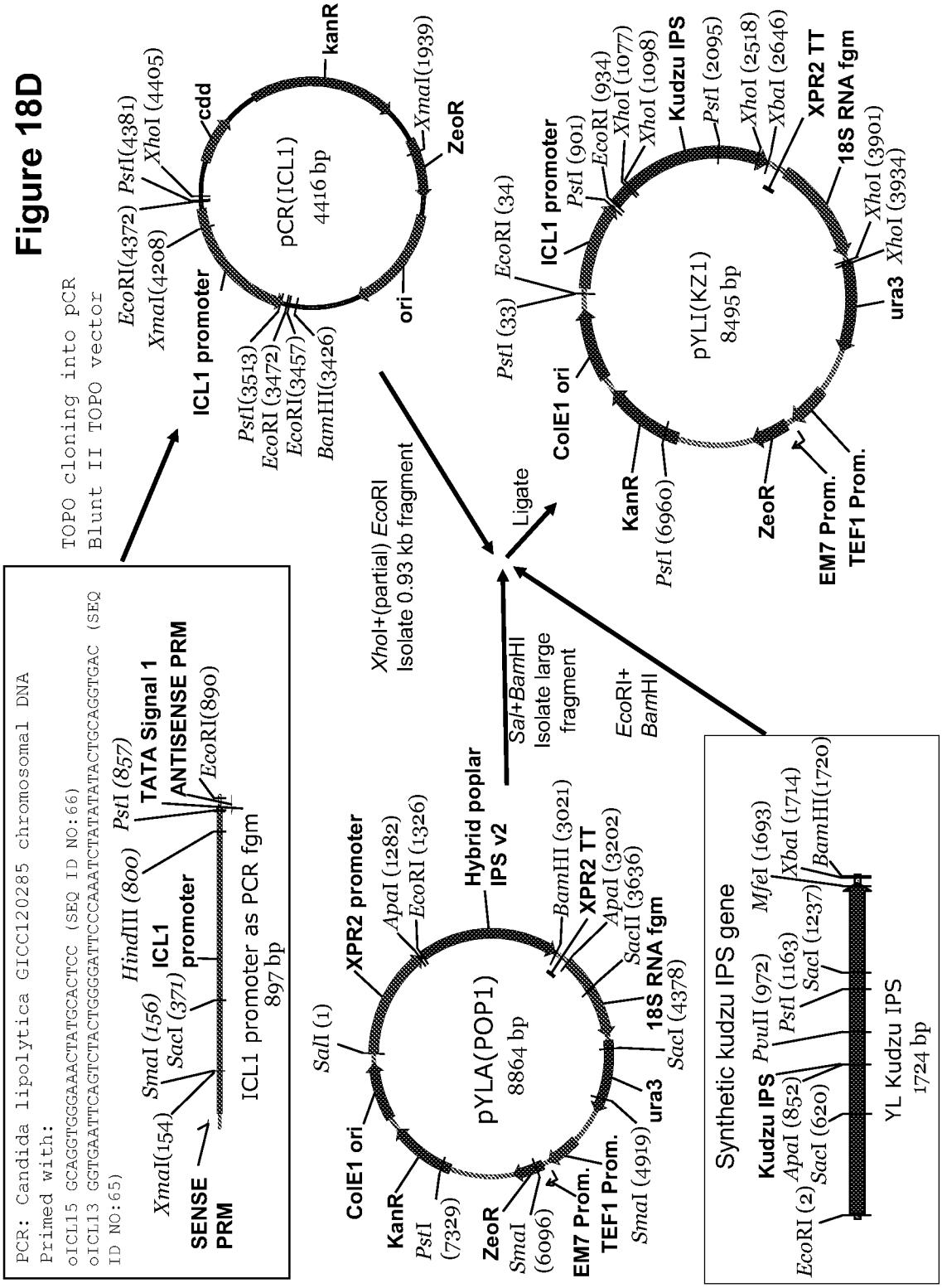


Figure 18D
PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with:
oICL15 GCAGGTGGGAAACTATGCACTCC (SEQ ID NO:66)
oICL13 GGTGAATTCAGTCTACTGGGGATTCCCAAATCTATATATACTGCAGGTGAC (SEQ ID NO:65)
XmaI(154)
SmaI (156)
SacI (371)
HindIII (800)
ICL1 promoter
PstI (857)
TATA Signal 1
ANTISENSE PRM
EcoRI(890)
SENSE PRM
ICL1 promoter as PCR fgm
897 bp
TOPO cloning into pCR
Blunt II TOPO vector
EcoRI(4372)
XmaI(4208)
PstI(4381)
XhoI (4405)
cdd
ICL1 promoter
pCR(ICL1)
4416 bp
kanR
XmaI(1939)
ZeoR
ori
PstI(3513)
EcoRI (3472)
EcoRI(3457)
BamHI(3426)
XhoI+(partial) EcoRI
Isolate 0.93 kb fragment
Ligate
Sal+BamHI
Isolate large fragment
EcoRI+ BamHI
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(POP1)
8864 bp
Synthetic kudzu IPS gene
Kudzu IPS
EcoRI (2)
ApaI (852)
SacI (620)
PvuII (972)
PstI (1163)
SacI (1237)
MfeI (1693)
XbaI (1714)
BamHI(1720)
YL Kudzu IPS
1724 bp
EcoRI (34)
PstI (33)
ICL1 promoter
PstI (901)
EcoRI (934)
XhoI (1077)
XhoI (1098)
Kudzu IPS
PstI (2095)
XhoI (2518)
XbaI (2646)
XPR2 TT
18S RNA fgm
XhoI (3901)
XhoI (3934)
ura3
ColE1 ori
pYLI(KZ1)
8495 bp
KanR
PstI (6960)
ZeoR
EM7 Prom.
TEF1 Prom.

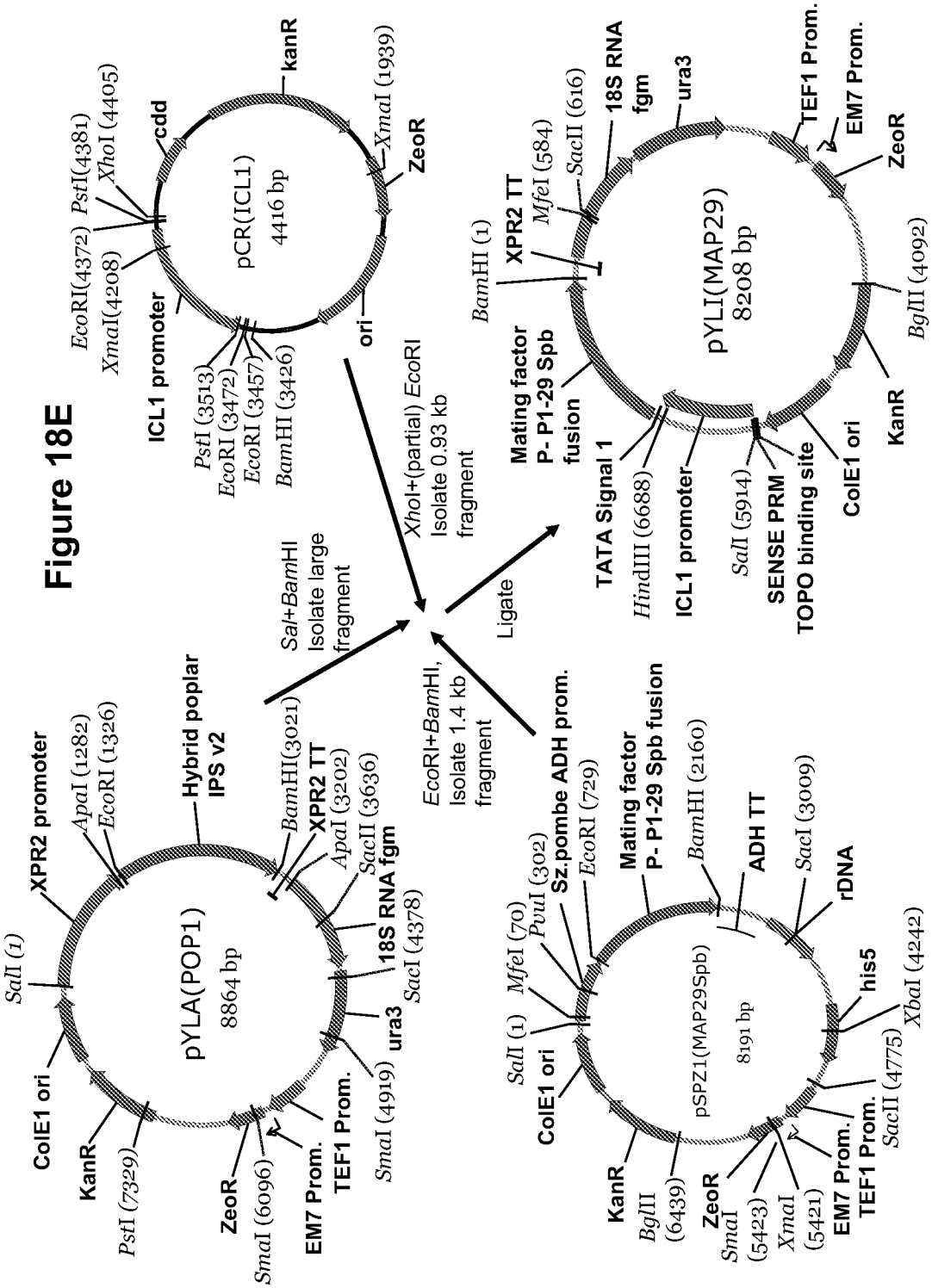


Figure 18E
pYLA(POP1)
8864 bp
ColE1 ori
KanR
ZeoR
EM7 Prom.
TEF1 Prom.
ura3
18S RNA fgm
XPR2 TT
Hybrid poplar IPS v2
XPR2 promoter
SalI (1)
ApaI (1282)
EcoRI (1326)
PstI (7329)
SmaI (6096)
SmaI (4919)
BamHI(3021)
ApaI (3202)
SacII (3636)
SacI (4378)
pCR(ICL1)
4416 bp
ICL1 promoter
cdd
kanR
ZeoR
ori
EcoRI(4372)
PstI(4381)
XhoI (4405)
XmaI(4208)
PstI (3513)
EcoRI (3472)
EcoRI (3457)
BamHI (3426)
XmaI (1939)
Sal+BamHI Isolate large fragment
XhoI+(partial) EcoRI Isolate 0.93 kb fragment
EcoRI+BamHI, Isolate 1.4 kb fragment
Ligate
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
SENSE PRM
TOPO binding site
SalI (5914)
ICL1 promoter
HindIII (6688)
TATA Signal 1
Mating factor P- P1-29 Spb fusion
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 (3009)
rDNA
his5
XbaI (4242)
SacII (4775)
TEF1 Prom.
EM7 Prom.
ZeoR
SmaI (5421)
XmaI (5423)
BglII (6439)
KanR
ColE1 ori

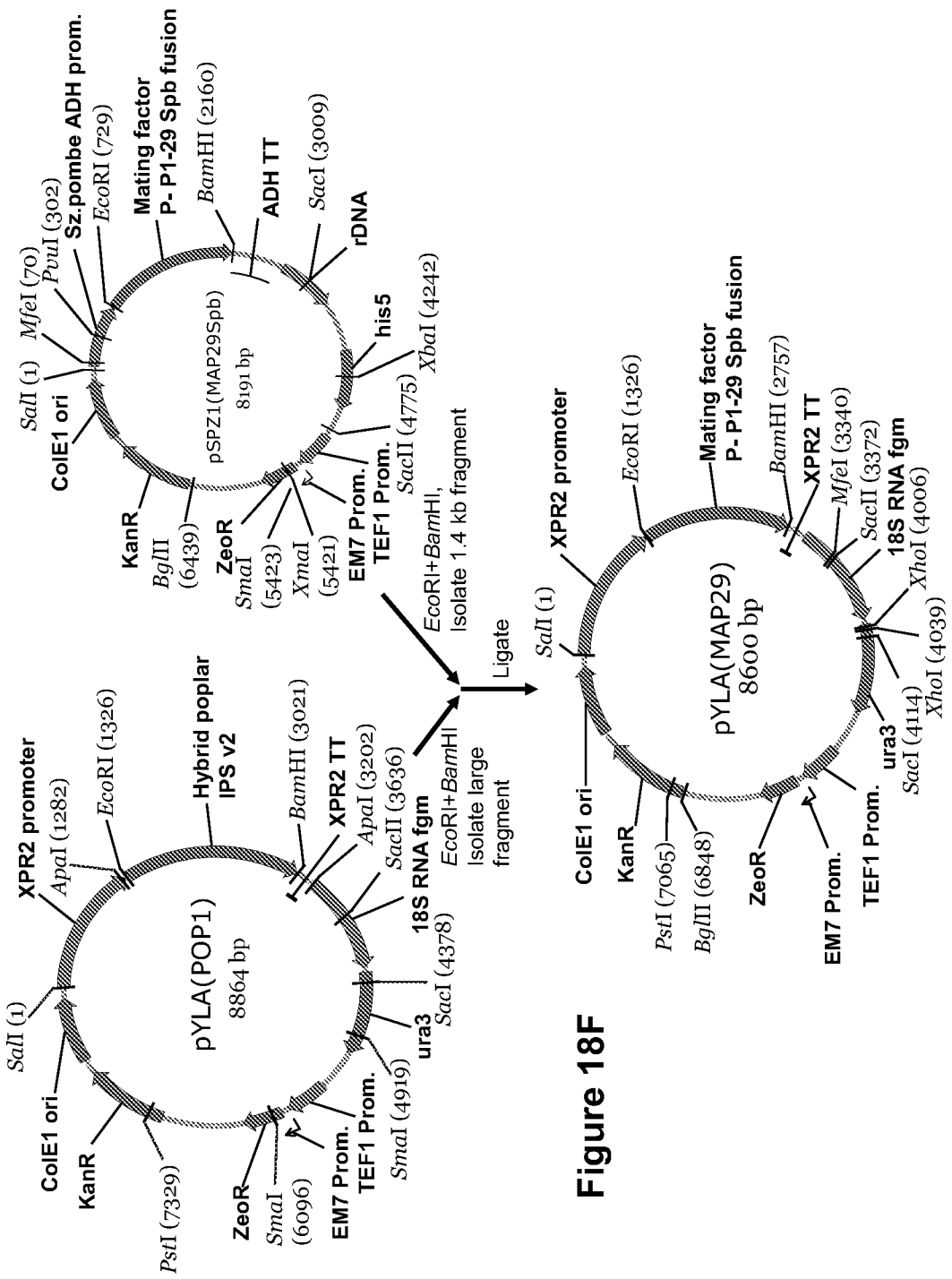

pYLA(POP1)
8864 bp
SalI (1)
ColE1 ori
KanR
PstI (7329)
ZeoR
SmaI (6096)
EM7 Prom.
TEF1 Prom.
SmaI (4919)
ura3
SacI (4378)
18S RNA fgm
EcoRI+BamHI
Isolate large fragment
XPR2 promoter
ApaI (1282)
EcoRI (1326)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
pSPZ1(MAP29Spb)
8191 bp
SalI (1)
ColE1 ori
MfeI (70)
PvuI (302)
Sz.pombe ADH prom.
EcoRI (729)
Mating factor P- P1-29 Spb fusion
BamHI (2160)
ADH TT
SacI (3009)
rDNA
his5
XbaI (4242)
SacII (4775)
EM7 Prom.
TEF1 Prom.
XmaI (5421)
SmaI (5423)
ZeoR
BglII (6439)
KanR
EcoRI+BamHI,
Isolate 1.4 kb fragment
Ligate
pYLA(MAP29)
8600 bp
SalI (1)
XPR2 promoter
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)
ura3
TEF1 Prom.
EM7 Prom.
ZeoR
BglII (6848)
PstI (7065)
KanR
ColE1 ori

Figure 18F

Figure 19A

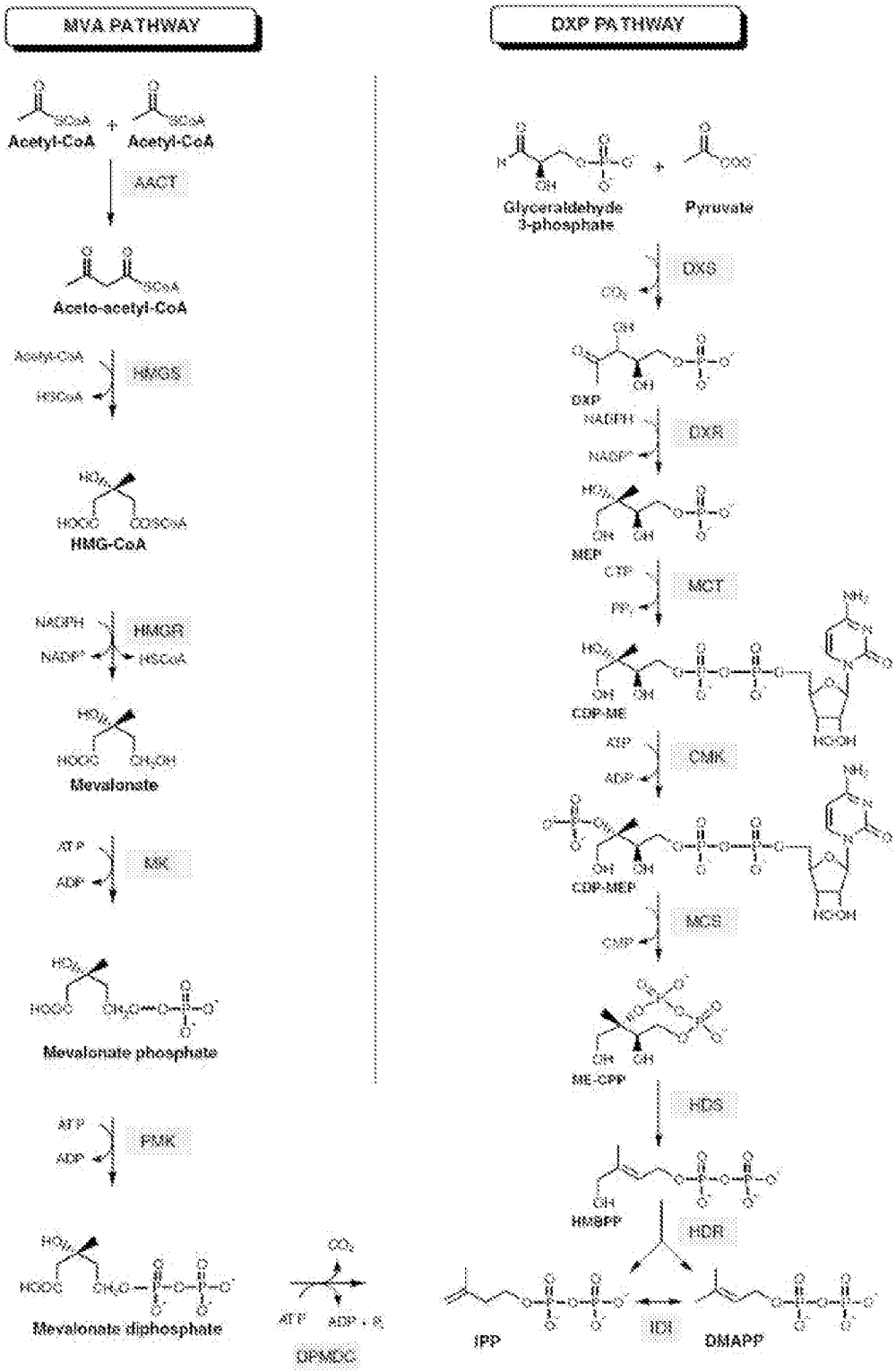

MVA PATHWAY
DXP PATHWAY
Acetyl-CoA
Acetyl-CoA
AACT
Aceto-acetyl-CoA
HMGS
HMG-CoA
HMGR
Mevalonate
MK
Mevalonate phosphate
PMK
Mevalonate diphosphate
MDC
IPP
IDI
DMAPP
Glyceraldehyde 3-phosphate
Pyruvate
DXS
DXP
DXR
MEP
MCT
CDP-ME
CMK
CDP-MEP
MCS
ME-CPP
HDS
HMBPP
HDR

Figure 19B

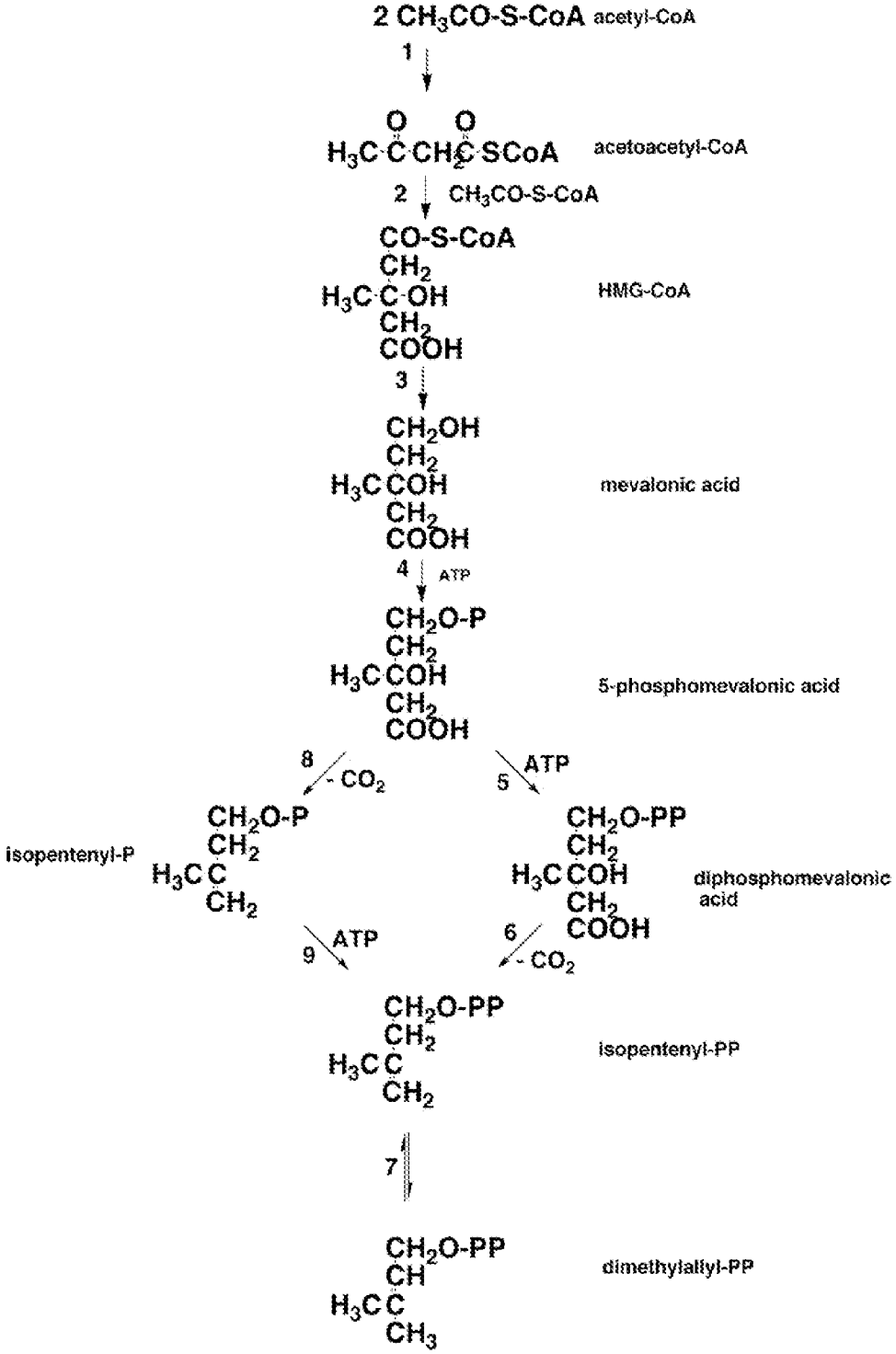

2 CH3CO-S-CoA
acetyl-CoA
1
acetoacetyl-CoA
2
CH3CO-S-CoA
HMG-CoA
3
mevalonic acid
4
ATP
5-phosphomevalonic acid
8
- CO2
5
ATP
isopentenyl-P
diphosphomevalonic acid
9
ATP
6
- CO2
isopentenyl-PP
7
dimethylallyl-PP

Figure 20A

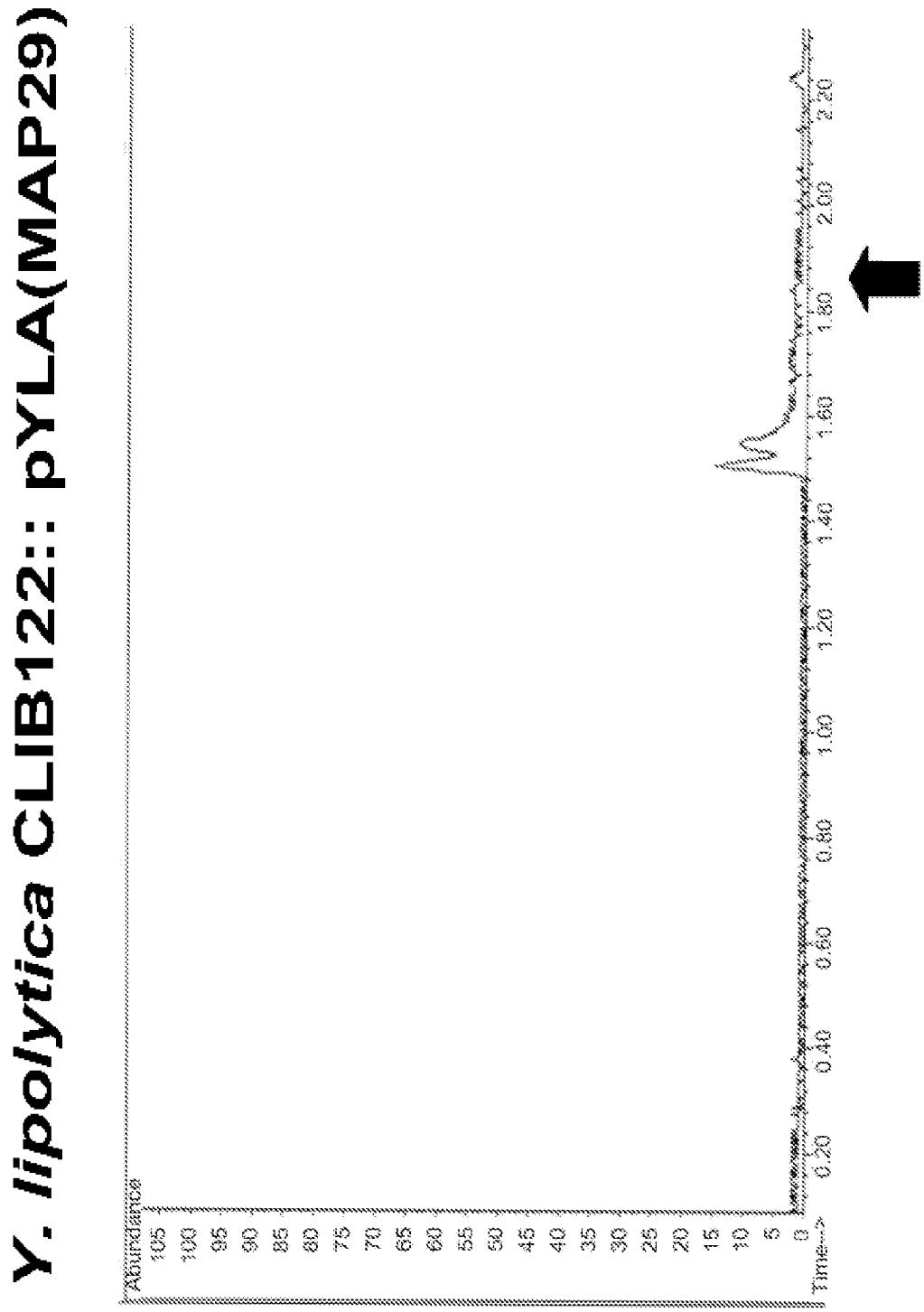

Y. lipolytica CLIB122:: pYLA(MAP29)
Abundance
Time-->
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
2.20

Figure 20B

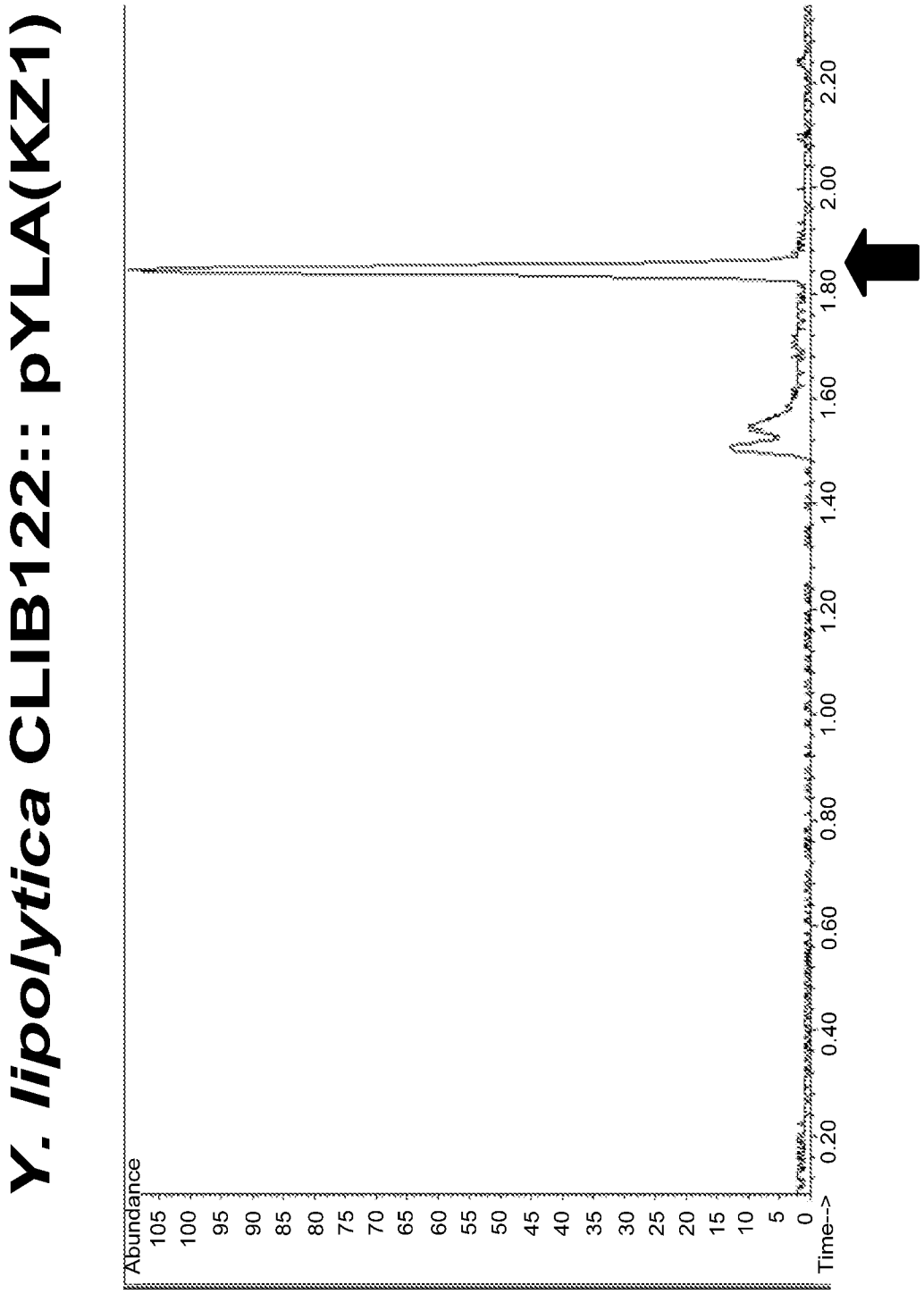

Y. lipolytica CLIB122:: pYLA(KZ1)
Abundance
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Time-->
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
2.20

Figure 21

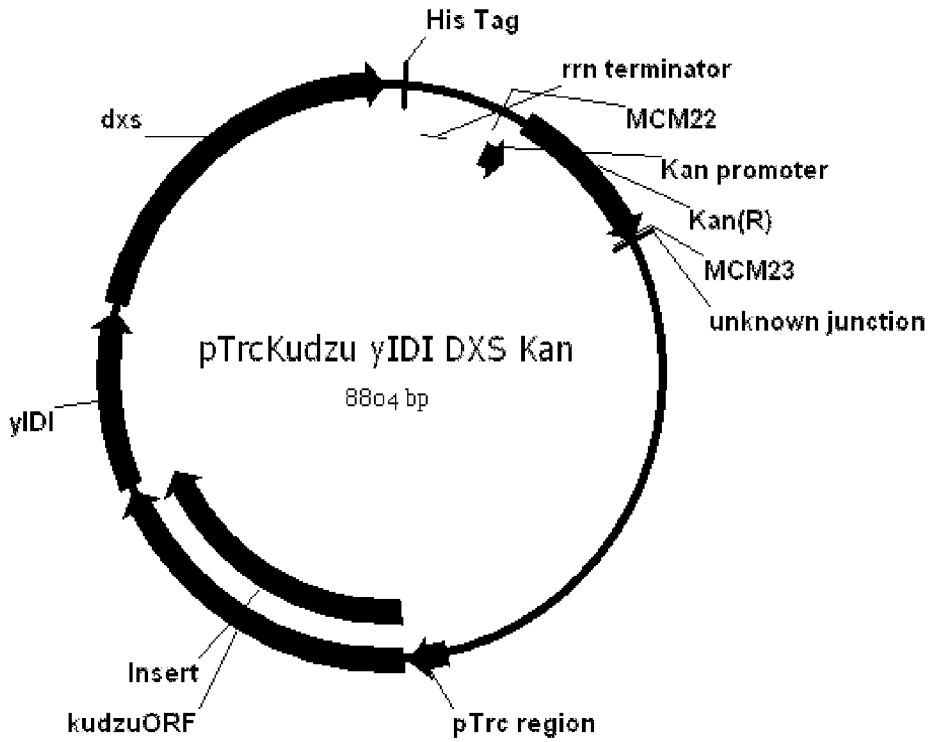

His Tag
rrn terminator
MCM22
Kan promoter
Kan(R)
MCM23
unknown junction
dxs
pTrcKudzu yIDI DXS Kan
8804 bp
yIDI
Insert
kudzuORF
pTrc region

Figure 22A

1
gctggtaccatatgggaattcgaagctttctagaacaaaaactcatctcagaagaggatctgaa
tagcgccgtcgaccatcatcatcatcatcattgagtttaaacggtctccagcttggctgttttg
gcggatgagagaagattttcagcctgatacagattaaatcagaacgcagaagcggtctgataaa
acagaatttgcctggcggcagtagcgcggtggtcccacctgaccccatgccgaactcagaagtg
aaacgccgtagcgccgatggtagtgtggggtctccccatgcgagagtagggaactgccaggcat
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acggatggcctttttgcgtttctacaaactcttttttgtttatttttctaaatacattcaaatat
gtatccgcttaaccggaattgccagctggggcgccctctggtaaggttgggaagccctgcaaag
taaactggatggctttctcgccgccaaggatctgatggcgcaggggatcaagctctgatcaaga
gacaggatgaggatcgtttcgcatgattgaacaagatggattgcacgcaggttctccggccgct
tgggtggagaggctattcggctatgactgggcacaacagacaatcggctgctctgatgccgccg
tgttccggctgtcagcgcaggggcgcccggttcttttgtcaagaccgacctgtccggtgccct
gaatgaactgcaagacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgca
gctgtgctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggc
aggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcg
gcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgag
cgagcacgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcagg
ggctcgcgccagccgaactgttcgccaggctcaaggcgagcatgcccgacggcgaggatctcgt
cgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgcttttctggattc
atcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgata
ttgctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcc
cgattcgcagcgcatcgccttctatcgccttcttgacgagttcttctgacatgaccaaaatccc
ttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttga
gatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtgg
tttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgca
gataccaaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgtagca
ccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgt
gtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacggg
gggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgt
gagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggca
gggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcc
tgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagc
ctatggaaaaacgccagcaacgcggcctttttacggttcctggccttttgctggccttttgctc
acatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagc
tgataccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcggaagag
cgcctgatgcggtatttctccttacgcatctgtgcggtatttcacaccgcatatggtgcactc
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tgggtcatggctgcgccccgacacccgccaacacccgctgacgcgccctgacgggcttgtctgc
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Figure 22B

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Figure 22C

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Figure 22D

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SEQ ID NO:10

Figure 23A

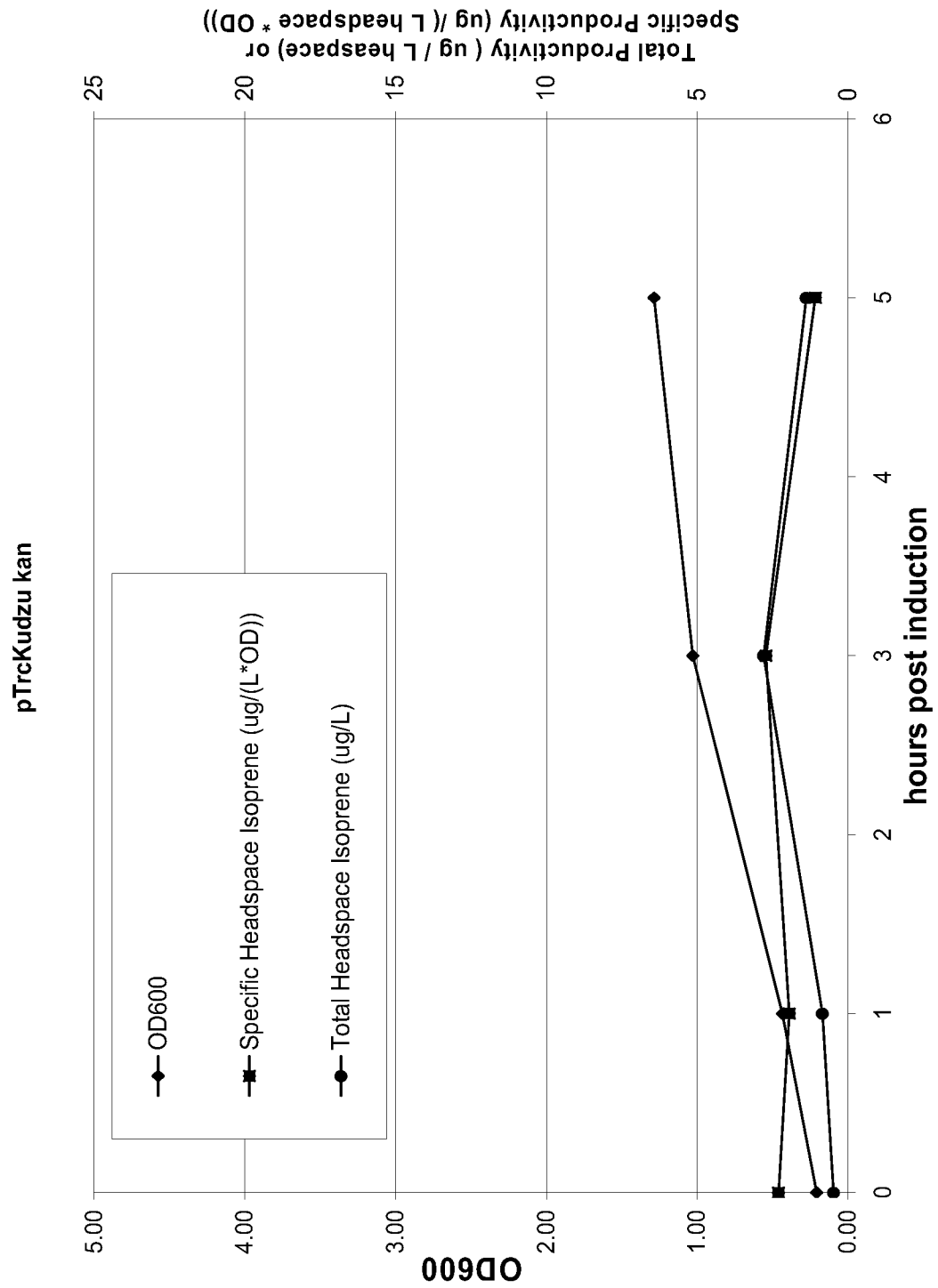

pTrcKudzu kan
OD600
Total Productivity (ug / L headspace) or Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23B

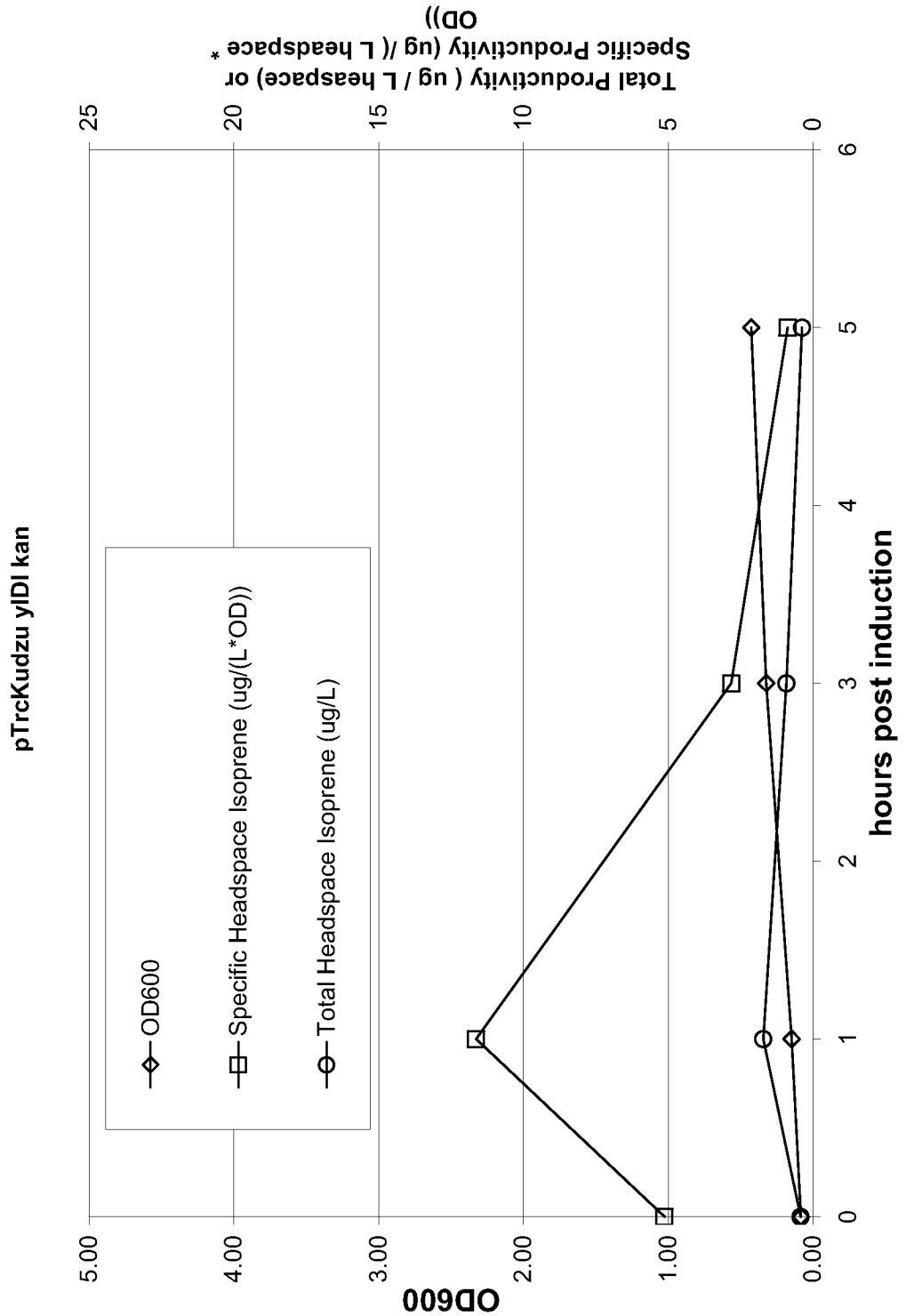

pTrcKudzu ylDI kan
OD600
hours post induction
Total Productivity (ug / L headspace) or Specific Productivity (ug /(L headspace * OD))
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23C

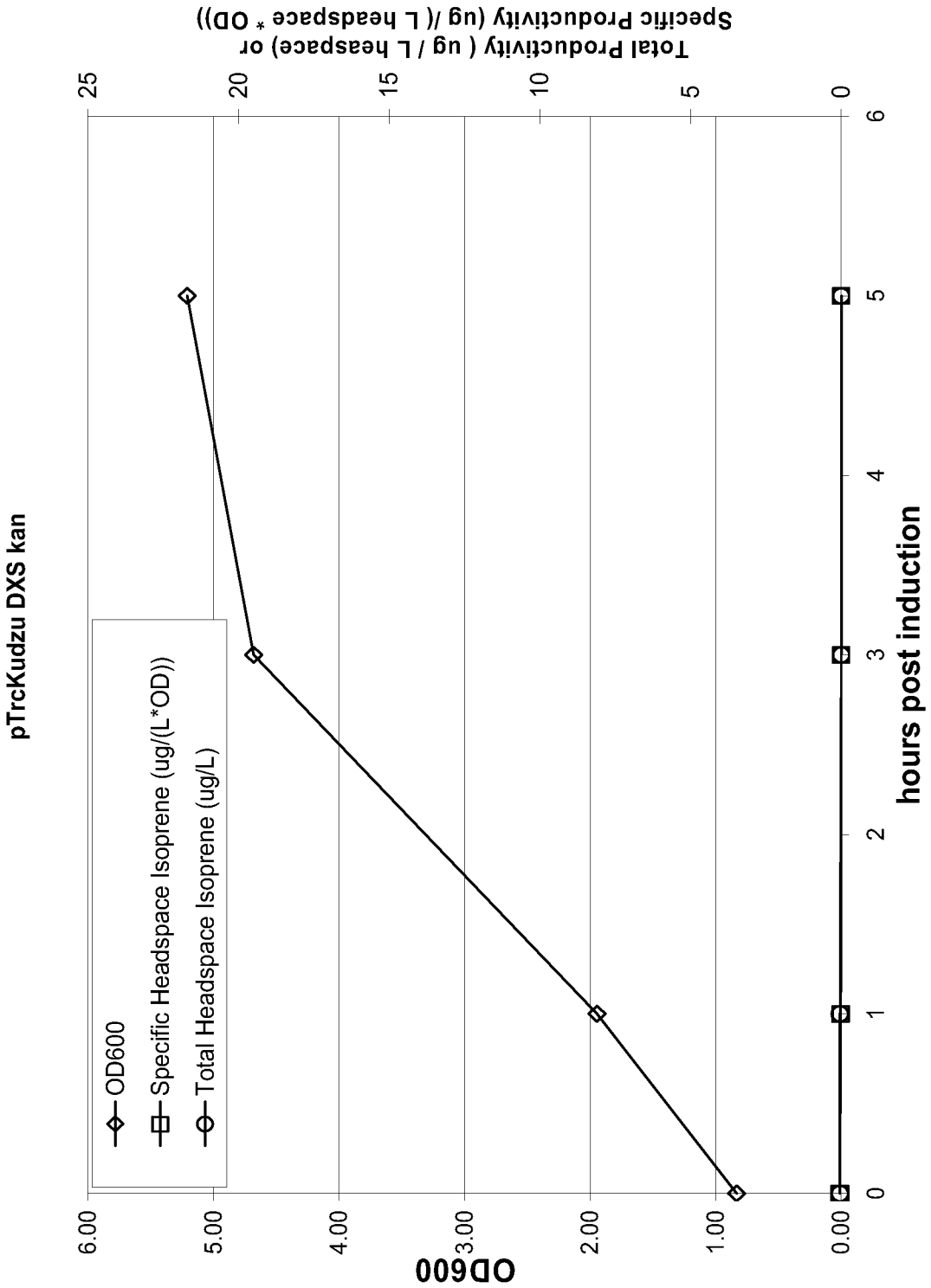

pTrcKudzu DXS kan
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
0.00
1.00
2.00
3.00
4.00
5.00
6.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23D

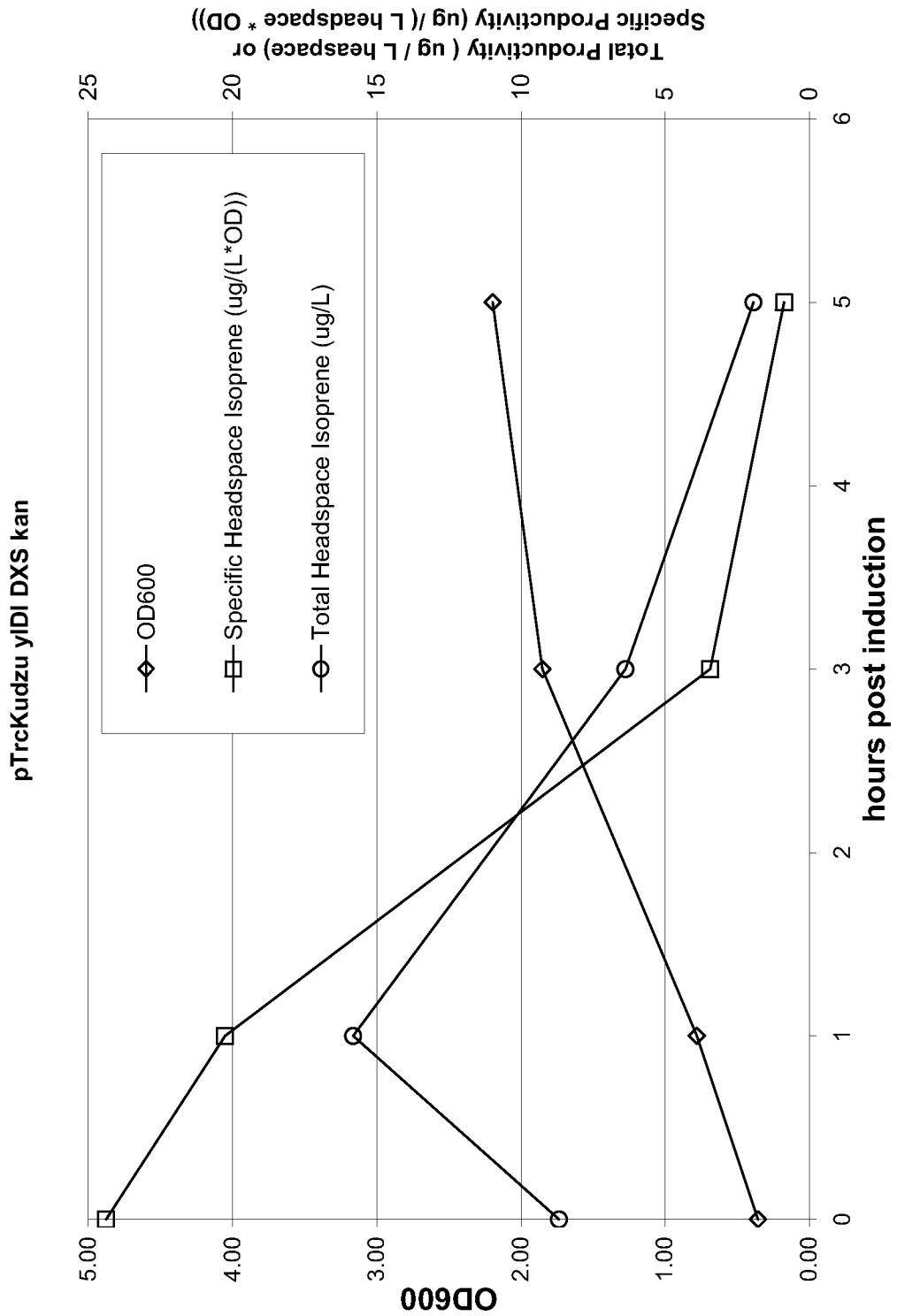

pTrcKudzu yIDI DXS kan
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
hours post induction
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23E

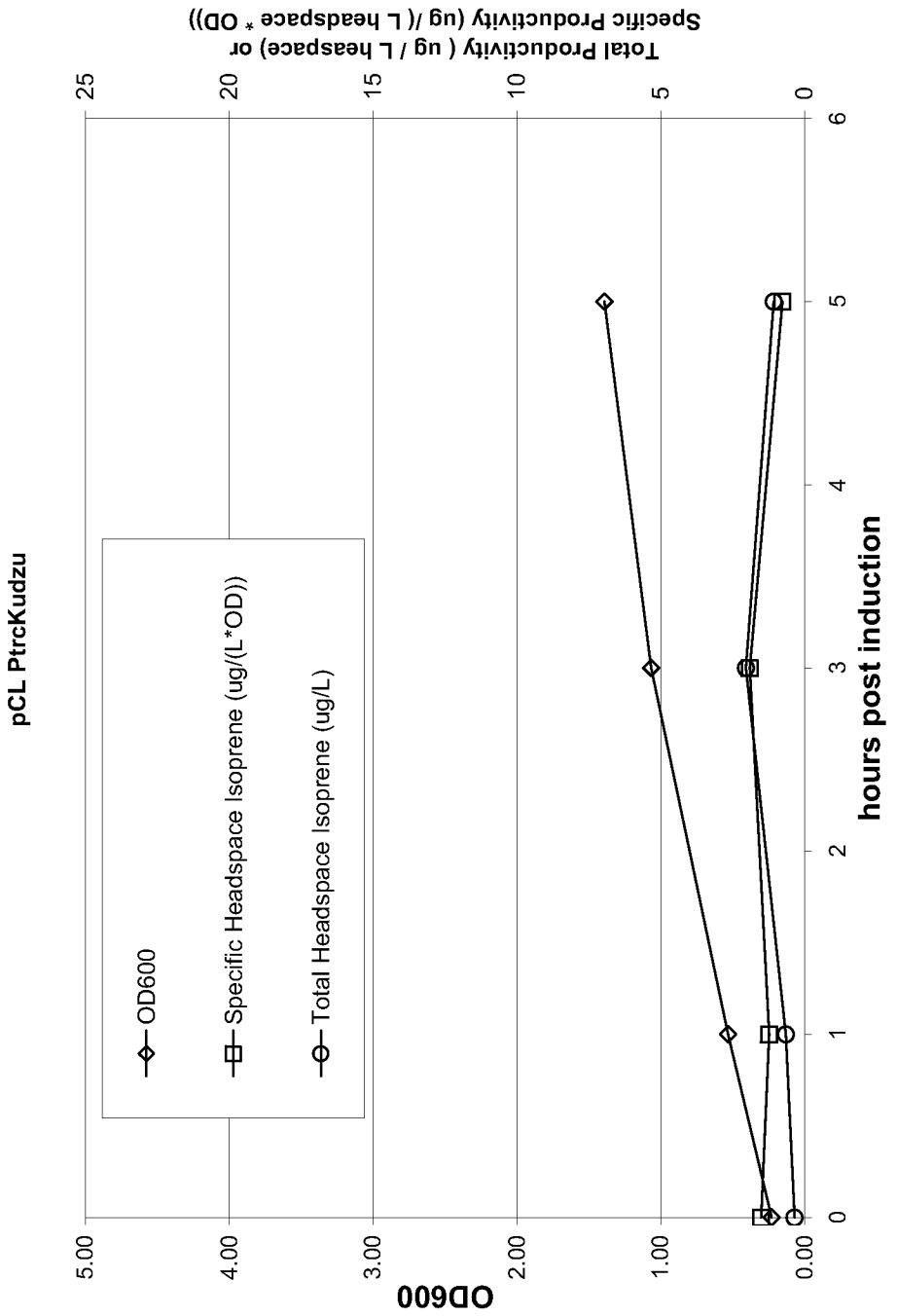

pCL PtrcKudzu
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
0
1
2
3
4
5
6

Figure 23F

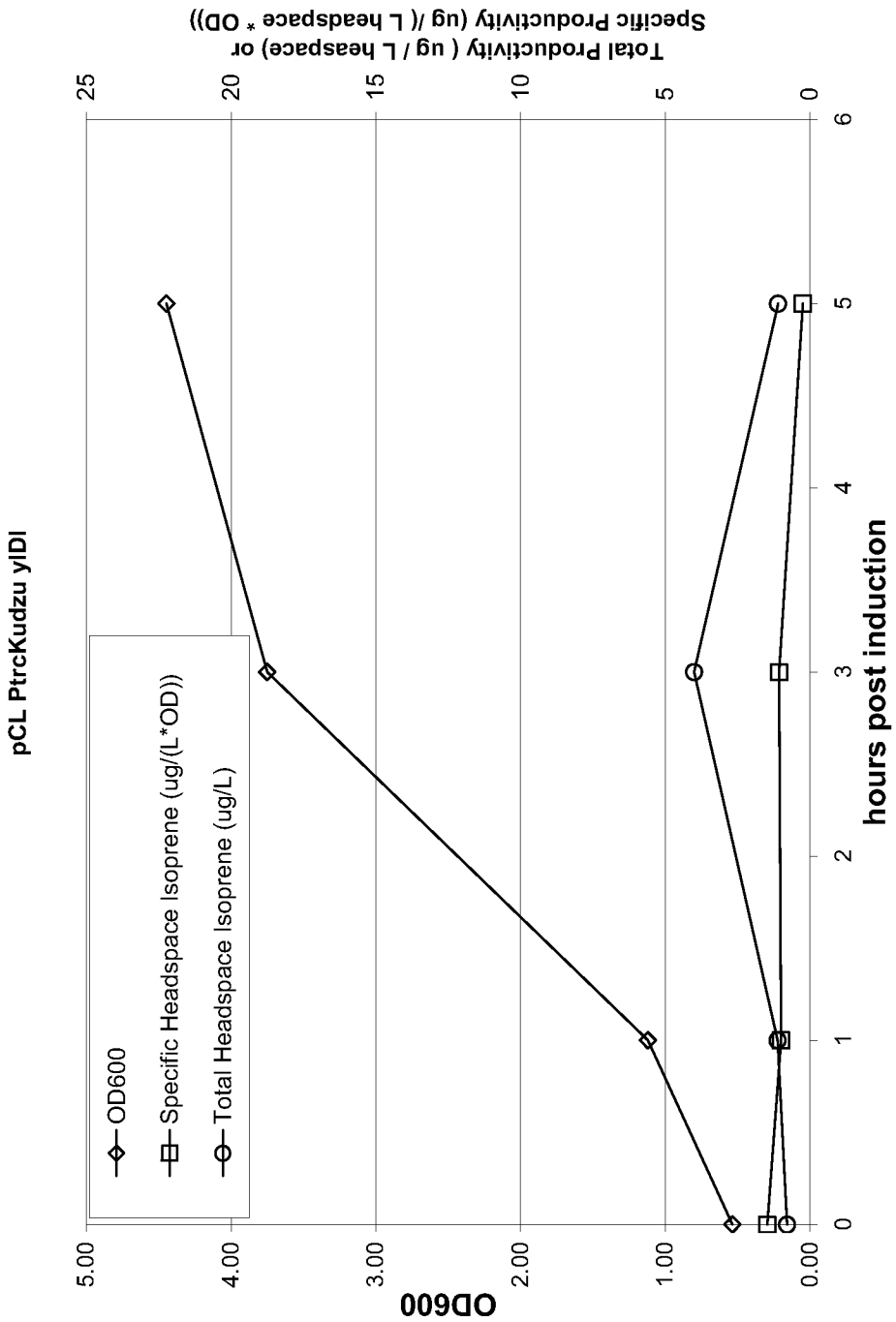

pCL PtrcKudzu ylDI
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
5.00
4.00
3.00
2.00
1.00
0.00
25
20
15
10
5
0
0
1
2
3
4
5
6

Figure 23G

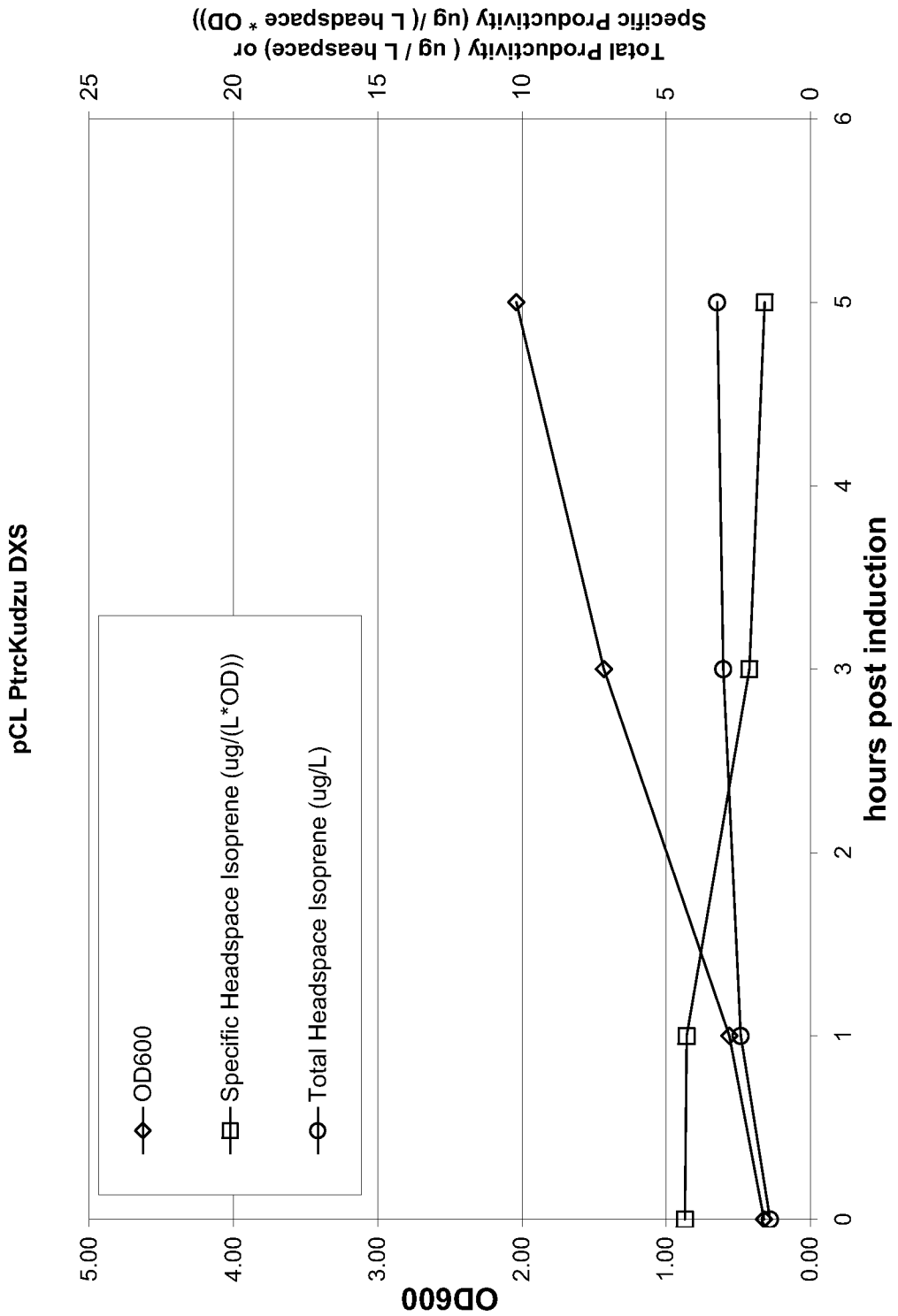

pCL PtrcKudzu DXS
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
5.00
4.00
3.00
2.00
1.00
0.00
25
20
15
10
5
0
0
1
2
3
4
5
6

Figure 23H

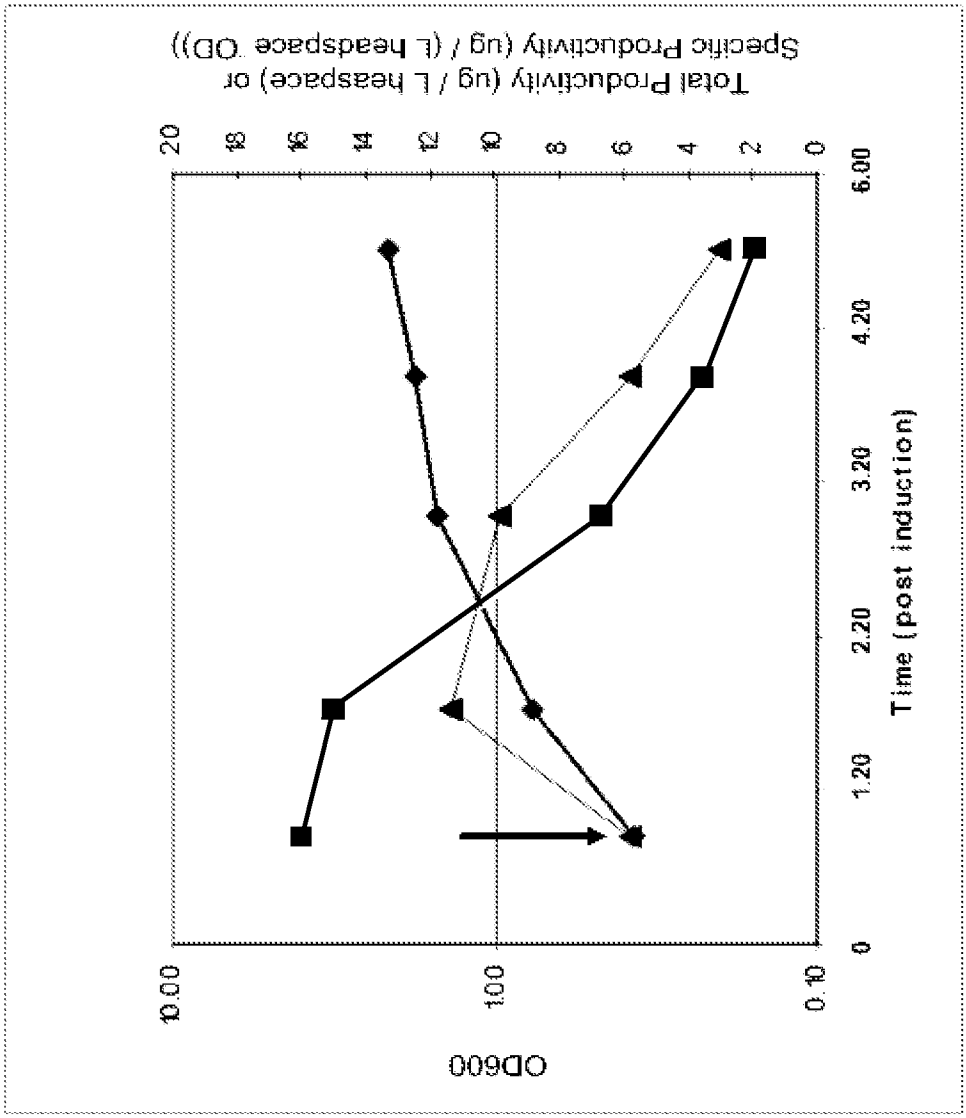
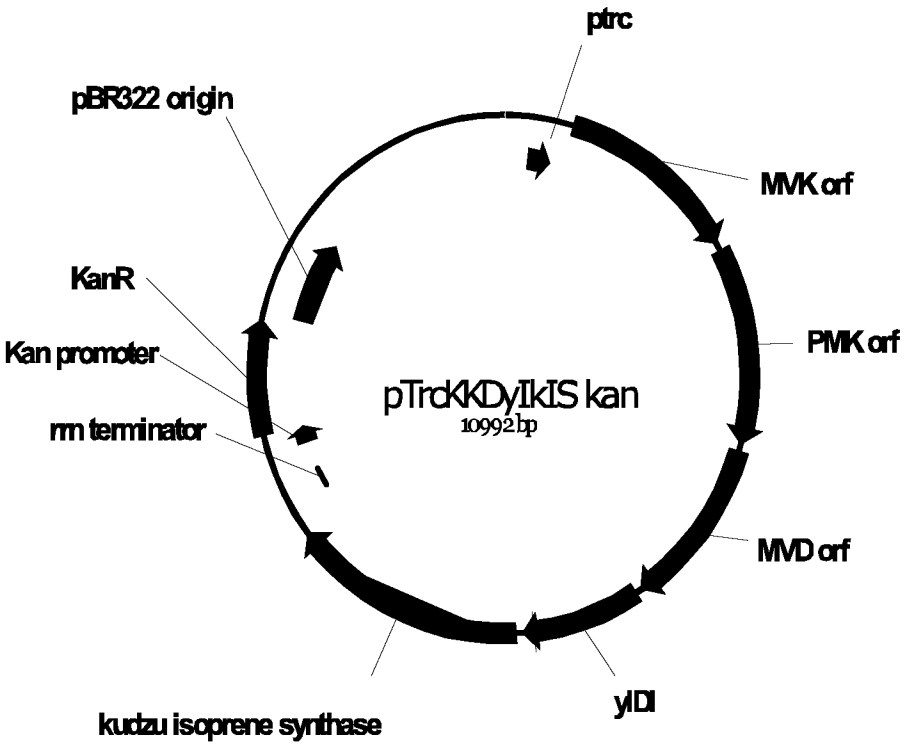

Total Productivity (ug / L headspace) or
Specific Productivity (ug / (L headspace *OD))
OD600
Time (post induction)
0
1.20
2.20
3.20
4.20
6.00
0.10
1.00
10.00
0
2
4
6
8
10
12
14
16
18
20

Figure 24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pBR322 origin
MVK orf
KanR
PMK orf
Kan promoter
pTrcKKDyIkIS kan
10992 bp
rrn terminator
MVD orf
yIDI
kudzu isoprene synthase

Figure 25A

5'-
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Figure 25B

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Figure 25C

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Figure 25D

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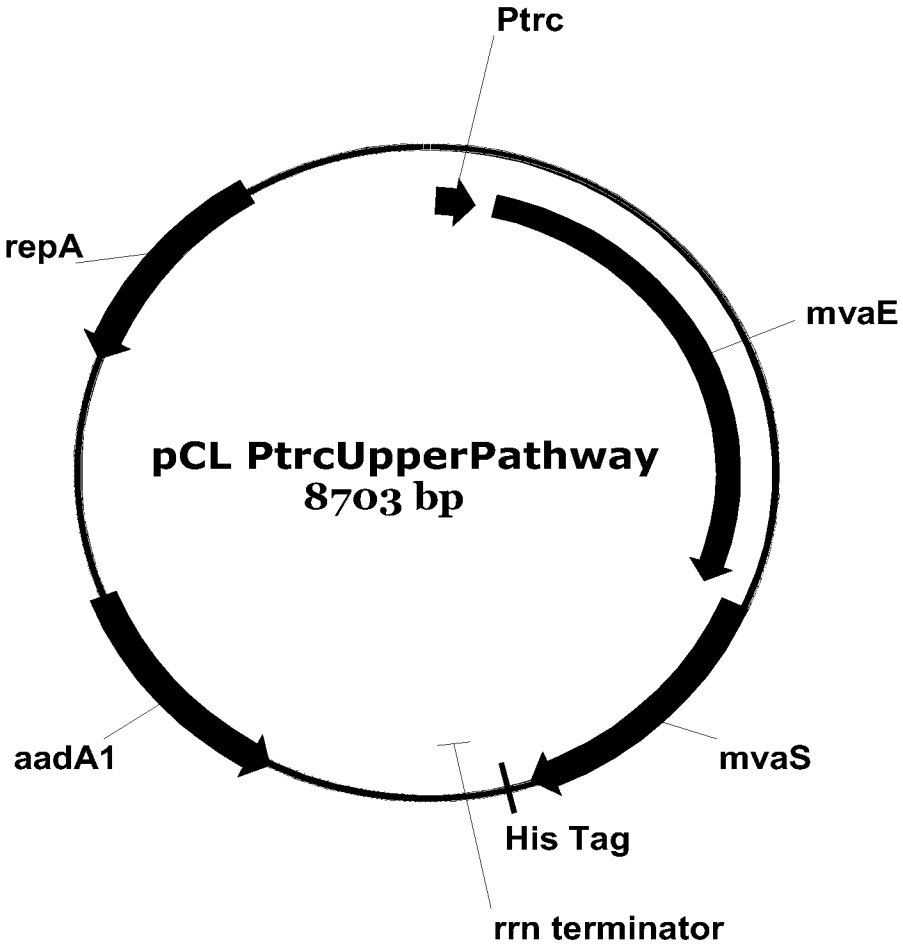
Figure 26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rrn terminator

Figure 27A

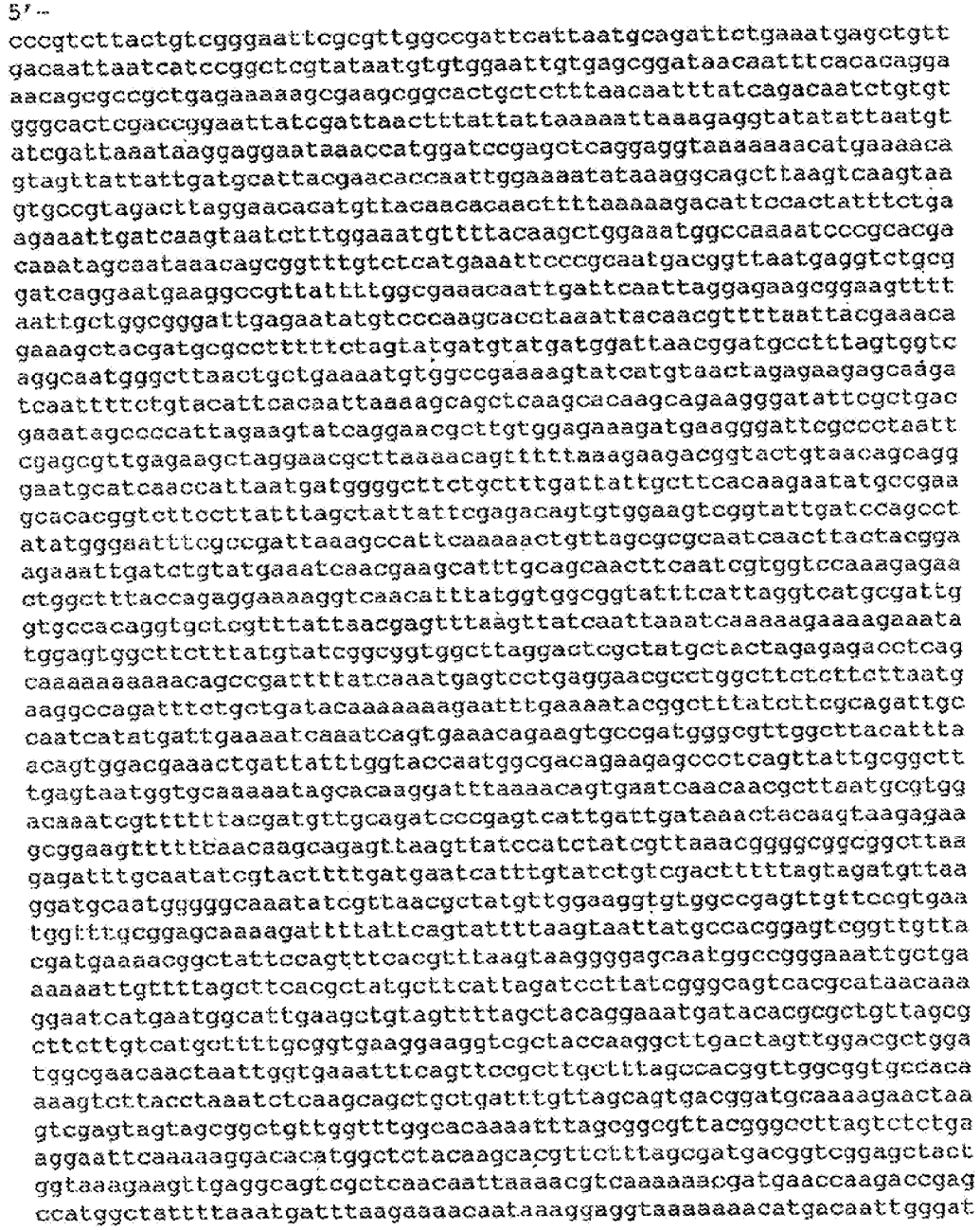
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Figure 27B

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Figure 27C

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Figure 27D

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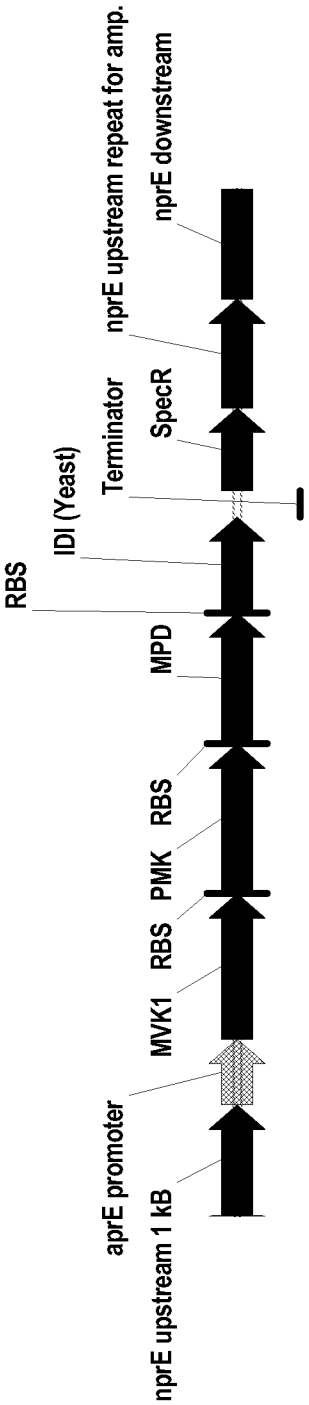

Lower Pathway Bacillus Cassette

9371 bp

Figure 29A

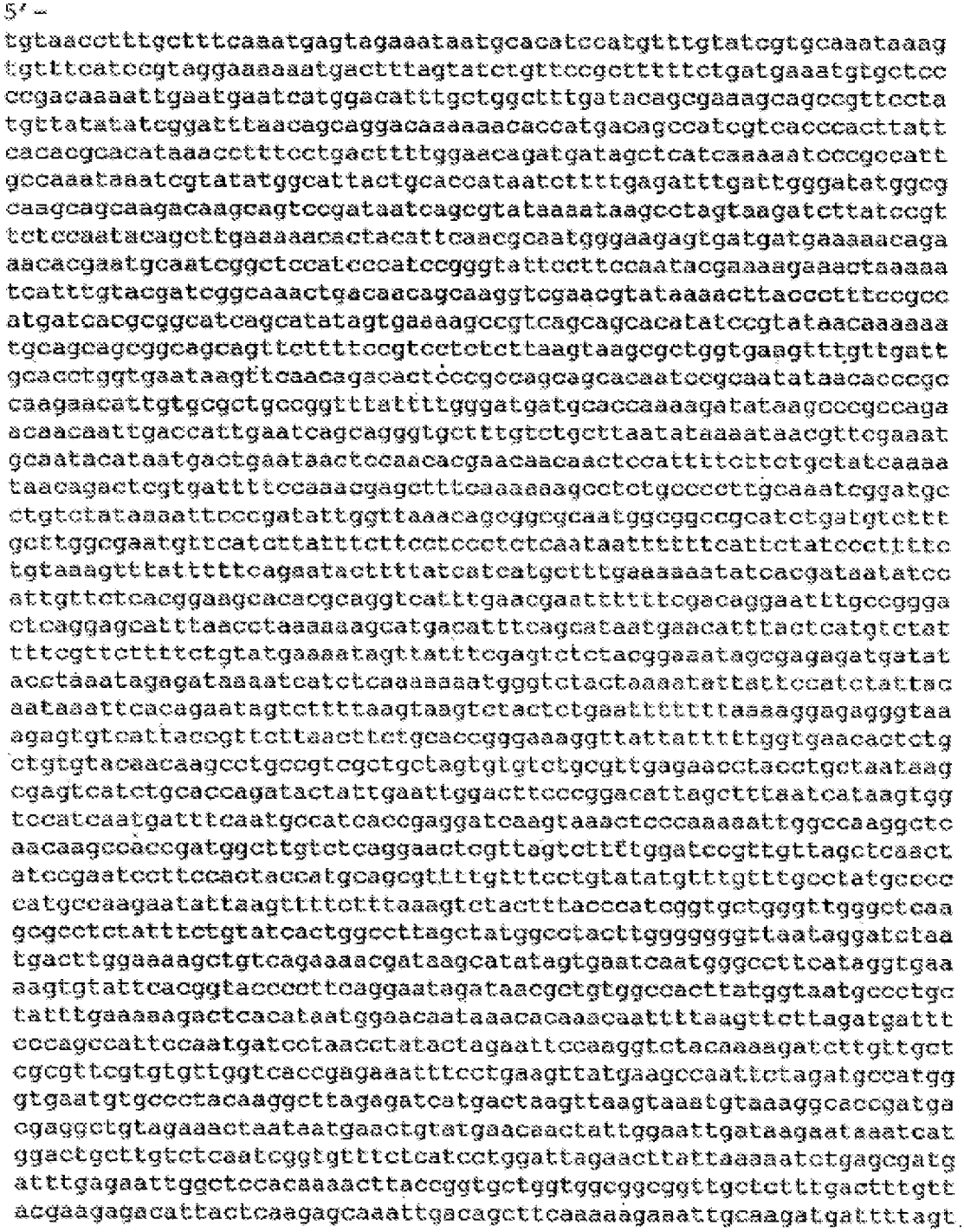
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Figure 29B

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Figure 29C

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Figure 29D

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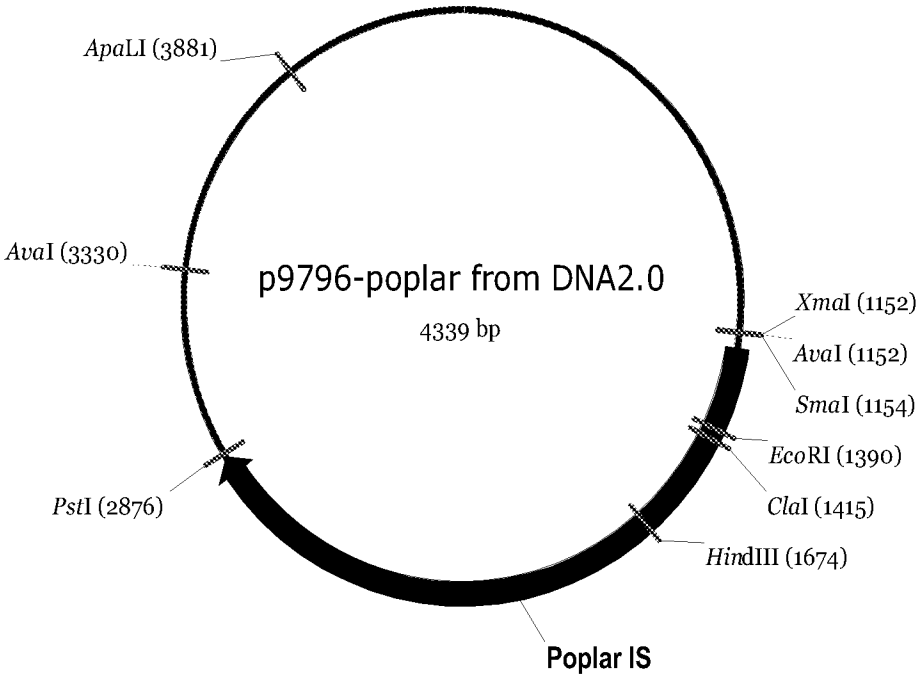
Figure 30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PstI (2876)
Poplar IS

Figure 31A

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Figure 31B

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Figure 32

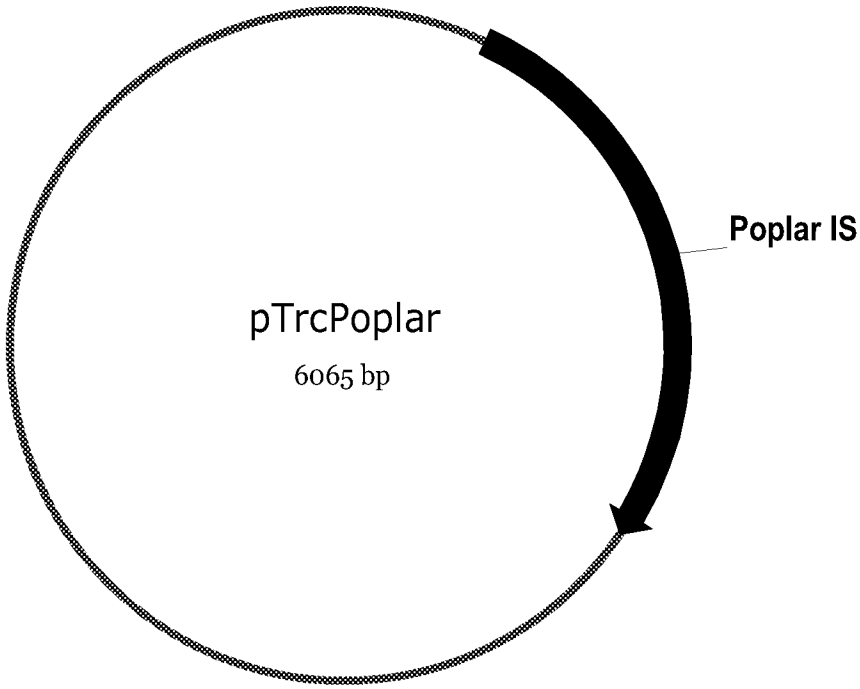

Figure 33A

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Figure 33B

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Figure 33C

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Figure 34

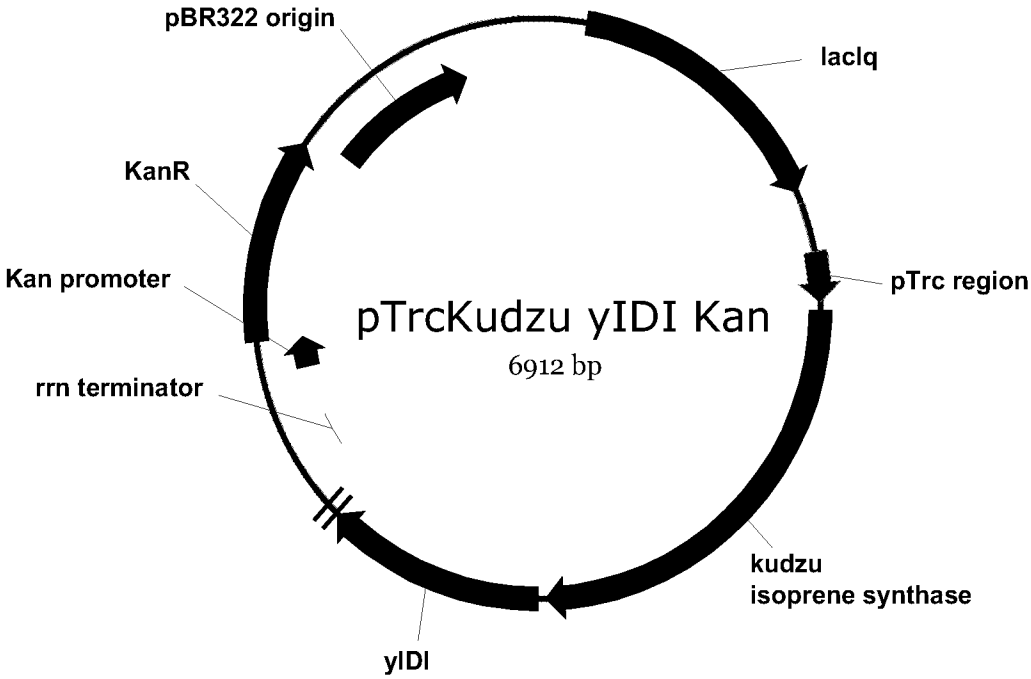
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Figure 35A

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Figure 35B

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Figure 35C

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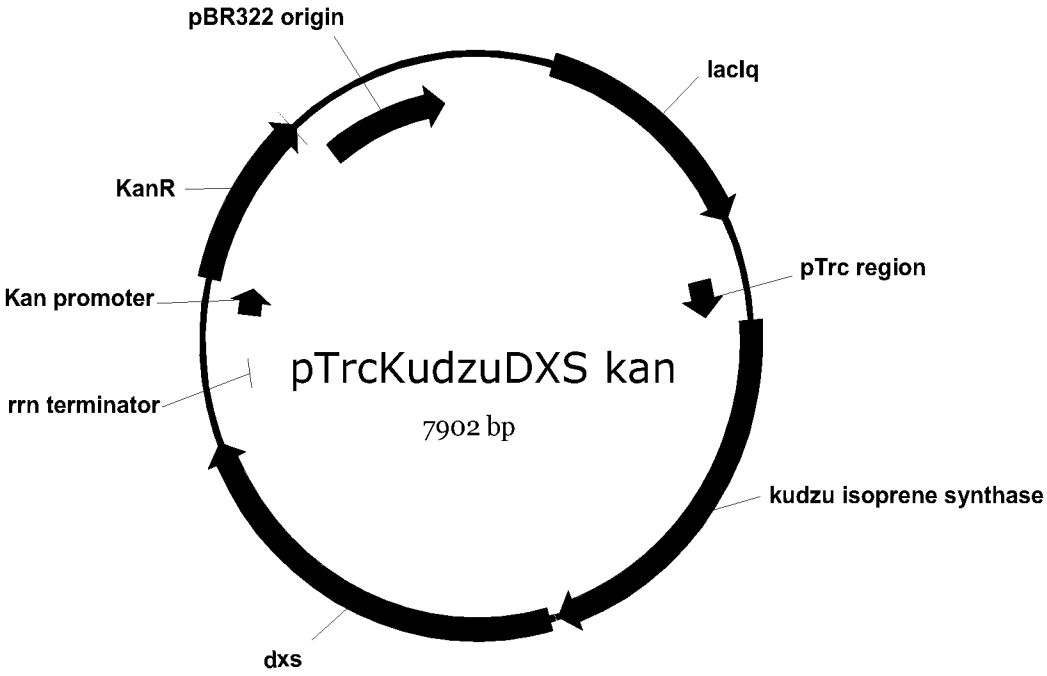
Figure 36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dxs

Figure 37A

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Figure 37B

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Figure 37C

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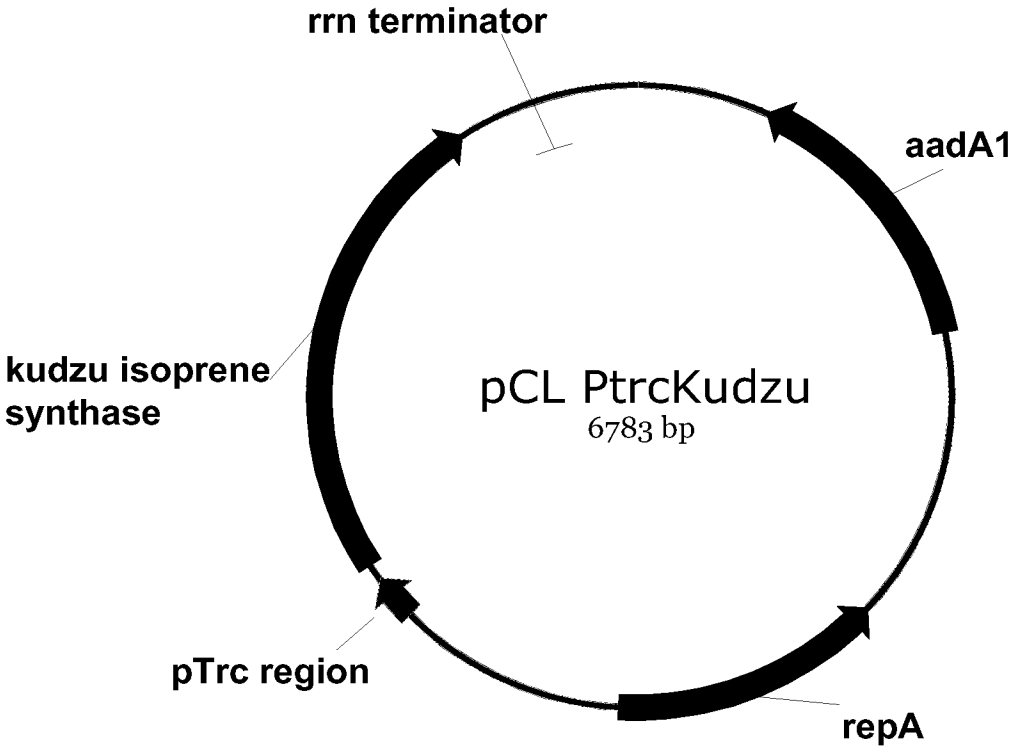
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Figure 39A

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Figure 39B

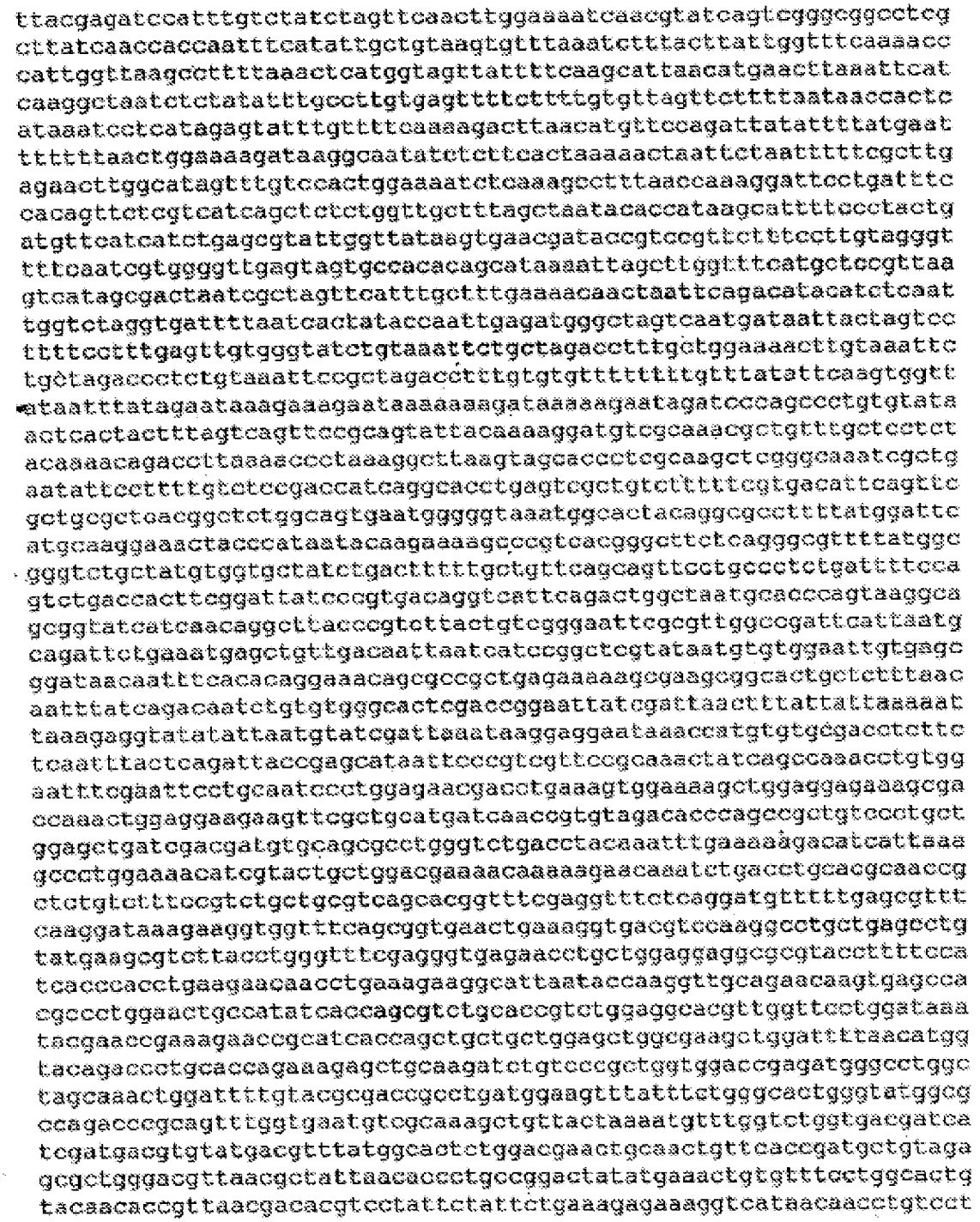
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Figure 39C

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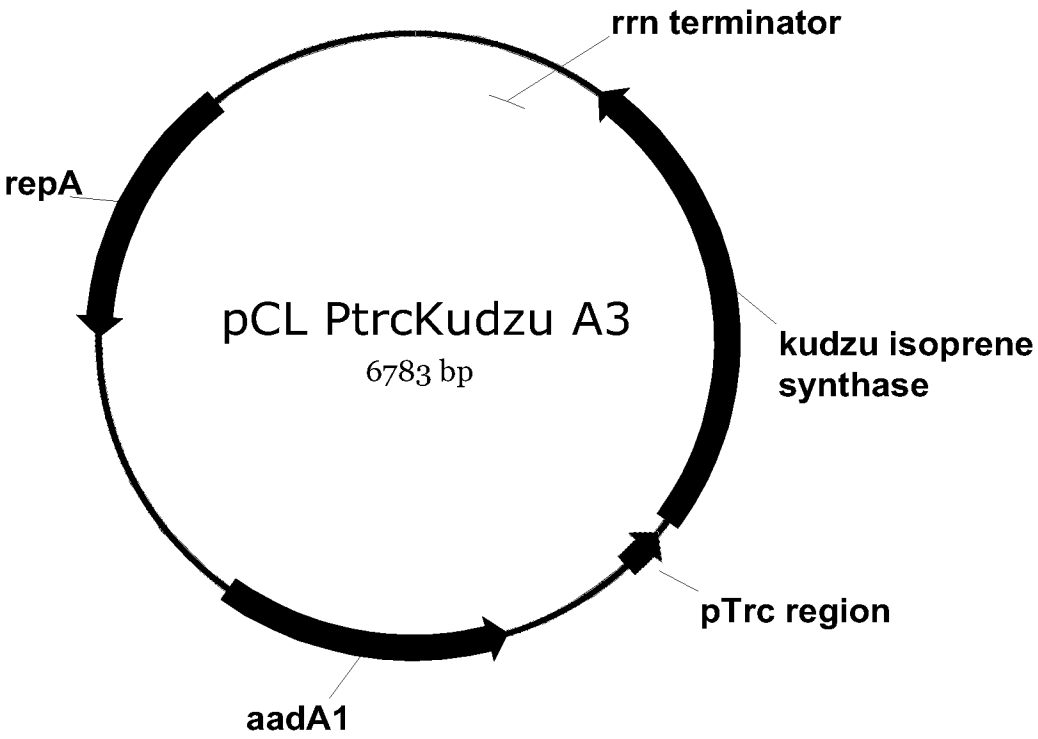
SEQ ID NO:18

Figure 40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pCL PtrcKudzu A3
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kudzu isoprene synthase
pTrc region
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Figure 41A

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Figure 41B

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Figure 41C

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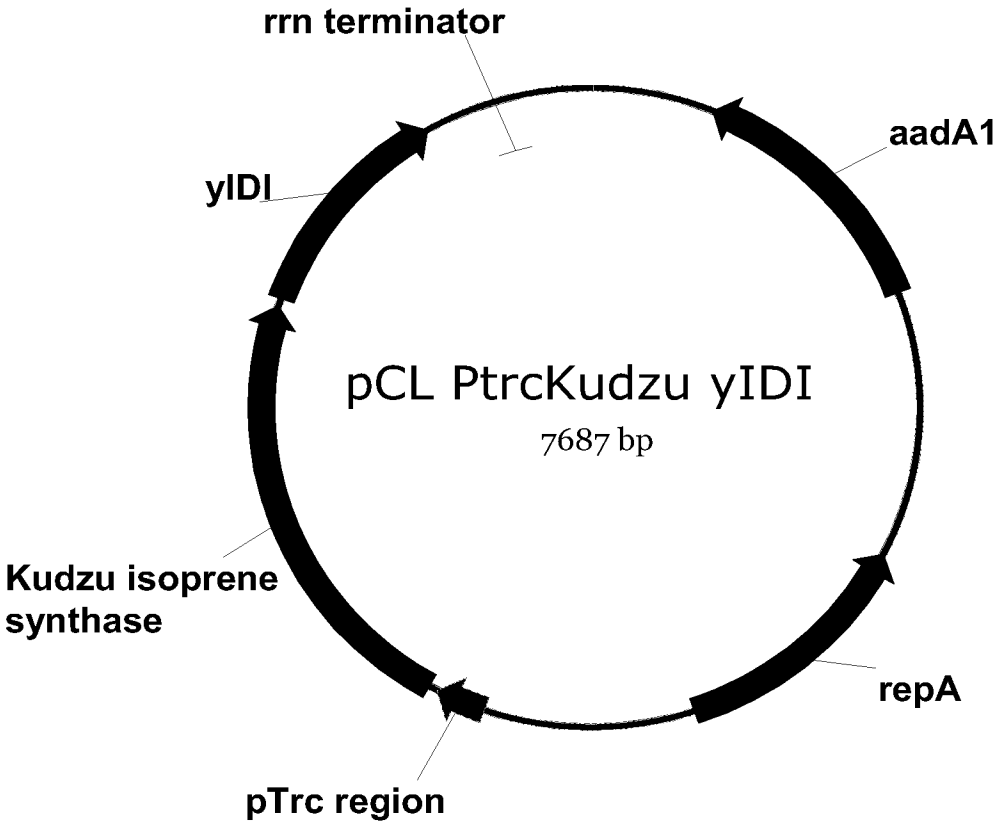
Figure 42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Kudzu isoprene synthase
repA
pTrc region

Figure 43A

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Figure 43B

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Figure 43C

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SEQ ID NO:20

Figure 44

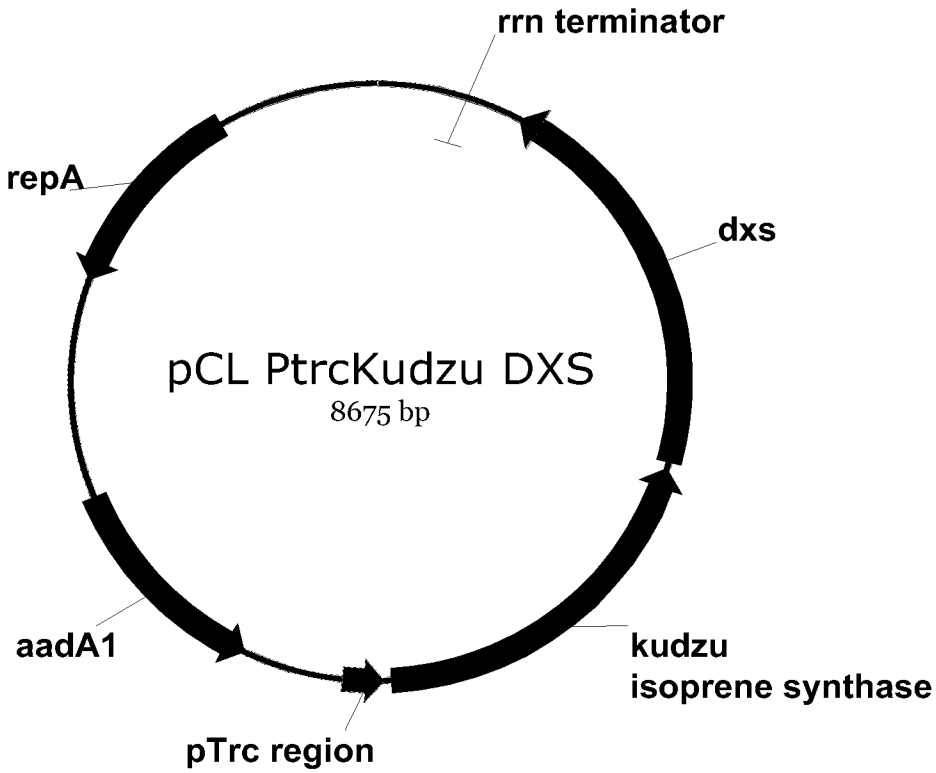
rrn terminator
repA
dxs
pCL PtrcKudzu DXS
8675 bp
aadA1
kudzu
isoprene synthase
pTrc region

Figure 45A

5'-
ccogtcttactgtcgggaattcgcgttggccgattcattaatgcagattattgaagcatttatc
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Figure 45B

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aagccgggataatttttgttgttggaccatttcgcctcttgcagaaaggctttgcacagttcacg
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Figure 45C

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Figure 45D

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gggcgttttatggcgggtctgctatgtggtgctatctgactttttgctgttcagcagttcctgc
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SEQ ID NO:21

Figure 46A

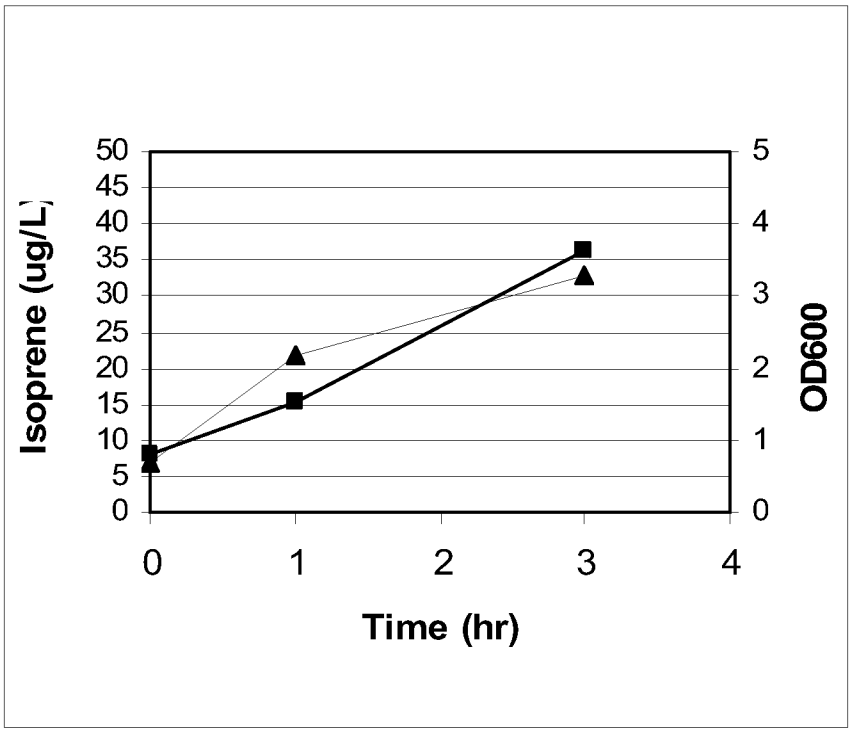
Isoprene (ug/L)
OD600
Time (hr)
50
45
40
35
30
25
20
15
10
5
0
5
4
3
2
1
0
0
1
2
3
4

Figure 46B

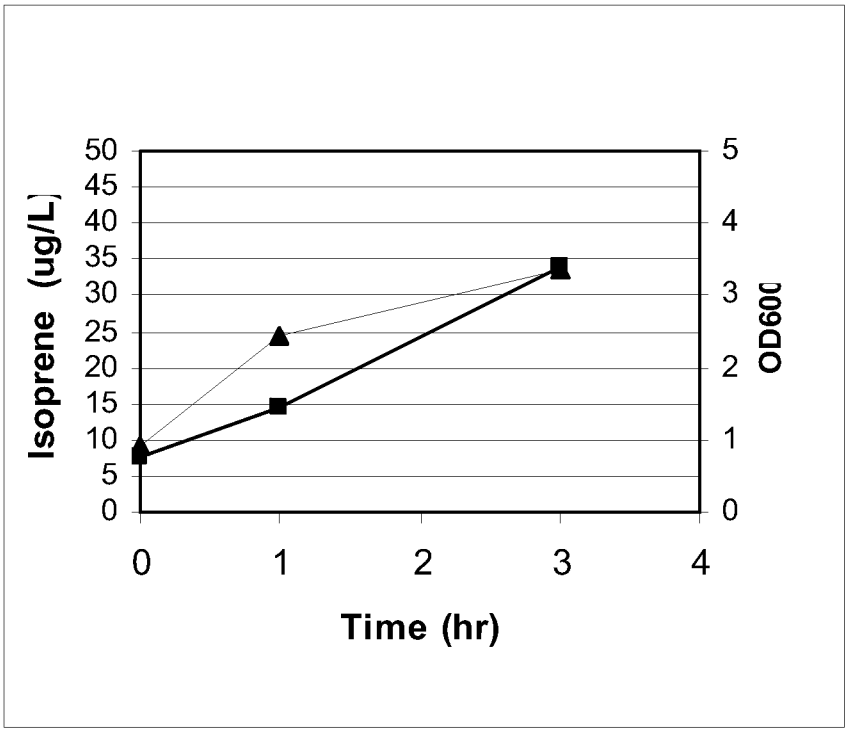
Isoprene (ug/L)
50
45
40
35
30
25
20
15
10
5
0
OD600
5
4
3
2
1
0
0
1
2
3
4
Time (hr)

Figure 46C

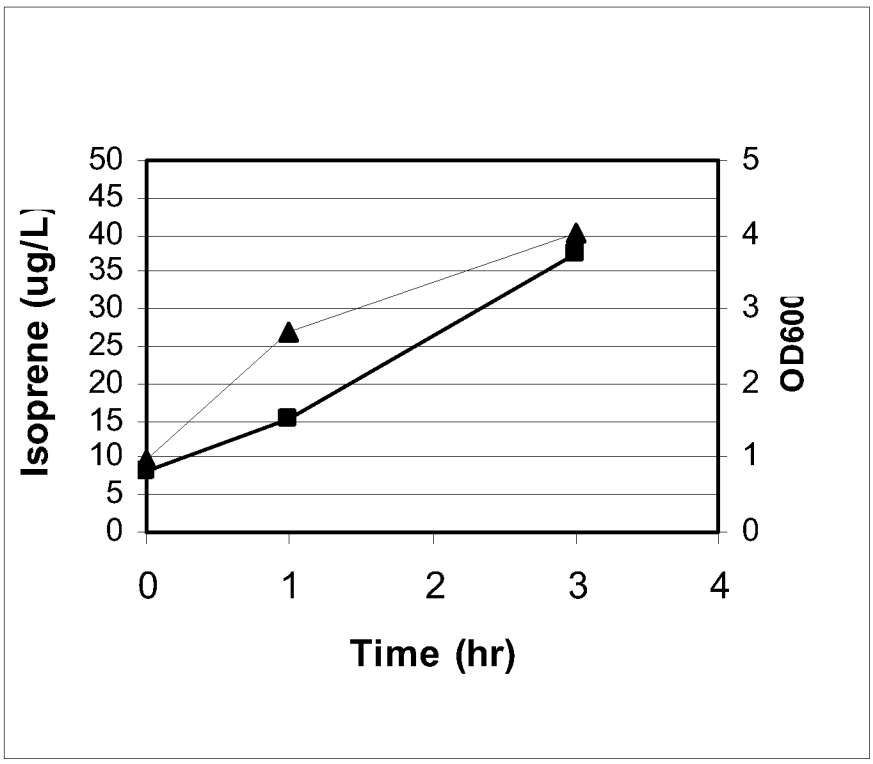
Isoprene (ug/L)
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
Time (hr)
5
4
3
2
1
0
OD600

Figure 46D

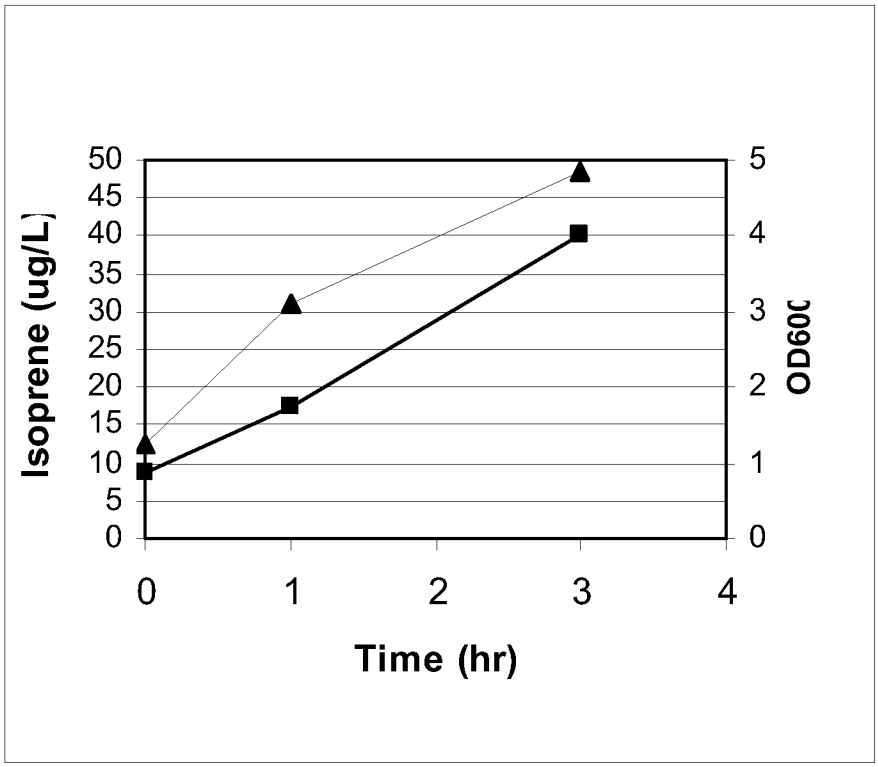
Isoprene (ug/L)
50
45
40
35
30
25
20
15
10
5
0
5
4
3
2
1
0
OD60
0
1
2
3
4
Time (hr)

Figure 46E

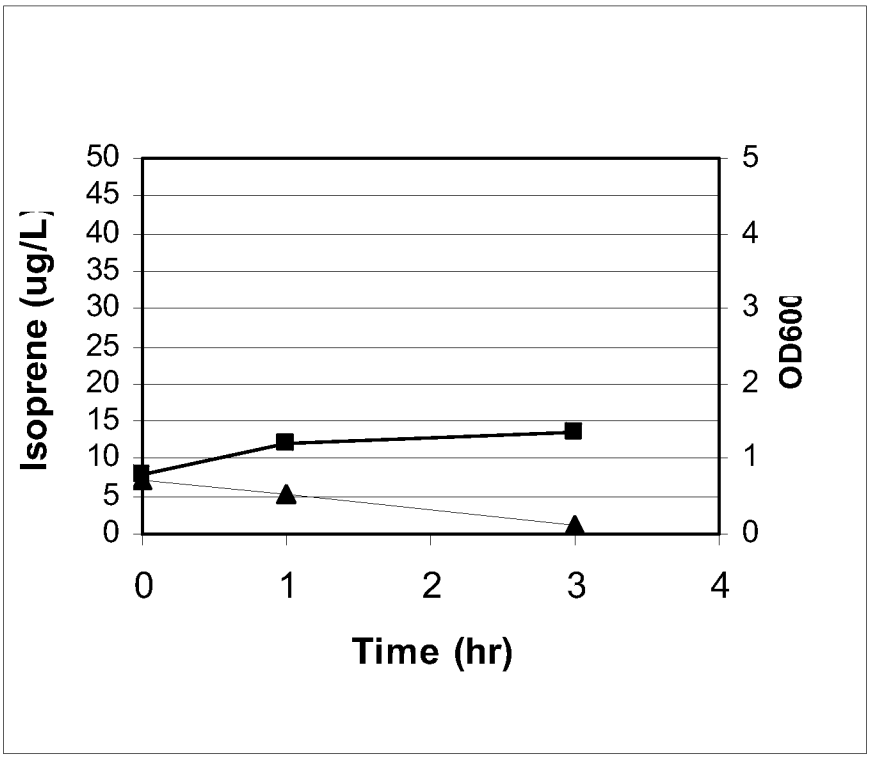
Isoprene (ug/L)
OD600
Time (hr)
50
45
40
35
30
25
20
15
10
5
0
5
4
3
2
1
0
0
1
2
3
4

Figure 47A

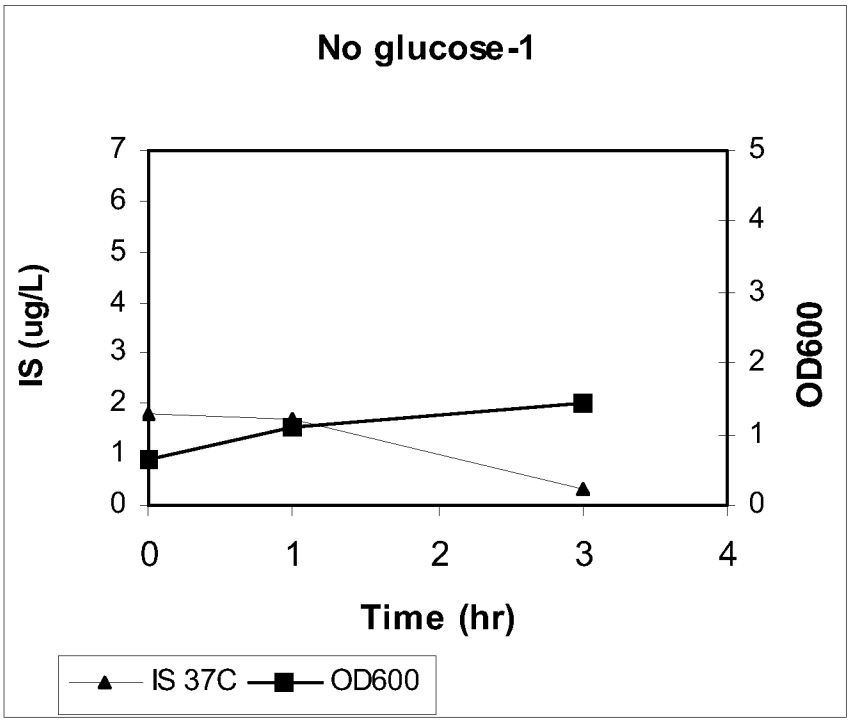

Figure 47B

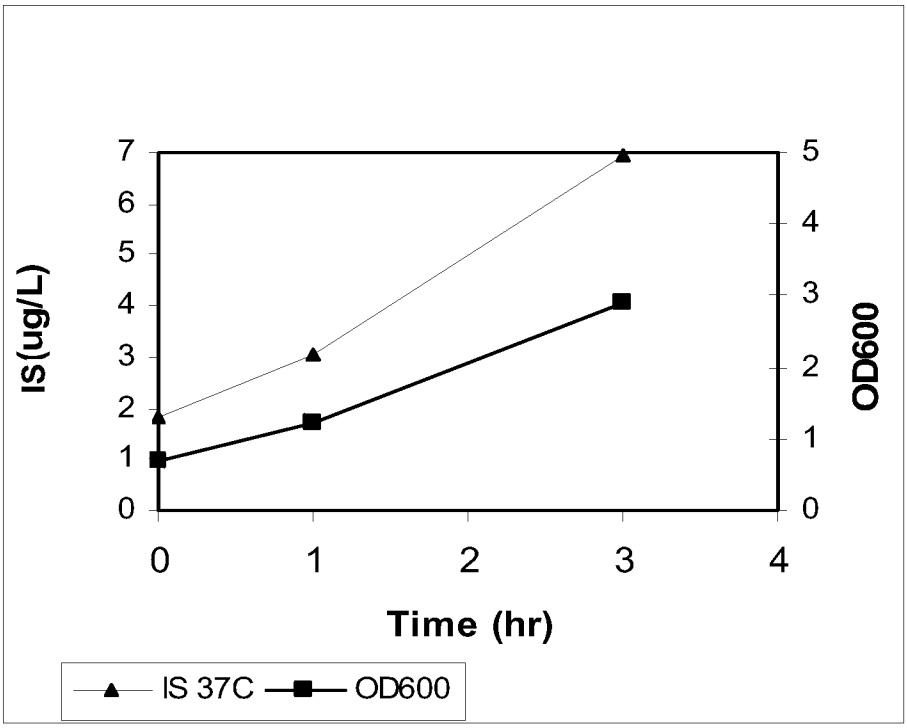

Figure 47C

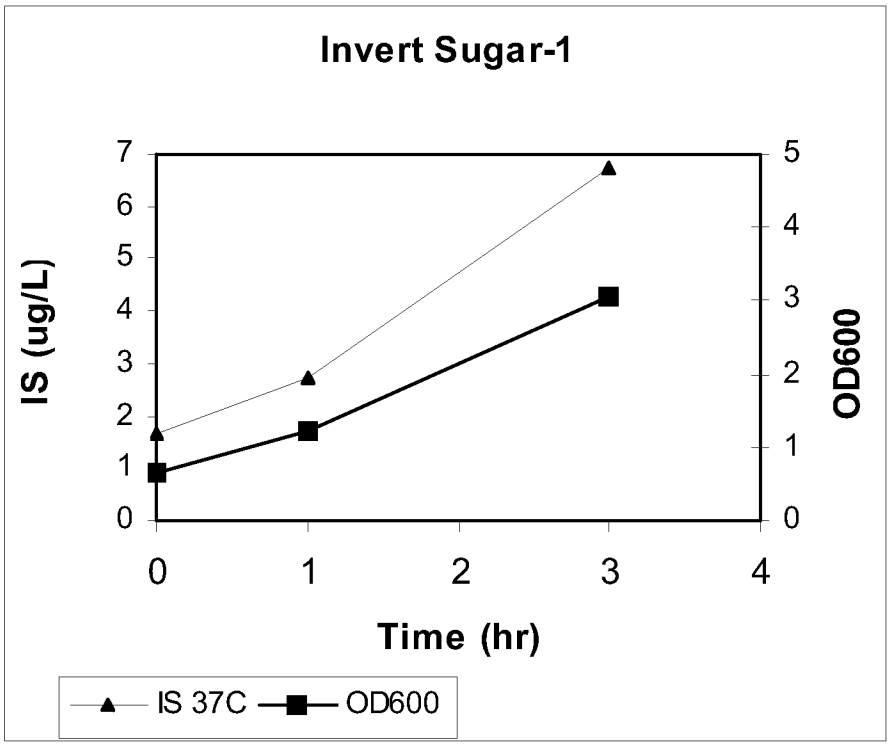

Figure 47D

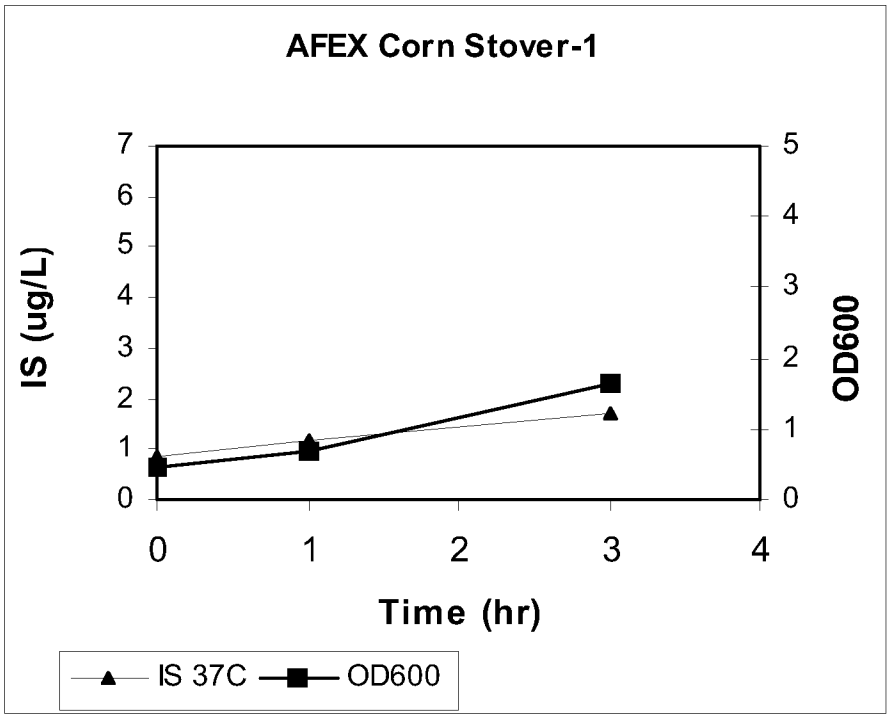

Figure 48A

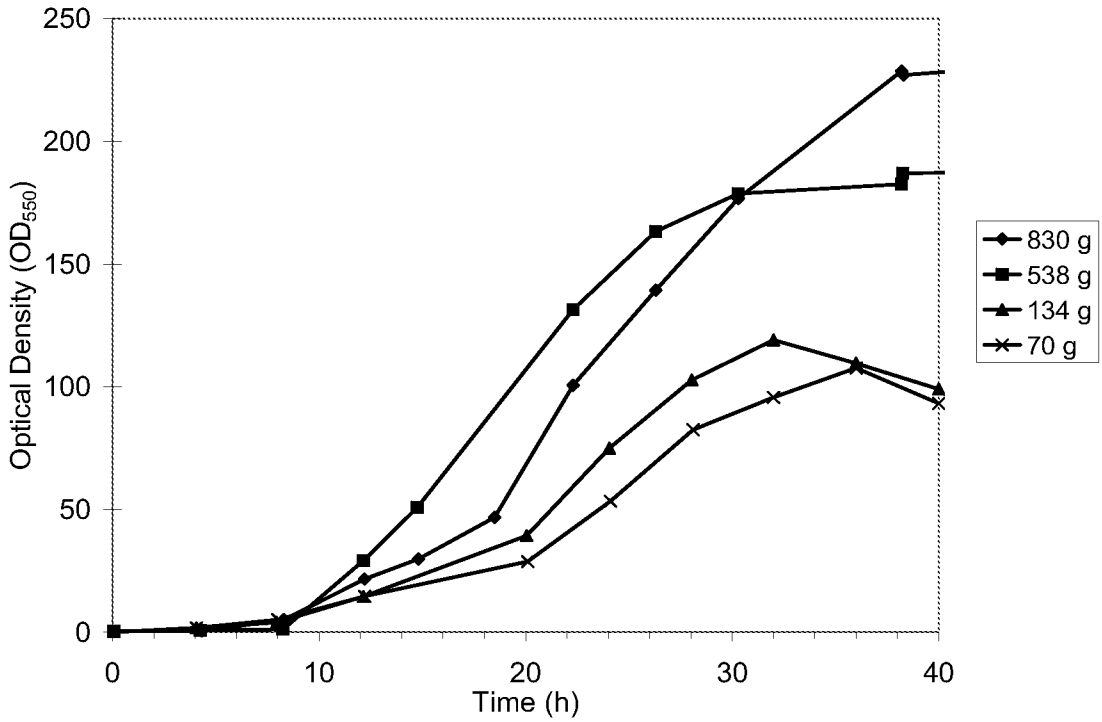
250
200
150
100
50
0
Optical Density (OD550)
0
10
20
30
40
Time (h)
830 g
538 g
134 g
70 g

Figure 48B

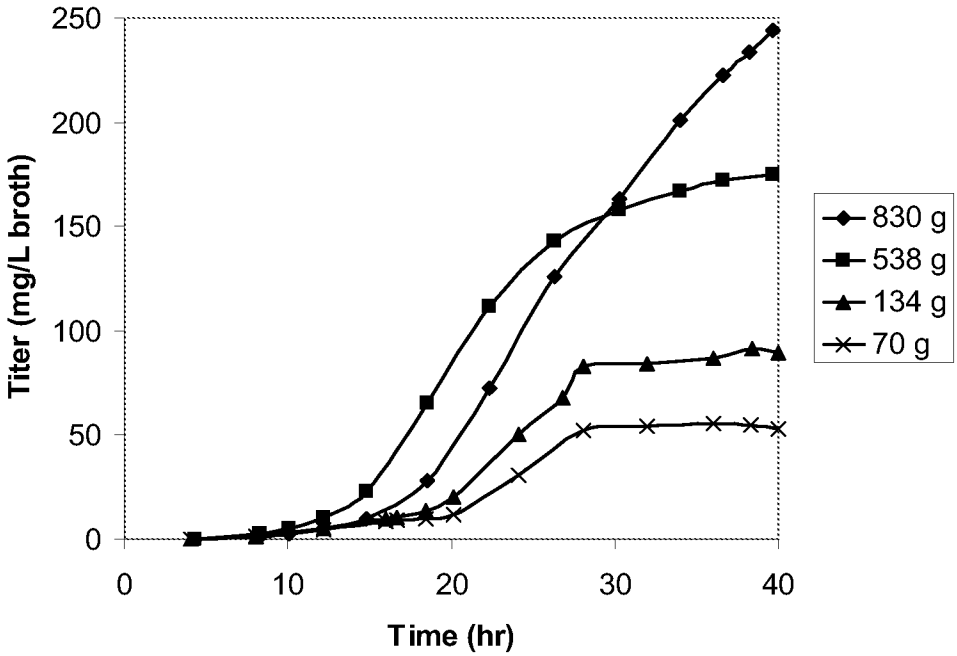
250
200
150
100
50
0
Titer (mg/L broth)
0
10
20
30
40
Time (hr)
830 g
538 g
134 g
70 g

Figure 48C

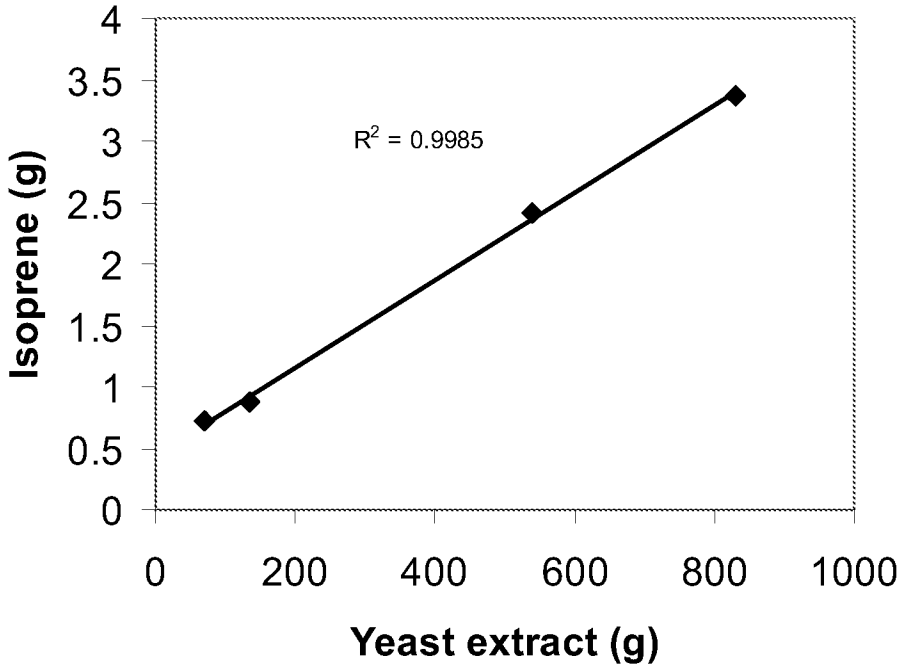
4
3.5
3
2.5
2
1.5
1
0.5
0
Isoprene (g)
R2 = 0.9985
0
200
400
600
800
1000
Yeast extract (g)

Figure 49A

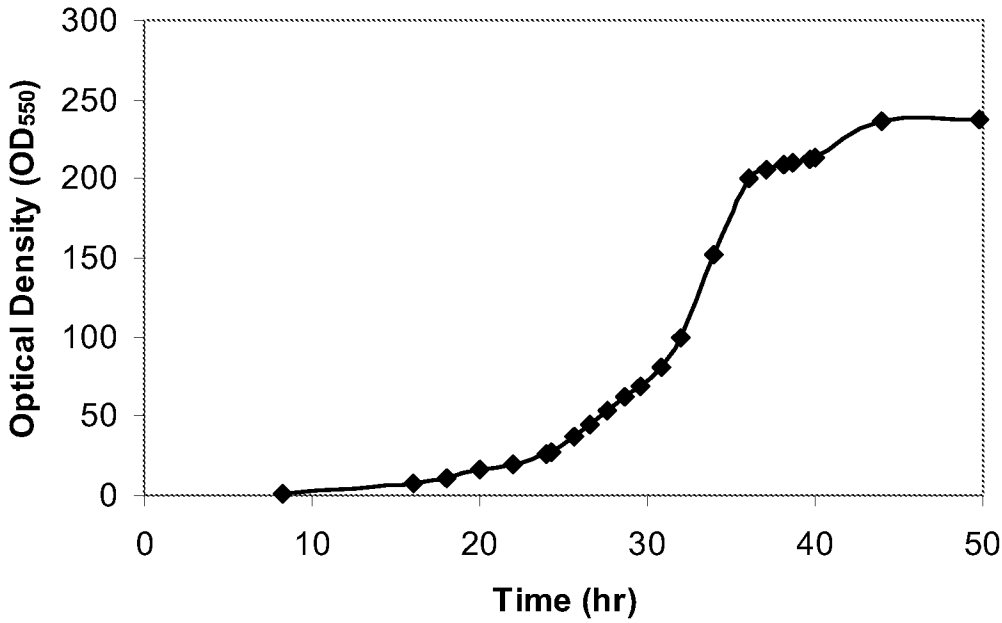
Optical Density (OD550)
300
250
200
150
100
50
0
0
10
20
30
40
50
Time (hr)

Figure 49B

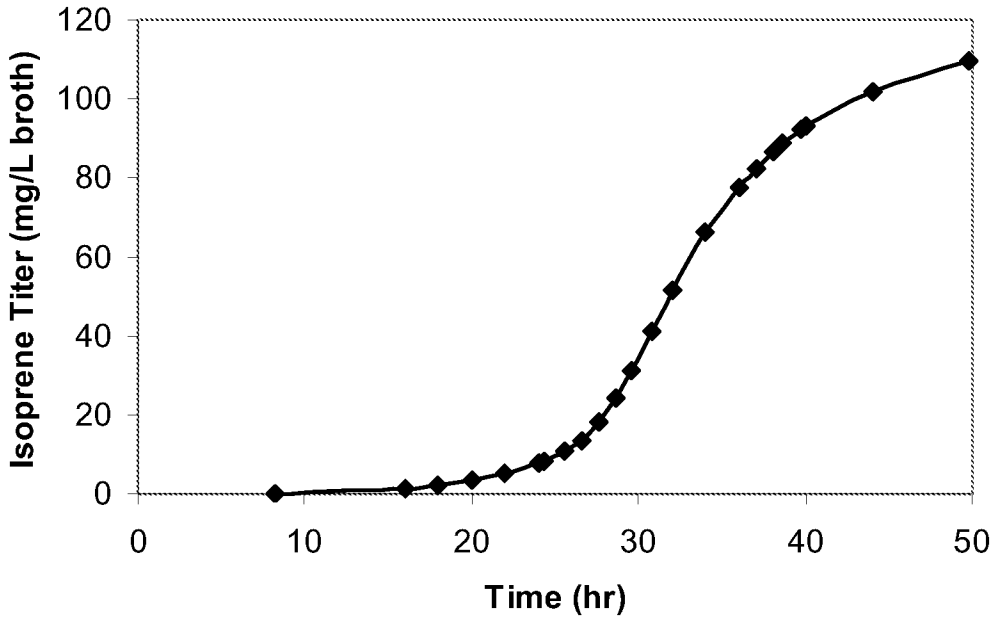
Isoprene Titer (mg/L broth)
120
100
80
60
40
20
0
0
10
20
30
40
50
Time (hr)

Figure 49C

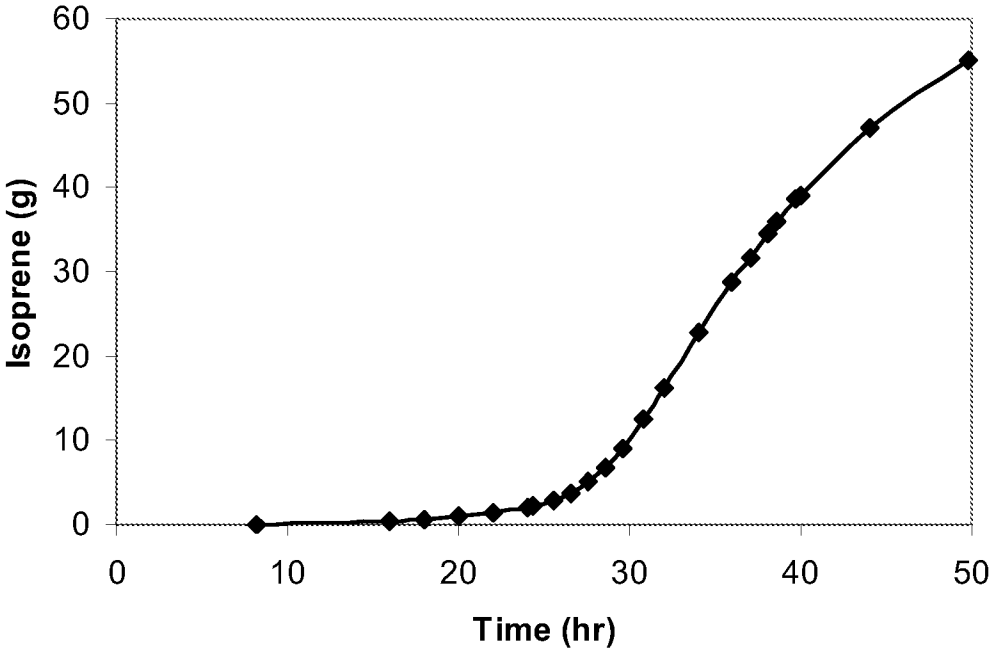

Figure 50

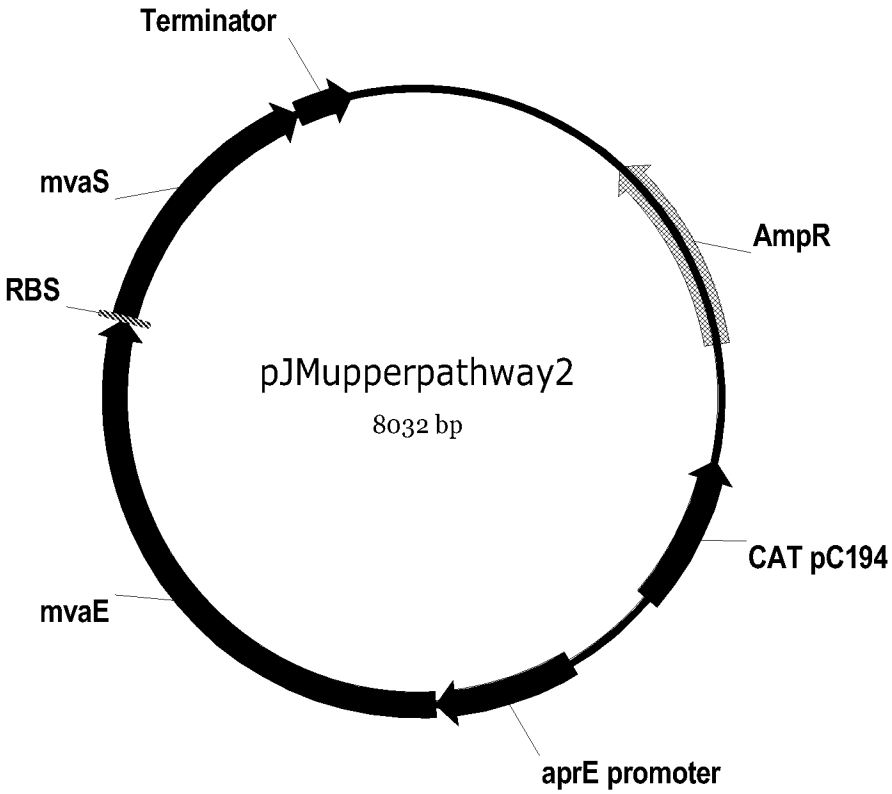
Terminator
mvaS
RBS
AmpR
pJMupperpathway2
8032 bp
CAT pC194
mvaE
aprE promoter

Figure 51A

5'-
tcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggt
tatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccag
gaaccgtaaaaaggccgcgttgctggcgtttttccataggctccgcccccctgacgagcatcac
aaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttc
cccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgc
ctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtg
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cactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtgg
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acggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaa
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tccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgttatcactcatggttatgg
cagcactgcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagta
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caactgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacaggaaggcaa
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aatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtattta
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ggctggcttaactatgcggcatcagagcagattgtactgagagtgcaccatagatctggagctg
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agagttcataaacaatcctgcatgataaccatcacaaacagaatgatgtacctgtaaagatagc
ggtaaatatattgaattacctttattaatgaatttttcctgctgtaataatgggtagaaggtaat
tactattattattgatatttaagttaaacccagtaaatgaagtccatggaataatagaaaagaga
aaaagcattttcaggtataggtgttttgggaaacaatttccccgaaccattatatttctctaca
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atgttttagatacaccatcaaaaattgtataaagtggctctaacttatcccaataacctaactc
tccgtcgctattgtaaccagttctaaaagctgtatttgagtttatcacccttgtcactaagaaa
atcaaatgcagggtaaaatttatatccttcttgttttatgtttc

Figure 51B

ggtataaaacactaatatcaatttctgtggttatactaaaagtcgtttgttggttcaaataatg
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tcctaaatttttatctaaagtgaatttaggaggcttacttgtctgctttcttcattagaatcaa
tcctttttttaaaagtcaatattactgtaacataaatatatatttttaaaaatatcccactttatc
caattttcgtttgttgaactaatgggtgctttagttgaagaataaaagacctatgcggtgtgaaa
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actgttgggaagggcgatcggtgcgggcctcttcgctattacgccagctggcgaaaggggatg
tgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacgacgttgtaaaacgacg
gccagtgccaagcttgcatgcctgcactccatttttcttctgctatcaaaataacagactcgtga
ttttccaaacgagctttcaaaaaagcctctgcccttgcaaatcggatgcctgtctataaaattt
cccgatattggttaaacagcggcgcaatggcggccgcatctgatgtctttgcttggcgaatgtt
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ttcagaatacttttatcatcatgctttgaaaaaatatcacgataatatccattgttctcacgga
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atagtcttttaagtaagtctactctgaatttttttaaaaggagagggtaaagagtgaaaacagt
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ttgctggcgggattgagaatatgtcccaagcacctaaattacaacgttttaattacgaaacaga
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gcaatgggcttaactgctgaaaatgtggccgaaaagtatcatgtaactagagaagagcaagatc
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agcgttgagaagctaggaacgcttaaaacagtttttaaagaagacggtactgtaacagcaggga
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acacggtcttccttatttagctattattcgagacagtgtggaagtcggtattgatccagcctat
atgggaatttcgccgattaaagccattcaaaaactgttagcgcgcaatcaacttactacggaag
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agtggacgaaactgattatttggtaccaatggcgacagaagagccctcagttattgcggctttg
agtaatggtgcaaaaatagcacaaggatttaaaacagtgaatcaacaacgcttaatgcgtggac
aaatcgtttttacgatgttgcagatcccgagtcattgattgataaactacaagtaagagaagc
ggaagtttttcaacaagcgagttaagttatccatctatcgttaaacggggcggcggcttaaga
gatttgcaatatcgtacttttgatgaatcatttgtatctgtcgacttttttagtagatgttaagg
atgcaatgggggcaaatatcgttaacgctatgttggaaggtgtg

Figure 51C

gccgagttgttccgtgaatggtttgcggagcaaaagattttattcagtattttaagtaattatg
ccacggagtcggttgttacgatgaaaacggctattccagtttcacgtttaagtaaggggagcaa
tggccgggaaattgctgaaaaaattgttttagcttcacgctatgcttcattagatcctttatcgg
gcagtcacgcataacaaaggaatcatgaatggcattgaagctgtagttttagctacaggaaatg
atacacgcgctgttagcgcttcttgtcatgcttttgcggtgaaggaaggtcgctaccaaggctt
gactagttggacgctggatggcgaacaactaattggtgaaatttcagttccgcttgctttagcc
acggttggcggtgccacaaaagtcttacctaaatctcaagcagctgctgatttgttagcagtga
cggatgcaaaagaactaagtcgagtagtagcggctgttggtttggcacaaaatttagcggcgtt
acgggccttagtctctgaaggaattcaaaaaggacacatggctctacaagcacgttctttagcg
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gacaattgggattgataaaattagtttttttgtgccccttattatattgatatgacggcactg
gctgaagccagaaatgtagaccctggaaaatttcatattggtattgggcaagaccaaatggcgg
tgaacccaatcagccaagatattgtgacatttgcagccaatgccgcagaagcgatcttgaccaa
agaagataaagaggccattgatatggtgattgtcgggactgagtccagtatcgatgagtcaaaa
gcggccgcagttgtcttacatcgtttaatgggattcaacctttcgctcgctctttcgaaatca
aggaagcttgttacggagcaacagcaggcttacagttagctaagaatcacgtagccttacatcc
agataaaaaagtcttggtcgtagcggcagatattgcaaaatatggcttaaattctggcggtgag
cctacacaaggagctggggcggttgcaatgttagttgctagtgaaccgcgcattttggctttaa
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tcctatggtcgatggtcctttgtcaaacgaaacctacatccaatcttttgcccaagtctgggat
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acacaaaaatgggcaaaaaagccttattagcaaaaatctccgaccaaactgaagcagaacagga
acgaattttagcccgttatgaagaaagtatcgtctatagtcgtcgcgtaggaaacttgtatacg
ggttcactttatctgggactcatttccctttagaaaatgcaacgactttaaccgcaggcaatc
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tggttatcaaaatcatttacaaaaagaaactcatttagcactgctggataatcggacagaactt
tctatcgctgaatatgaagccatgtttgcagaaactttagacacagacattgatcaaacgttag
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aaaaccggccttggccccgccggttttttattatttttcttcctccgcatgttcaatccgctcc
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ccgtaacggccaagtcctgaaacgtctcaatcgccgcttcccggtttccggtcagctcaatgcc
gtaacggtcggcggcgttttcctgataccgggagacggcattcgtaatcgggatcccccgggtac
cgagctcgaattcgtaatcatgtcatagctgtttcctgtgtgaaattgttatccgctcacaatt
ccacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagctaac
tcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgca
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ctcactgac

SEQ ID NO:22

Figure 52

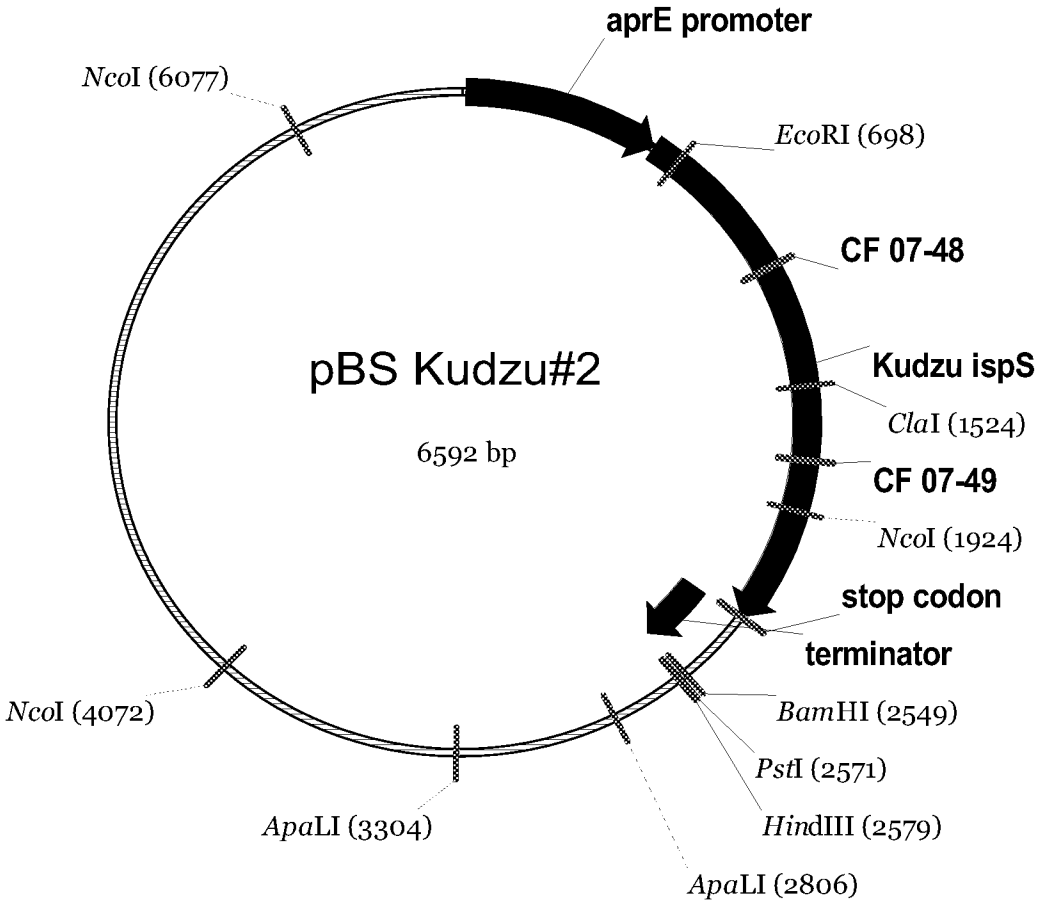

aprE promoter
NcoI (6077)
EcoRI (698)
CF 07-48
pBS Kudzu#2
6592 bp
Kudzu ispS
ClaI (1524)
CF 07-49
NcoI (1924)
stop codon
terminator
NcoI (4072)
BamHI (2549)
PstI (2571)
HindIII (2579)
ApaLI (3304)
ApaLI (2806)

Figure 53A

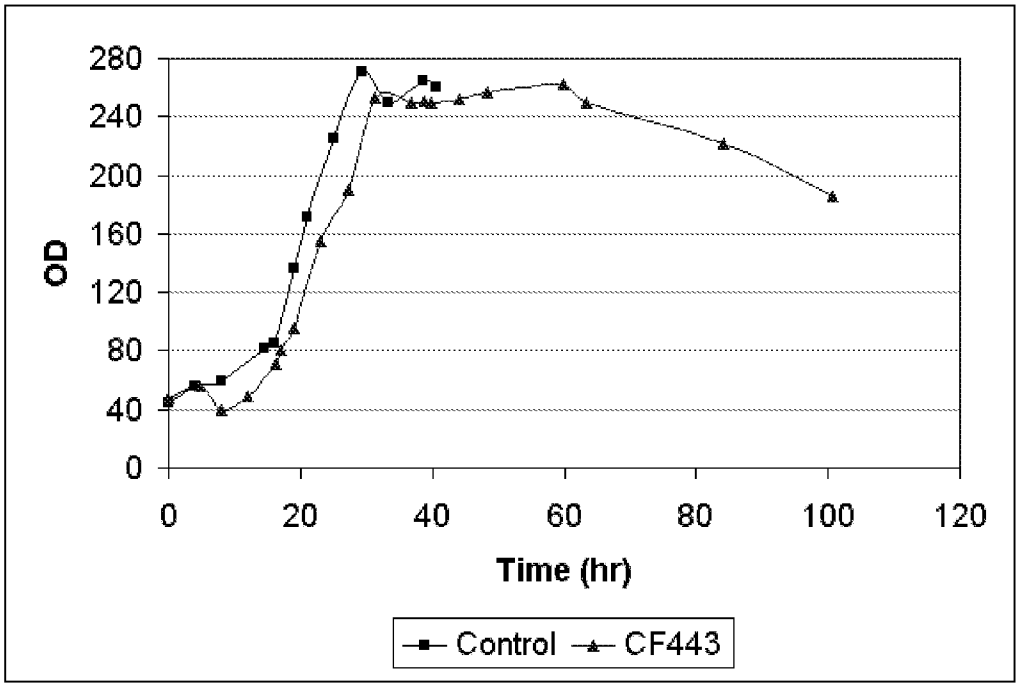

280
240
200
160
120
80
40
0
OD
0
20
40
60
80
100
120
Time (hr)
Control
CF443

Figure 53B

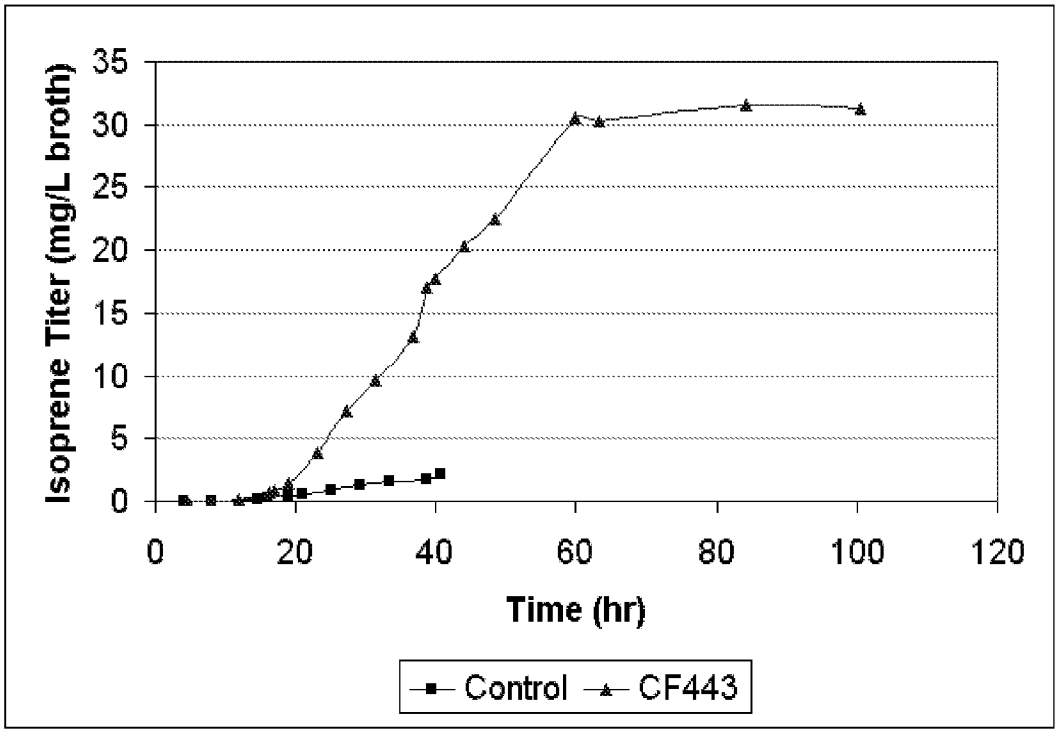

Isoprene Titer (mg/L broth)
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
Time (hr)
Control
CF443

Figure 54

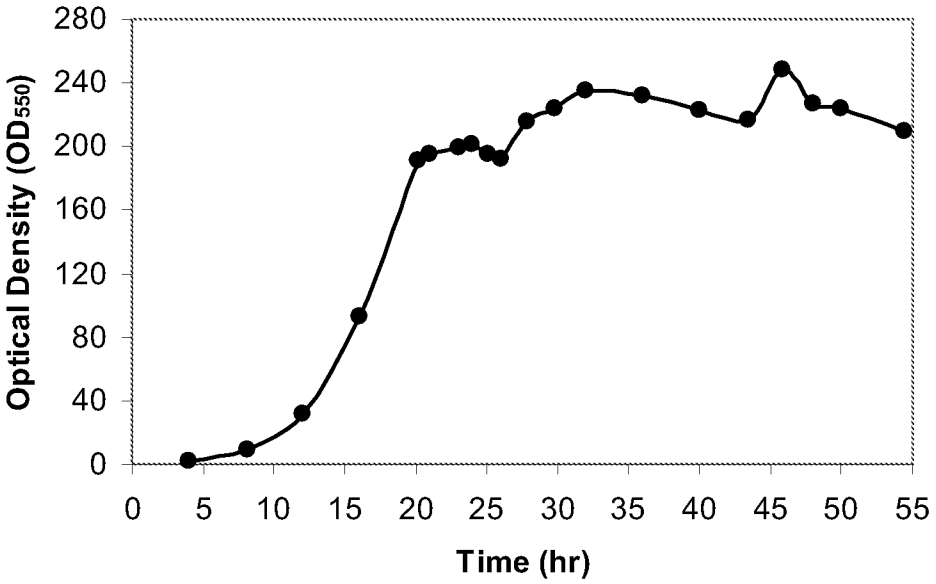

Optical Density (OD550)
280
240
200
160
120
80
40
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 55

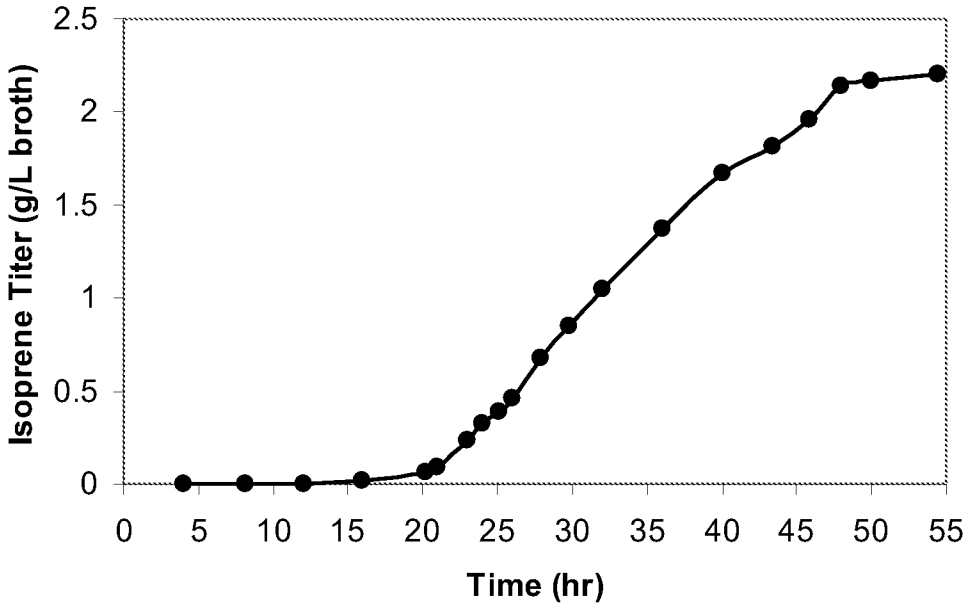

Isoprene Titer (g/L broth)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 56

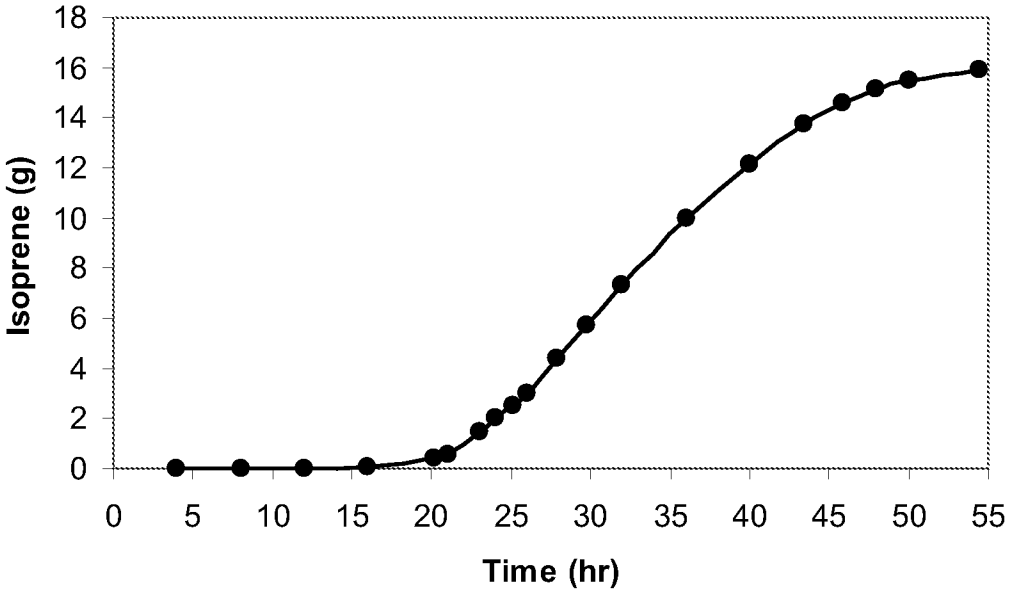

Isoprene (g)
Time (hr)
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25
30
35
40
45
50
55

Figure 57

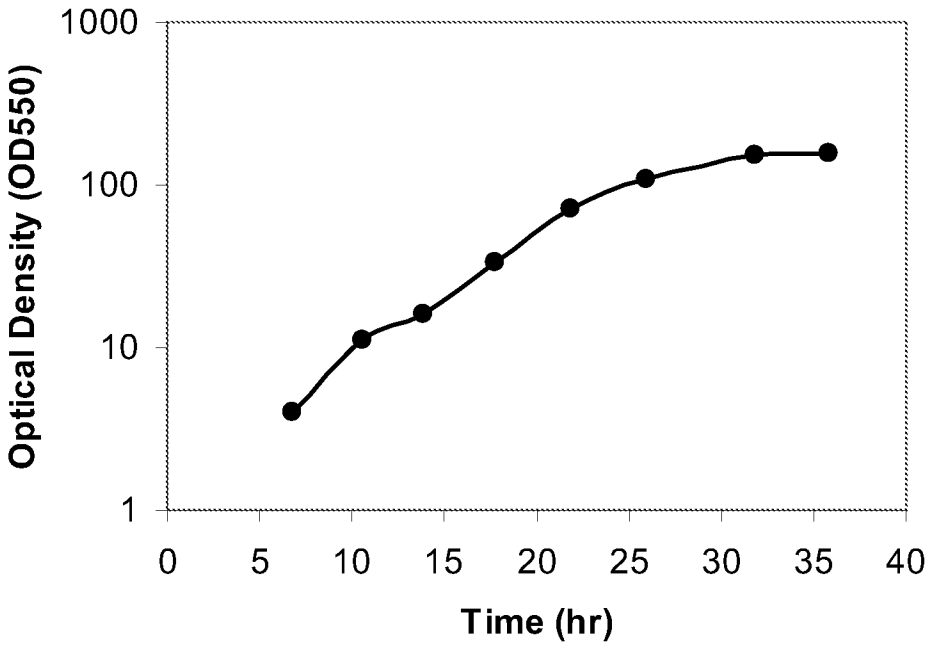

1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
Time (hr)

Figure 58

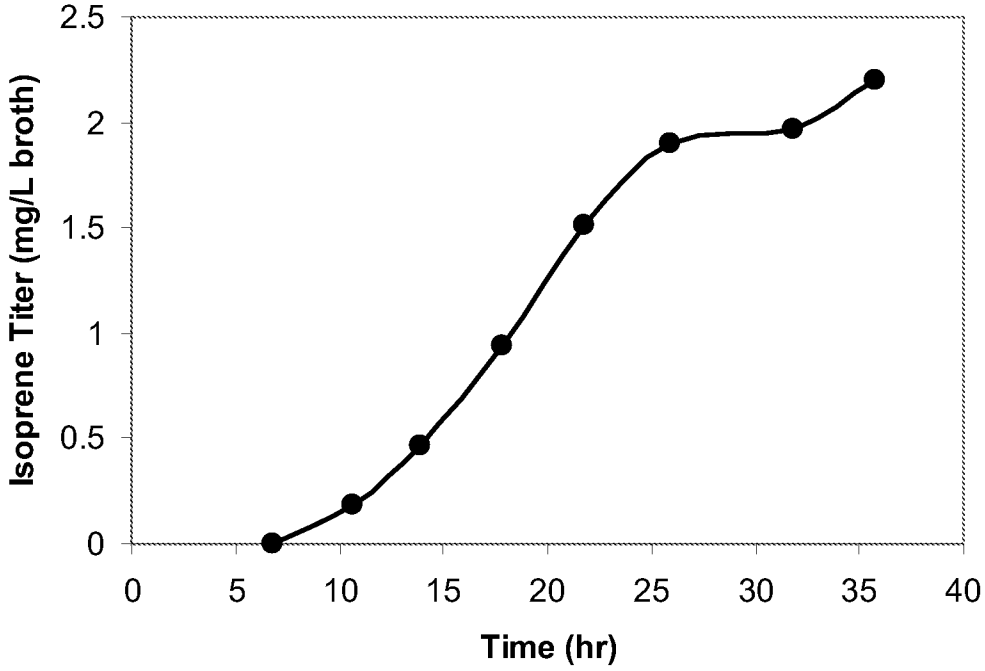

Isoprene Titer (mg/L broth)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40
Time (hr)

Figure 59

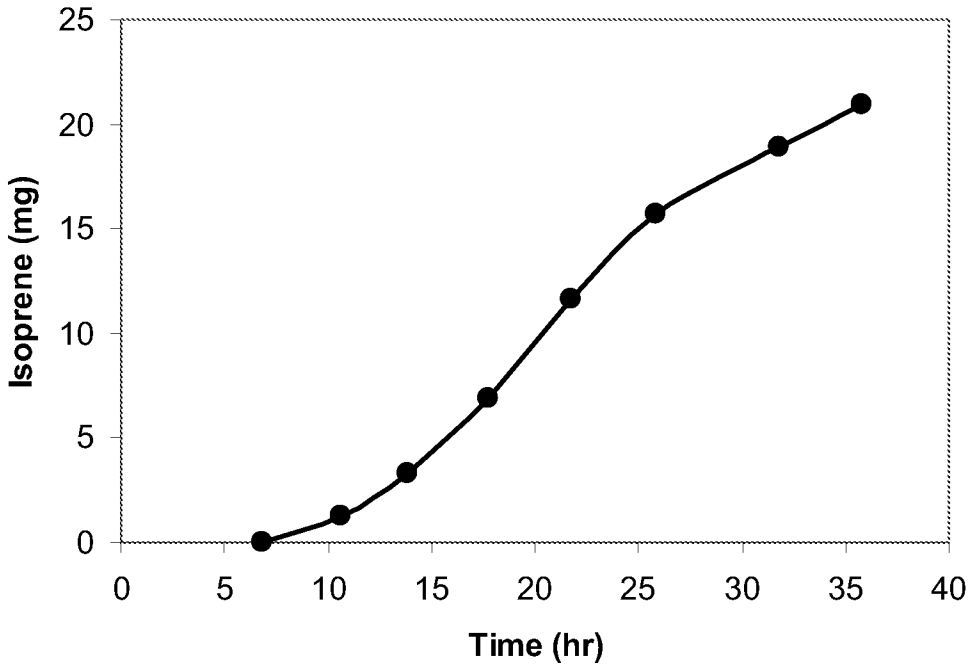


Figure 60A

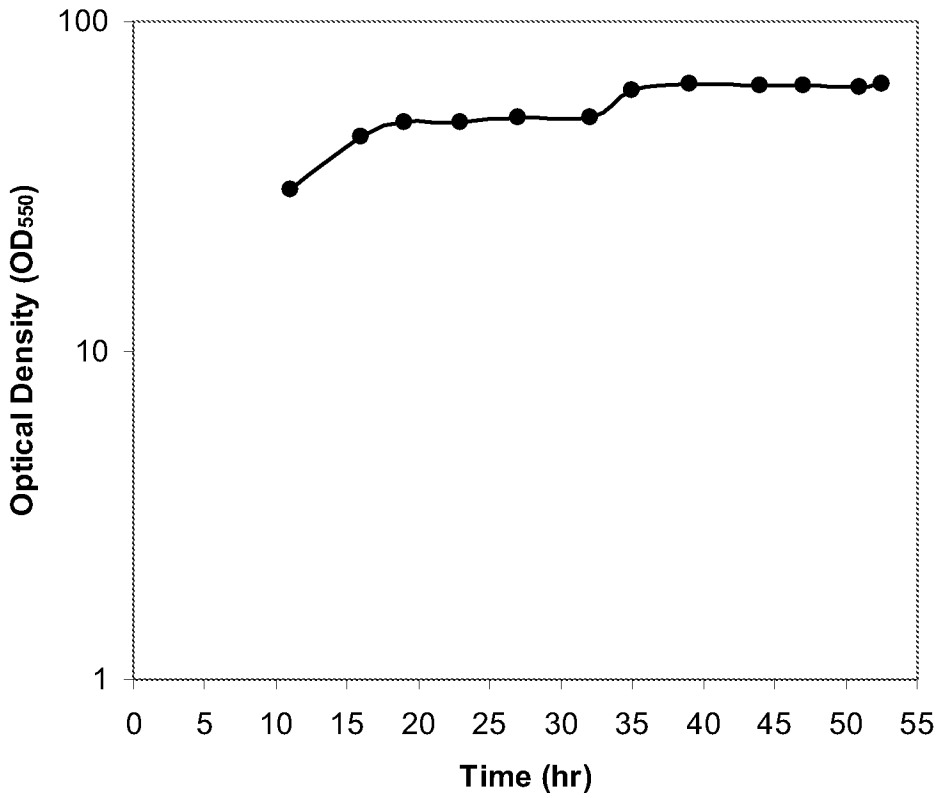

100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 60B

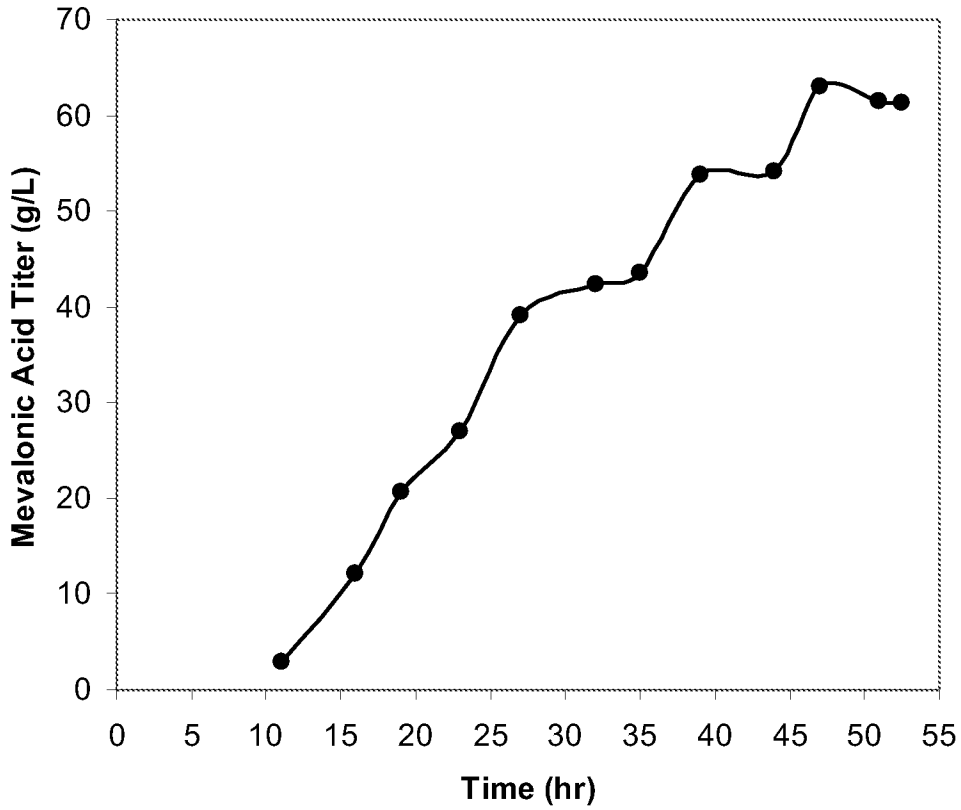

Mevalonic Acid Titer (g/L)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 60C

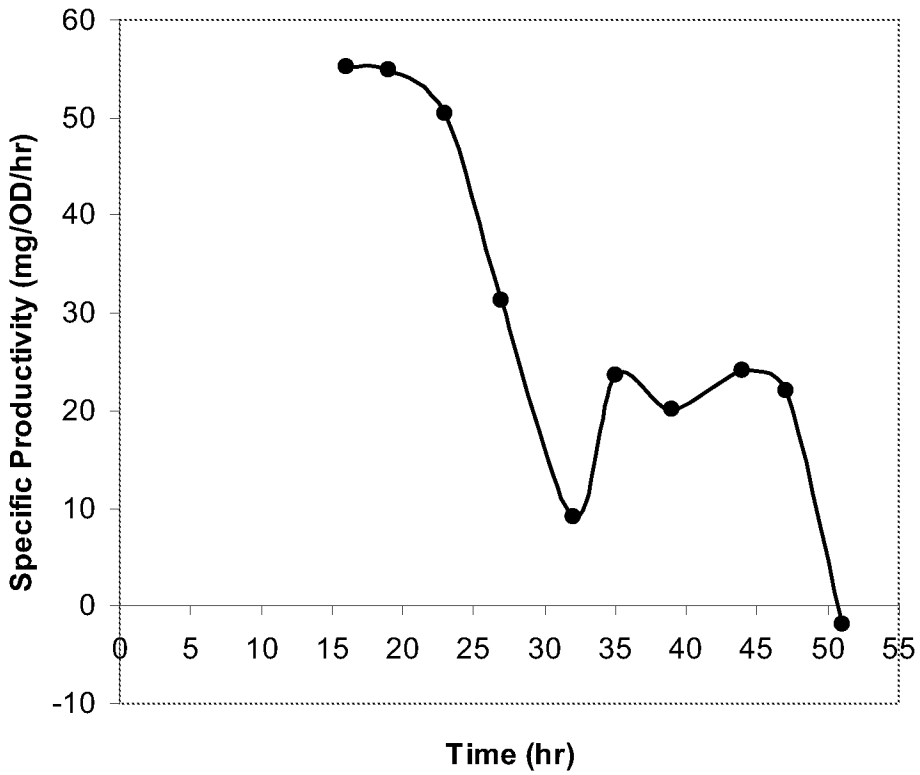

Specific Productivity (mg/OD/hr)
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 61A

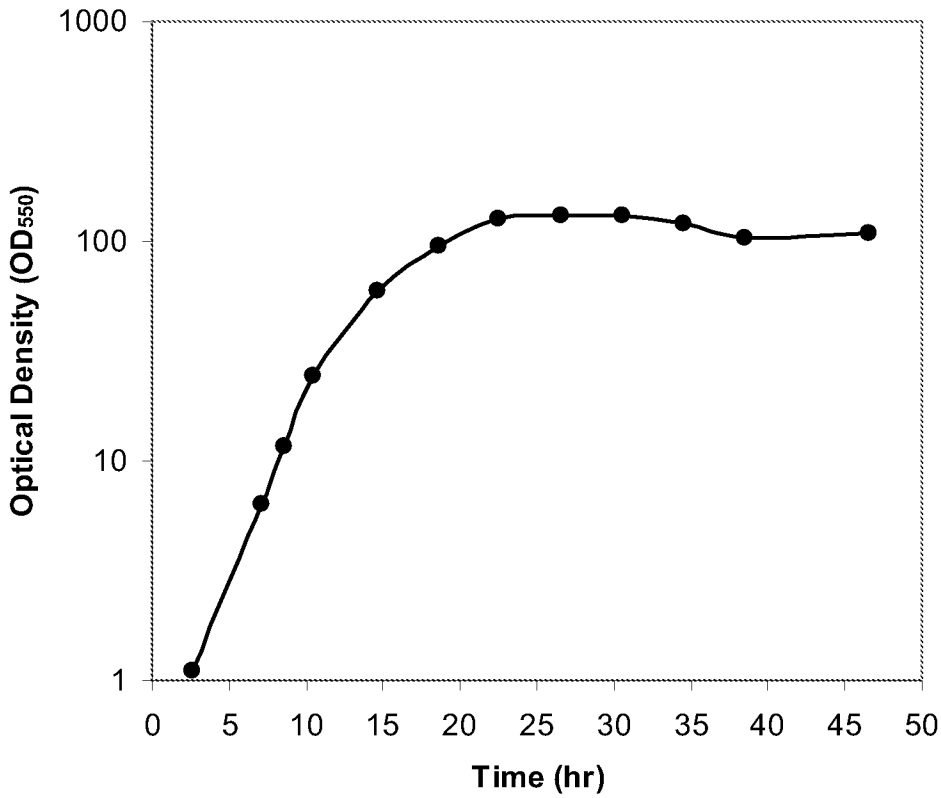

1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
Time (hr)

Figure 61B

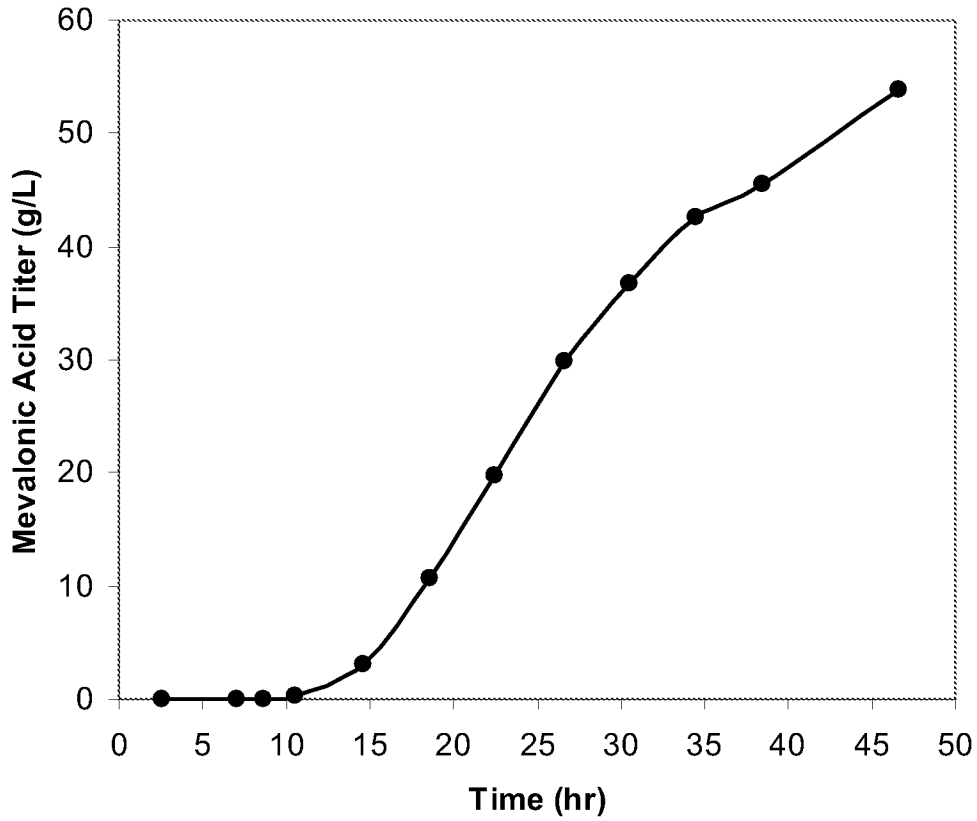

Mevalonic Acid Titer (g/L)
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
Time (hr)

Figure 61C

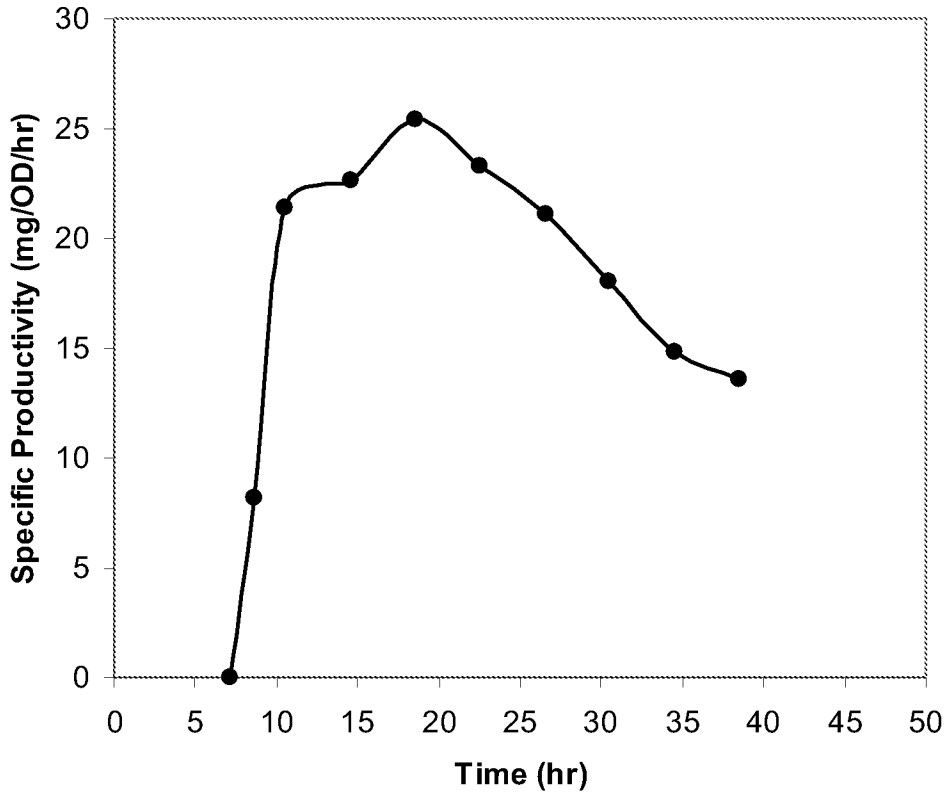

Specific Productivity (mg/OD/hr)
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
45
50
Time (hr)

Figure 62A

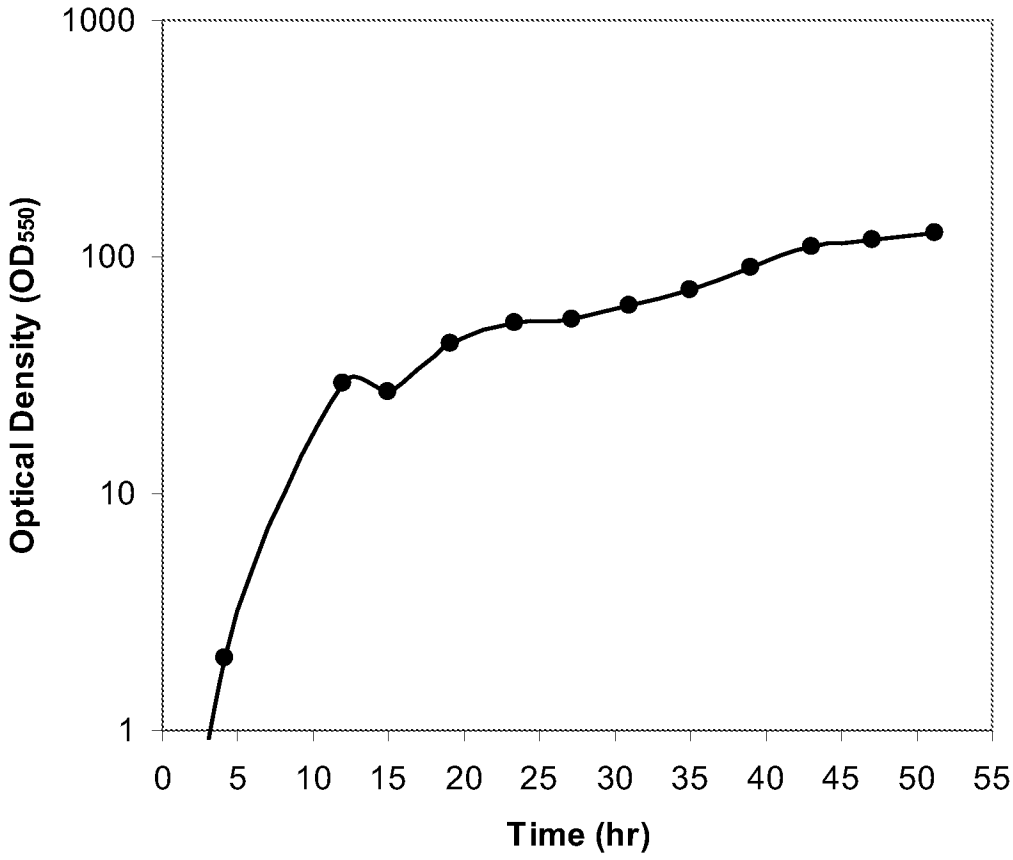

1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 62B

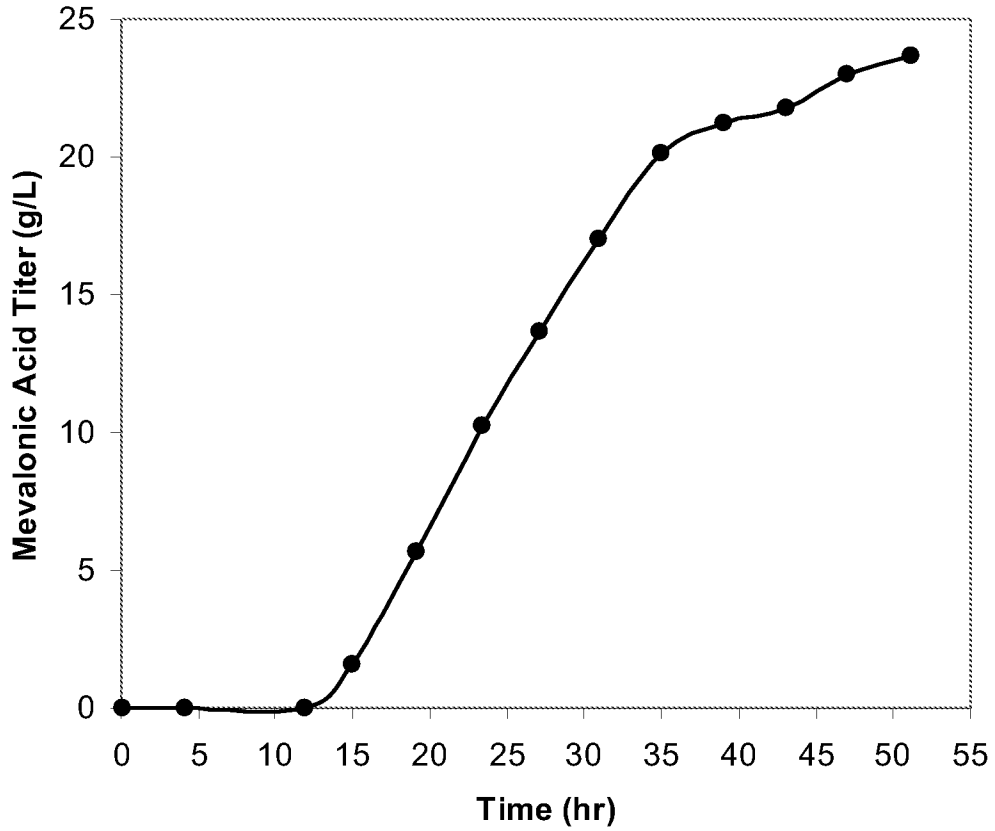

Mevalonic Acid Titer (g/L)
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 62C

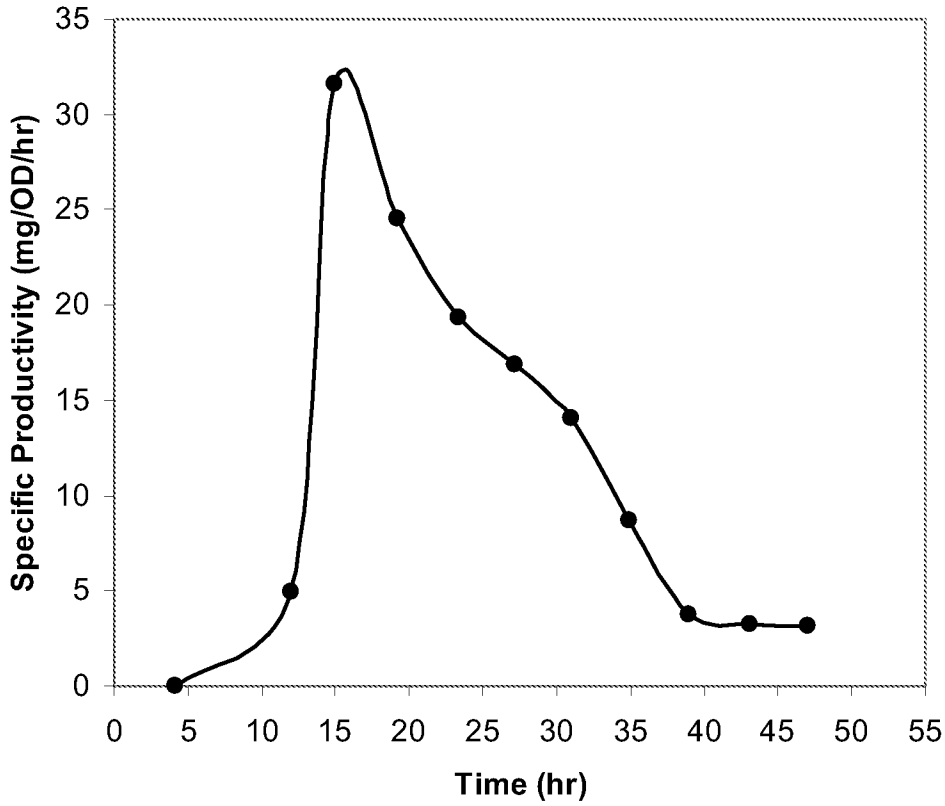

Specific Productivity (mg/OD/hr)
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 63A

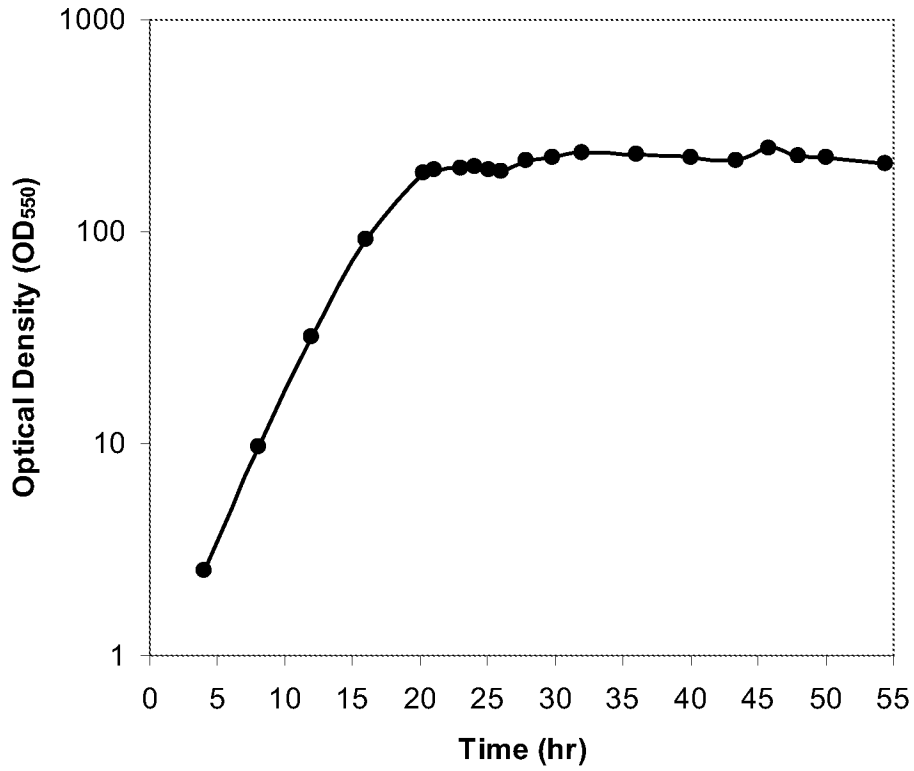

1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 63B

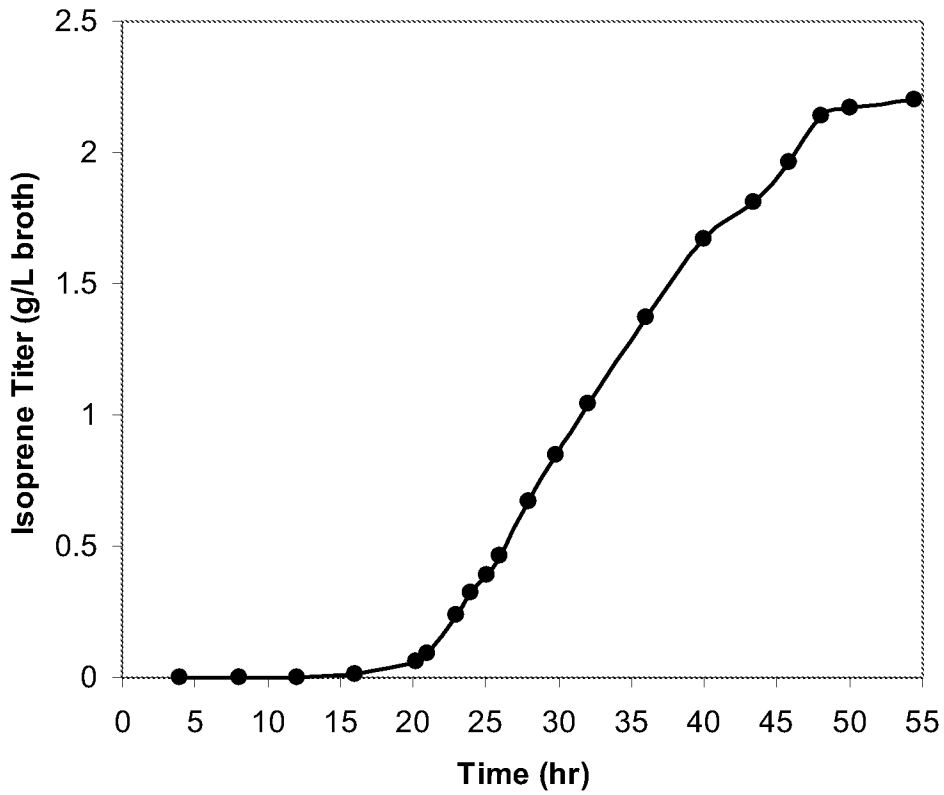

Isoprene Titer (g/L broth)
2.5
2
1.5
1
0.5
0
0 5 10 15 20 25 30 35 40 45 50 55
Time (hr)

Figure 63C

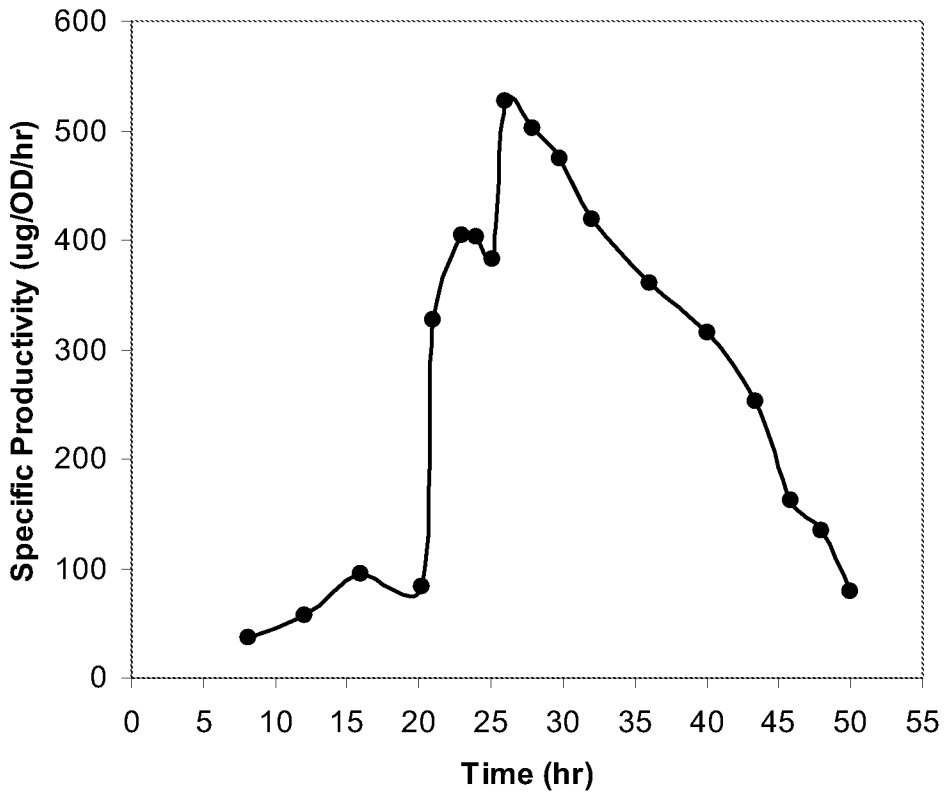

Specific Productivity (ug/OD/hr)
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 64A

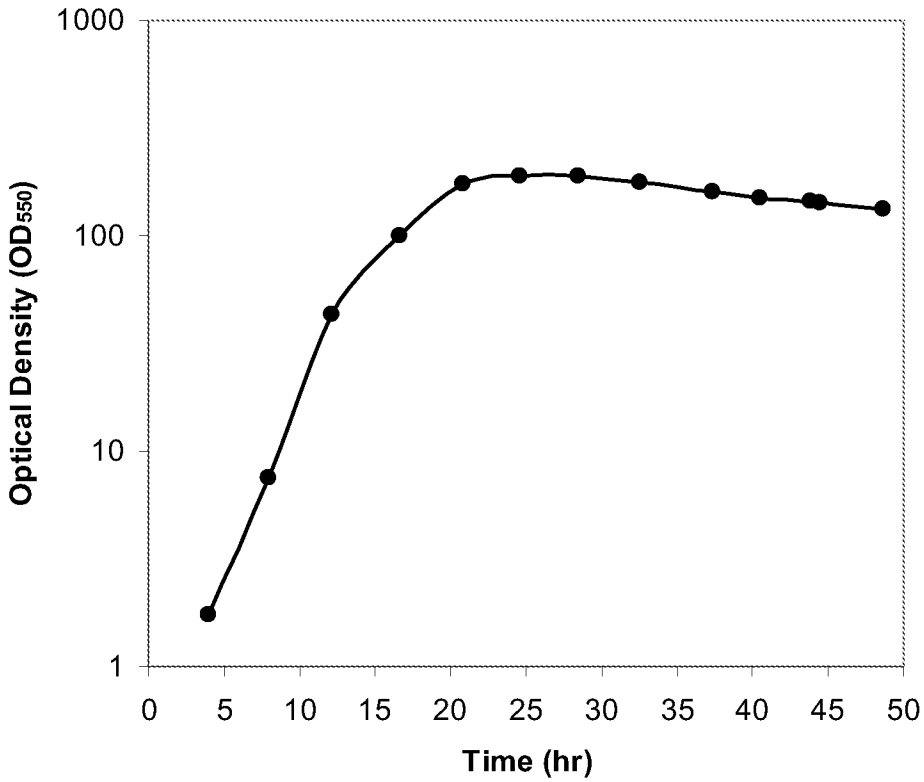

1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
Time (hr)

Figure 64B

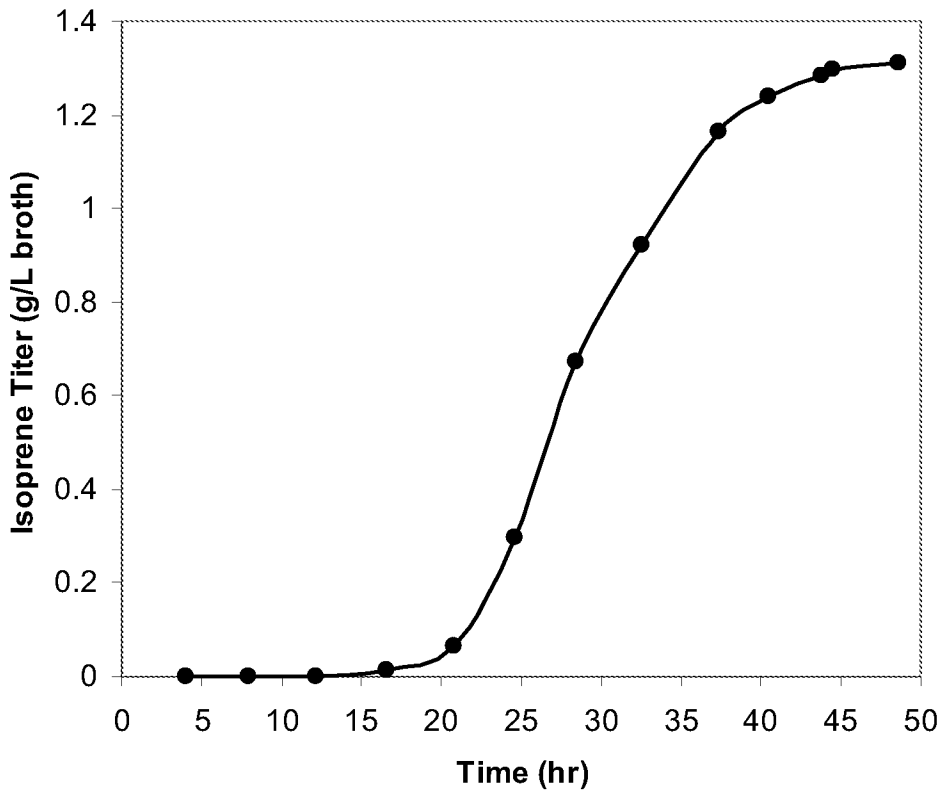

Isoprene Titer (g/L broth)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
45
50
Time (hr)

Figure 64C

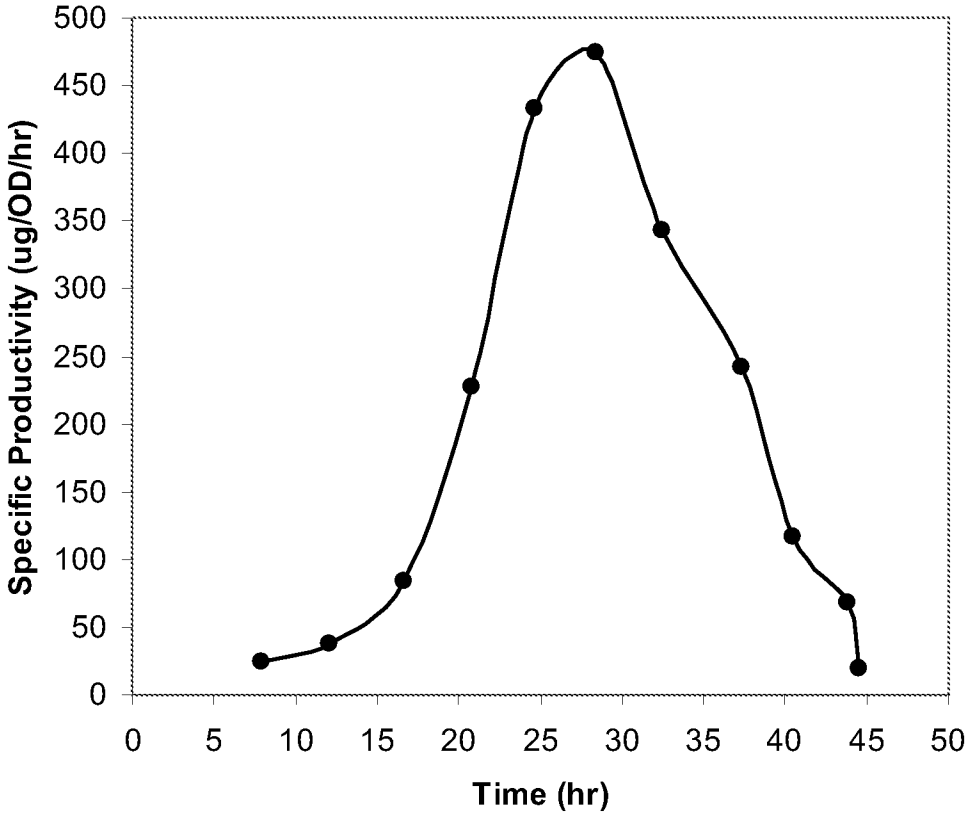

Specific Productivity (ug/OD/hr)
500
450
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
45
50
Time (hr)

Figure 65A

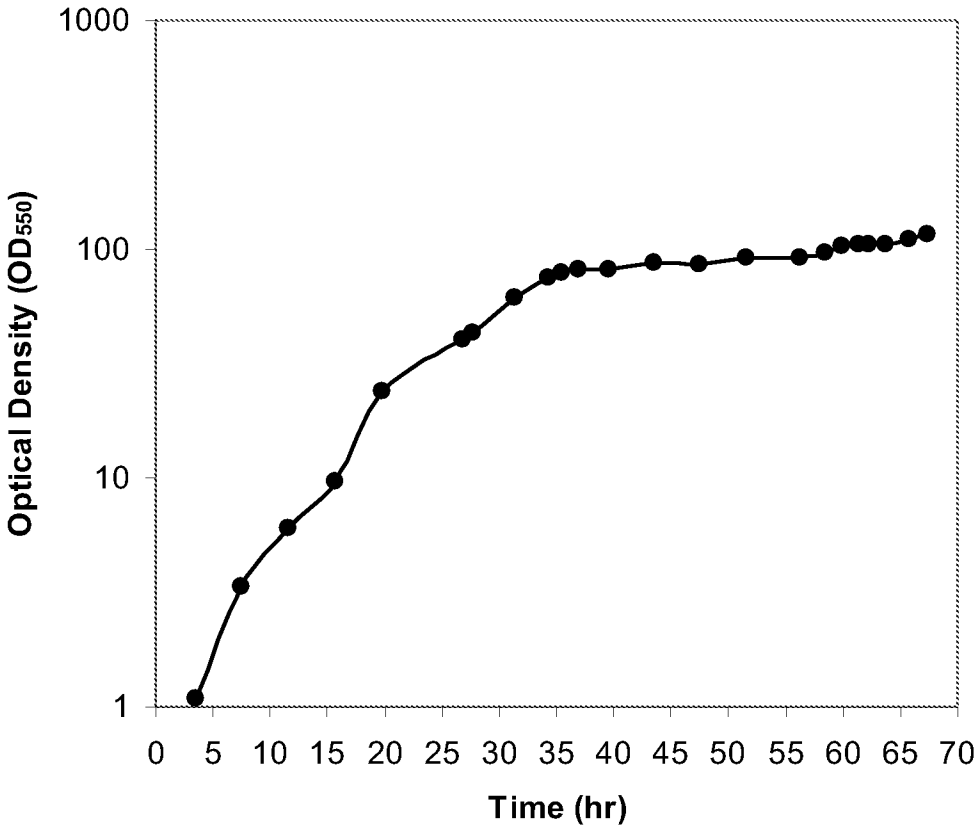

1000
100
10
1
Optical Density (OD550)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time (hr)

Figure 65B

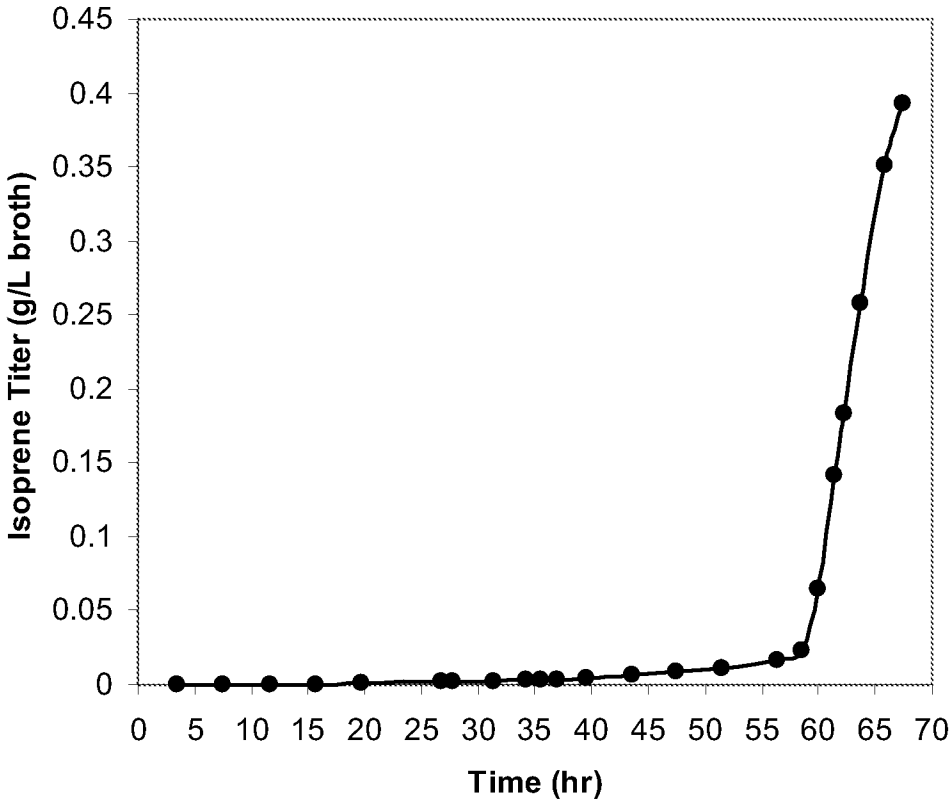

Isoprene Titer (g/L broth)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time (hr)

Figure 65C

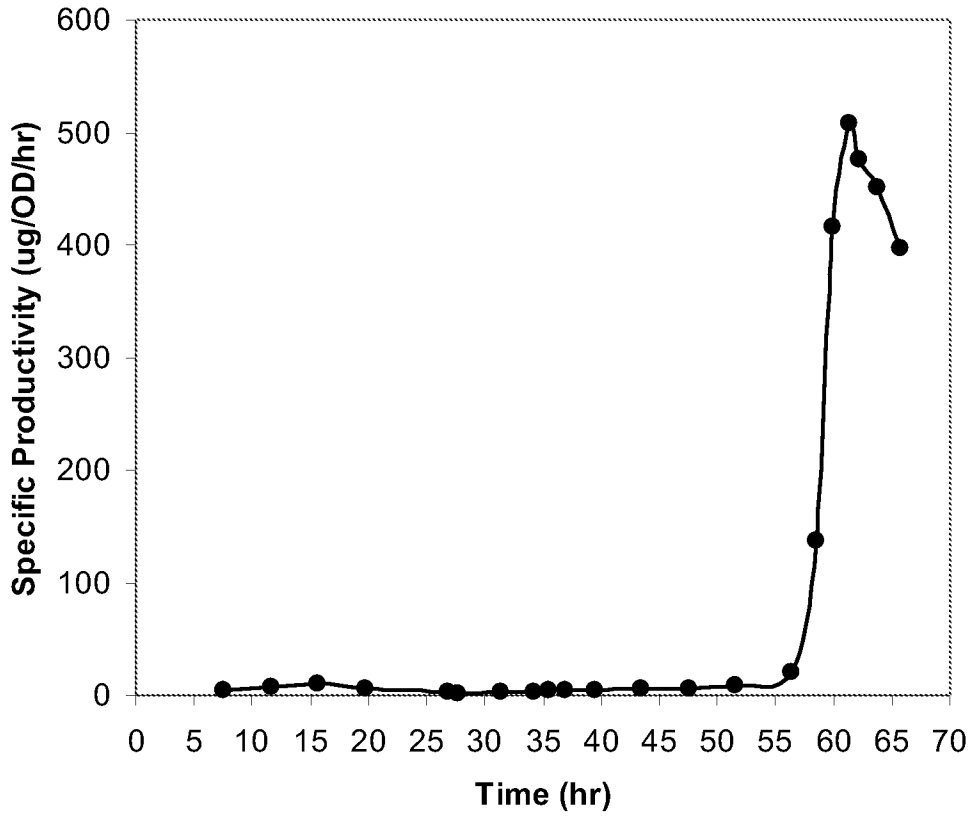

Specific Productivity (ug/OD/hr)
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time (hr)

Figure 66A

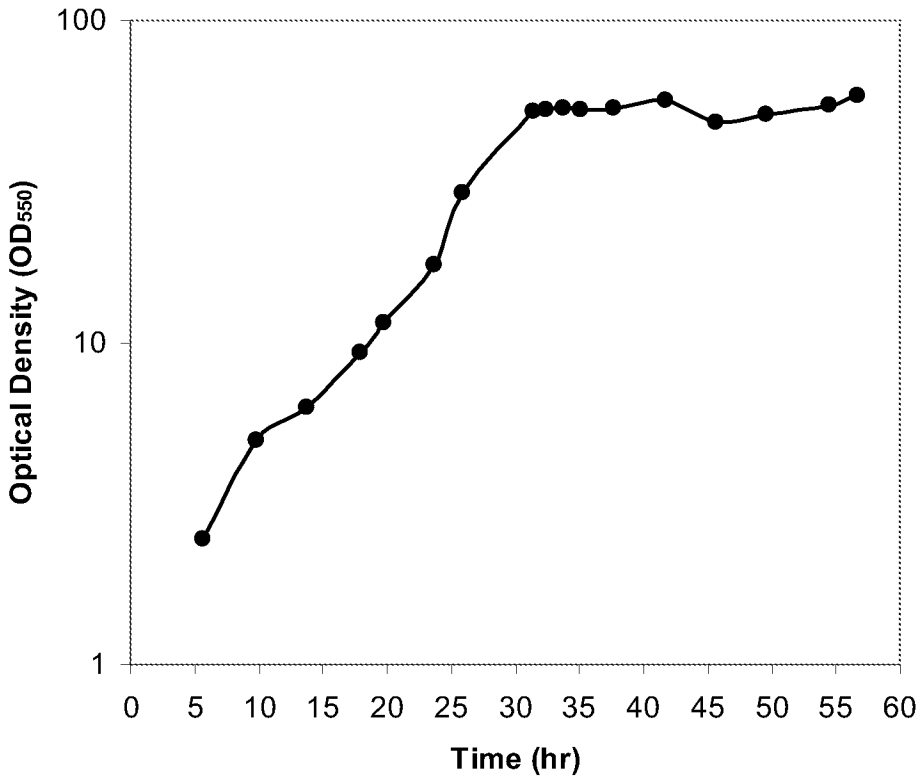

100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 66B

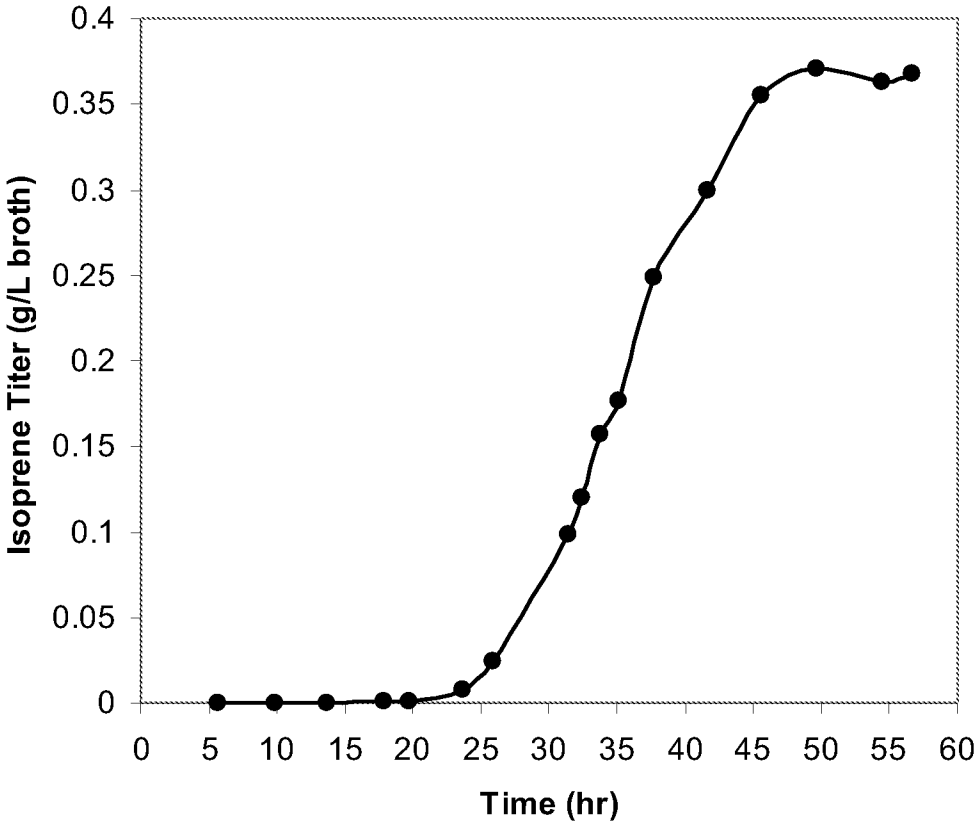

Isoprene Titer (g/L broth)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (hr)

Figure 66C

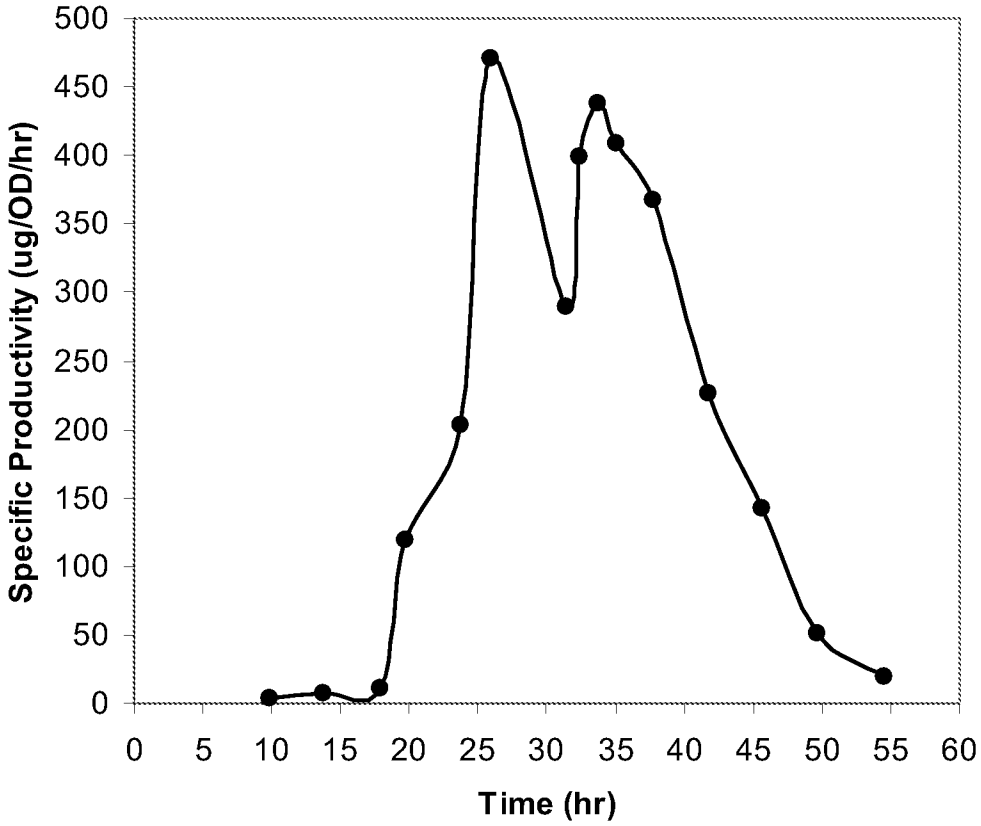

Specific Productivity (ug/OD/hr)
500
450
400
350
300
250
200
150
100
50
0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (hr)

Figure 67A

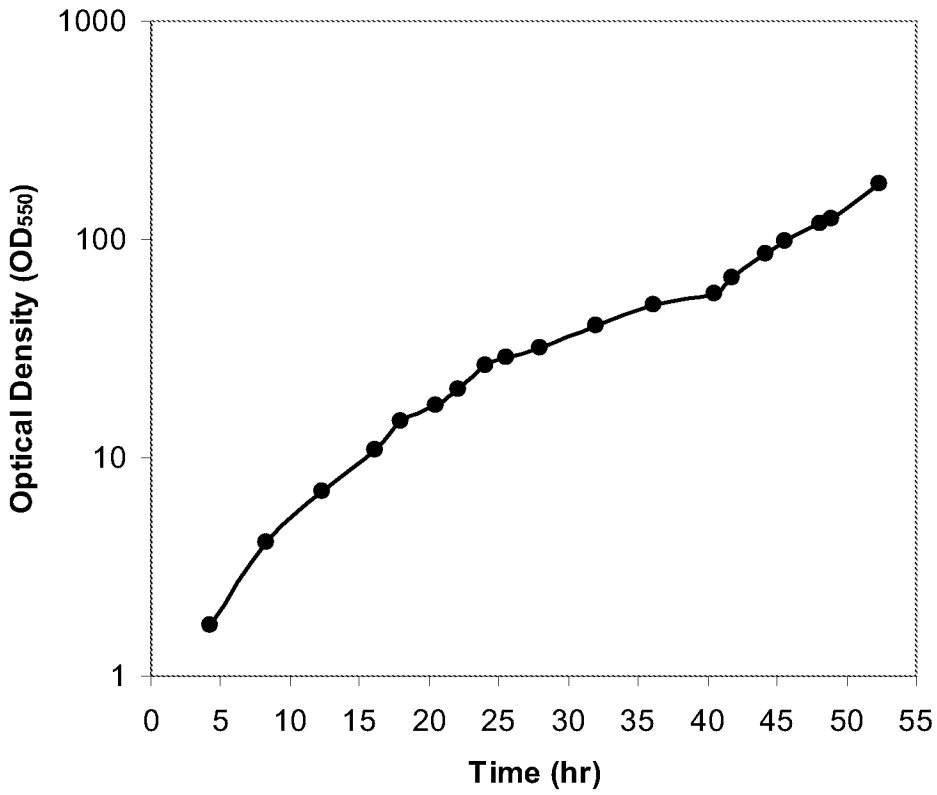

1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 67B

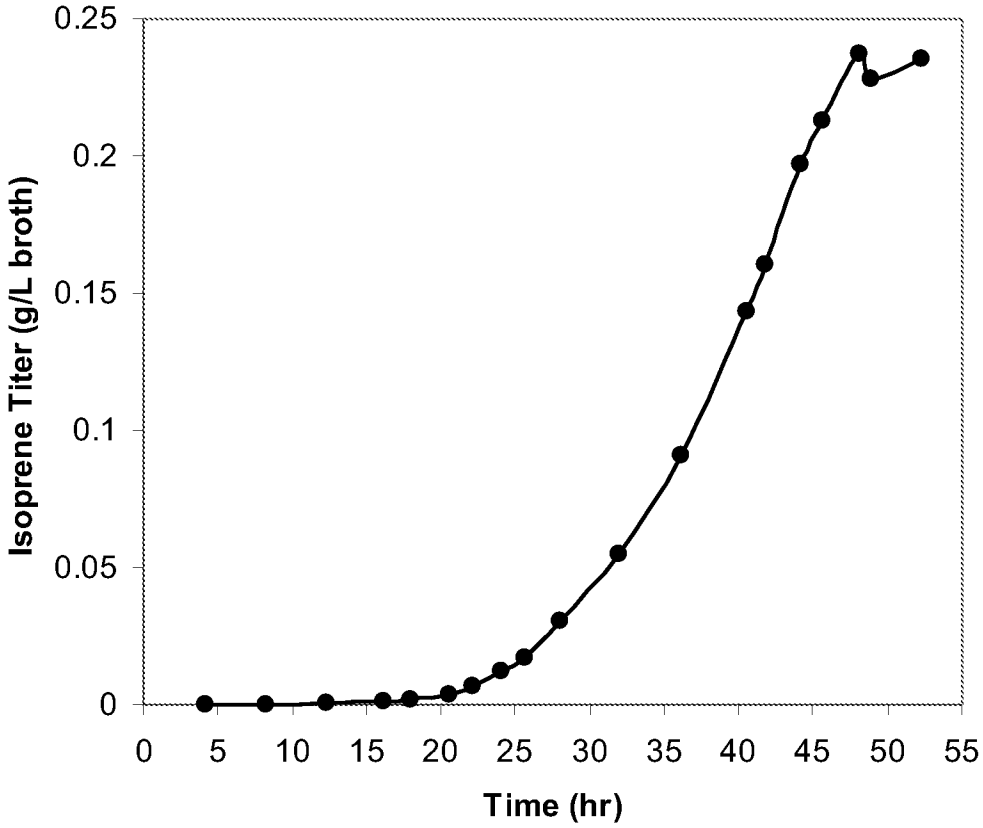

0.25
0.2
0.15
0.1
0.05
0
Isoprene Titer (g/L broth)
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 67C

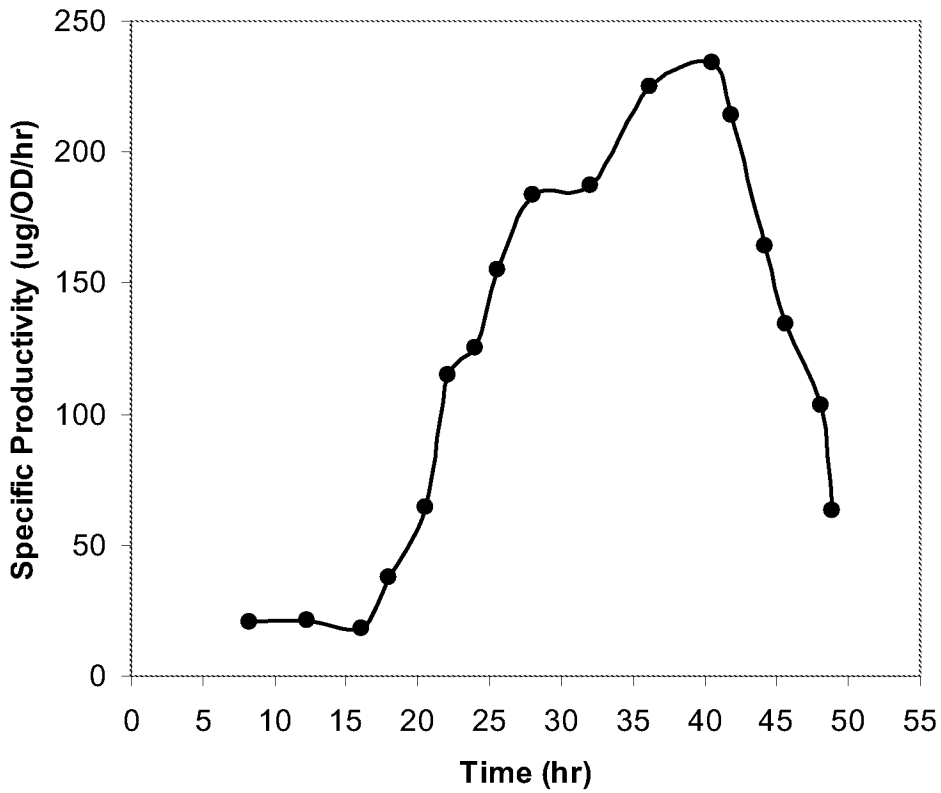

Specific Productivity (ug/OD/hr)
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 68

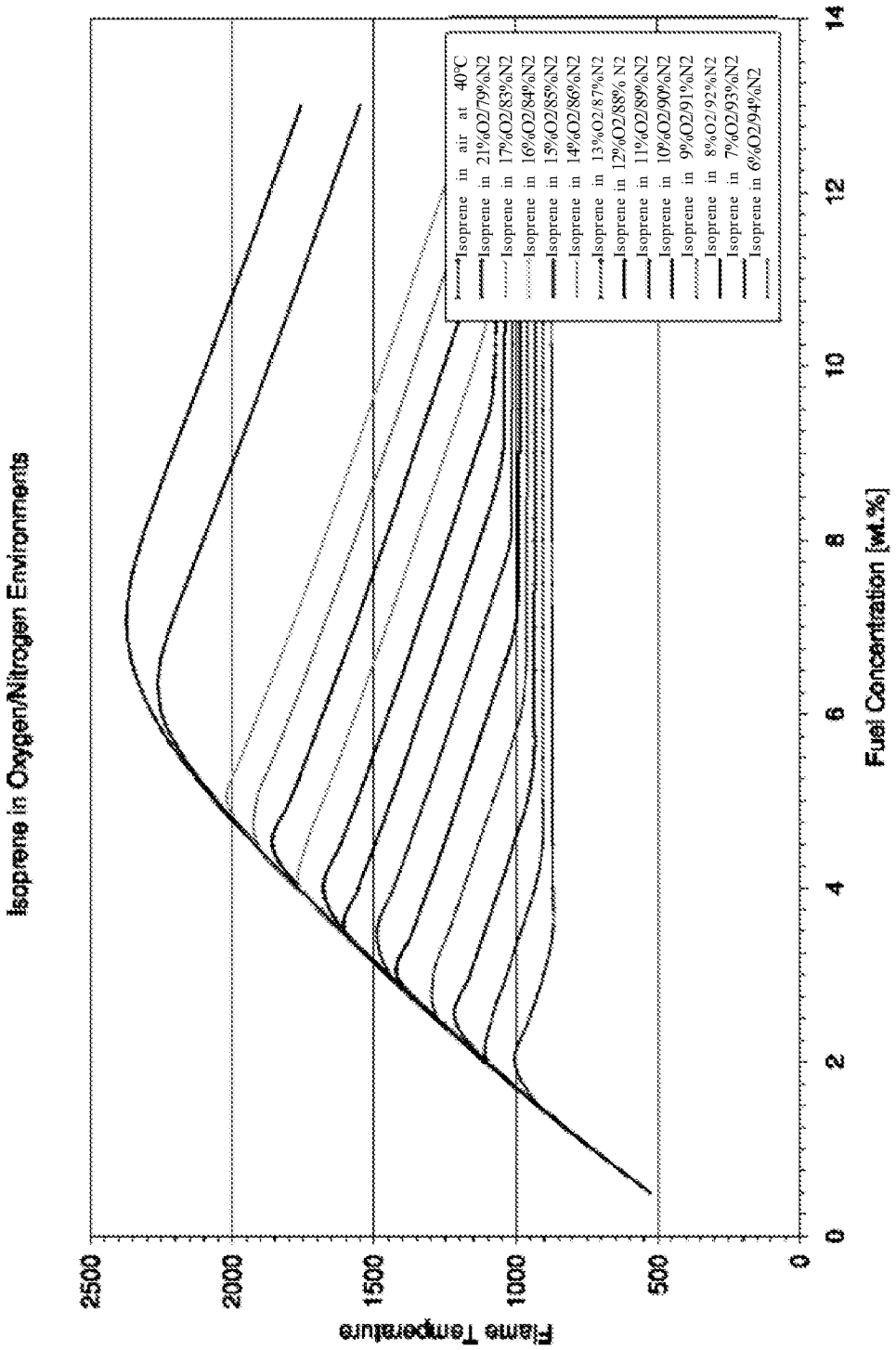

Isoprene in Oxygen/Nitrogen Environments
Flame Temperature
Fuel Concentration [wt.%]
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Isoprene in air at 40°C
Isoprene in 21%O2/79%N2
Isoprene in 17%O2/83%N2
Isoprene in 16%O2/84%N2
Isoprene in 15%O2/85%N2
Isoprene in 14%O2/86%N2
Isoprene in 13%O2/87%N2
Isoprene in 12%O2/88% N2
Isoprene in 11%O2/89%N2
Isoprene in 10%O2/90%N2
Isoprene in 9%O2/91%N2
Isoprene in 8%O2/92%N2
Isoprene in 7%O2/93%N2
Isoprene in 6%O2/94%N2

Figure 69

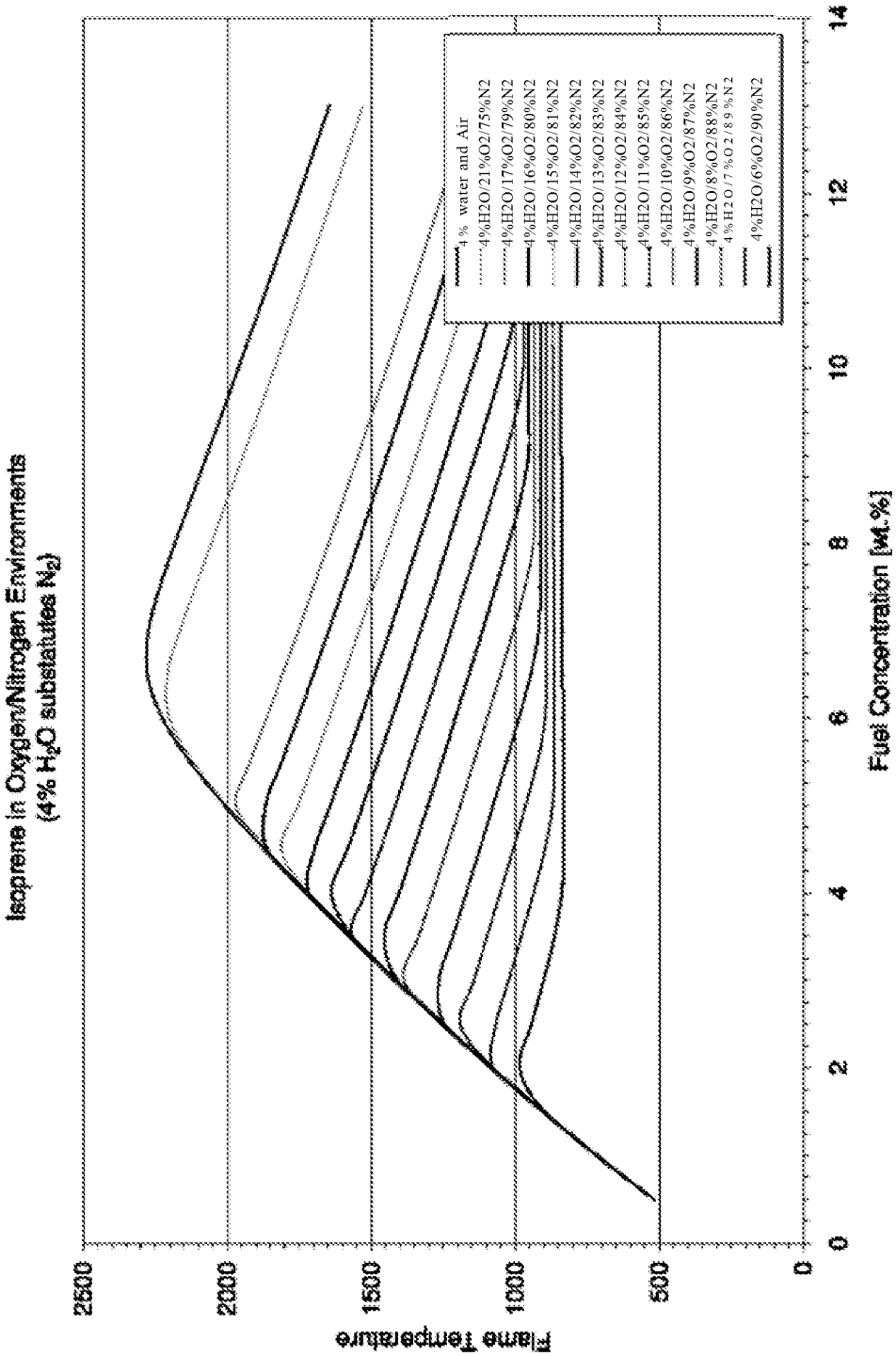

Isoprene in Oxygen/Nitrogen Environments
(4% H2O substatutes N2)
Flame Temperature
Fuel Concentration [M.%]
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
4% water and Air
4%H2O/21%O2/75%N2
4%H2O/17%O2/79%N2
4%H2O/16%O2/80%N2
4%H2O/15%O2/81%N2
4%H2O/14%O2/82%N2
4%H2O/13%O2/83%N2
4%H2O/12%O2/84%N2
4%H2O/11%O2/85%N2
4%H2O/10%O2/86%N2
4%H2O/9%O2/87%N2
4%H2O/8%O2/88%N2
4%H2O/7%O2/89%N2
4%H2O/6%O2/90%N2

Figure 70

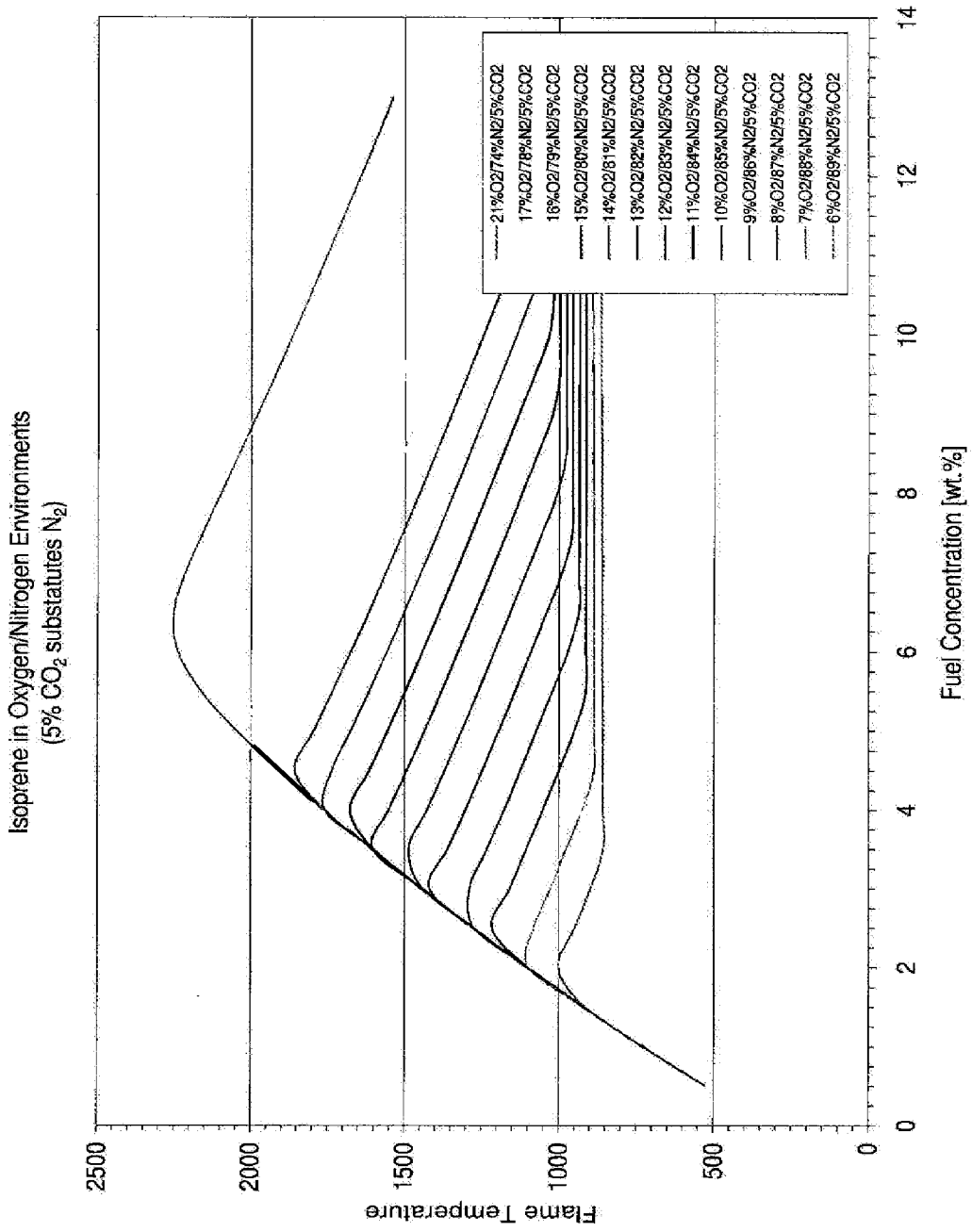

Isoprene in Oxygen/Nitrogen Environments
(5% CO_2 substatutes N_2)
Flame Temperature
Fuel Concentration [wt.%]
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
21%O2/74%N2/5%CO2
17%O2/78%N2/5%CO2
16%O2/79%N2/5%CO2
15%O2/80%N2/5%CO2
14%O2/81%N2/5%CO2
13%O2/82%N2/5%CO2
12%O2/83%N2/5%CO2
11%O2/84%N2/5%CO2
10%O2/85%N2/5%CO2
9%O2/86%N2/5%CO2
8%O2/87%N2/5%CO2
7%O2/88%N2/5%CO2
6%O2/89%N2/5%CO2

Figure 71

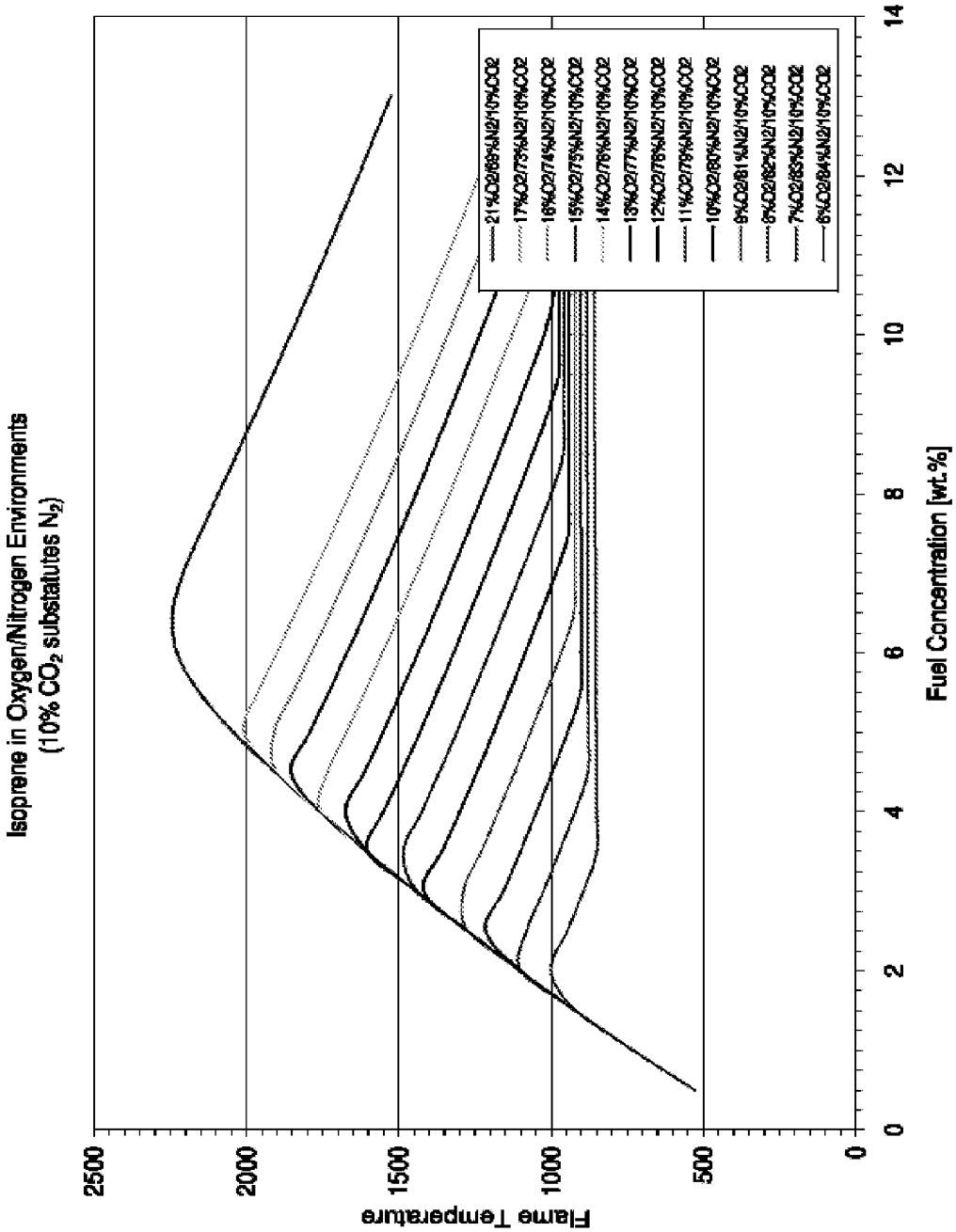

Isoprene in Oxygen/Nitrogen Environments
(10% CO_2 substitutes N_2)
Flame Temperature
Fuel Concentration [wt.%]
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
21%O2/89%N2/10%CO2
17%O2/73%N2/10%CO2
18%O2/74%N2/10%CO2
15%O2/75%N2/10%CO2
14%O2/76%N2/10%CO2
13%O2/77%N2/10%CO2
12%O2/78%N2/10%CO2
11%O2/79%N2/10%CO2
10%O2/80%N2/10%CO2
9%O2/81%N2/10%CO2
8%O2/82%N2/10%CO2
7%O2/83%N2/10%CO2
6%O2/84%N2/10%CO2

Figure 72

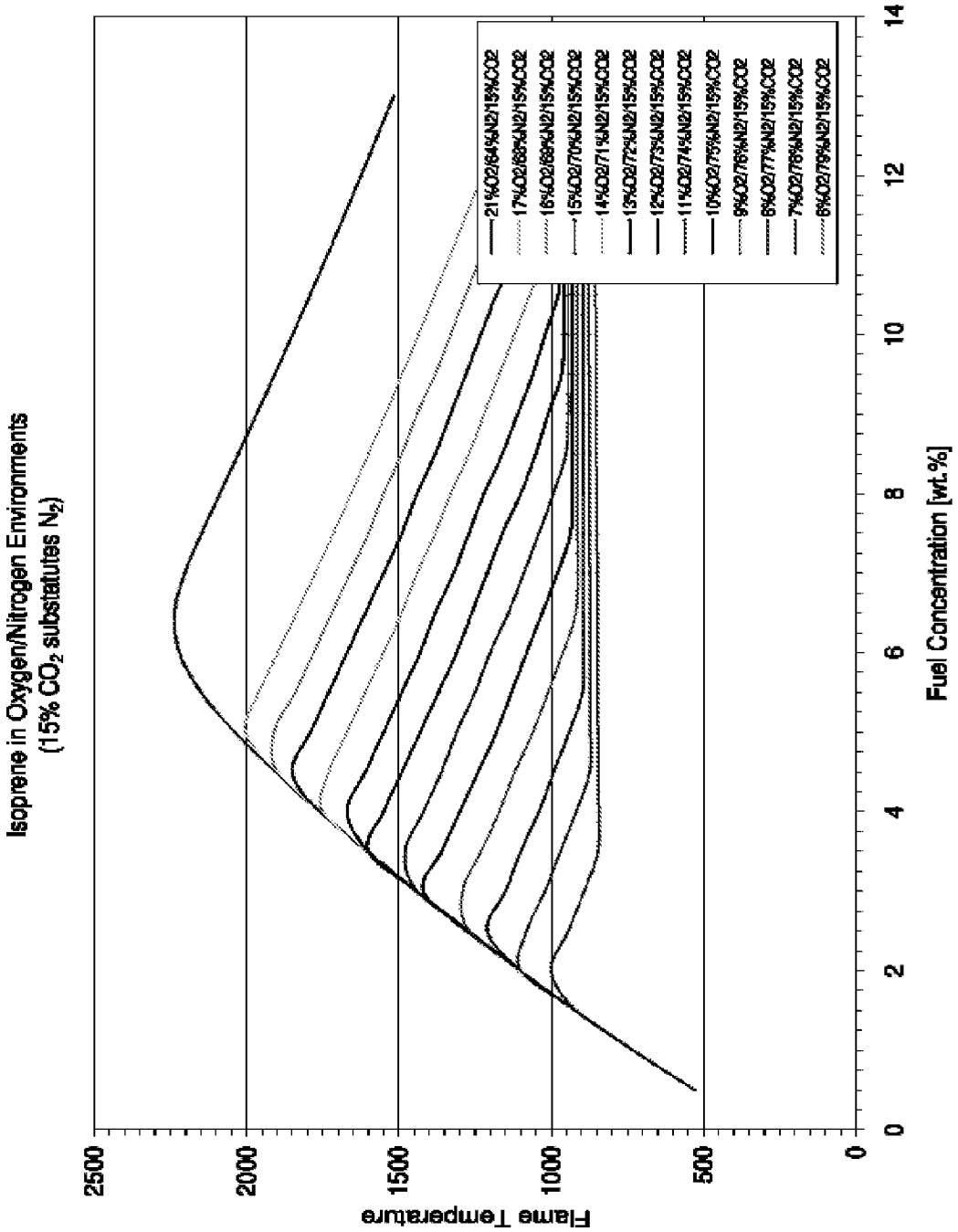

Isoprene in Oxygen/Nitrogen Environments
(15% CO_2 substitutes N_2)
Flame Temperature
Fuel Concentration [wt.%]
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
21%O2/64%N2/15%CO2
17%O2/68%N2/15%CO2
16%O2/69%N2/15%CO2
15%O2/70%N2/15%CO2
14%O2/71%N2/15%CO2
13%O2/72%N2/15%CO2
12%O2/73%N2/15%CO2
11%O2/74%N2/15%CO2
10%O2/75%N2/15%CO2
9%O2/76%N2/15%CO2
8%O2/77%N2/15%CO2
7%O2/78%N2/15%CO2
6%O2/79%N2/15%CO2

Figure 73

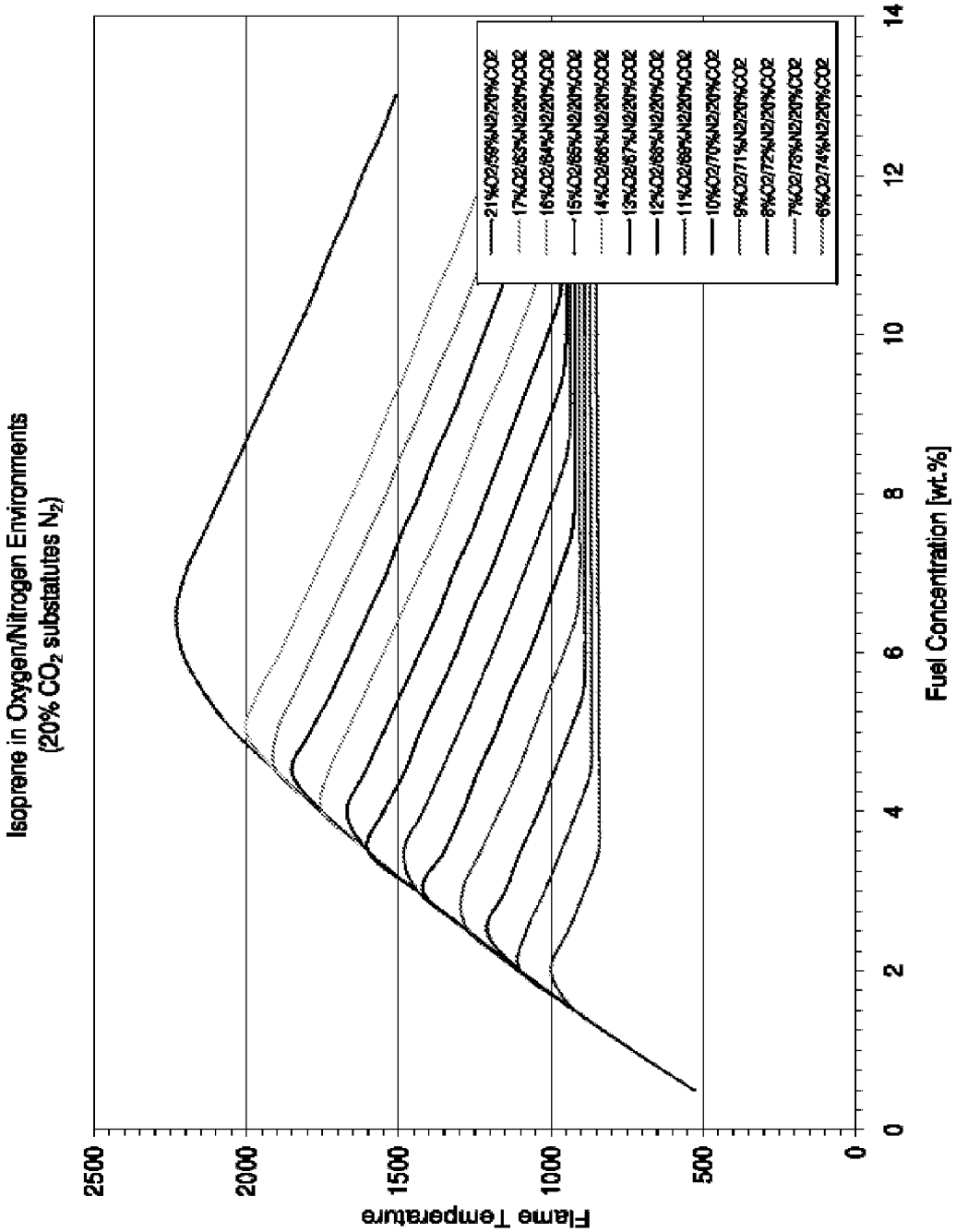

Isoprene in Oxygen/Nitrogen Environments
(20% CO_2 substitutes N_2)
Flame Temperature
Fuel Concentration [wt.%]
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
21%O2/0%N2/20%CO2
17%O2/4%N2/20%CO2
16%O2/5%N2/20%CO2
15%O2/6%N2/20%CO2
14%O2/7%N2/20%CO2
13%O2/8%N2/20%CO2
12%O2/9%N2/20%CO2
11%O2/9%N2/20%CO2
10%O2/70%N2/20%CO2
9%O2/71%N2/20%CO2
8%O2/72%N2/20%CO2
7%O2/73%N2/20%CO2
6%O2/74%N2/20%CO2

Figure 74

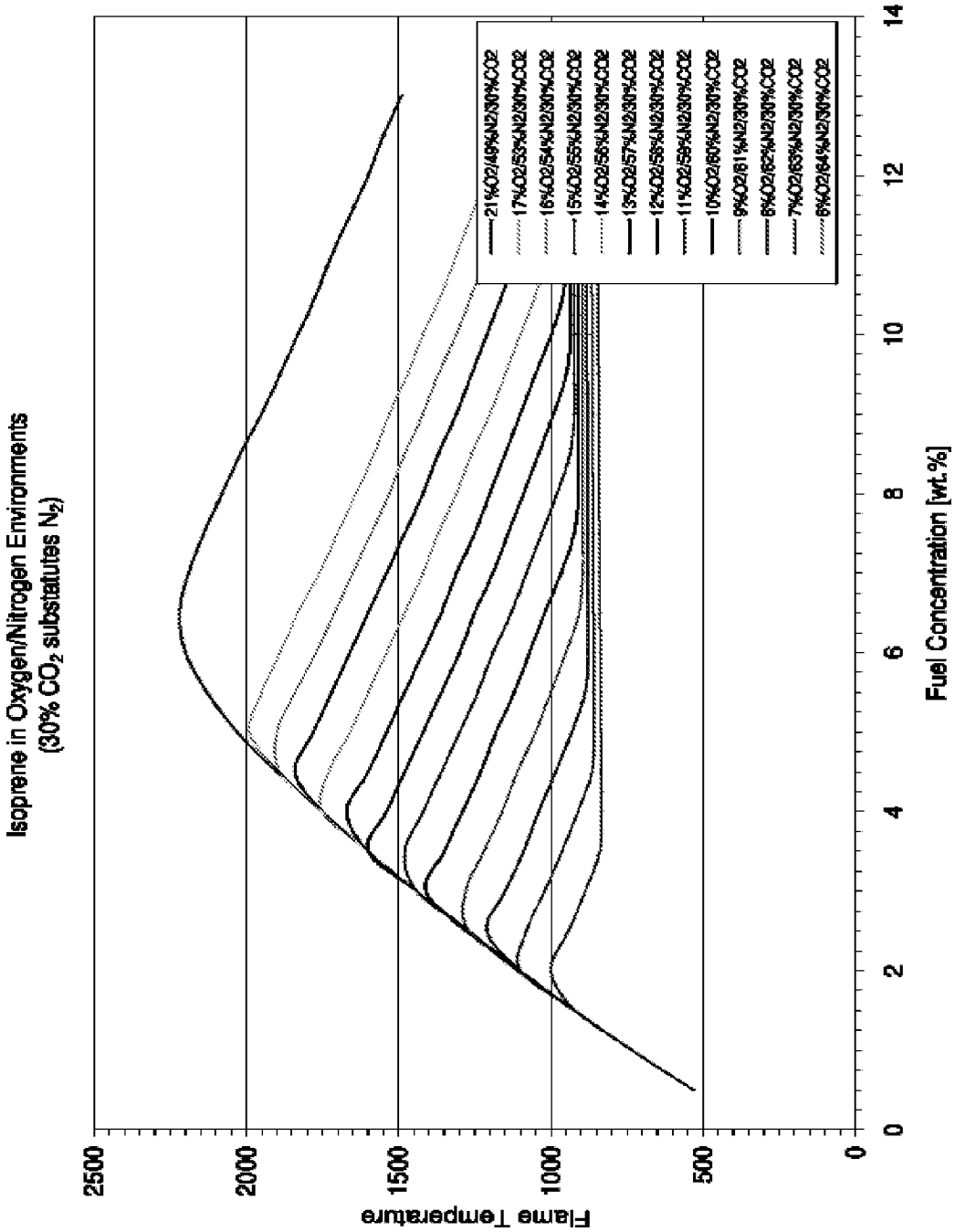
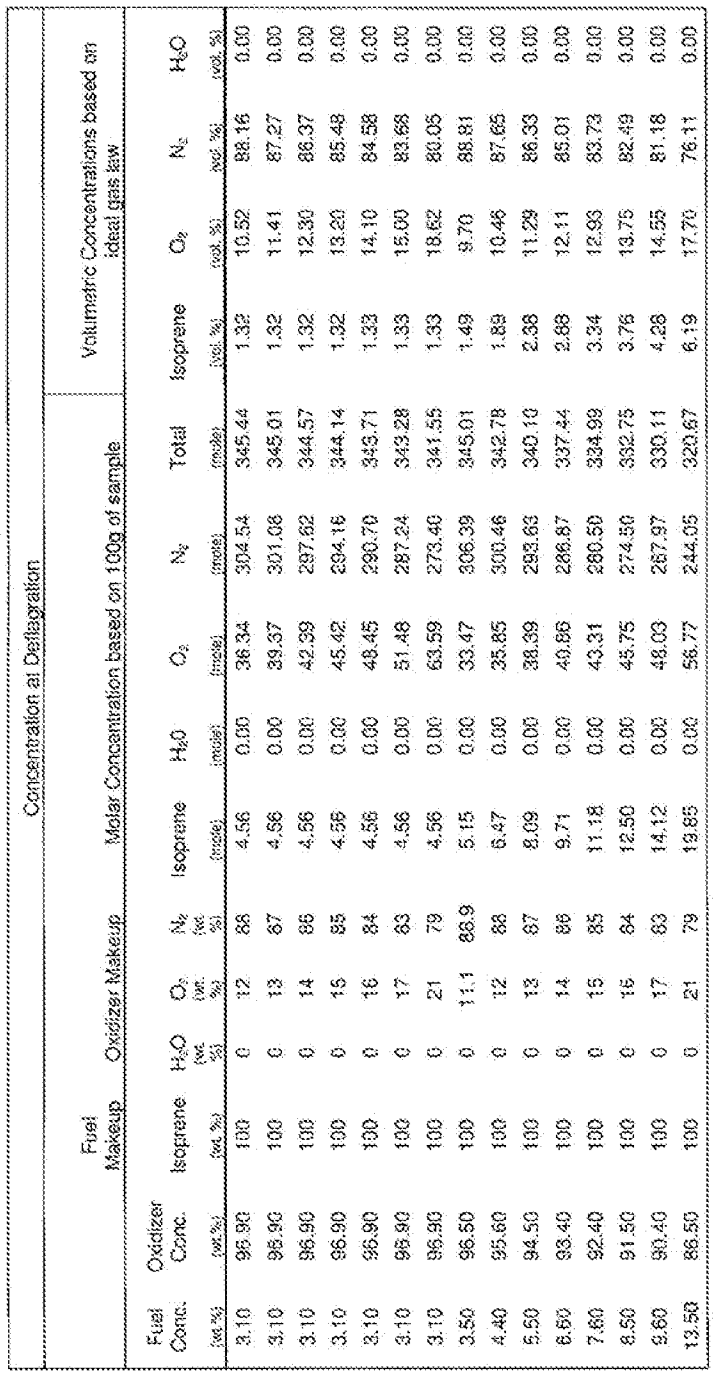

Isoprene in Oxygen/Nitrogen Environments
(30% CO_2 substitutes N_2)
Flame Temperature
Fuel Concentration [wt.%]
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
21%O2/49%N2/30%CO2
17%O2/53%N2/30%CO2
16%O2/54%N2/30%CO2
15%O2/55%N2/30%CO2
14%O2/56%N2/30%CO2
13%O2/57%N2/30%CO2
12%O2/58%N2/30%CO2
11%O2/59%N2/30%CO2
10%O2/60%N2/30%CO2
9%O2/61%N2/30%CO2
8%O2/62%N2/30%CO2
7%O2/63%N2/30%CO2
6%O2/64%N2/30%CO2

Figure 75A



Concentration at Deflagration																			
		Fuel Makeup	Oxidizer Makeup				Molar Concentration based on 100g of sample						Volumetric Concentrations based on ideal gas law						
Fuel Conc.	Oxidizer Conc.	Isoprene	H_2O	O_2	N_2	Isoprene	H_2O	O_2	N_2	Total	Isoprene	O_2	N_2	H_2O					
(vol.%)	(vol.%)	(vol. %)	(wt. %)	(wt. %)	(wt. %)	(mole)	(mole)	(mole)	(mole)	(mole)	(vol. %)	(vol. %)	(vol. %)	(vol. %)					
3.10	96.90	100	0	12	88	4.56	0.00	36.34	304.54	345.44	1.32	10.52	88.16	0.00					
3.10	96.90	100	0	13	87	4.56	0.00	39.37	301.08	345.01	1.32	11.41	87.27	0.00					
3.10	96.90	100	0	14	86	4.56	0.00	42.39	297.62	344.57	1.32	12.30	86.37	0.00					
3.10	96.90	100	0	15	85	4.56	0.00	45.42	294.16	344.14	1.32	13.20	85.48	0.00					
3.10	96.90	100	0	16	84	4.56	0.00	48.45	290.70	343.71	1.33	14.10	84.58	0.00					
3.10	96.90	100	0	17	83	4.56	0.00	51.48	287.24	343.28	1.33	15.00	83.68	0.00					
3.10	96.90	100	0	21	79	4.56	0.00	63.59	273.40	341.55	1.33	18.62	80.05	0.00					
3.50	96.50	100	0	11.1	88.9	5.15	0.00	33.47	306.39	345.01	1.49	9.70	88.81	0.00					
4.40	95.60	100	0	12	88	6.47	0.00	35.85	300.46	342.78	1.89	10.46	87.65	0.00					
5.50	94.50	100	0	13	87	8.09	0.00	38.39	293.63	340.10	2.38	11.29	86.33	0.00					
6.60	93.40	100	0	14	86	9.71	0.00	40.86	286.87	337.44	2.88	12.11	85.01	0.00					
7.60	92.40	100	0	15	85	11.18	0.00	43.31	280.50	334.99	3.34	12.93	83.73	0.00					
8.50	91.50	100	0	16	84	12.50	0.00	45.75	274.50	332.75	3.76	13.75	82.49	0.00					
9.60	90.40	100	0	17	83	14.12	0.00	48.03	267.97	330.11	4.28	14.55	81.18	0.00					
12.50	86.50	100	0	21	79	19.85	0.00	56.77	244.05	320.67	6.19	17.70	76.11	0.00					

Figure 75B

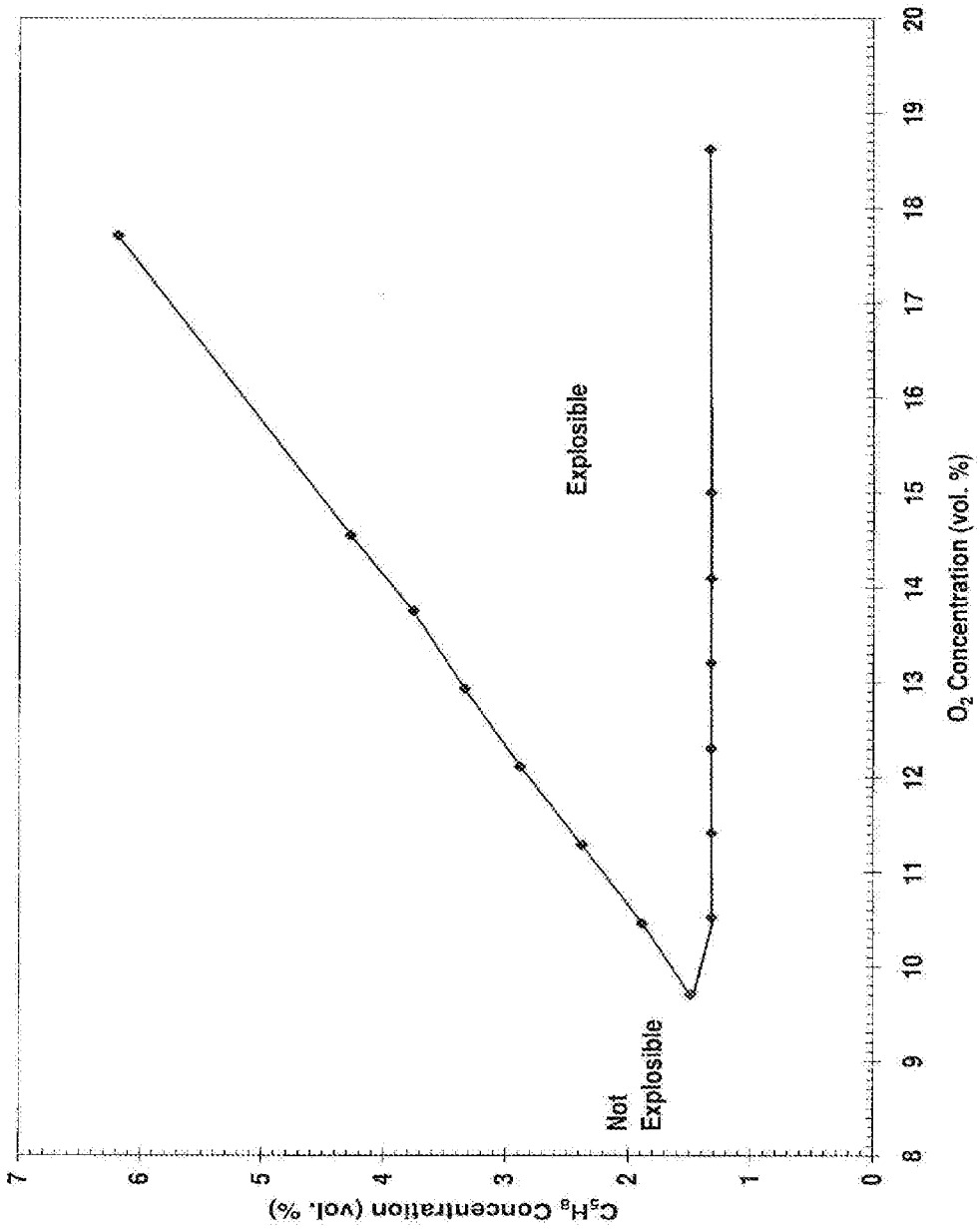
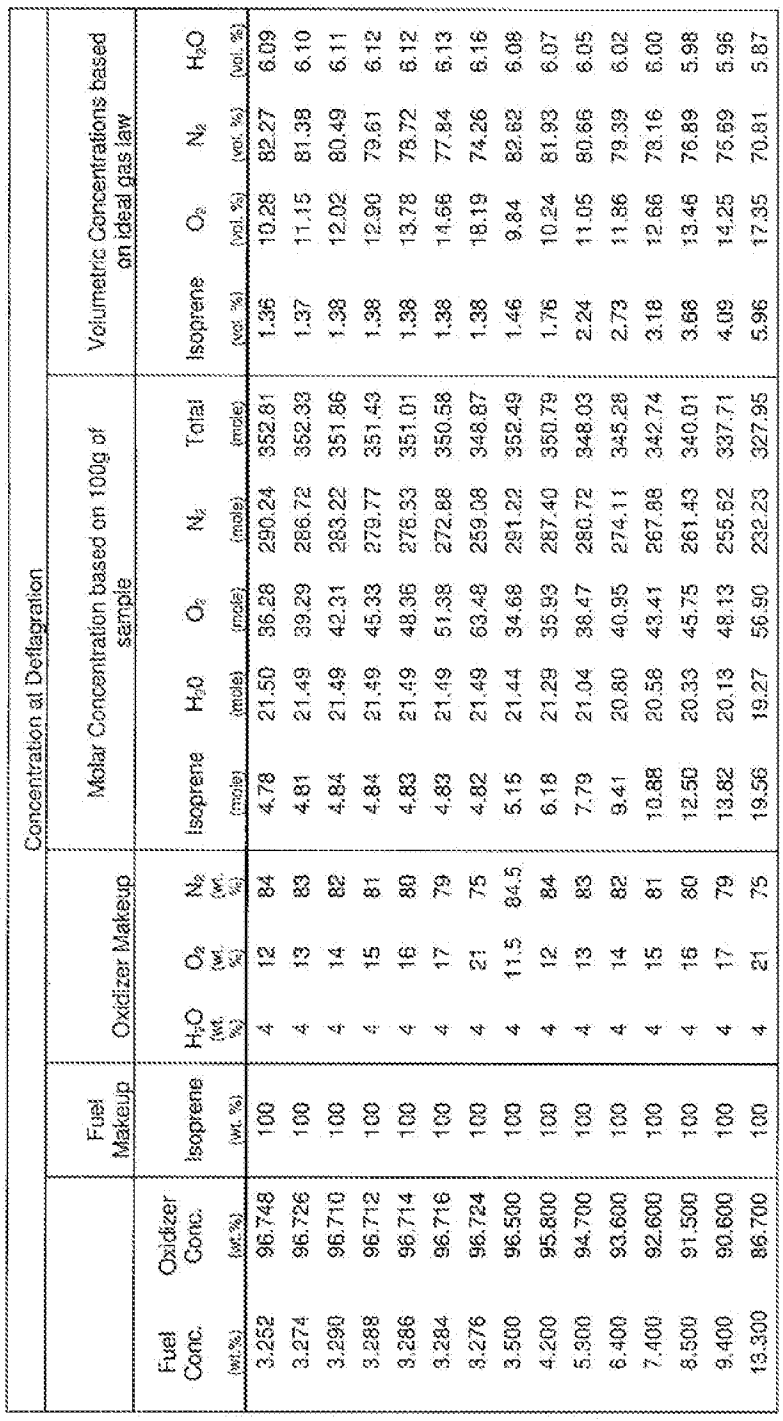

Explosible
Not Explosible
C_3H_8 Concentration (vol. %)
O_2 Concentration (vol. %)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Figure 76A



Concentration at Deflagration																				
		Fuel Makeup	Oxidizer Makeup				Molar Concentration based on 100g of sample						Volumetric Concentrations based on ideal gas law							
Fuel Conc.	Oxidizer Conc.	Isoprene	H_2O	O_2	N_2	Isoprene	H_2O	O_2	N_2	Total	Isoprene	O_2	N_2	H_2O						
(wt.%)	(wt.%)	(wt. %)	(wt. %)	(wt. %)	(wt. %)	(mole)	(mole)	(mole)	(mole)	(mole)	(vol. %)	(vol. %)	(vol. %)	(vol. %)						
3.252	96.748	100	4	12	84	4.78	21.50	36.28	290.24	352.81	1.36	10.28	82.27	6.09						
3.274	96.726	100	4	13	83	4.81	21.49	39.29	286.72	352.33	1.37	11.15	81.38	6.10						
3.290	96.710	100	4	14	82	4.84	21.49	42.31	283.22	351.86	1.38	12.02	80.49	6.11						
3.288	96.712	100	4	15	81	4.84	21.49	45.33	279.77	351.43	1.38	12.90	79.61	6.12						
3.286	96.714	100	4	16	80	4.83	21.49	48.36	276.33	351.01	1.38	13.78	78.72	6.12						
3.284	96.716	100	4	17	79	4.83	21.49	51.38	272.88	350.58	1.38	14.66	77.84	6.13						
3.276	96.724	100	4	21	75	4.82	21.49	63.48	259.08	348.87	1.38	18.19	74.26	6.16						
3.500	96.500	100	4	11.5	84.5	5.15	21.44	34.69	291.22	352.49	1.46	9.84	82.62	6.08						
4.200	95.800	100	4	12	84	6.18	21.29	35.93	287.40	350.79	1.76	10.24	81.93	6.07						
5.300	94.700	100	4	13	83	7.79	21.04	38.47	280.72	348.03	2.24	11.05	80.66	6.05						
6.400	93.600	100	4	14	82	9.41	20.80	40.95	274.11	345.28	2.73	11.86	79.39	6.02						
7.400	92.600	100	4	15	81	10.88	20.58	43.41	267.88	342.74	3.18	12.66	78.16	6.00						
8.500	91.500	100	4	16	80	12.50	20.33	45.75	261.43	340.01	3.66	13.46	76.89	5.98						
9.400	90.600	100	4	17	79	13.82	20.13	48.13	255.62	337.71	4.09	14.25	75.69	5.96						
13.300	86.700	100	4	21	75	19.56	19.27	56.90	232.23	327.95	5.96	17.35	70.81	5.87						

Figure 76B

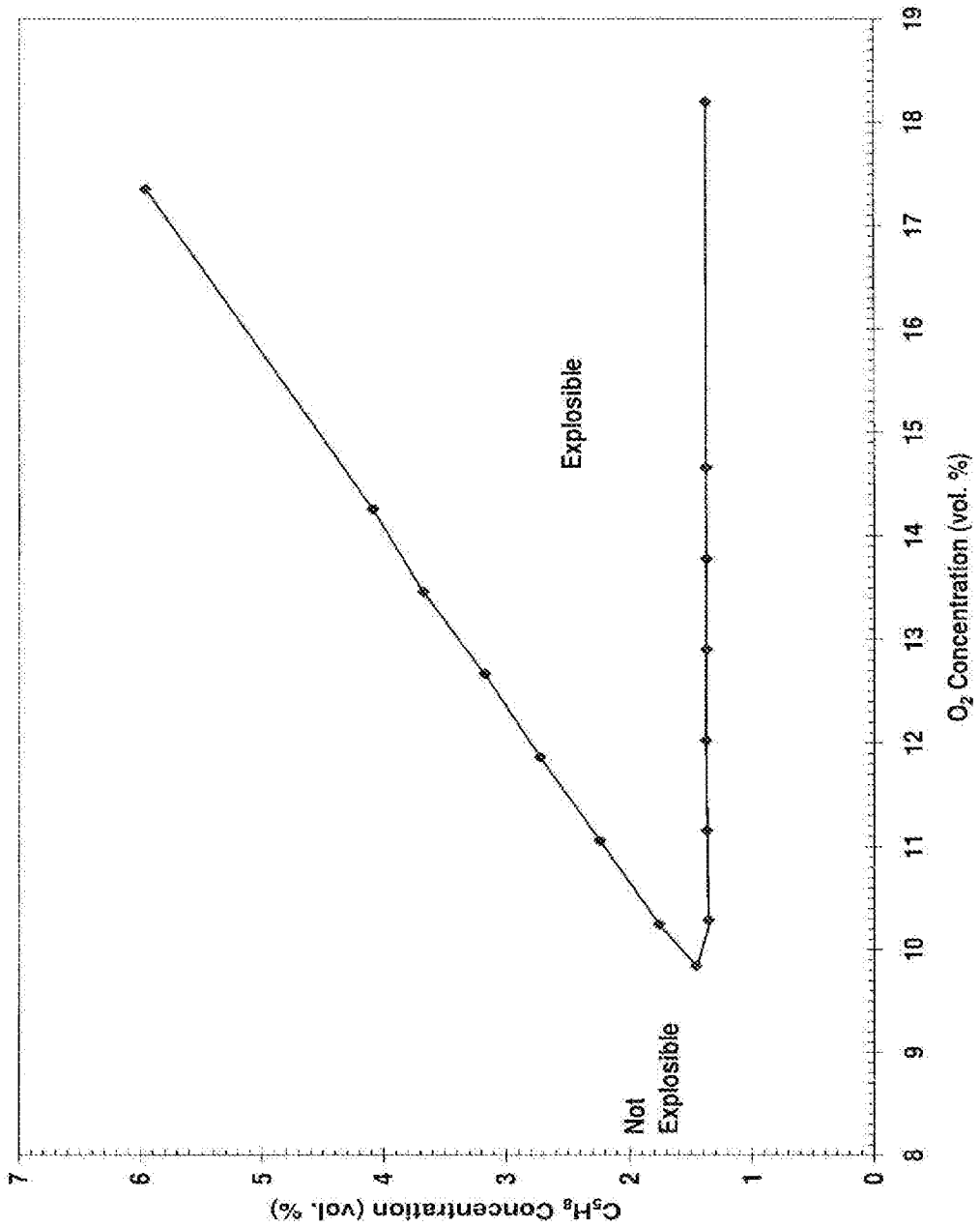

Explosible
Not Explosible
C_3H_8 Concentration (vol. %)
O_2 Concentration (vol. %)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Figure 77

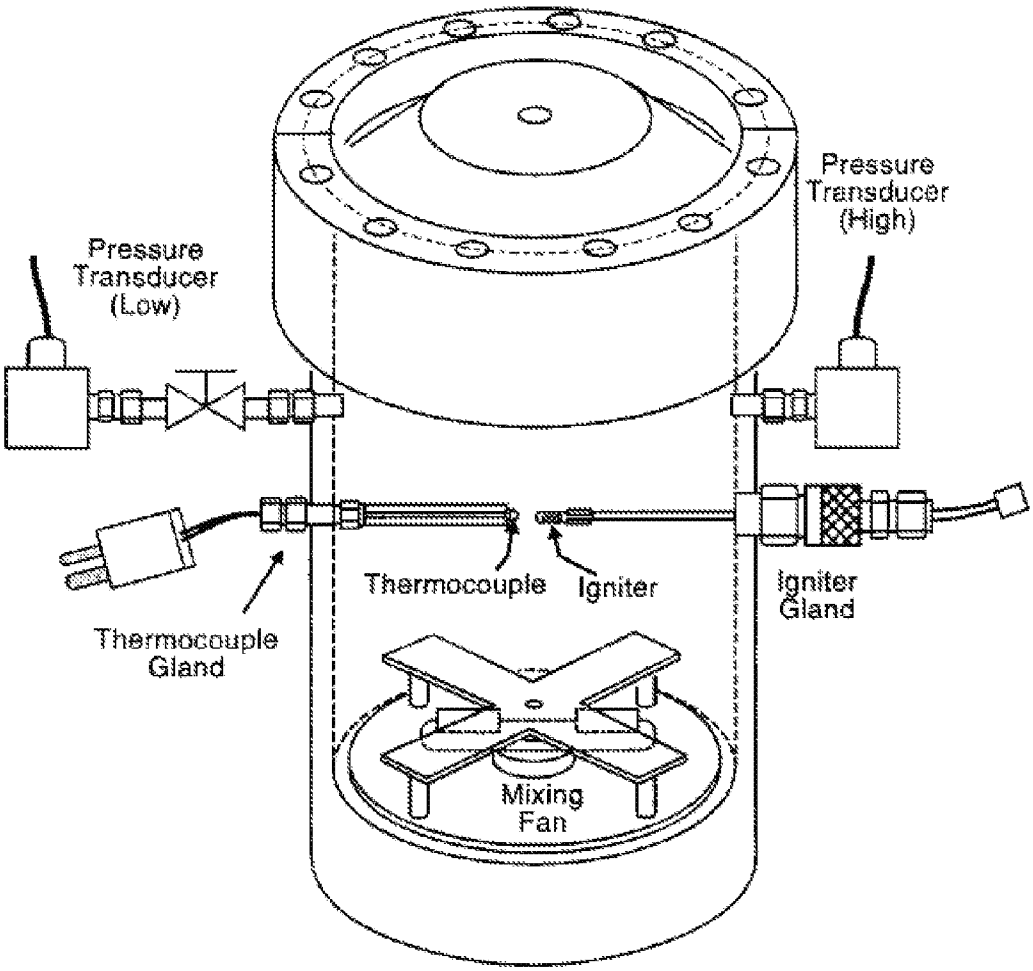

Pressure Transducer (High)
Pressure Transducer (Low)
Thermocouple
Igniter
Igniter Gland
Thermocouple Gland
Mixing Fan

Figure 78A

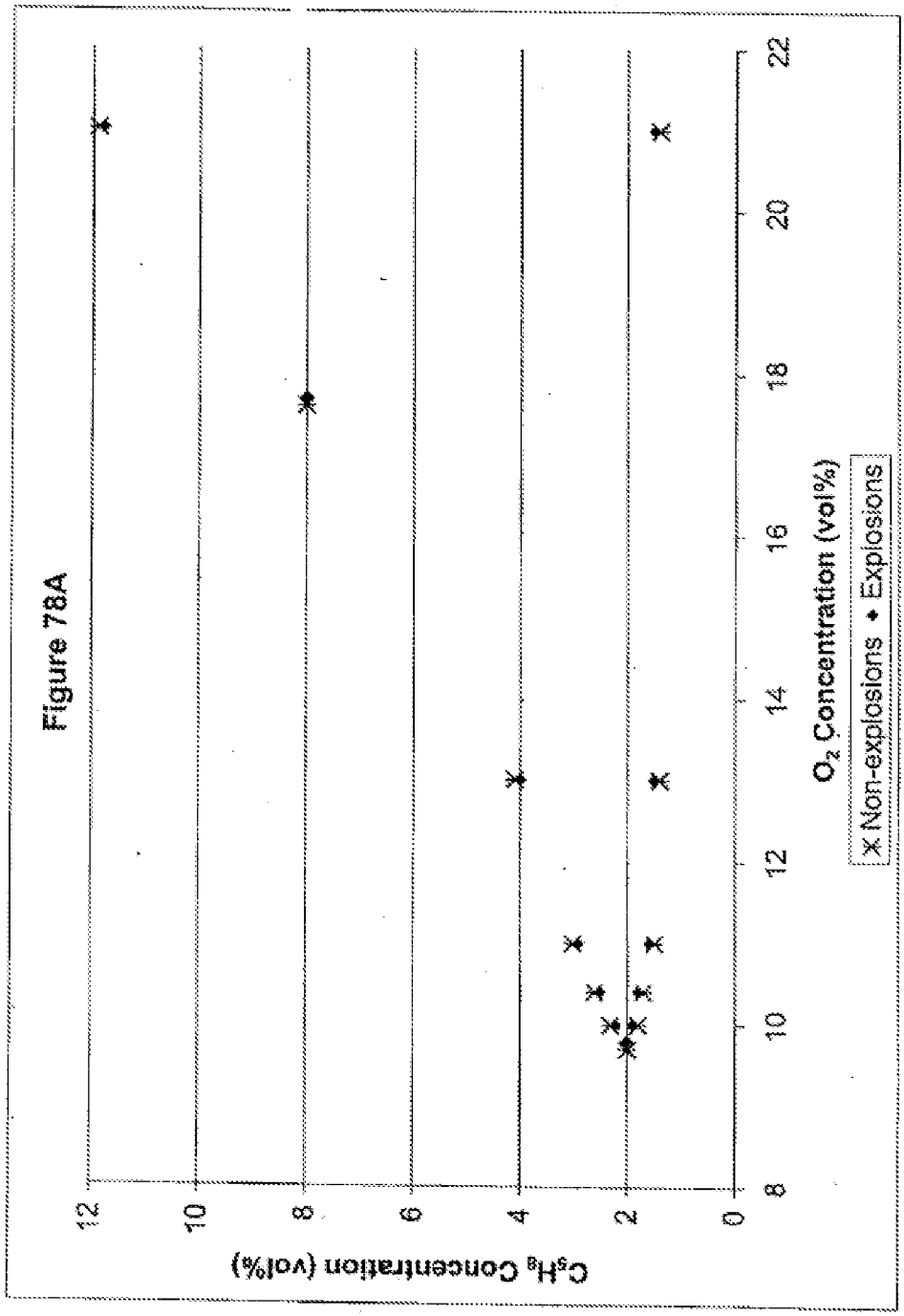


Figure 78A
C3H8 Concentration (vol%)
0
2
4
6
8
10
12
8
10
12
14
16
18
20
22
O2 Concentration (vol%)
Non-explosions
Explosions

Figure 78B

Explosions		Non-explosions	
O2 Concentration (vol. %)	**C5H8 Concentration (vol. %)**	**O2 Concentration (vol. %)**	**C5H8 Concentration (vol. %)**
21.0	1.5	21.0	1.4
13.0	1.5	13.0	1.4
11.0	1.6	11.0	1.5
10.4	1.8	10.4	1.7
10.0	1.9	10.0	1.8
9.8	2	9.7	2
10.0	2.2	10.0	2.3
10.4	2.5	10.4	2.6
11.0	2.9	11.0	3.0
13.0	4.0	13.0	4.1
17.7	8.0	17.6	8.0
21.0	11.8	21.0	11.9

Figure 78C

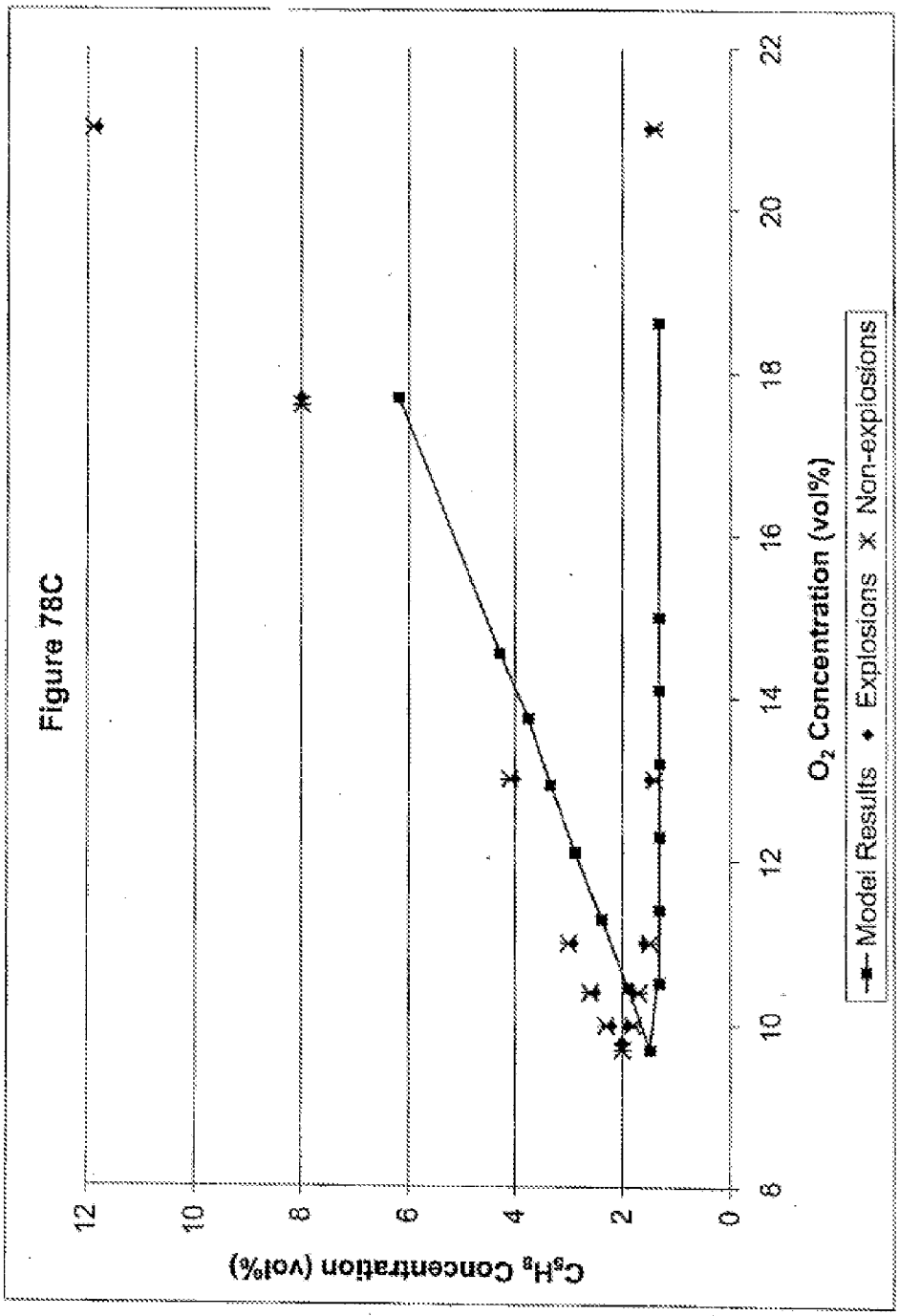


Figure 78C
C3H8 Concentration (vol%)
O2 Concentration (vol%)
Model Results
Explosions
Non-explosions
0
2
4
6
8
10
12
8
10
12
14
16
18
20
22

Figure 79A

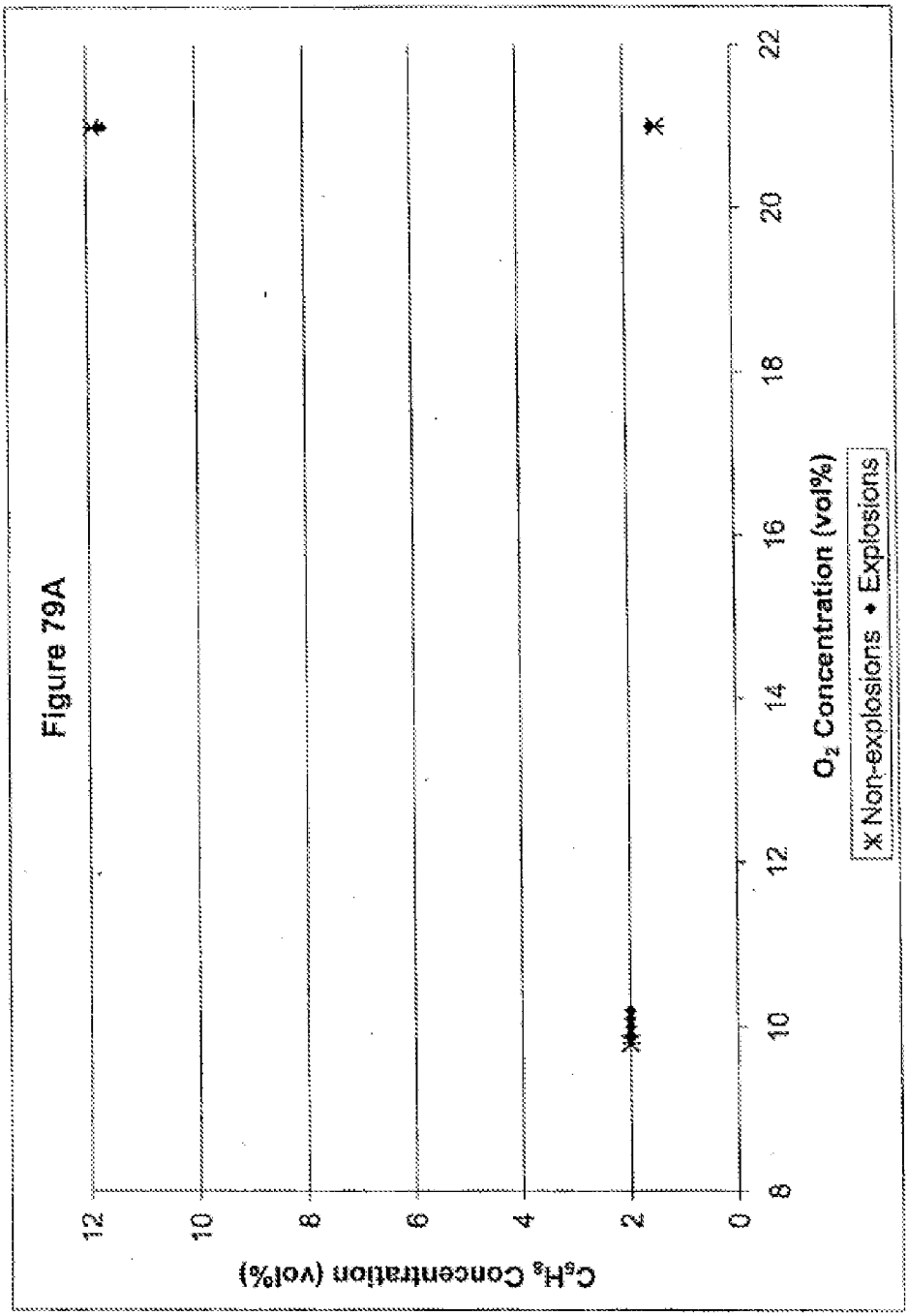


Figure 79A
C3H8 Concentration (vol%)
0
2
4
6
8
10
12
8
10
12
14
16
18
20
22
O2 Concentration (vol%)
x Non-explosions
Explosions

Figure 79B

Explosions		Non-explosions	
O2 Concentration (vol. %)	**C5H8 Concentration (vol. %)**	**O2 Concentration (vol. %)**	**C5H8 Concentration (vol. %)**
21.0	11.7	21.0	11.9
21.0	11.8	21.0	11.9
21.0	11.8	21.0	11.9
21.0	1.5	21.0	1.4
21.0	1.5	21.0	1.4
10.2	2.0	21.0	1.4
10.1	2.0	9.8	2.0
10.0	2.0	9.8	2.0
9.9	2.0	9.8	2.0

Figure 79C

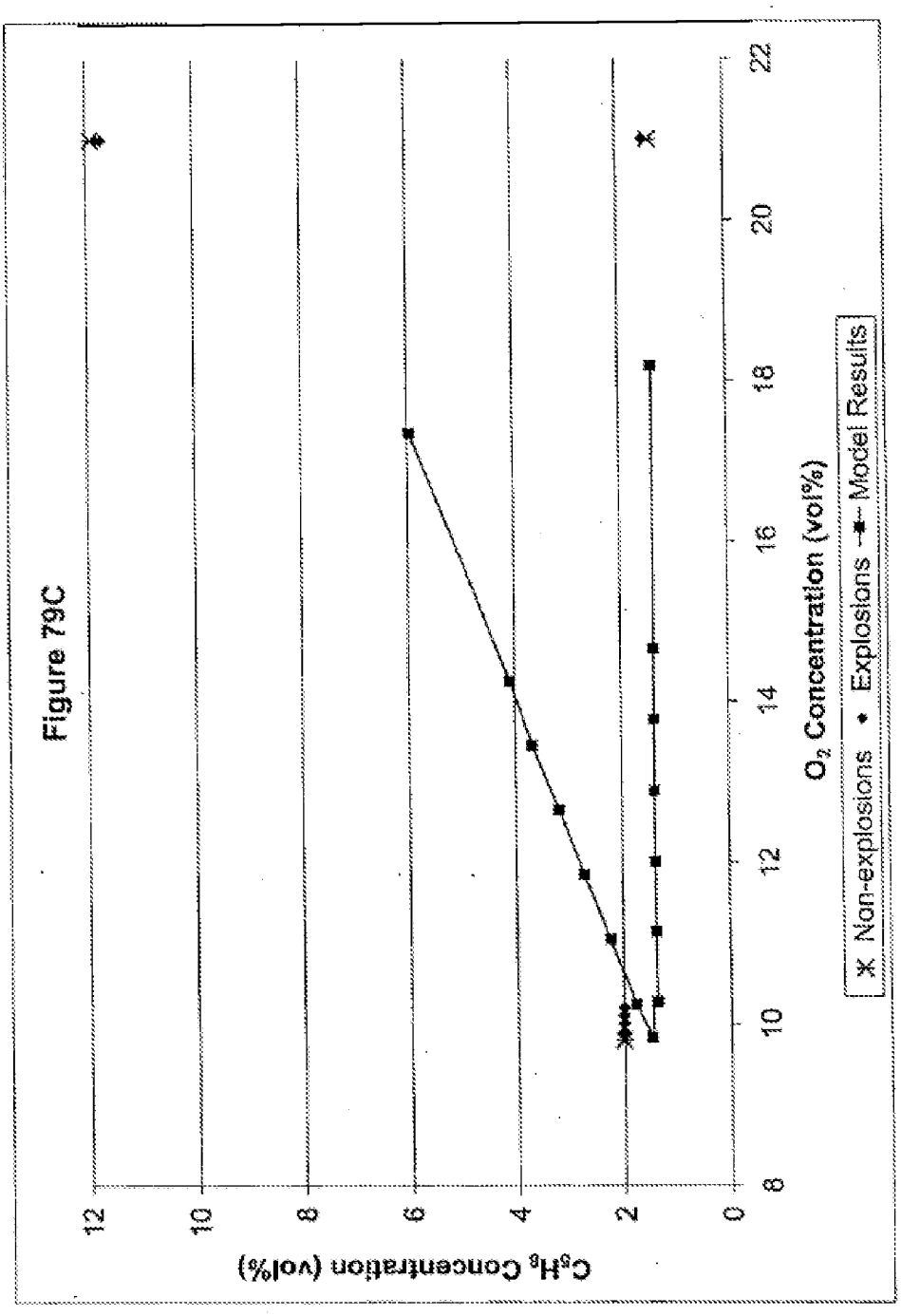


Figure 79C
C3H8 Concentration (vol%)
O2 Concentration (vol%)
Non-explosions
Explosions
Model Results
0
2
4
6
8
10
12
8
10
12
14
16
18
20
22

Figure 80A

TEST SERIES 1

Test	Data File Name	Temp	Initial Pressure	Partial Pressures			Concentrations			Result	Pex
				C_2H_6	N_2	O_2	C_2H_6	N_2	O_2		
		°C	bara	mbar	mbar	mbar	vol. %	vol. %	vol. %		bara
1	T11120700	40	1.012	12	787	213	1.2	77.8	21.0	Non-Explosion	1.05
2	T11120701	40	1.016	16	787	213	1.6	77.5	21.0	Explosion	5.5
3	T11120702	40	1.015	14	788	213	1.4	77.6	21.0	Non-Explosion	<1.02
4	T11120703	40	1.014	15	786	213	1.5	77.5	21.0	Non-Explosion	<1.02
5	T11120704	40	1.014	15	786	213	1.5	77.5	21.0	Explosion	4.31
6	T11120705	40	1.017	18	785	214	1.8	77.2	21.0	Explosion	5.47
7	T11120706	40	1.014	15	786	213	1.5	77.5	21.0	Explosion	4.51
8	T11120707	40	1.014	14	787	213	1.4	77.6	21.0	Non-Explosion	<1.02
9	T11120708	40	1.014	14	787	213	1.4	77.6	21.0	Non-Explosion	1.05
10	T11120709	40	1.015	102	700	213	10.0	69.0	21.0	Explosion	1.45
11	T11120710	40	1.014	102	699	213	10.1	68.9	21.0	Explosion	1.39
12	T11120711	40	1.014	106	695	213	10.5	68.5	21.0	Explosion	1.34
13	T11120712	40	1.014	113	688	213	11.1	67.9	21.0	Explosion	1.29
14	T11120713	40	1.014	122	679	213	12.0	67.0	21.0	Non-Explosion	<1.02
15	T11120714	40	1.014	117	684	213	11.5	67.5	21.0	Explosion	1.32
16	T11120715	40	1.014	120	681	213	11.8	67.2	21.0	Non-Explosion	1.06
17	T11130700	40	1.014	120	681	213	11.8	67.2	21.0	Explosion	1.09
18	T11130701	40	1.014	121	680	213	11.9	67.1	21.0	Non-Explosion	1.07
19	T11130702	40	1.015	121	681	213	11.9	67.1	21.0	Non-Explosion	1.06
20	T11130703	40	1.015	121	681	213	11.9	67.1	21.0	Non-Explosion	1.07
21	T11130704	40	1.015	30	853	132	3.0	84.0	13.0	Explosion	1.61
22	T11130705	40	1.014	36	846	132	3.6	83.4	13.0	Explosion	1.28
23	T11130706	40	1.014	39	843	132	3.8	83.1	13.0	Explosion	1.12
24	T11130707	40	1.015	41	842	132	4.0	83.0	13.0	Explosion	1.09
25	T11130708	40	1.014	42	840	132	4.1	82.8	13.0	Non-Explosion	1.06
26	T11130709	40	1.015	42	841	132	4.1	82.9	13.0	Non-Explosion	1.06
27	T11130710	40	1.014	42	840	132	4.1	82.8	13.0	Non-Explosion	1.05
28	T11130711	40	1.014	15	867	132	1.5	85.5	13.0	Non-Explosion	1.03
29	T11130712	40	1.014	16	866	132	1.6	85.4	13.0	Explosion	4.81
30	T11130713	40	1.014	15	867	132	1.5	85.5	13.0	Explosion	4
31	T11130714	40	1.014	14	868	132	1.4	85.6	13.0	Non-Explosion	1.03
32	T11130715	40	1.014	14	868	132	1.4	85.6	13.0	Non-Explosion	<1.02
33	T11130716	40	1.014	14	868	132	1.4	85.6	13.0	Non-Explosion	1.03
34	T11130717	40	1.015	20	883	112	2.0	87.0	11.0	Explosion	1.7
35	T11130718	40	1.014	28	874	112	2.8	86.2	11.0	Non-Explosion	1.08
36	T11130719	40	1.014	28	874	112	2.8	86.2	11.0	Non-Explosion	1.08
37	T11130720	40	1.014	28	874	112	2.8	86.2	11.0	Explosion	1.13
38	T11130721	40	1.015	29	874	112	2.9	86.1	11.0	Non-Explosion	1.08
39	T11130722	40	1.014	29	873	112	2.9	86.1	11.0	Explosion	1.1

Figure 80B

Test	Data File Name	Temp	Initial Pressure	Partial Pressures			Concentrations			Result	Pex
				C_5H_8	N_2	O_2	C_5H_8	N_2	O_2		
		°C	bara	mbar	mbar	mbar	vol. %	vol. %	vol. %		bara
40	T11130723	40	1.014	30	872	112	3.0	86.0	11.0	Non-Explosion	1.08
41	T11130724	40	1.014	30	872	112	3.0	86.0	11.0	Non-Explosion	1.05
42	T11130725	40	1.014	30	872	112	3.0	86.0	11.0	Non-Explosion	1.05
43	T11130726	40	1.014	15	887	112	1.5	87.5	11.0	Non-Explosion	<1.02
44	T11130727	40	1.014	15	887	112	1.5	87.5	11.0	Non-Explosion	<1.02
45	T11140700	40	1.014	16	886	112	1.6	87.4	11.0	Non-Explosion	<1.02
46	T11140701	40	1.014	17	885	112	1.7	87.3	11.0	Explosion	1.81
47	T11140702	40	1.014	16	886	112	1.6	87.4	11.0	Explosion	1.54
48	T11140703	40	1.014	15	887	112	1.5	87.5	11.0	Non-Explosion	<1.02
49	T11140704	40	1.015	20	899	96	2.0	88.6	9.5	Non-Explosion	1.05
50	T11140705	40	1.014	20	898	96	2.0	88.6	9.5	Non-Explosion	1.05
51	T11140706	40	1.014	23	890	101	2.3	87.8	10.0	Non-Explosion	1.05
52	T11140707	40	1.015	23	886	106	2.3	87.3	10.4	Explosion	1.19
53	T11140708	40	1.014	25	884	105	2.5	87.2	10.4	Explosion	1.09
54	T11140709	40	1.014	26	883	105	2.6	87.1	10.4	Non-Explosion	1.05
55	T11140710	40	1.014	26	883	105	2.6	87.1	10.4	Non-Explosion	1.06
56	T11140711	40	1.014	26	883	105	2.6	87.1	10.4	Non-Explosion	1.07
57	T11140712	40	1.014	20	889	105	2.0	87.7	10.4	Explosion	1.21
58	T11140713	40	1.014	17	892	105	1.7	88.0	10.4	Non-Explosion	1.04
59	T11140714	40	1.014	18	891	105	1.8	87.9	10.4	Explosion	1.21
60	T11140715	40	1.014	17	892	105	1.7	88.0	10.4	Non-Explosion	1.03
61	T11140716	40	1.014	17	892	105	1.7	88.0	10.4	Non-Explosion	1.03
62	T11140717	40	1.014	21	890	103	2.1	87.8	10.2	Explosion	1.1
63	T11140718	40	1.014	21	891	102	2.1	87.9	10.1	Explosion	1.09
64	T11140719	40	1.014	21	892	101	2.1	88.0	10.0	Explosion	1.09
65	T11140720	40	1.014	22	891	101	2.2	87.9	10.0	Explosion	1.1
66	T11140721	40	1.014	23	890	101	2.3	87.8	10.0	Non-Explosion	1.06
67	T11140722	40	1.014	23	890	101	2.3	87.8	10.0	Non-Explosion	1.08
68	T11140723	40	1.014	19	894	101	1.9	88.2	10.0	Explosion	1.12
69	T11140724	40	1.014	18	895	101	1.8	88.3	10.0	Non-Explosion	1.06
70	T11140725	40	1.014	18	895	101	1.8	88.3	10.0	Non-Explosion	1.03
71	T11140726	40	1.014	18	895	101	1.8	88.3	10.0	Non-Explosion	1.04
72	T11140727	40	1.014	20	895	99	2.0	88.3	9.8	Non-Explosion	1.08
73	T11140728	40	1.014	20	895	99	2.0	88.3	9.8	Explosion	1.1
74	T11140729	40	1.014	20	896	98	2.0	88.4	9.7	Non-Explosion	1.06
75	T11140730	40	1.014	20	896	98	2.0	88.4	9.7	Non-Explosion	1.08
76	T11140731	40	1.014	20	896	98	2.0	88.4	9.7	Non-Explosion	1.07
77	T11140732	40	1.014	81	761	172	8.0	75.0	17.0	Non-Explosion	1.04
78	T11140733	40	1.014	81	750	183	8.0	74.0	18.0	Explosion	1.3
79	T11140734	40	1.014	81	754	179	8.0	74.4	17.7	Explosion	1.24
80	T11140735	40	1.014	81	757	176	8.0	74.7	17.4	Non-Explosion	1.03
81	T11140736	40	1.014	81	755	178	8.0	74.5	17.6	Non-Explosion	1.05
82	T11140737	40	1.014	81	755	178	8.0	74.5	17.6	Non-Explosion	1.03
83	T11140738	40	1.014	81	755	178	8.0	74.5	17.6	Non-Explosion	1.03

Figure 81

TEST SERIES 2

Test	Data File Name	Temp	Initial Pressure	Partial Pressures				Concentrations				Result	Pex
				H_2O	C_2H_6	N_2	O_2	H_2O	C_2H_6	N_2	O_2		
		°C	bara	mbar	mbar	mbar	mbar	vol. %	vol. %	vol. %	vol. %		bara
1	T11150700	40	1.014	41	119	641	213	4.0	11.7	63.2	21.0	Explosion	1.33
2	T11150701	40	1.014	40	121	640	213	3.9	11.9	63.1	21.0	Non-explosion	1.07
3	T11150702	40	1.014	41	120	640	213	4.0	11.8	63.1	21.0	Explosion	1.09
4	T11150703	40	1.014	40	121	640	213	3.9	11.9	63.1	21.0	Non-explosion	1.06
5	T11150704	40	1.014	40	120	641	213	3.9	11.8	63.2	21.0	Explosion	1.09
6	T11150705	40	1.014	40	121	640	213	3.9	11.9	63.1	21.0	Non-explosion	1.08
7	T11150706	40	1.014	40	15	746	213	3.9	1.5	73.6	21.0	Explosion	4.68
8	T11150707	40	1.014	41	15	745	213	4.0	1.5	73.5	21.0	Explosion	5.27
9	T11150708	40	1.014	41	14	746	213	4.0	1.4	73.6	21.0	Non-explosion	1.03
10	T11150709	40	1.014	42	14	745	213	4.1	1.4	73.5	21.0	Non-explosion	1.03
11	T11160700	40	1.014	41	14	746	213	4.0	1.4	73.6	21.0	Non-explosion	1.03
12	T11160701	40	1.014	41	20	850	103	4.0	2.0	83.8	10.2	Explosion	1.11
13	T11160702	40	1.014	41	20	851	102	4.0	2.0	83.9	10.1	Explosion	1.11
14	T11160703	40	1.014	41	20	852	101	4.0	2.0	84.0	10.0	Explosion	1.09
15	T11160704	40	1.014	41	20	853	100	4.0	2.0	84.1	9.9	Explosion	1.09
16	T11160705	40	1.014	41	20	854	99	4.0	2.0	84.2	9.8	Non-explosion	1.07
17	T11160706	40	1.014	40	20	855	99	3.9	2.0	84.3	9.8	Non-explosion	1.06
18	T11160707	40	1.014	41	20	854	99	4.0	2.0	84.2	9.8	Non-explosion	1.08

Figure 82

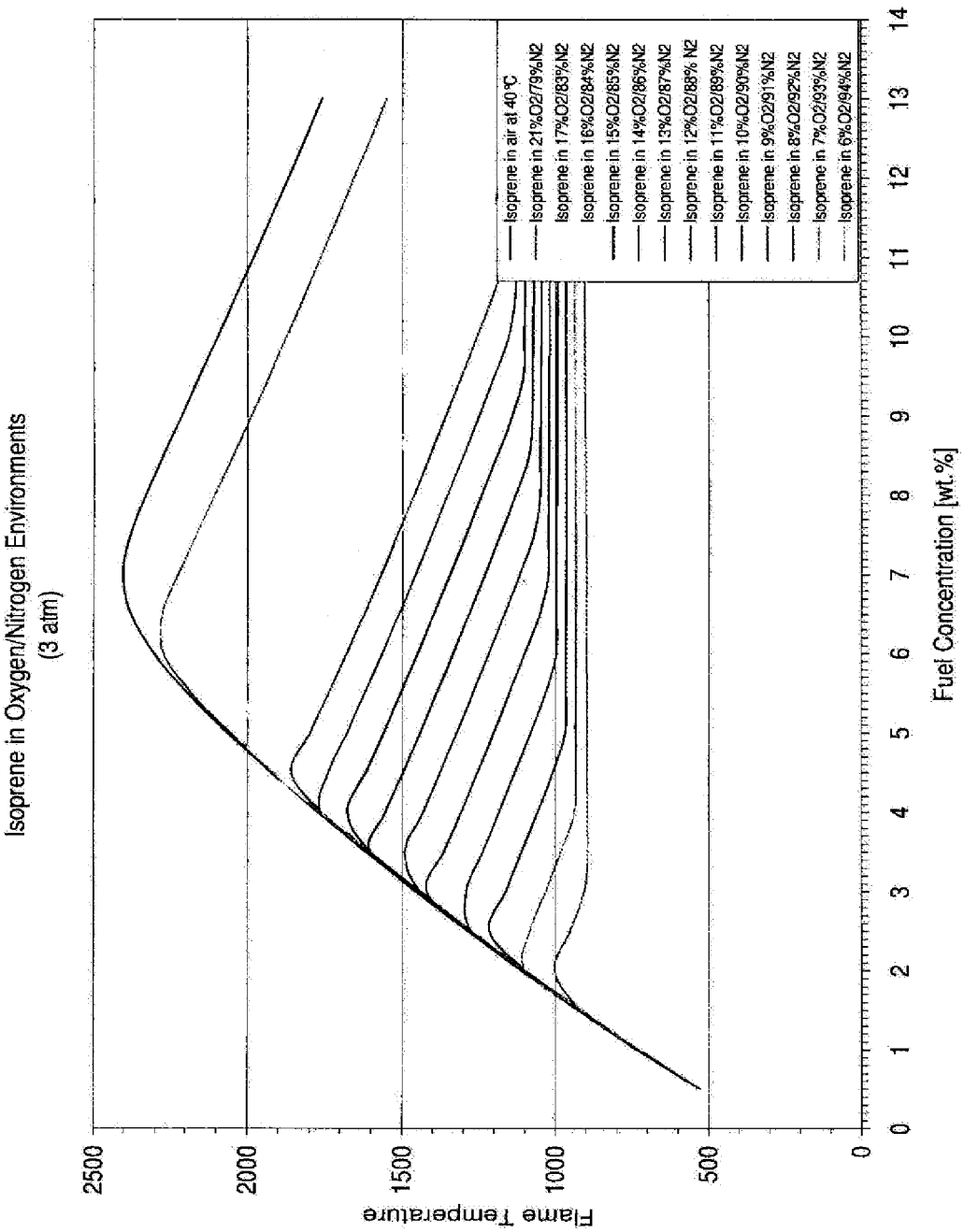

Isoprene in Oxygen/Nitrogen Environments
(3 atm)
Flame Temperature
Fuel Concentration [wt.%]
2500
2000
1500
1000
500
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Isoprene in air at 40°C
Isoprene in 21%O2/79%N2
Isoprene in 17%O2/83%N2
Isoprene in 16%O2/84%N2
Isoprene in 15%O2/85%N2
Isoprene in 14%O2/86%N2
Isoprene in 13%O2/87%N2
Isoprene in 12%O2/88% N2
Isoprene in 11%O2/89%N2
Isoprene in 10%O2/90%N2
Isoprene in 9%O2/91%N2
Isoprene in 8%O2/92%N2
Isoprene in 7%O2/93%N2
Isoprene in 6%O2/94%N2

Figure 83

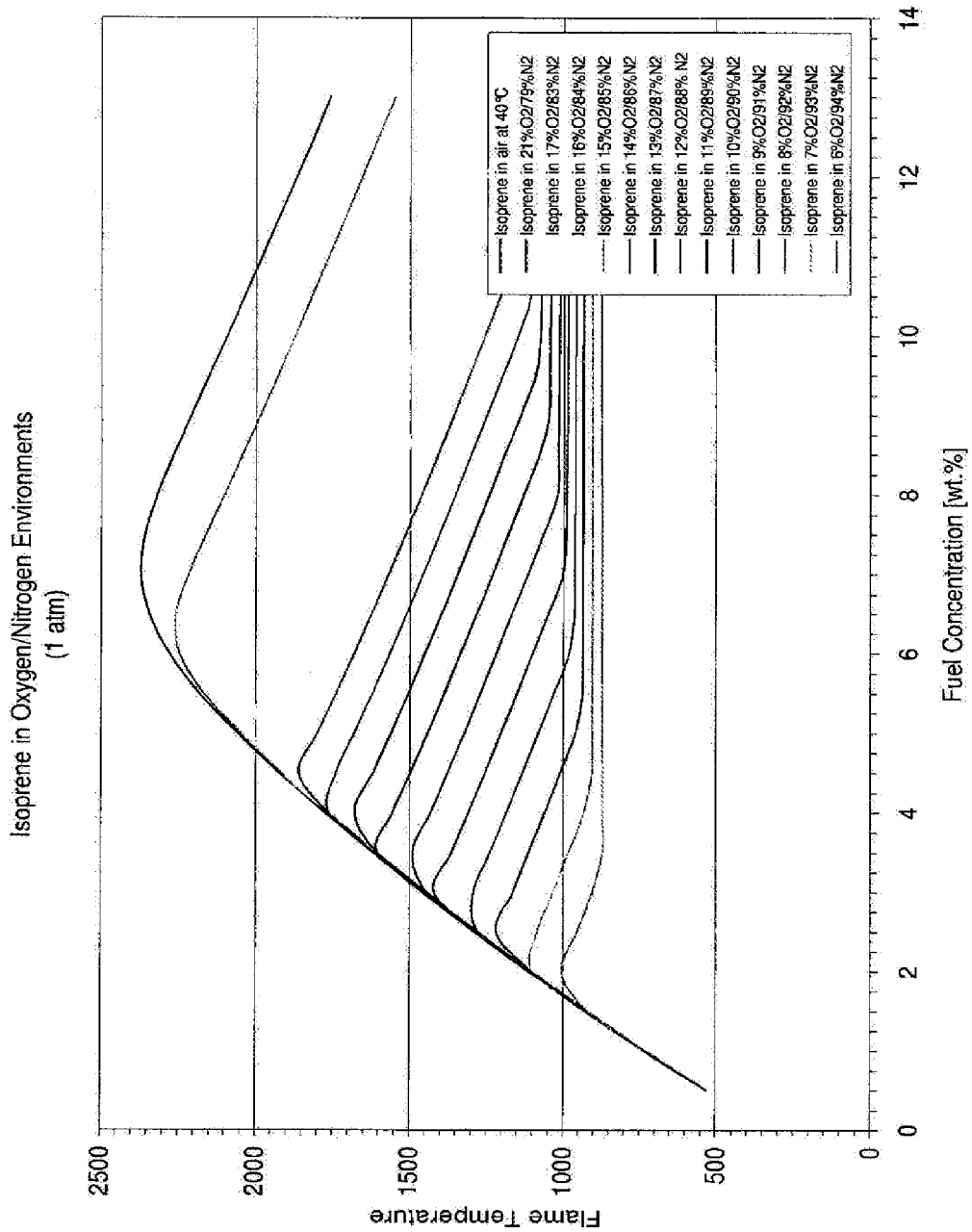

Isoprene in Oxygen/Nitrogen Environments
(1 atm)
Flame Temperature
Fuel Concentration [wt.%]
Isoprene in air at 40°C
Isoprene in 21%O2/79%N2
Isoprene in 17%O2/83%N2
Isoprene in 16%O2/84%N2
Isoprene in 15%O2/85%N2
Isoprene in 14%O2/86%N2
Isoprene in 13%O2/87%N2
Isoprene in 12%O2/88%.N2
Isoprene in 11%O2/89%N2
Isoprene in 10%O2/90%N2
Isoprene in 9%O2/91%N2
Isoprene in 8%O2/92%N2
Isoprene in 7%O2/93%N2
Isoprene in 6%O2/94%N2
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14

Figure 84

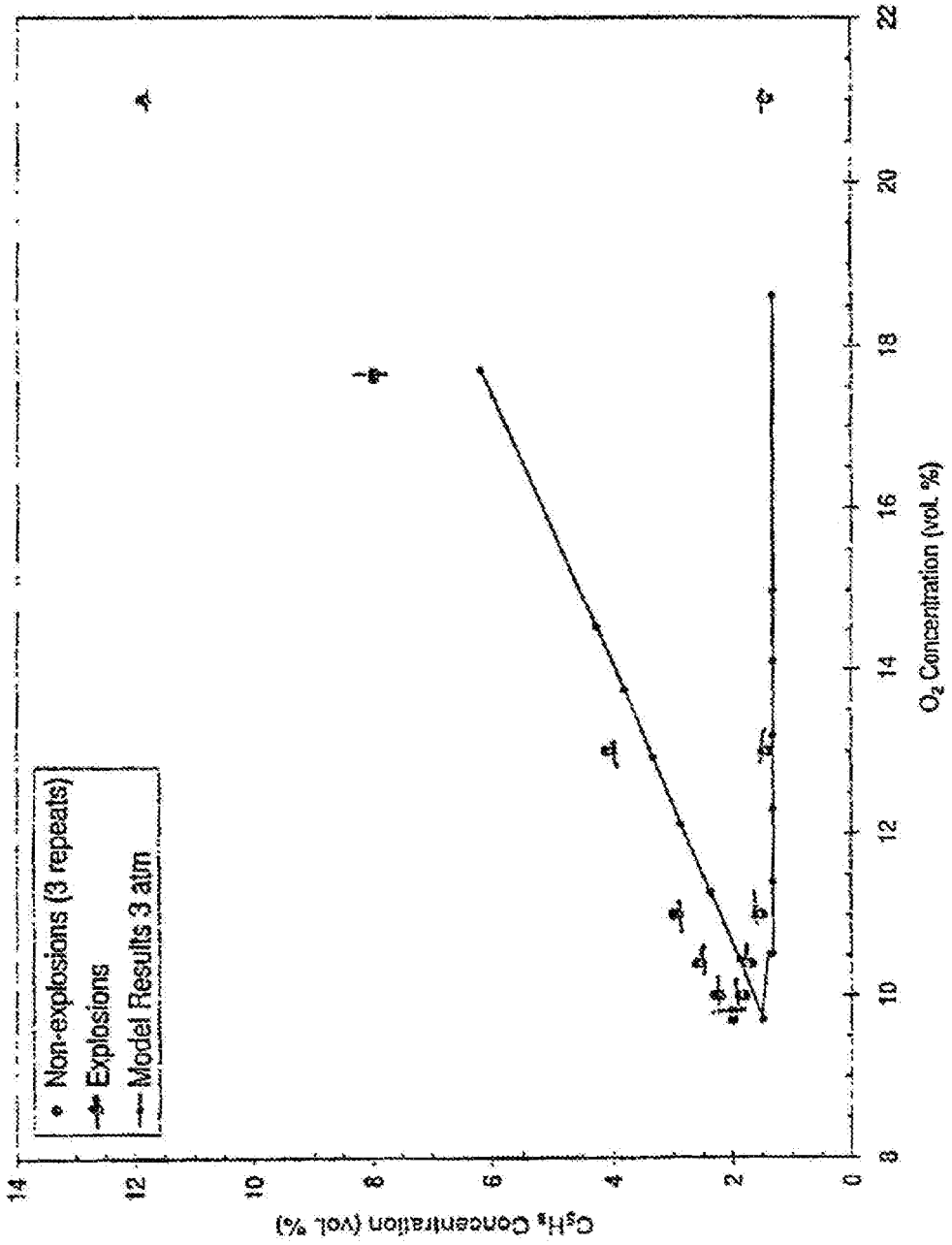

Non-explosions (3 repeats)
Explosions
Model Results 3 atm
C_3H_8 Concentration (vol. %)
O_2 Concentration (vol. %)
0
2
4
6
8
10
12
14
8
10
12
14
16
18
20
22

Figure 85

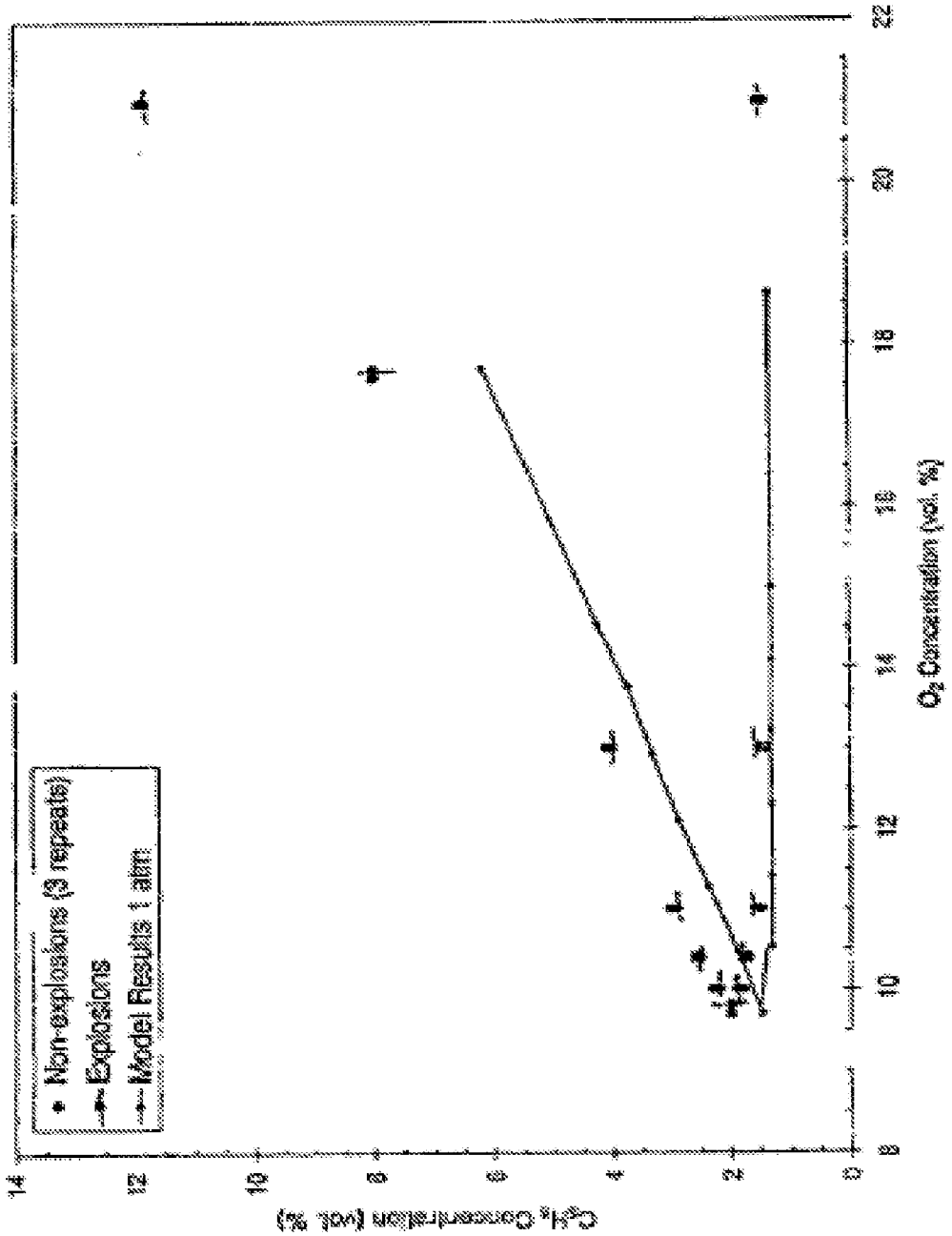

Non-explosions (3 repeats)
Explosions
Model Results 1 atm
C_2H_2 Concentration (vol. %)
O_2 Concentration (vol. %)
0
2
4
6
8
10
12
14
8
10
12
14
16
18
20
22

Figure 86A

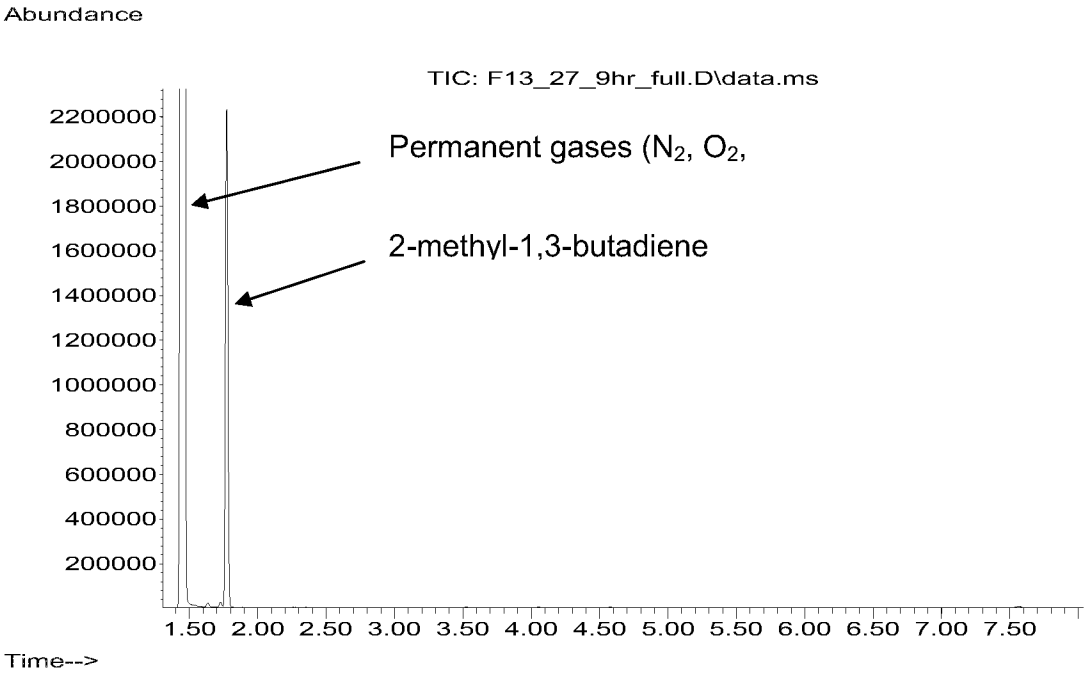

Abundance
TIC: F13_27_9hr_full.D\data.ms
2200000
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
Permanent gases (N_2, O_2,
2-methyl-1,3-butadiene
1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50 7.00 7.50
Time-->

Figure 86B

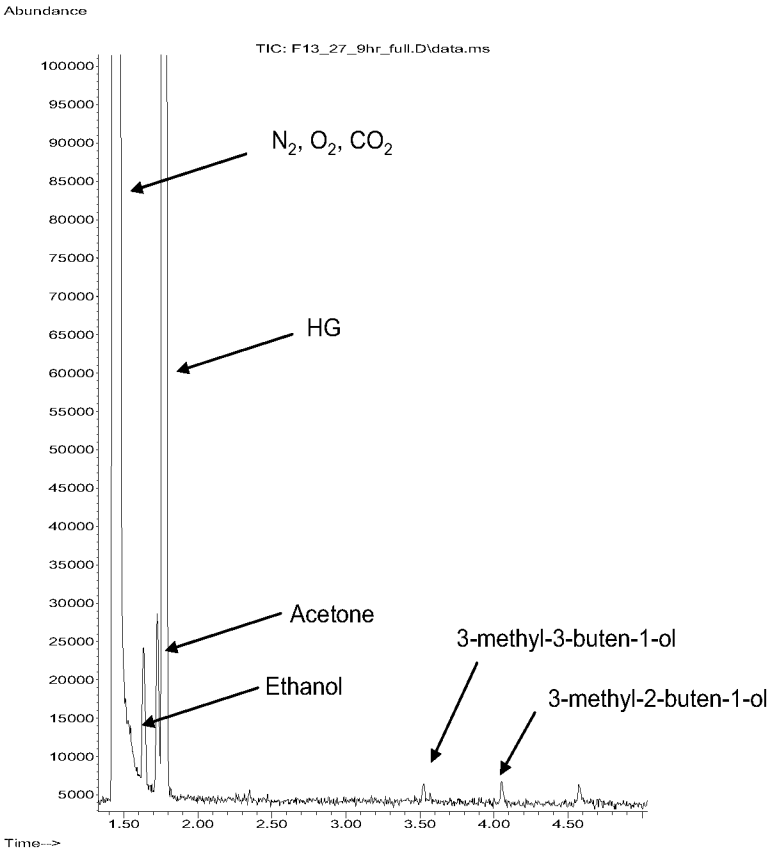

Abundance
TIC: F13_27_9hr_full.D\data.ms
100000
95000
90000
85000
80000
75000
70000
65000
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
1.50
2.00
2.50
3.00
3.50
4.00
4.50
Time-->
N2, O2, CO2
HG
Acetone
Ethanol
3-methyl-3-buten-1-ol
3-methyl-2-buten-1-ol

Figure 87A

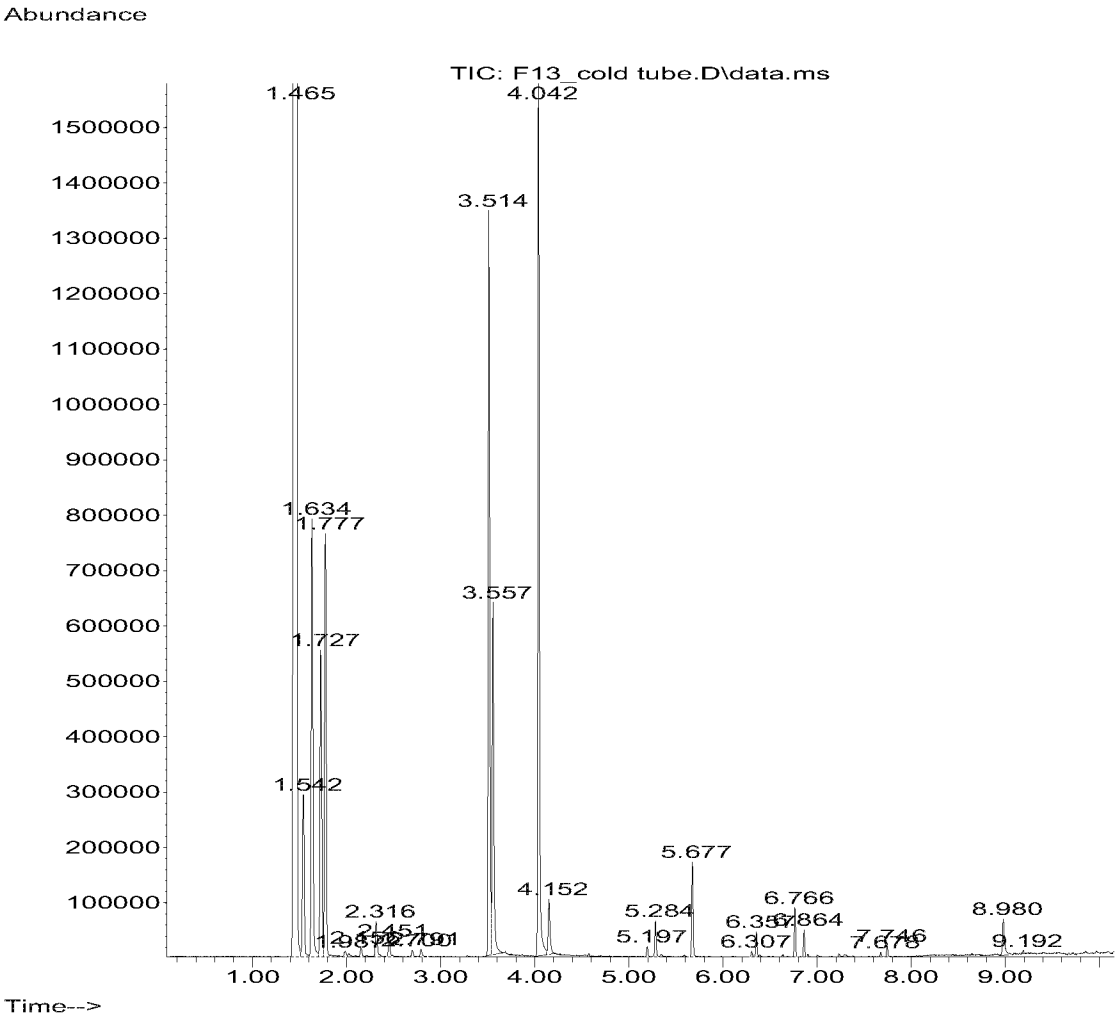

Abundance
TIC: F13_cold tube.D\data.ms
1.465
4.042
3.514
1.634
1.777
3.557
1.727
1.542
5.677
4.152
6.766
2.316
5.284
8.980
5.197
6.307
9.192
1500000
1400000
1300000
1200000
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Time-->

Figure 87B

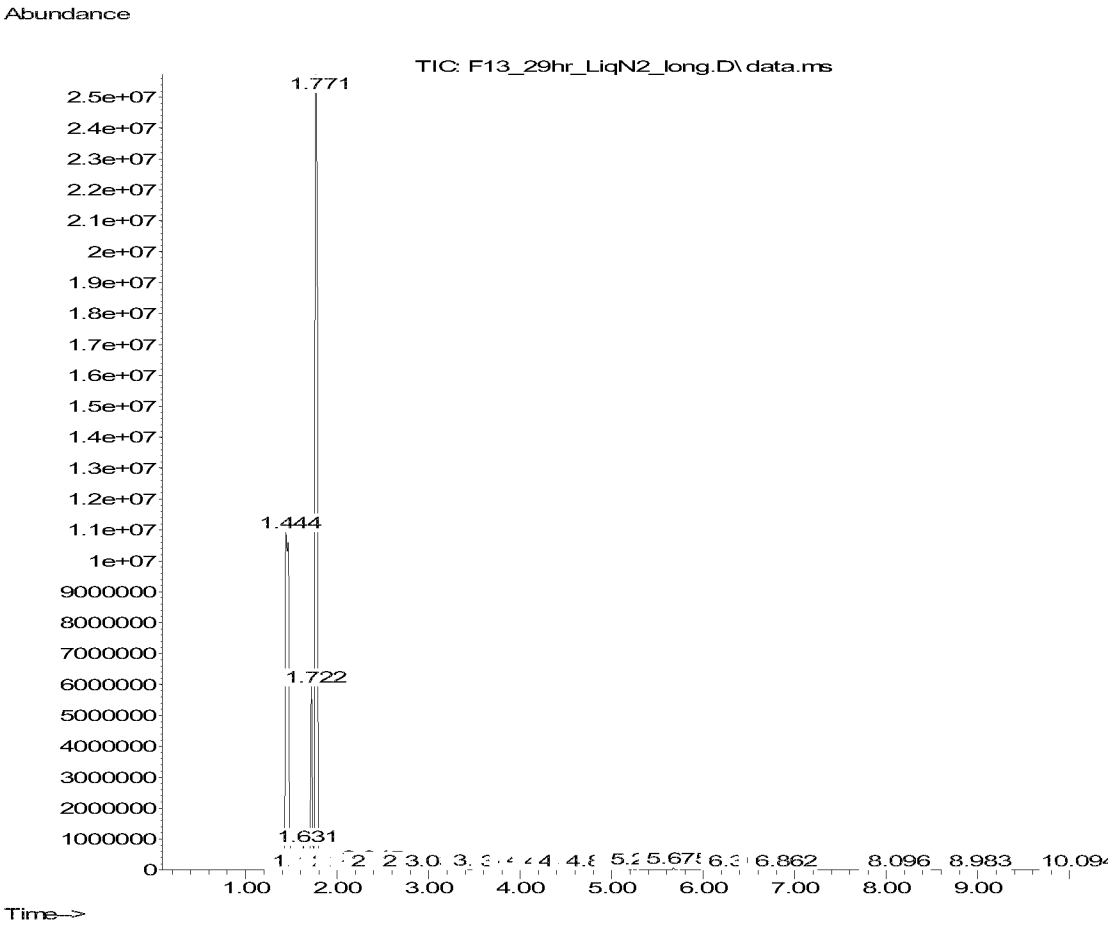

Abundance
TIC: F13_29hr_LiqN2_long.D\data.ms
1.771
1.444
1.722
1.631
2.5e+07
2.4e+07
2.3e+07
2.2e+07
2.1e+07
2e+07
1.9e+07
1.8e+07
1.7e+07
1.6e+07
1.5e+07
1.4e+07
1.3e+07
1.2e+07
1.1e+07
1e+07
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
6.862
8.096
8.983
Time-->

Figure 87C

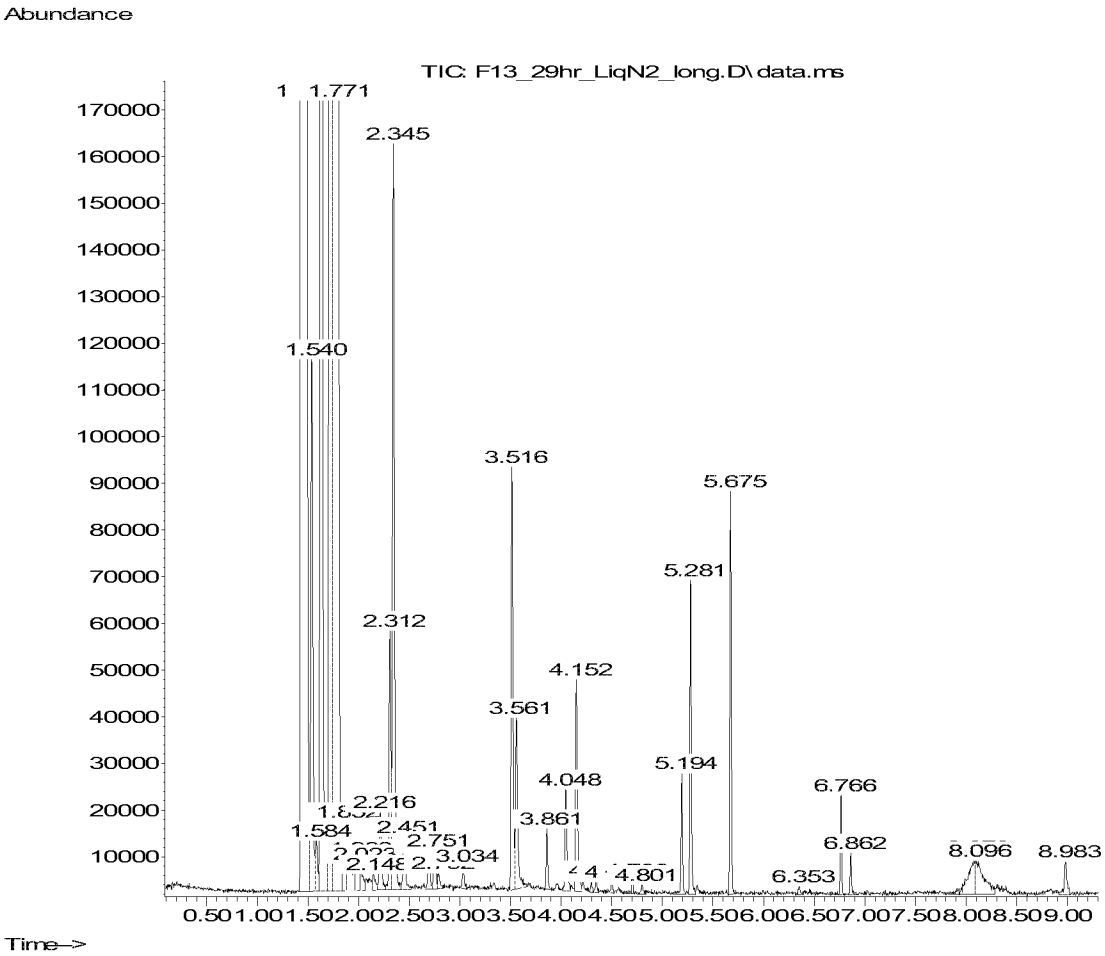

Abundance
TIC: F13_29hr_LiqN2_long.D\data.ms
1.771
2.345
1.540
3.516
5.675
5.281
2.312
4.152
3.561
5.194
4.048
6.766
2.216
3.861
6.862
8.096
8.983
1.584
2.451
2.751
3.034
6.353
4.801
Time-->

Figure 87D

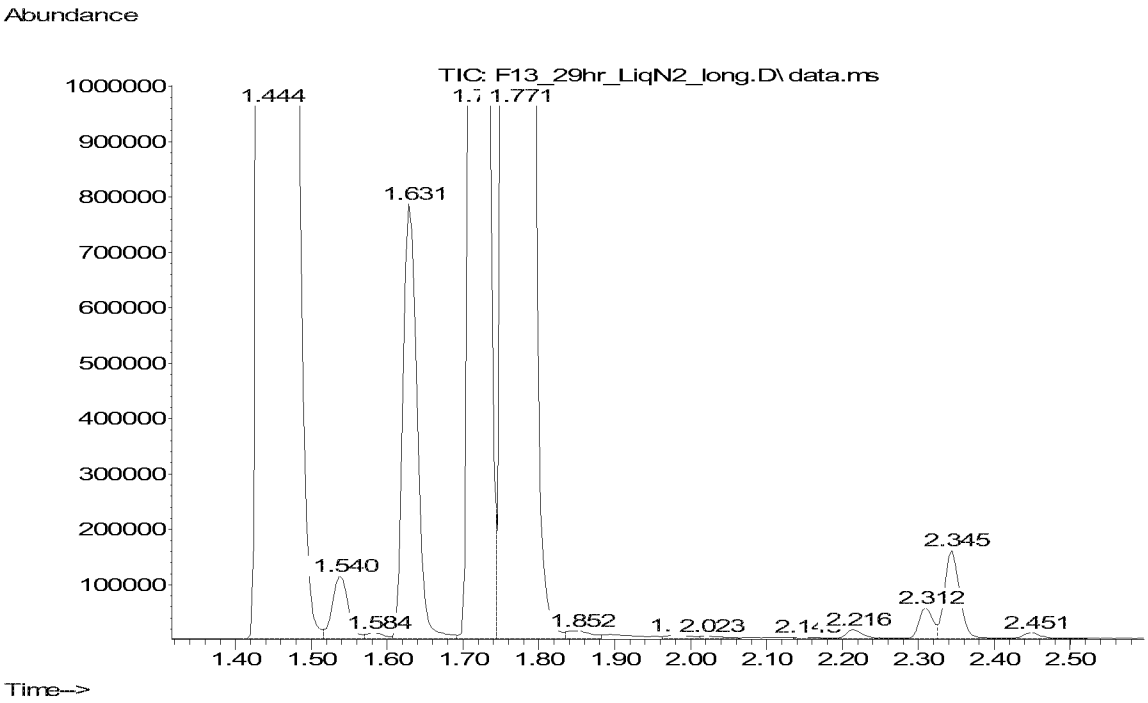

Abundance
TIC: F13_29hr_LiqN2_long.D\data.ms
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
1.444
1.631
1.771
1.540
1.584
1.852
2.345
2.312
2.216
2.451
1.40
1.50
1.60
1.70
1.80
1.90
2.00
2.10
2.20
2.30
2.40
2.50
Time-->

Figure 88A

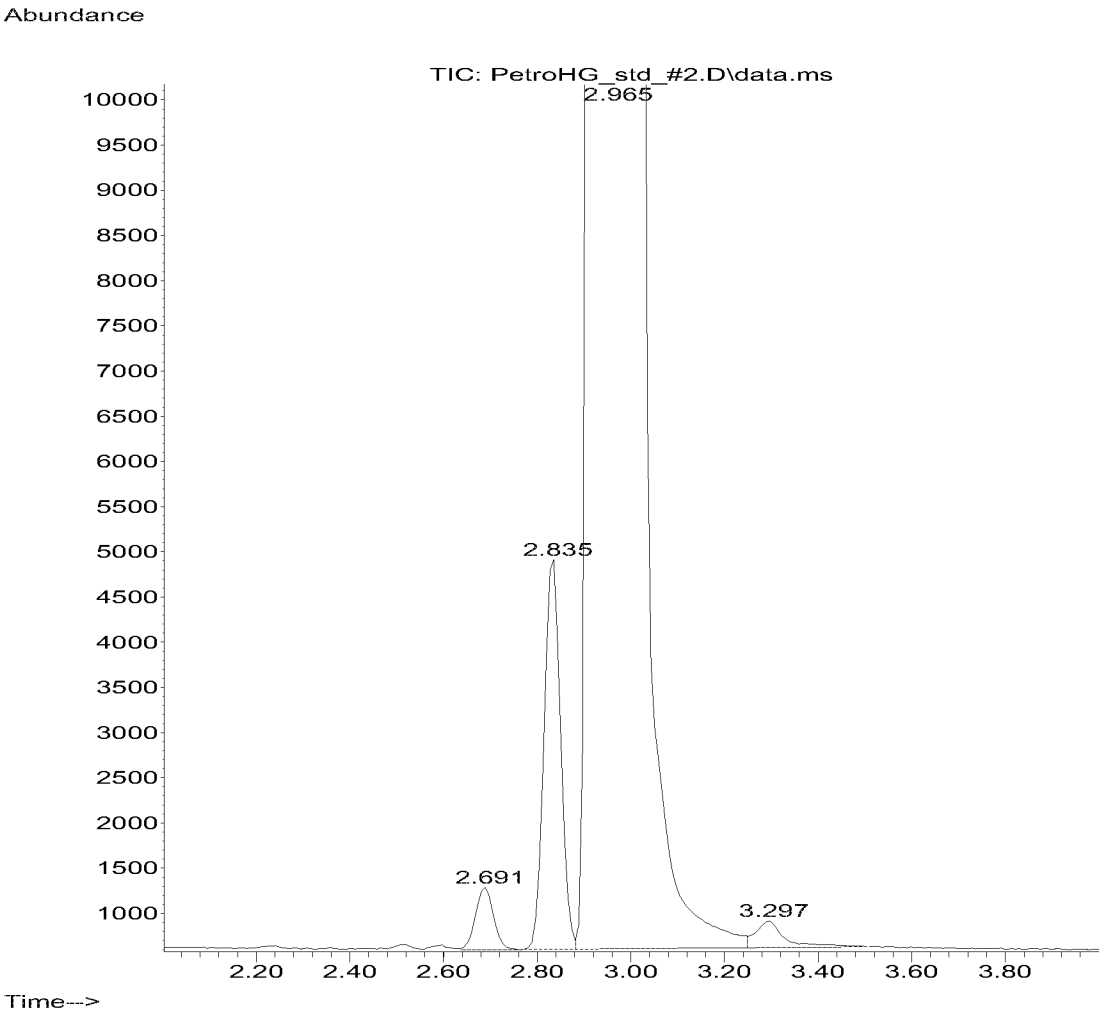

Abundance
TIC: PetroHG_std_#2.D\data.ms
2.965
2.835
2.691
3.297
10000
9500
9000
8500
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
2.20
2.40
2.60
2.80
3.00
3.20
3.40
3.60
3.80
Time--->

Figure 88B

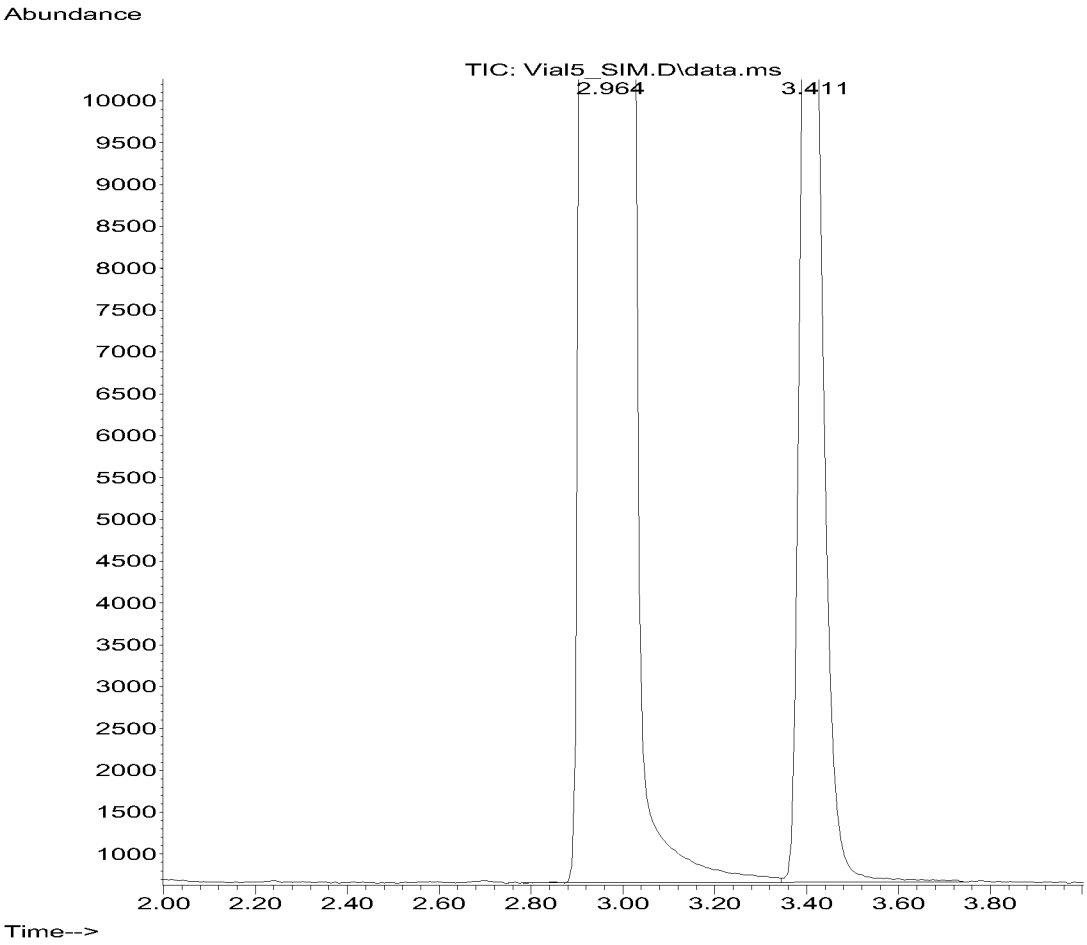

Abundance
TIC: Vial5_SIM.D\data.ms
2.964
3.411
10000
9500
9000
8500
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
2.00
2.20
2.40
2.60
2.80
3.00
3.20
3.40
3.60
3.80
Time-->

Figure 89

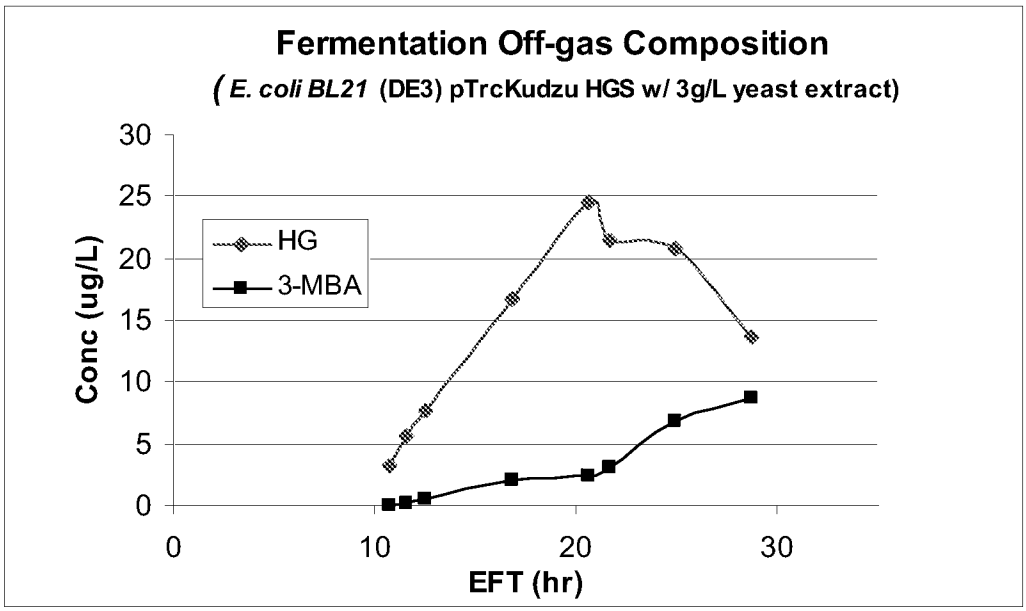
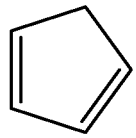
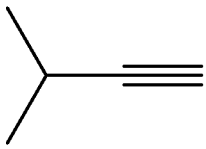

Fermentation Off-gas Composition
(E. coli BL21 (DE3) pTrcKudzu HGS w/ 3g/L yeast extract)
Conc (ug/L)
EFT (hr)
HG
3-MBA
0
5
10
15
20
25
30
0
10
20
30

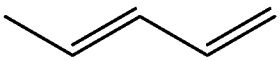
Figure 90



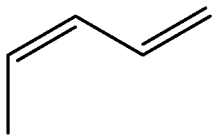
cyclopentadiene



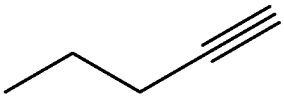
"isopryne" = 3-Me-1-butyne



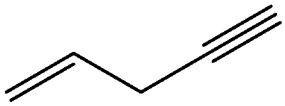
trans-piperylene



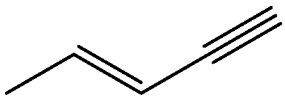
cis-piperylene



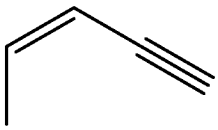
1-pentyne



pent-4-ene-1-yne



trans-pent-3-ene-1-yne



cis-pent-3-ene-1-yne

Figure 91

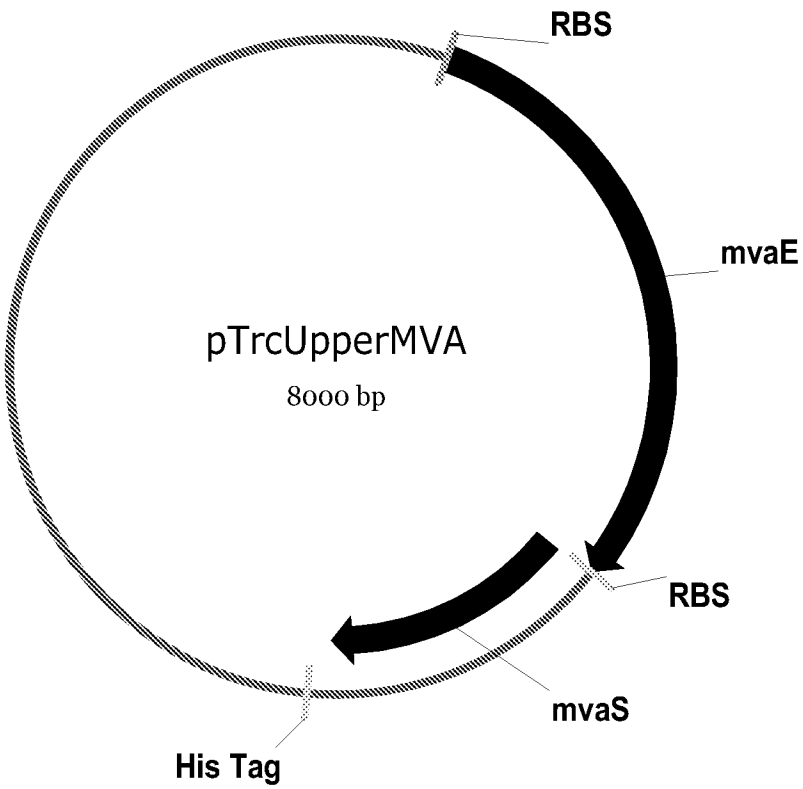


Figure 92A

1-
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agcgccgctgagaaaaagcgaagcggcactgctctttaacaatttatcagacaatctgtgtgggcactcgaccggaattatcgat
taacttattattaaaaattaaagaggtatatattaatgtatcgattaaataaggaggaataaaccatggatcgagctcaggaggta
aaaaaacatgaaaacagtagttattattgatgcattacgaacaccaattggaaaatataaaggcagcttaagtcaagtaagtgccg
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tgaggtctgcggatcaggaatgaaggccgttatttttggcgaaacaattgattcaattaggagaagcggaagttttaattgctggcg
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Figure 92B

tagctggttatcaaaatcatttacaaaaagaaactcatttagcactgctggataatcggacagaactttctatcgctgaatatgaagc
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cttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatct
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cctccagtctggccctgcacgcgccgtcgcaaattgtcgcggcgattaaatctcgcgccgatcaactgggtgccagcgtggtgg
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aatcgcgctgttagcgggcccattaagttctgtctcggcgcgtctgcgtctggctggctggcataaatatctcactcgcaatcaaat
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Figure 92C

ttcccactgcgatgctggttgccaacgatcagatggcgctgggcgcaatgcgcgccattaccgagtccgggctgcgcgttggtg
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SEQ ID NO:23

Figure 93

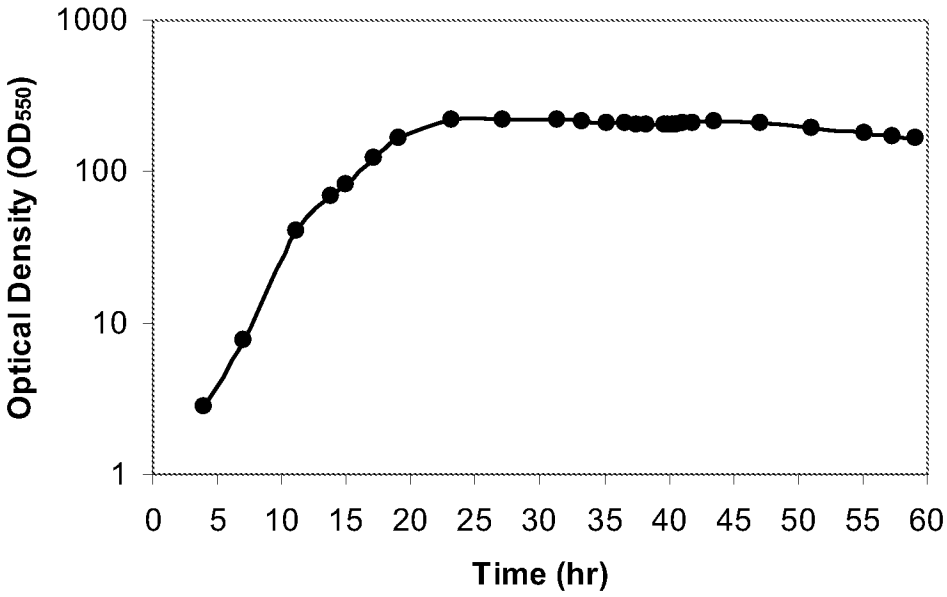
1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 94

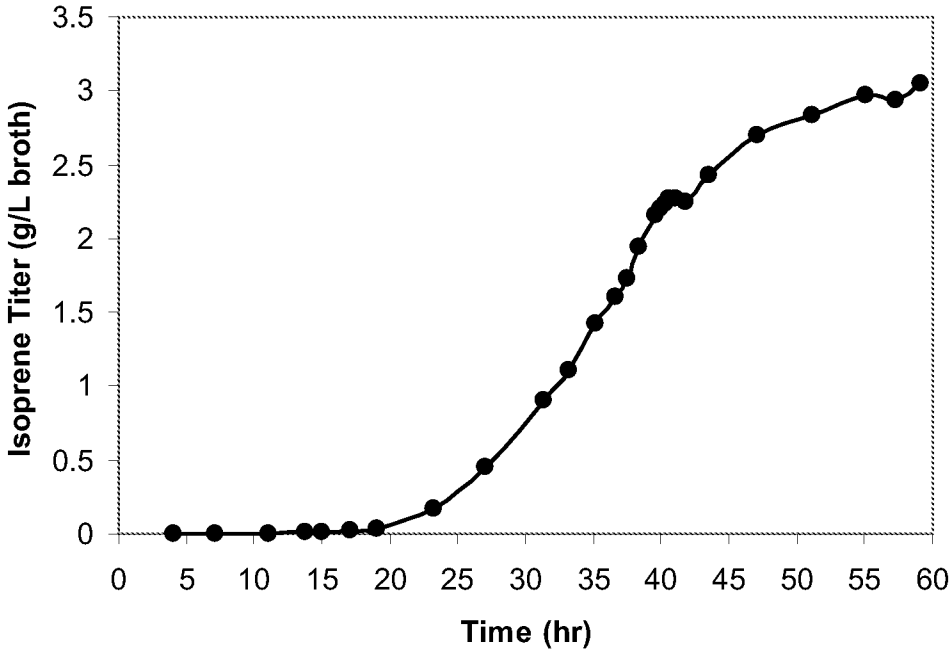
Isoprene Titer (g/L broth)
3.5
3
2.5
2
1.5
1
0.5
0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (hr)

Figure 95

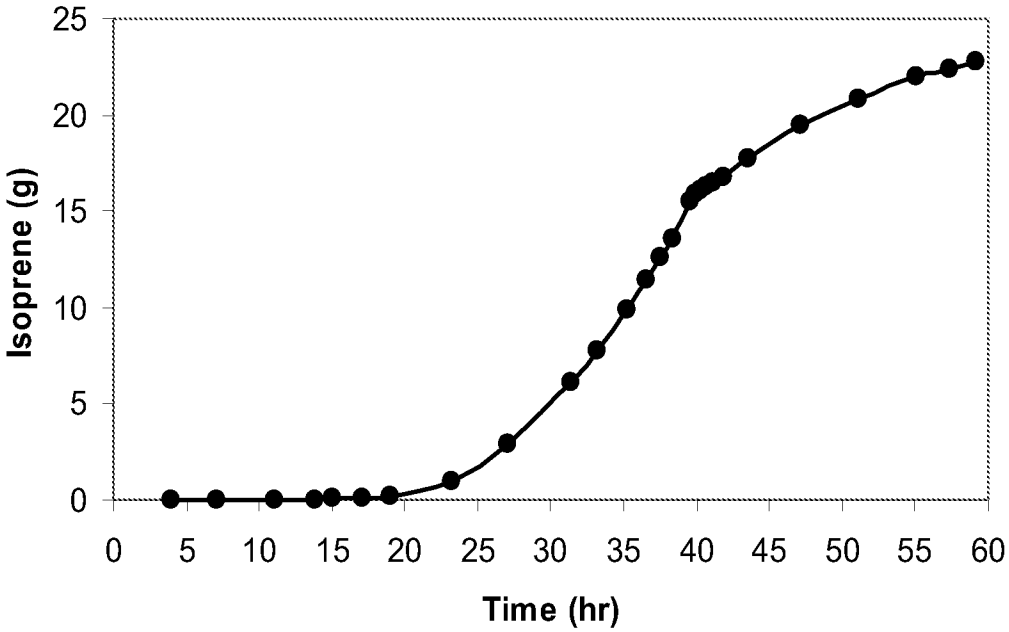

Figure 96

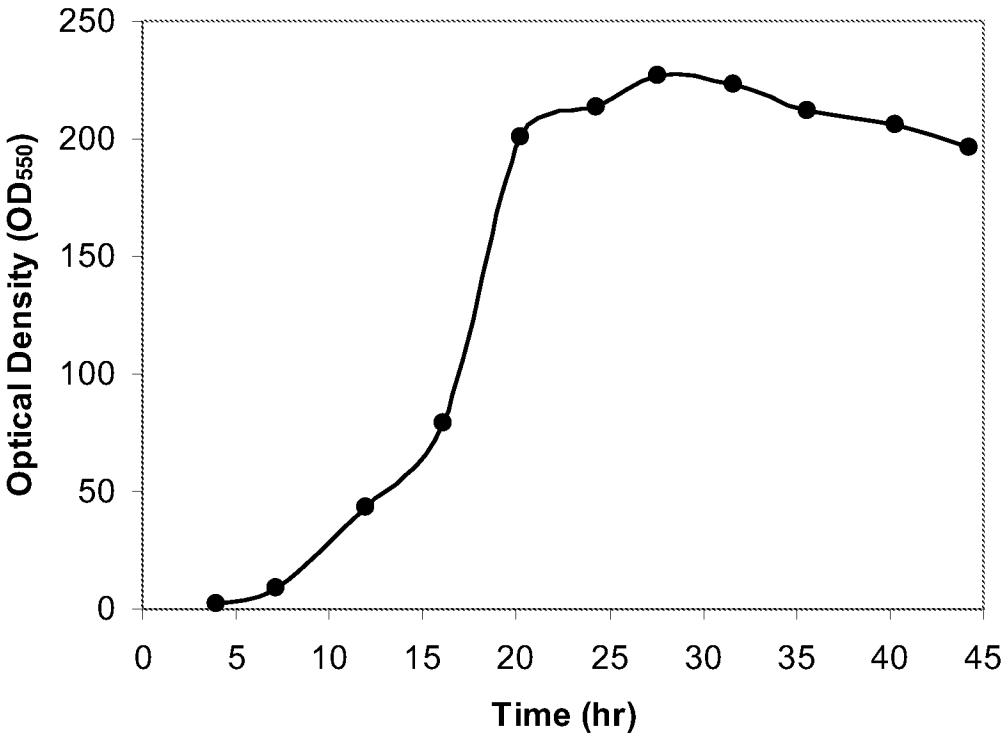
250
200
150
100
50
0
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
Time (hr)

Figure 97

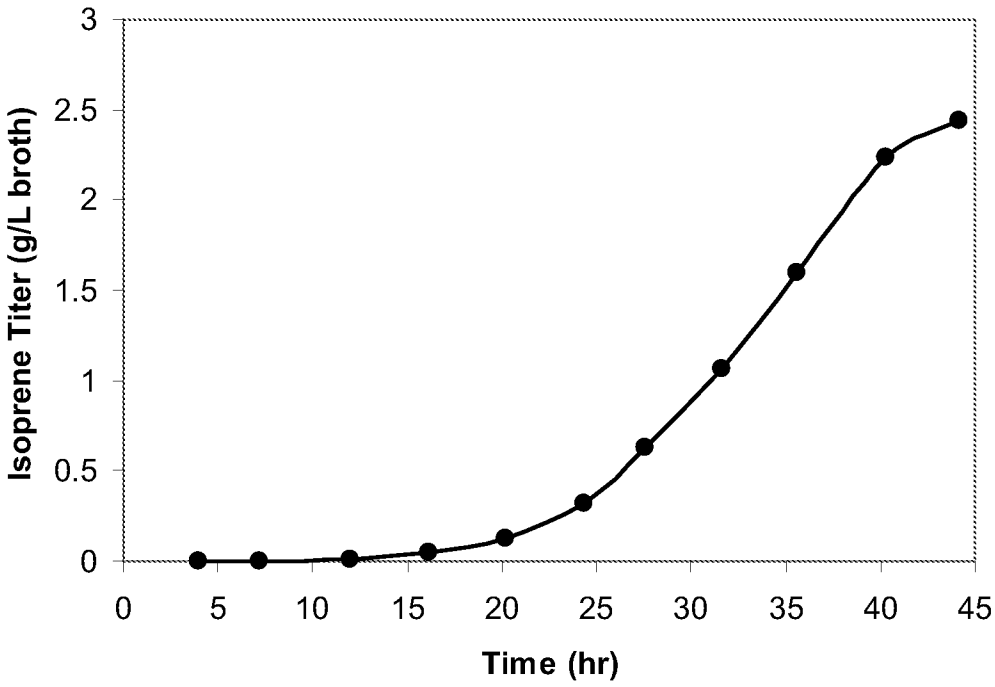
Isoprene Titer (g/L broth)
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40
45
Time (hr)

Figure 98

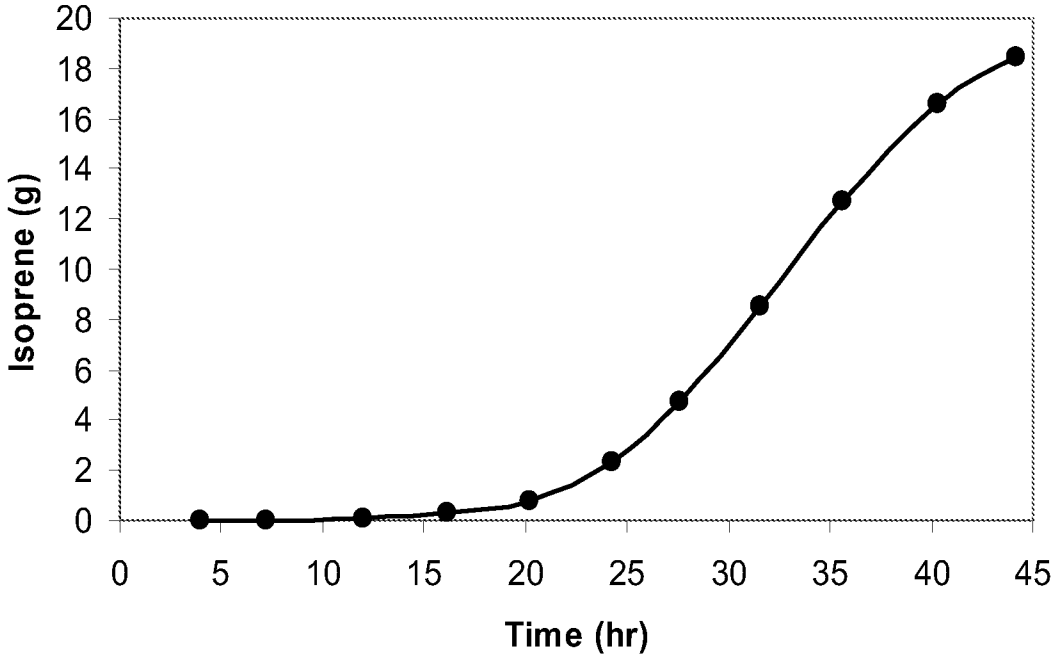
Isoprene (g)
Time (hr)
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
25
30
35
40
45

Figure 99

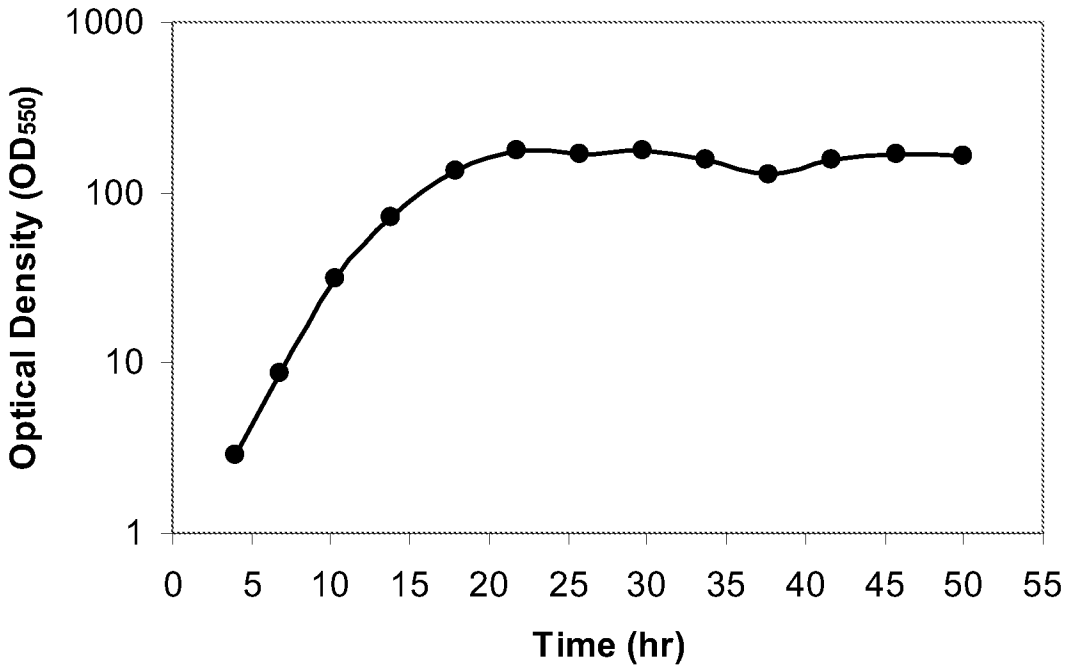
1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 100

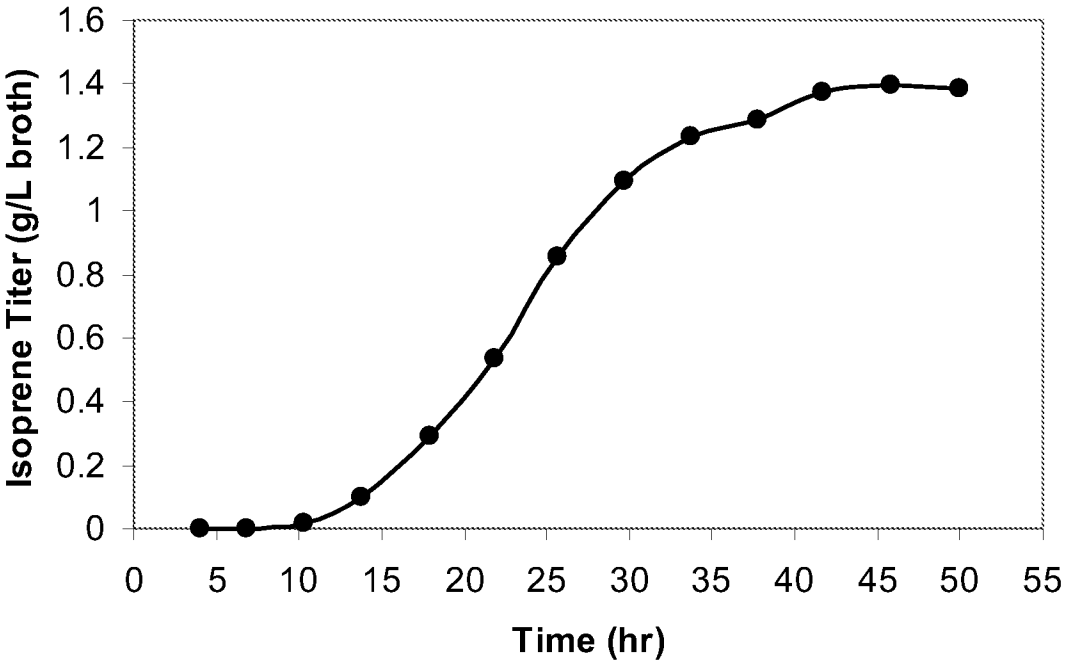
Isoprene Titer (g/L broth)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (hr)

Figure 101

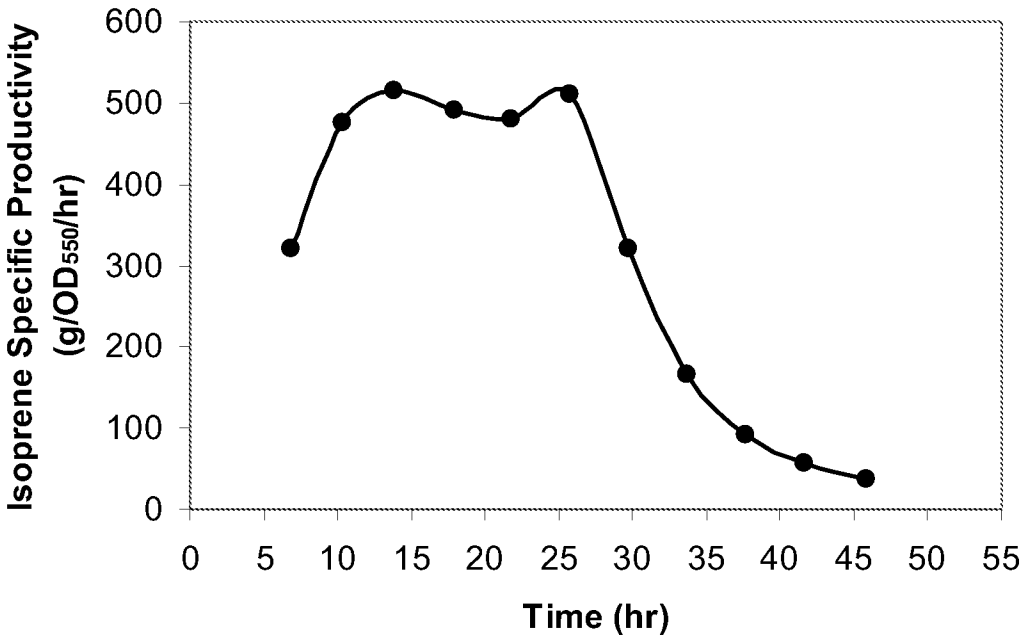
Isoprene Specific Productivity (g/OD550/hr)
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40 45 50 55
Time (hr)

Figure 102

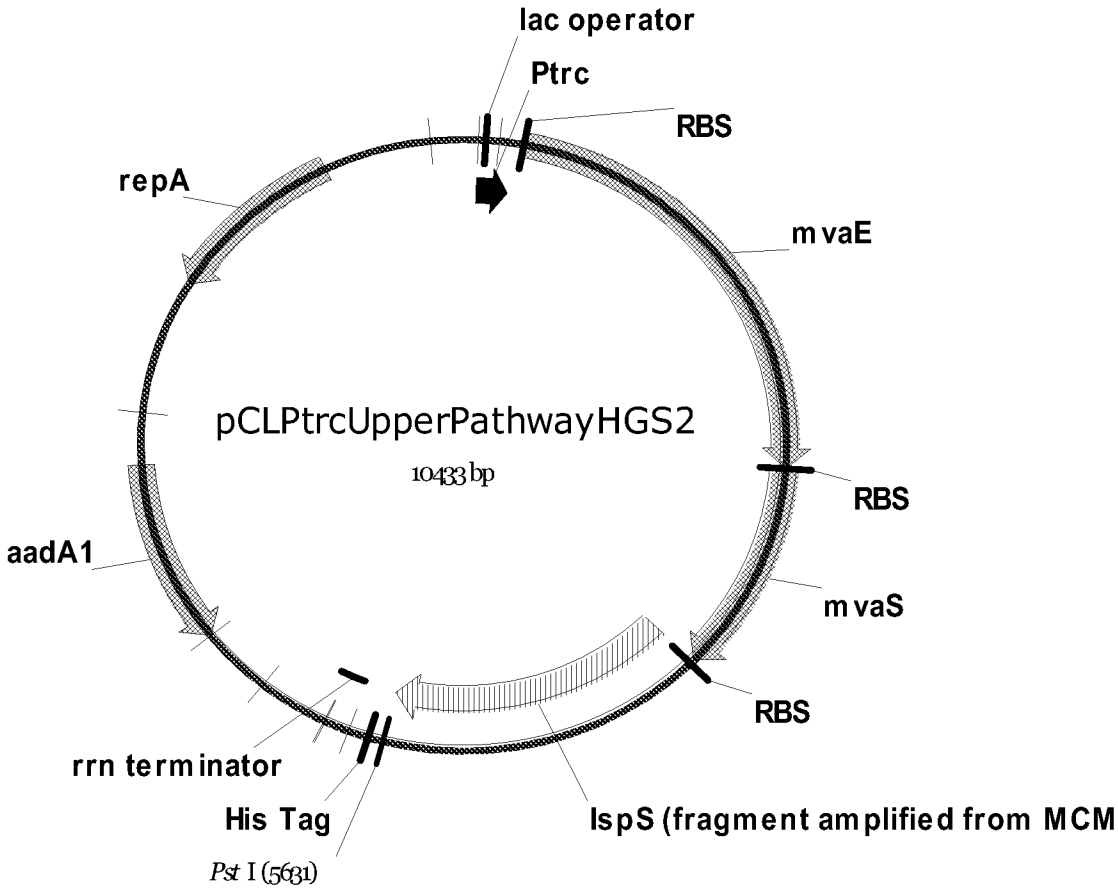
lac operator
Ptrc
RBS
repA
mvaE
pCLPtrcUpperPathwayHGS2
10433 bp
RBS
aadA1
mvaS
RBS
rrn terminator
His Tag
IspS (fragment amplified from MCM
Pst I (5631)

Figure 103A

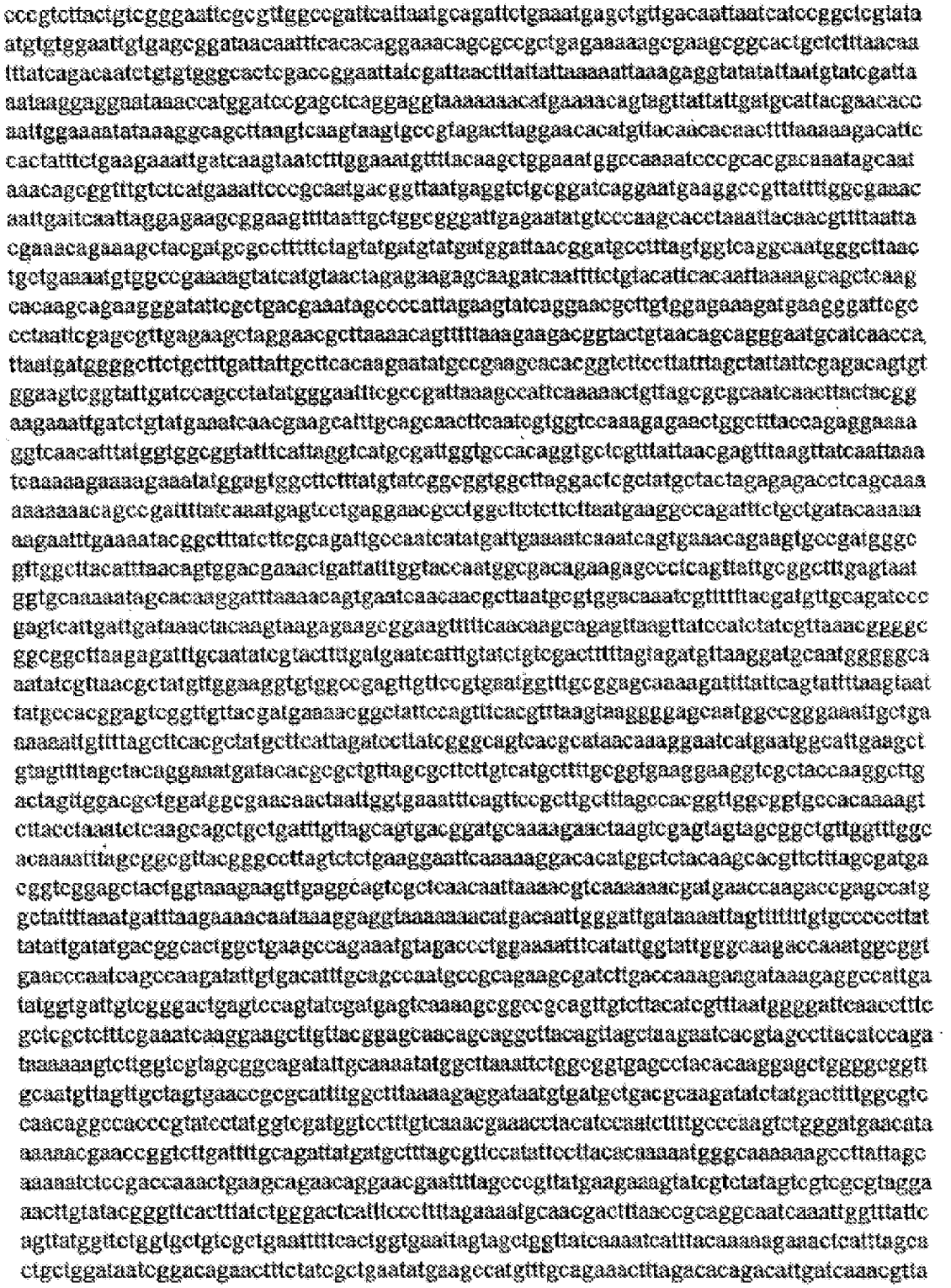

Figure 103B

Figure 103C

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SEQ ID NO:24

Figure 104

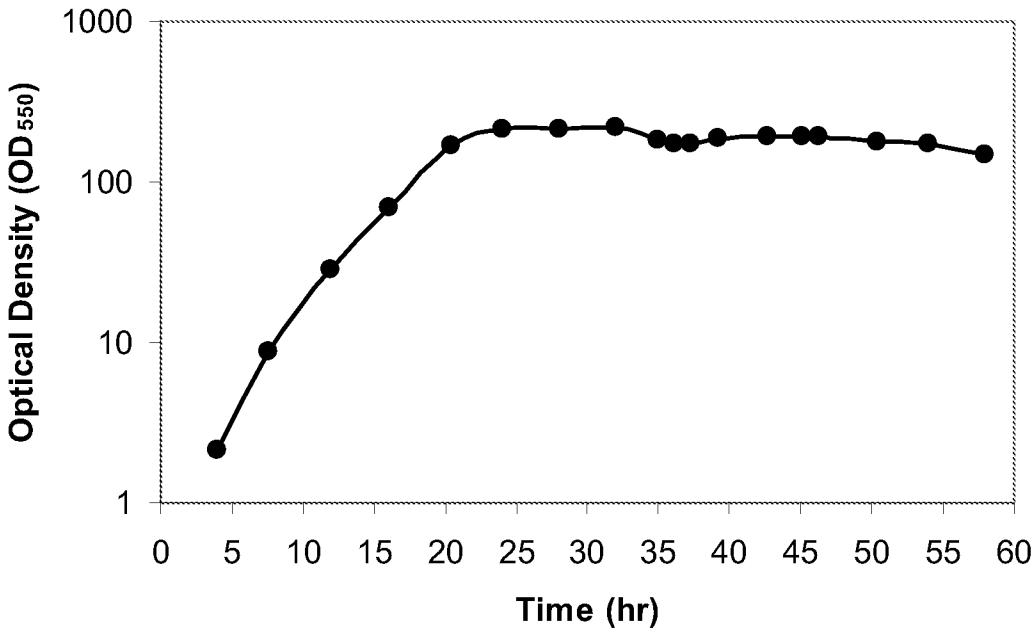
1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 105

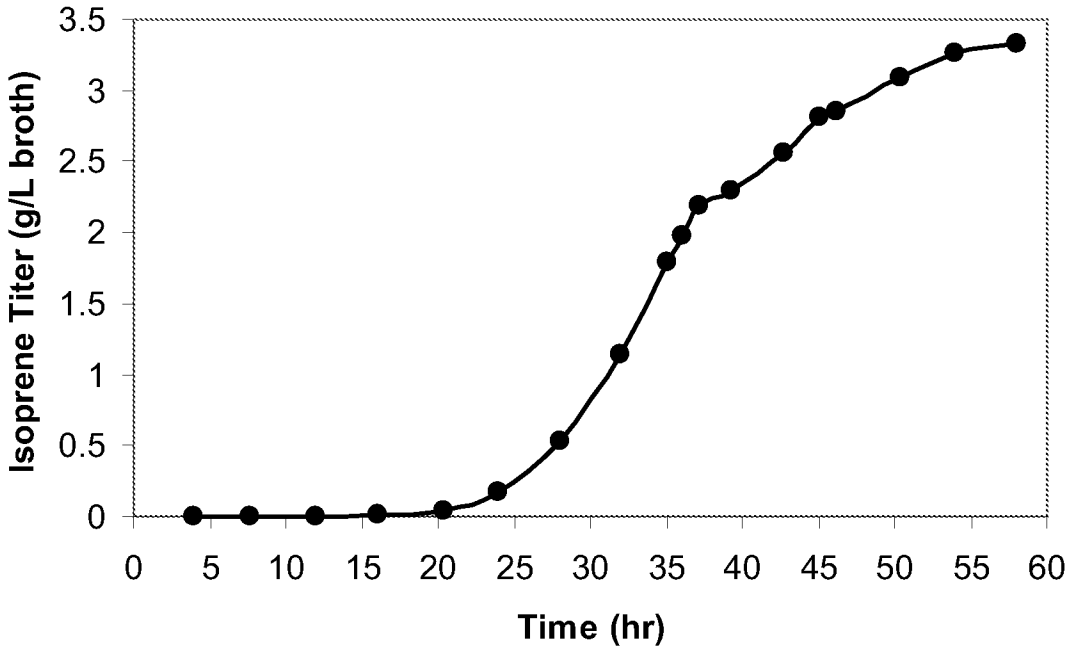
Isoprene Titer (g/L broth)
3.5
3
2.5
2
1.5
1
0.5
0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (hr)

Figure 106

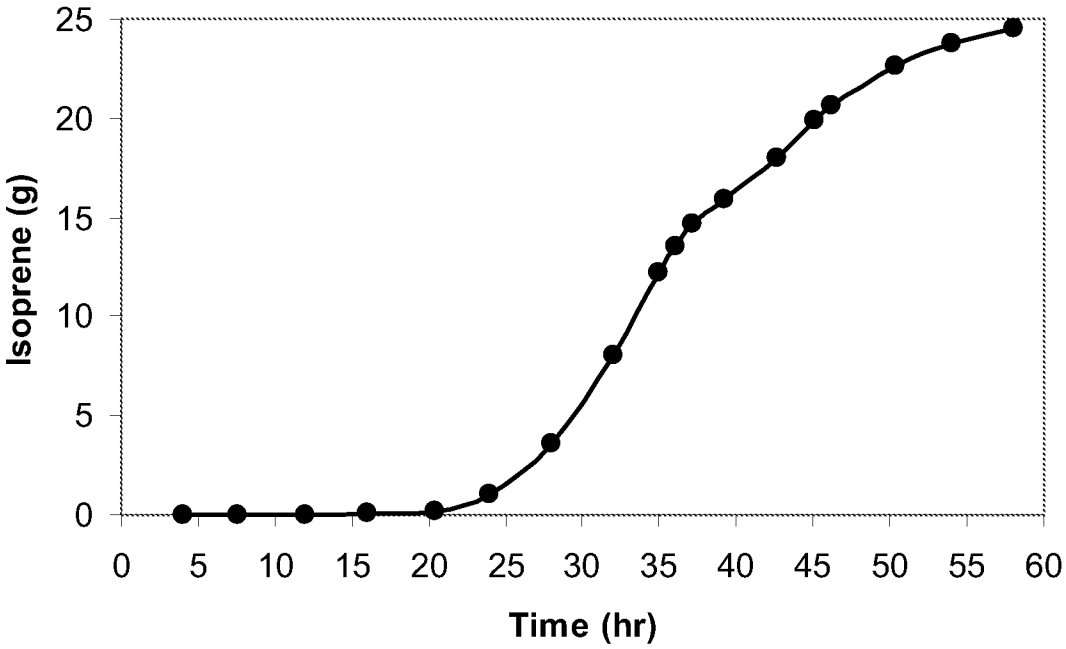

Figure 107

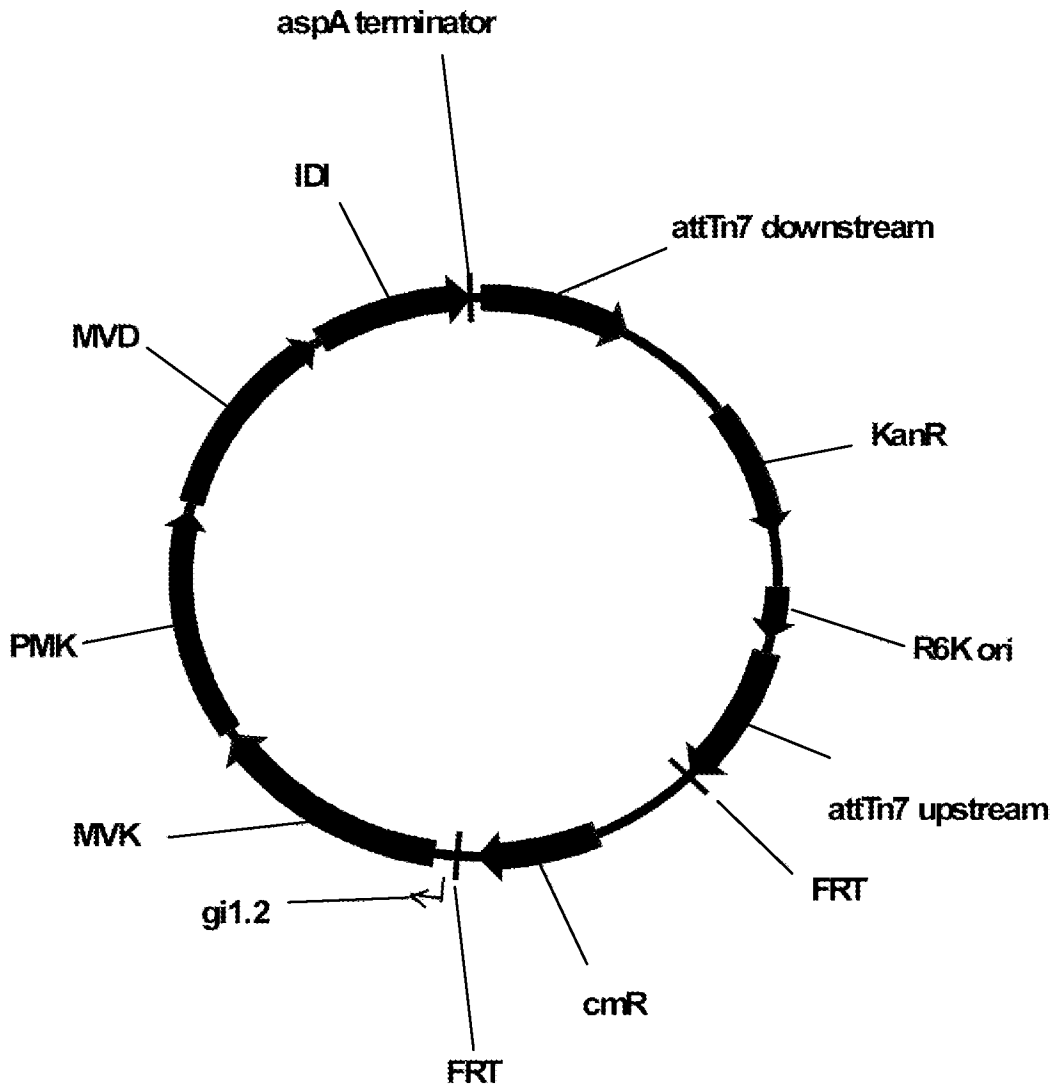
aspA terminator
IDI
attTn7 downstream
MVD
KanR
PMK
R6K ori
attTn7 upstream
MVK
FRT
gi1.2
cmR
FRT
MCM330 - FRT-cm-FRT-gi1.2-KKDyI at attTn7
10356 bp

Figure 108A

1
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Figure 108B

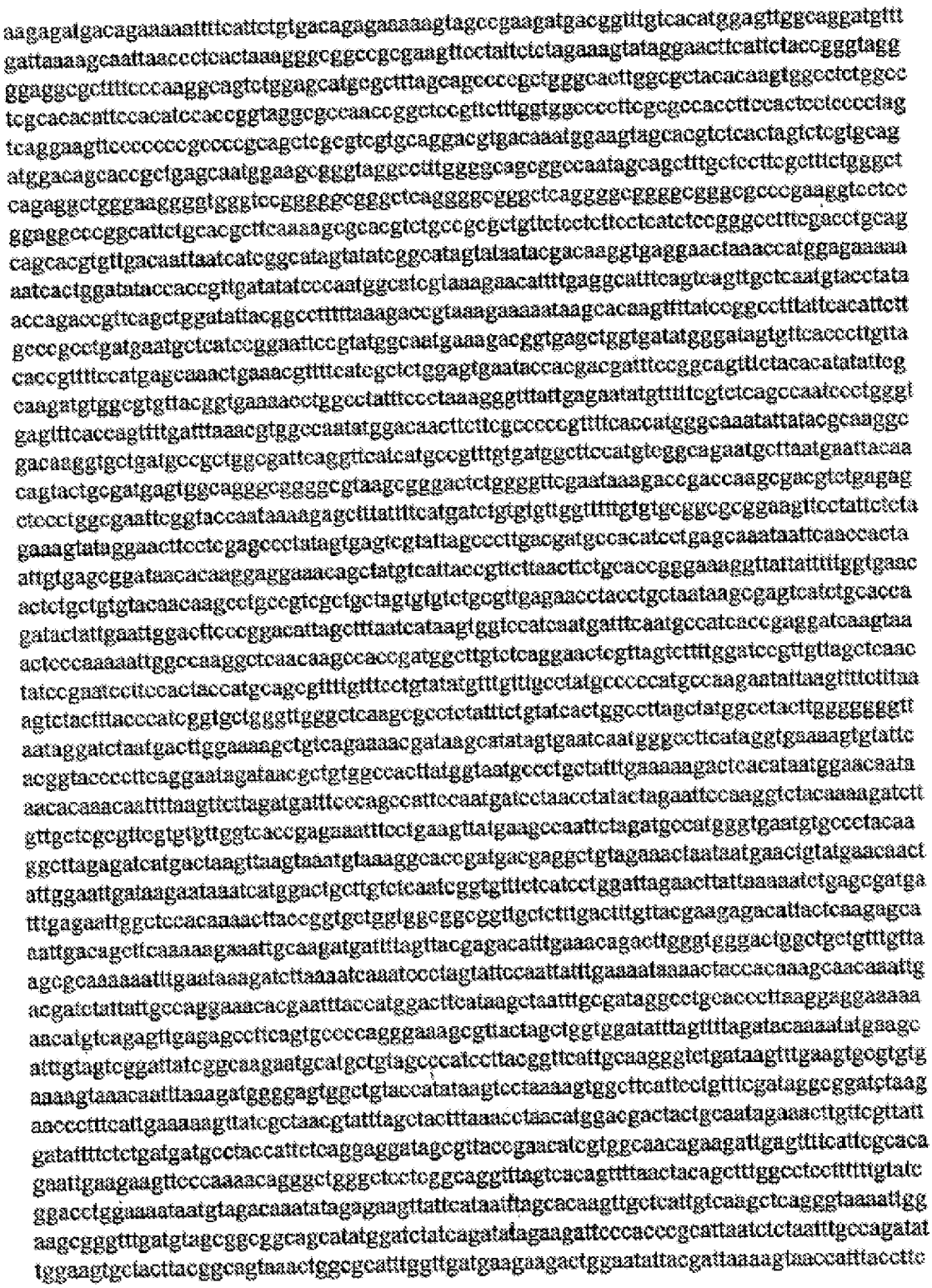
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Figure 108C

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Figure 109

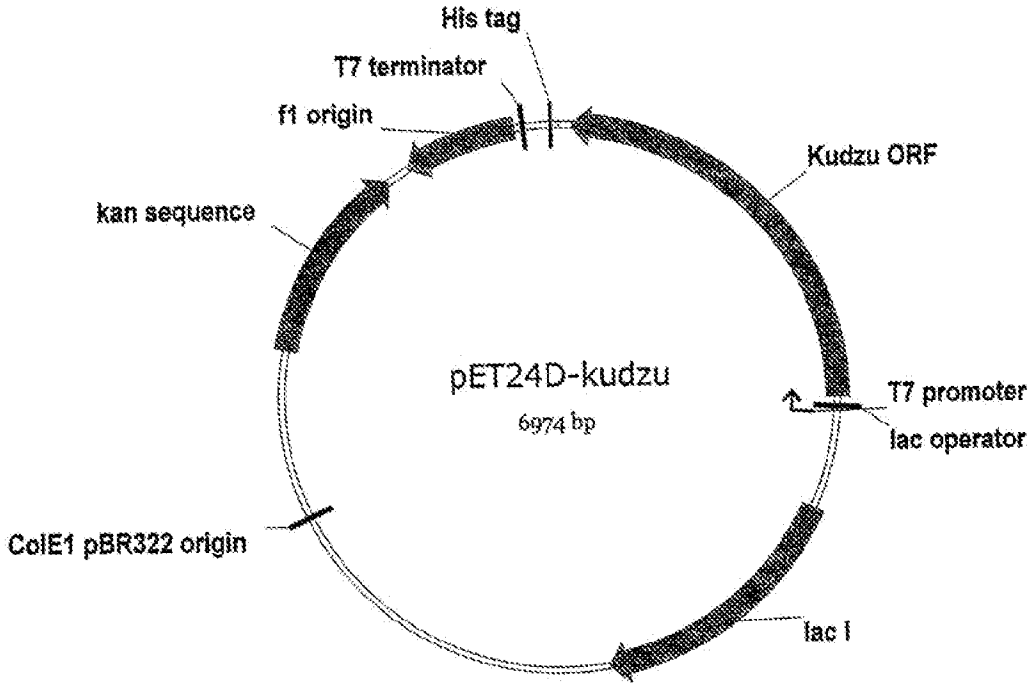
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Figure 110A

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Figure 110B

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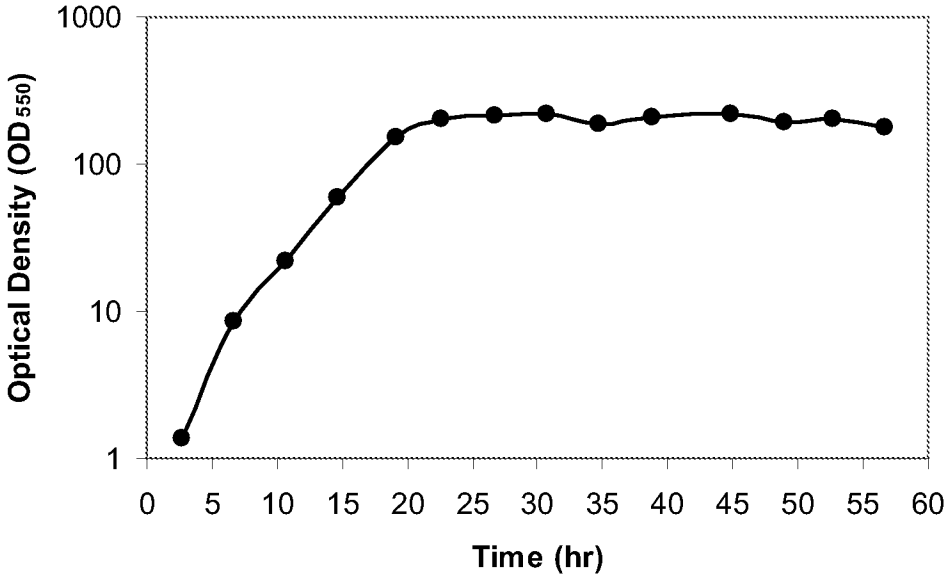
Figure 111A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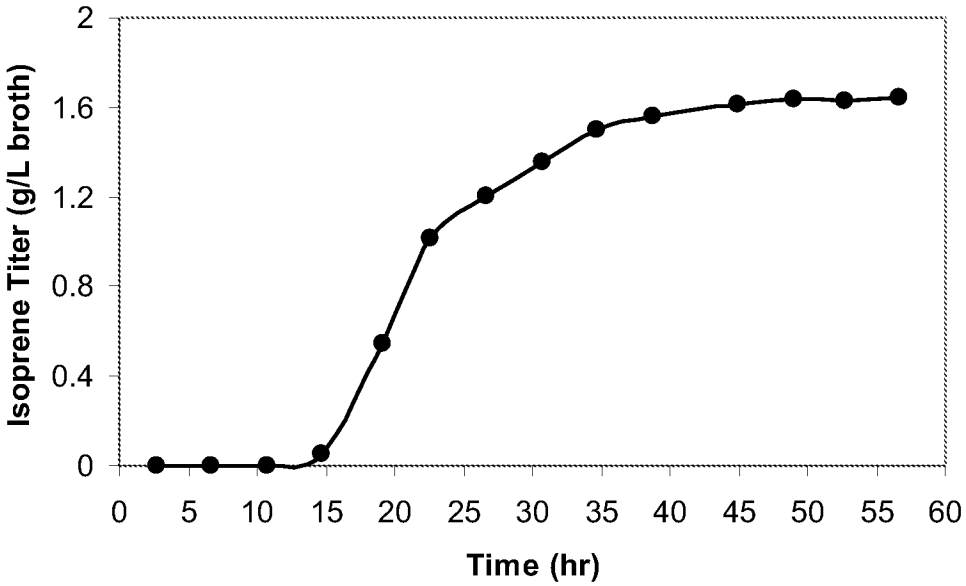
50
55
60
Time (hr)

Figure 111B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1.2
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0.4
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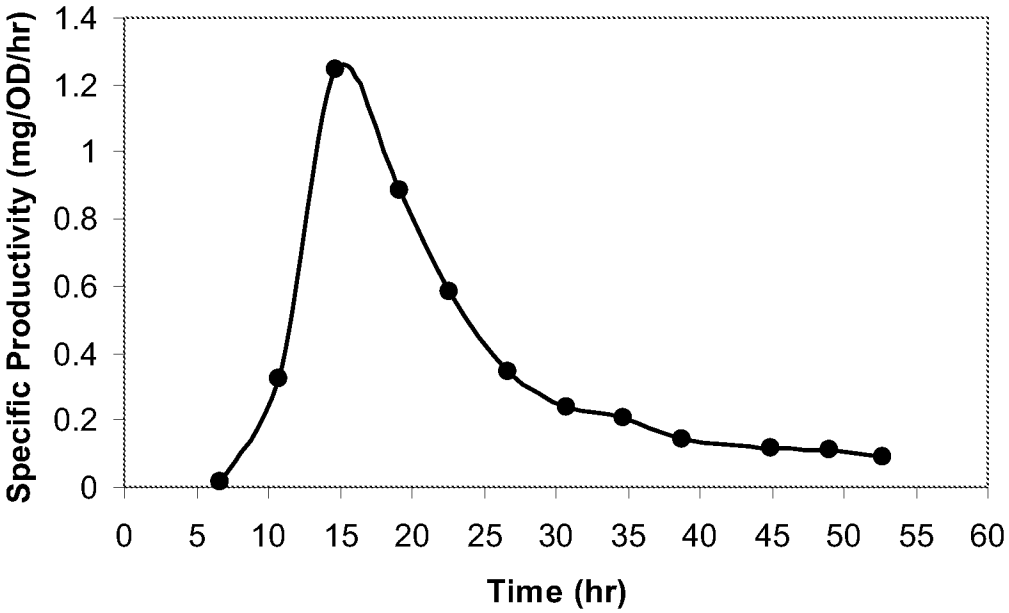
Time (hr)

Figure 111C


Specific Productivity (mg/OD/hr)
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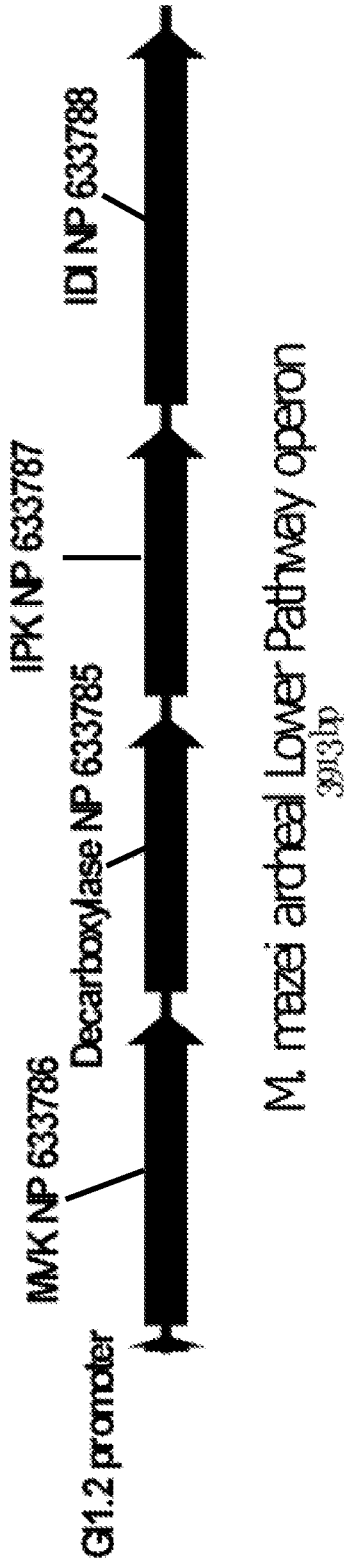
Time (hr)

Figure 112A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Decarboxylase NP 633785
IPK NP 633787
IDI NP 633788
M. mazei archeal Lower Pathway operon
3913bp

Figure 112B

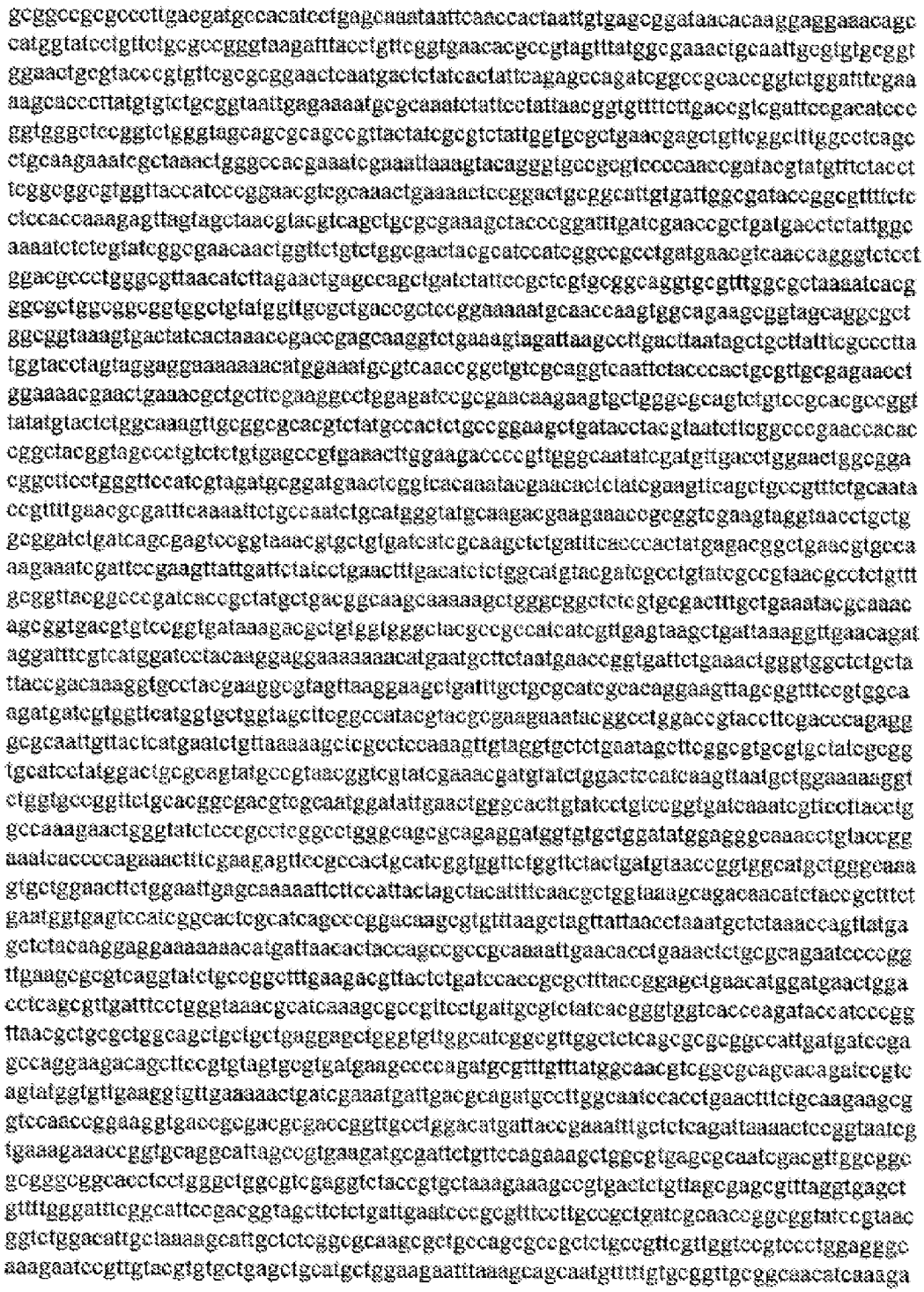

Figure 112C

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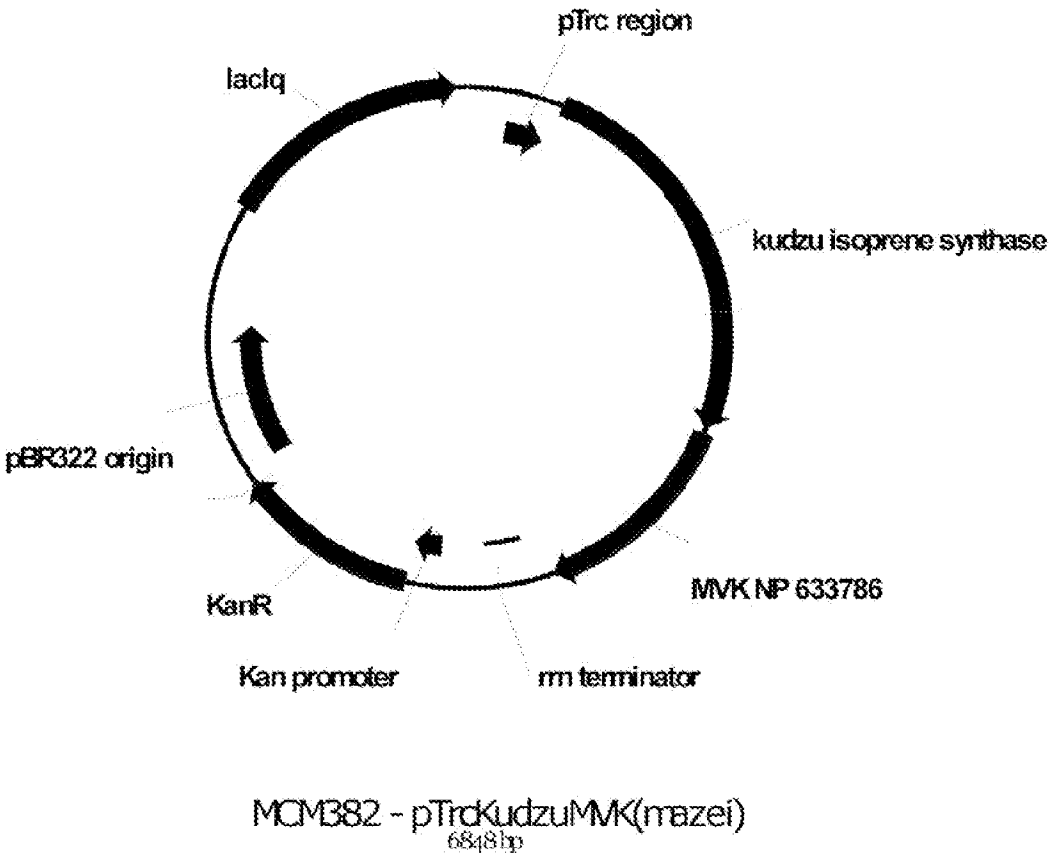
Figure 113A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MVK NP 633786
KanR
Kan promoter
rrn terminator
MCM382 - pTrcKudzuMVK(mazei)
6848 bp

Figure 113B

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Figure 113C

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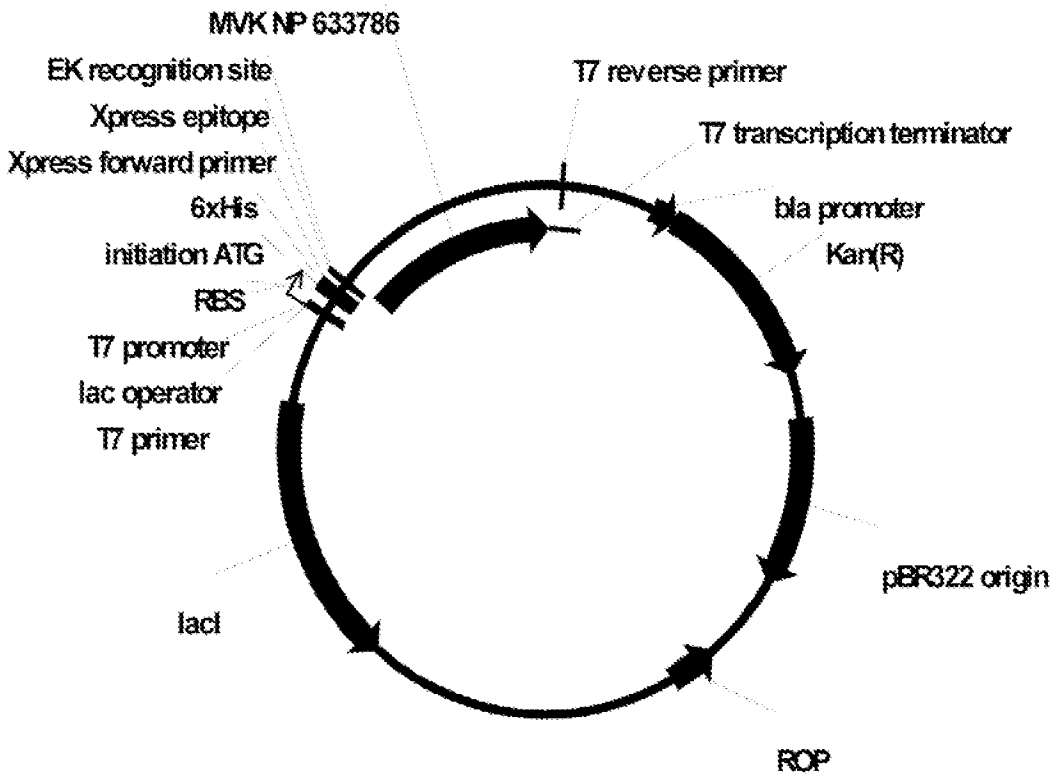
Figure 114A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bla promoter
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MCM376 - MVK from M. mazei archeal Lowerin pET200D
6647 bp

Figure 114B

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Figure 114C

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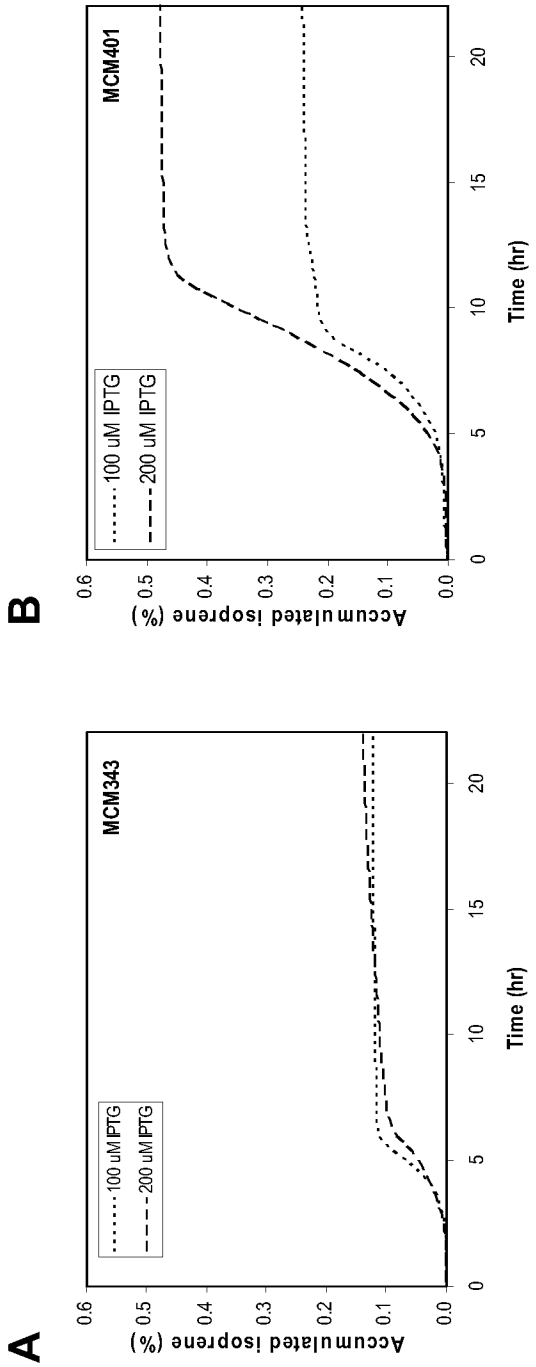

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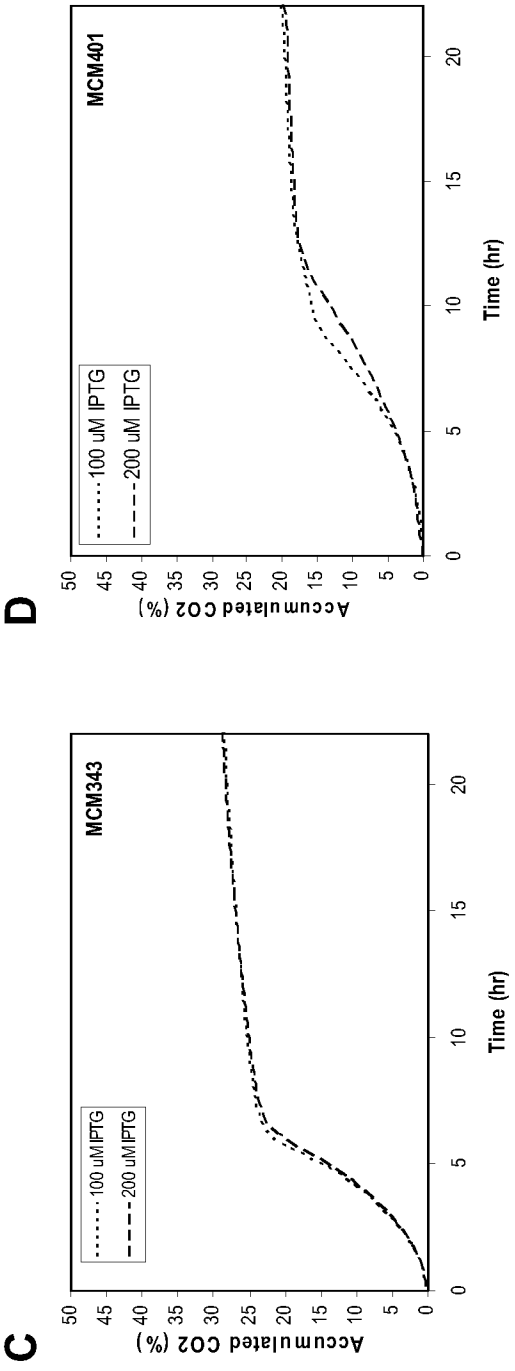
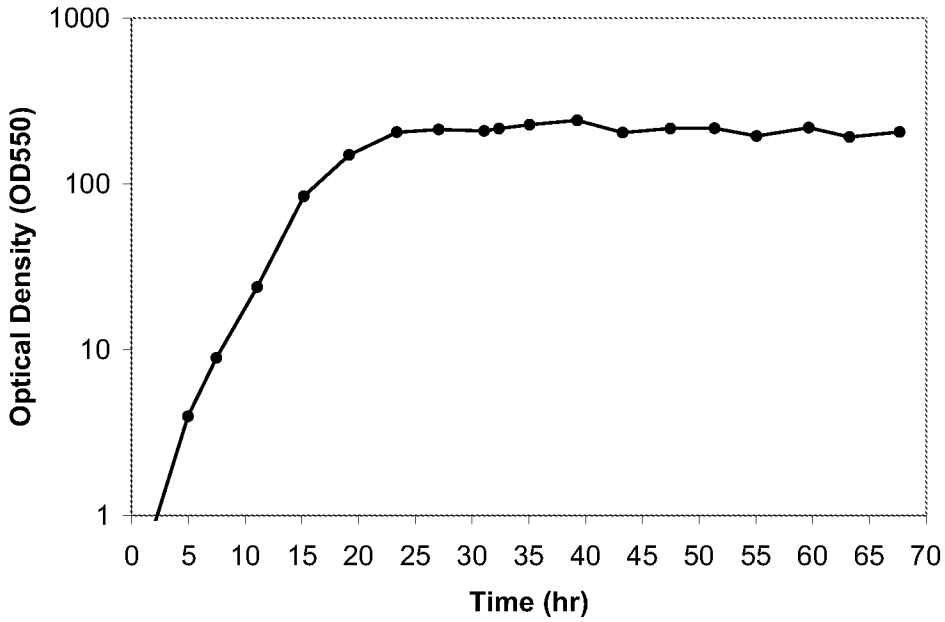


Figure 116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Time (hr)

Figure 117

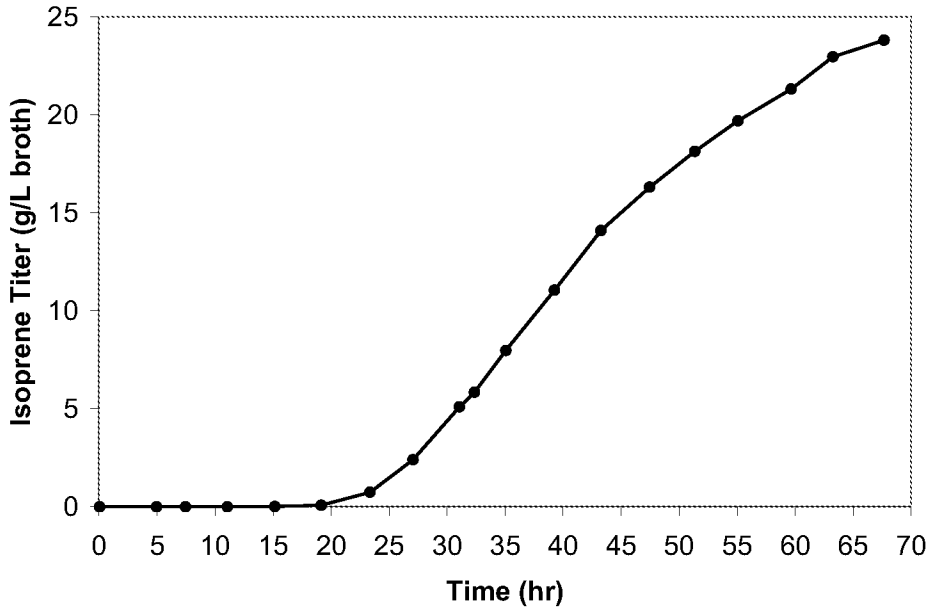
Isoprene Titer (g/L broth)
25
20
15
10
5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time (hr)

Figure 118

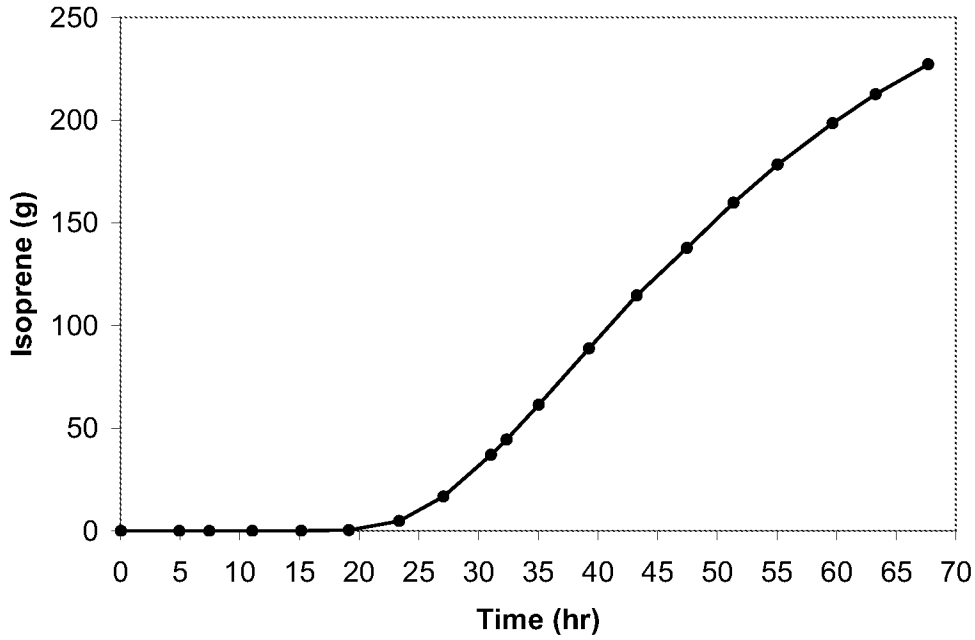
250
200
150
100
50
0
Isoprene (g)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time (hr)

Figure 119

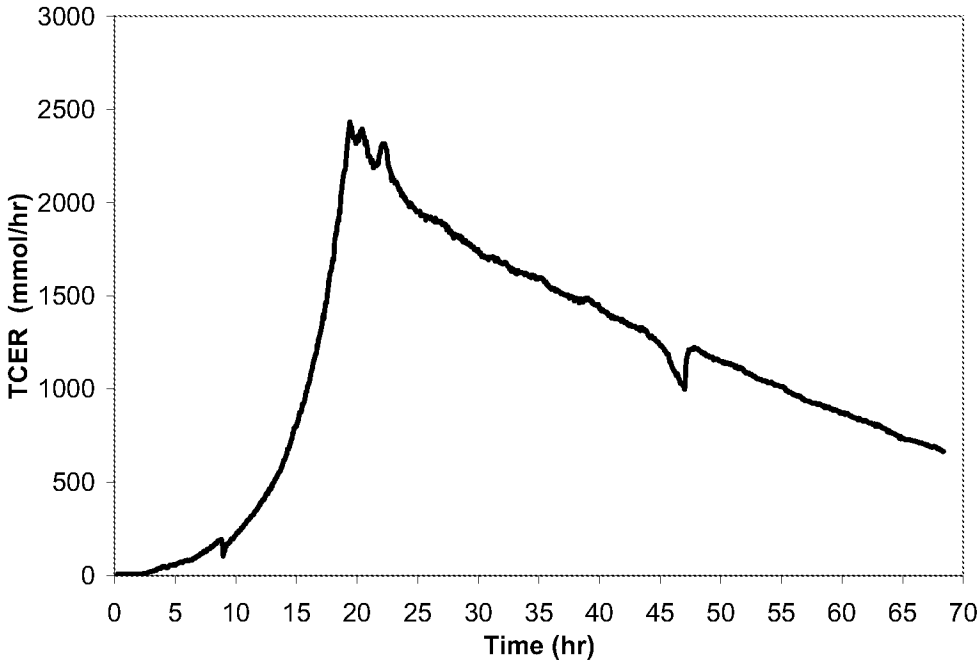
3000
2500
2000
1500
1000
500
0
TCER (mmol/hr)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time (hr)

Figure 120

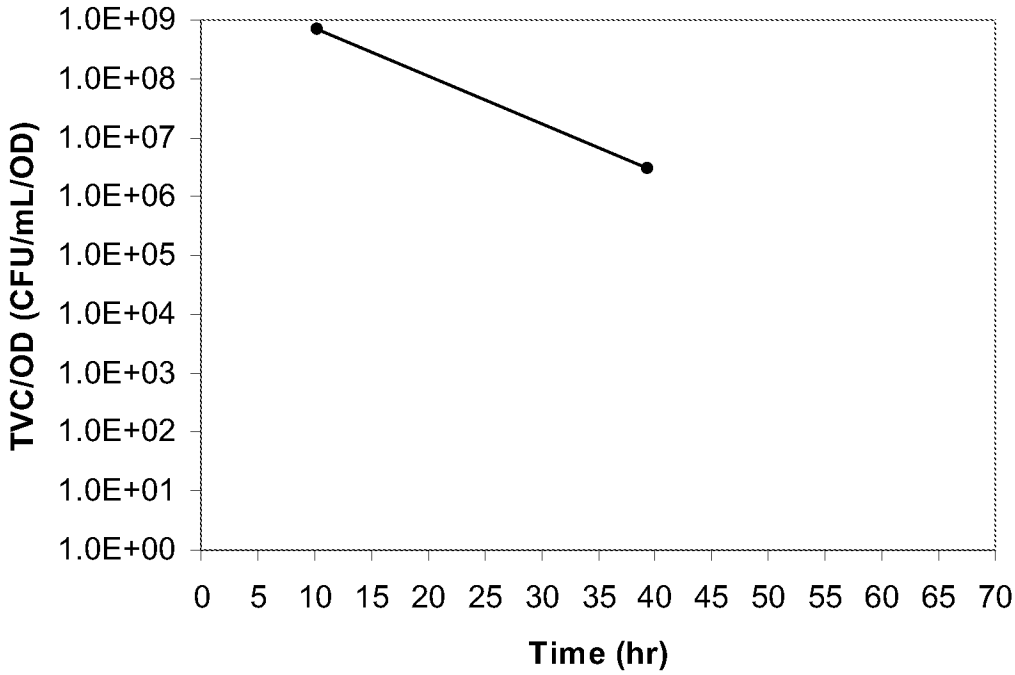
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
TVC/OD (CFU/mL/OD)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time (hr)

Figure 121

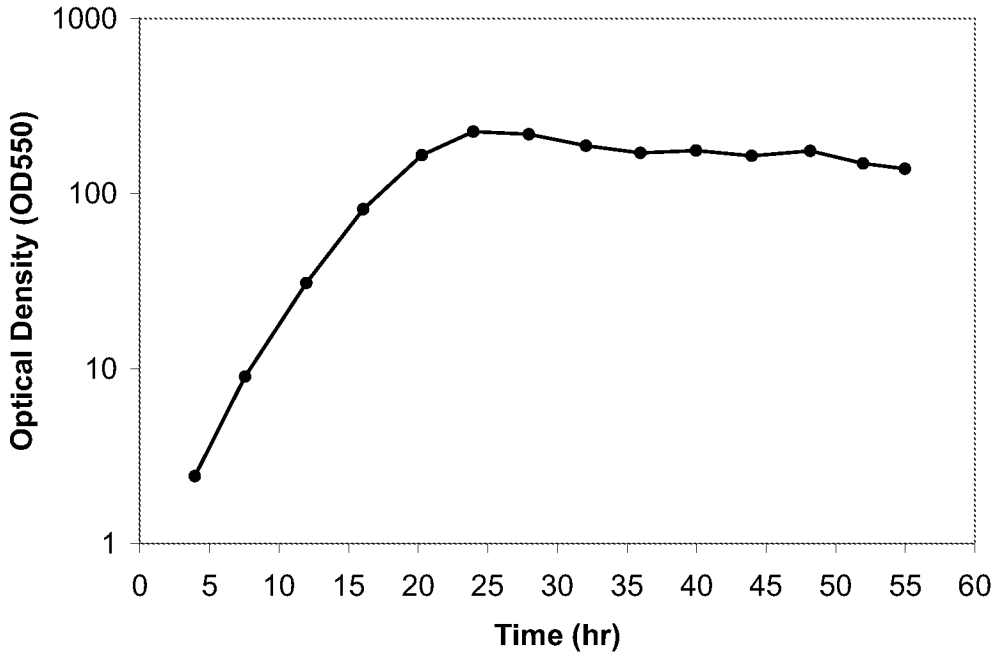
1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 122

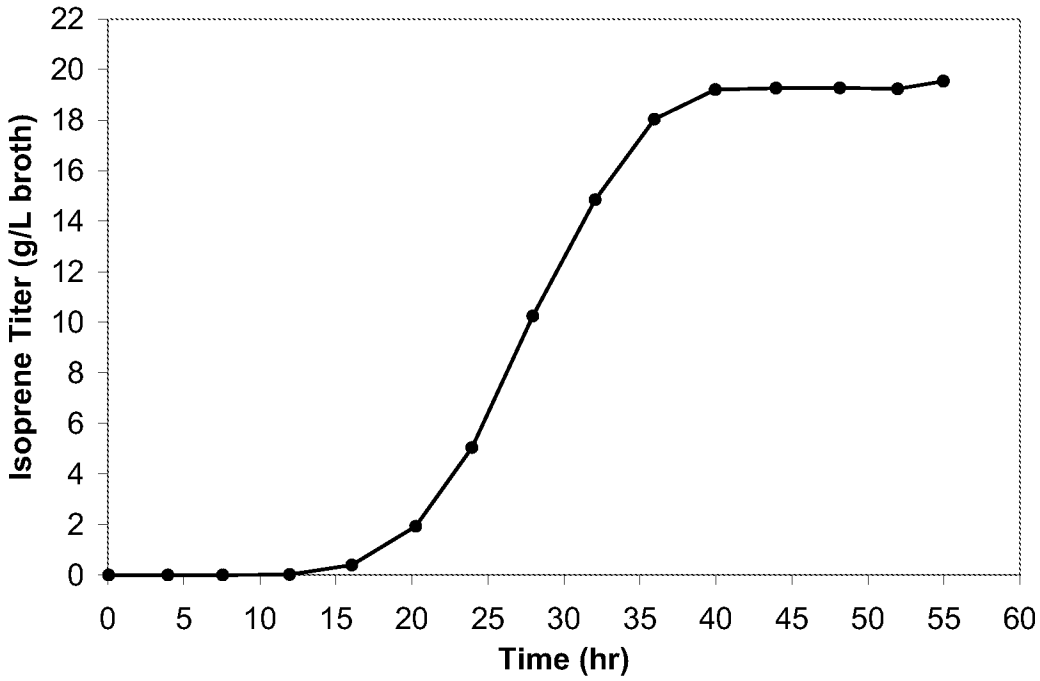
Isoprene Titer (g/L broth)
22
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 123

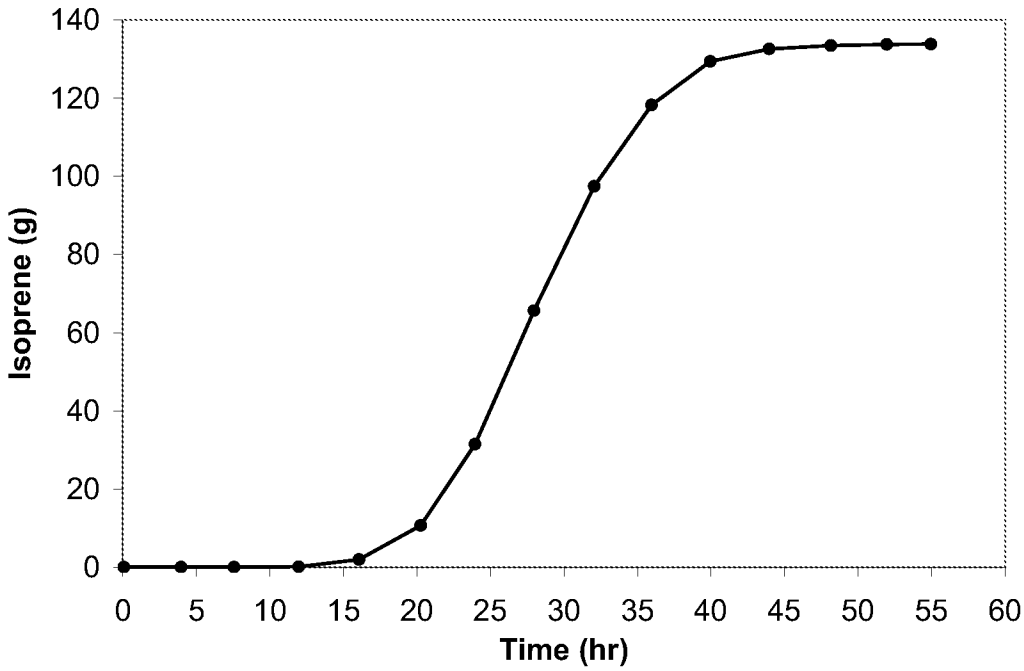
140
120
100
80
60
40
20
0
Isoprene (g)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 124

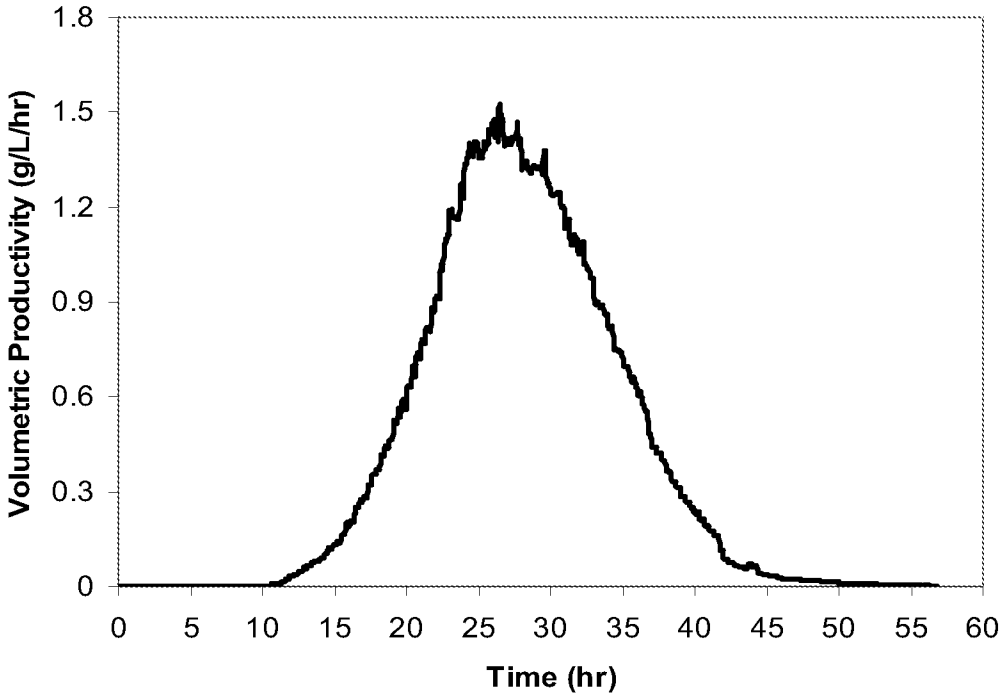
Volumetric Productivity (g/L/hr)
1.8
1.5
1.2
0.9
0.6
0.3
0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (hr)

Figure 125

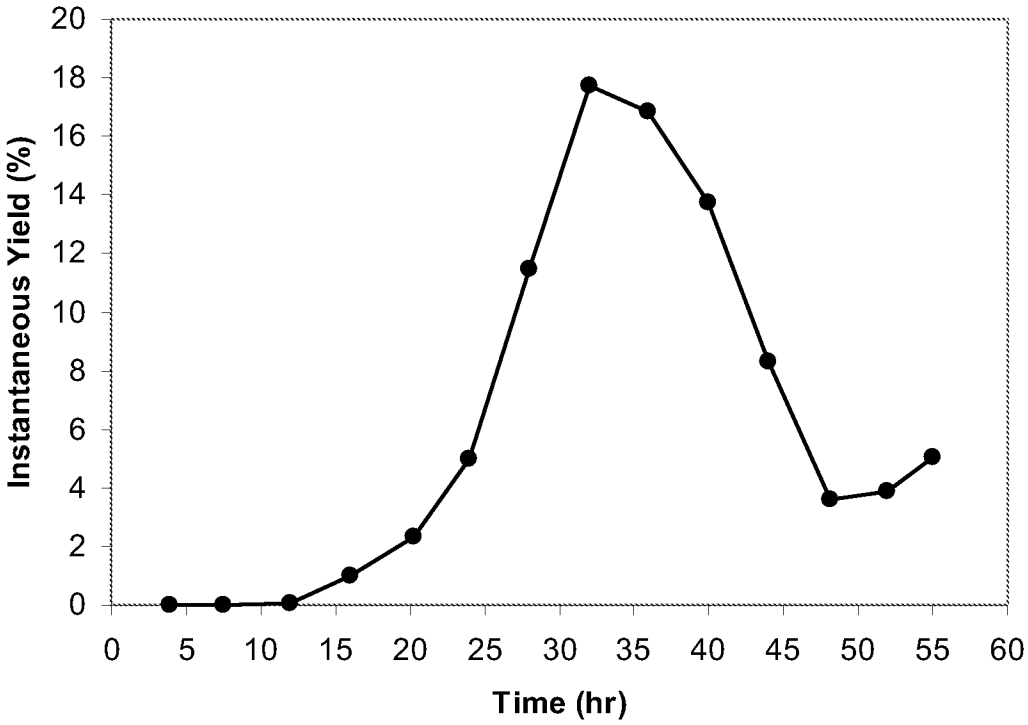
Instantaneous Yield (%)
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 126

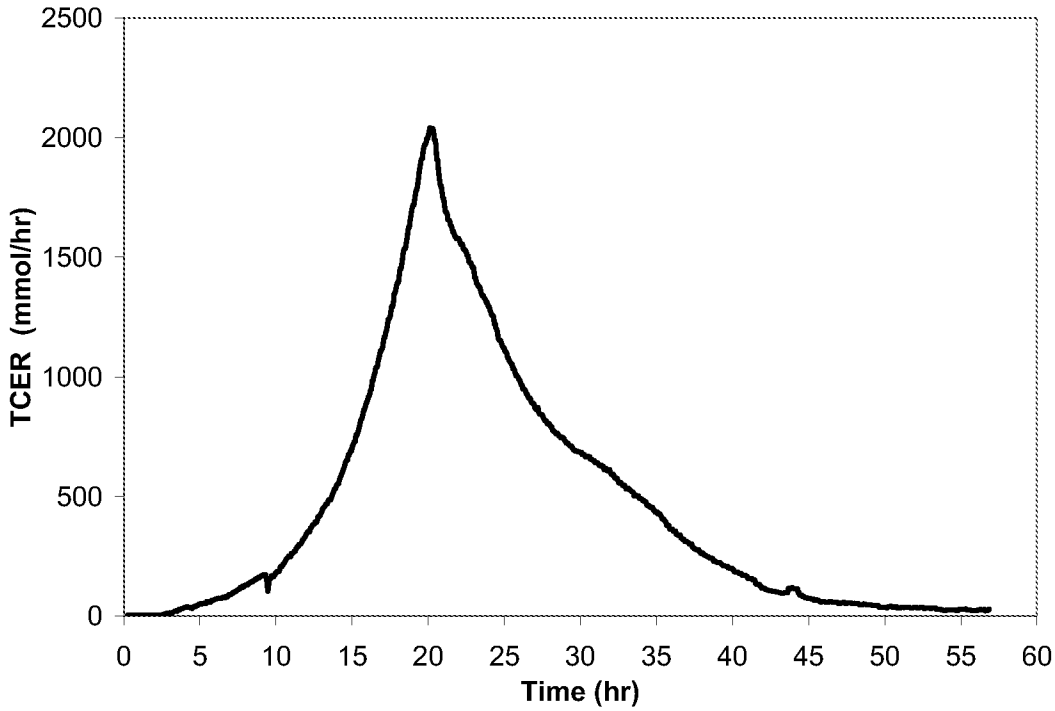
2500
2000
1500
1000
500
0
TCER (mmol/hr)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 127

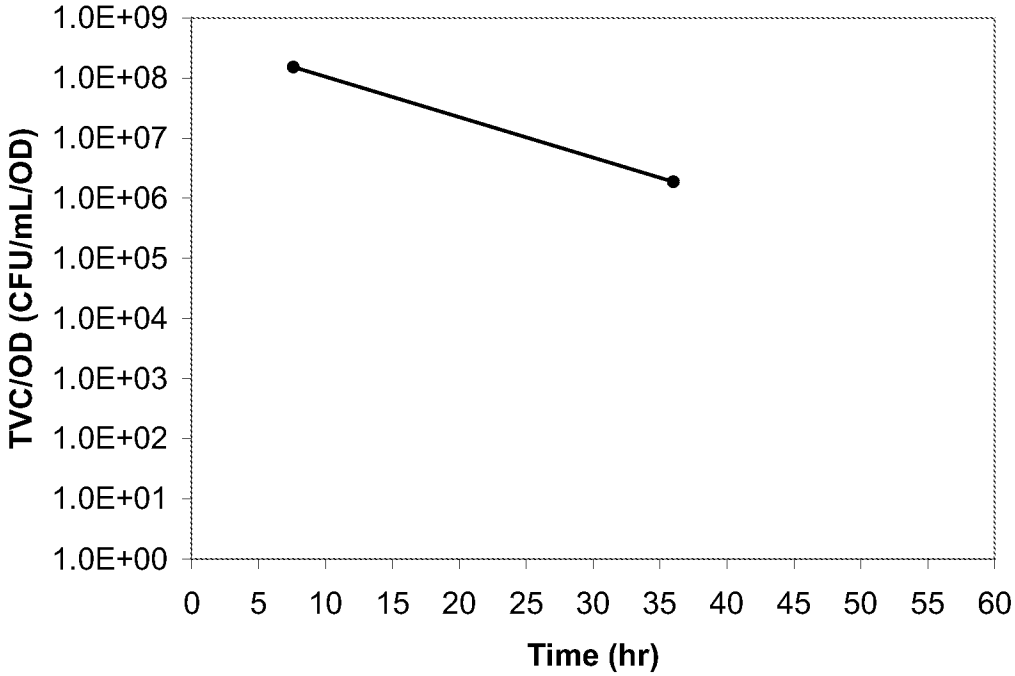
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
TVC/OD (CFU/mL/OD)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 128

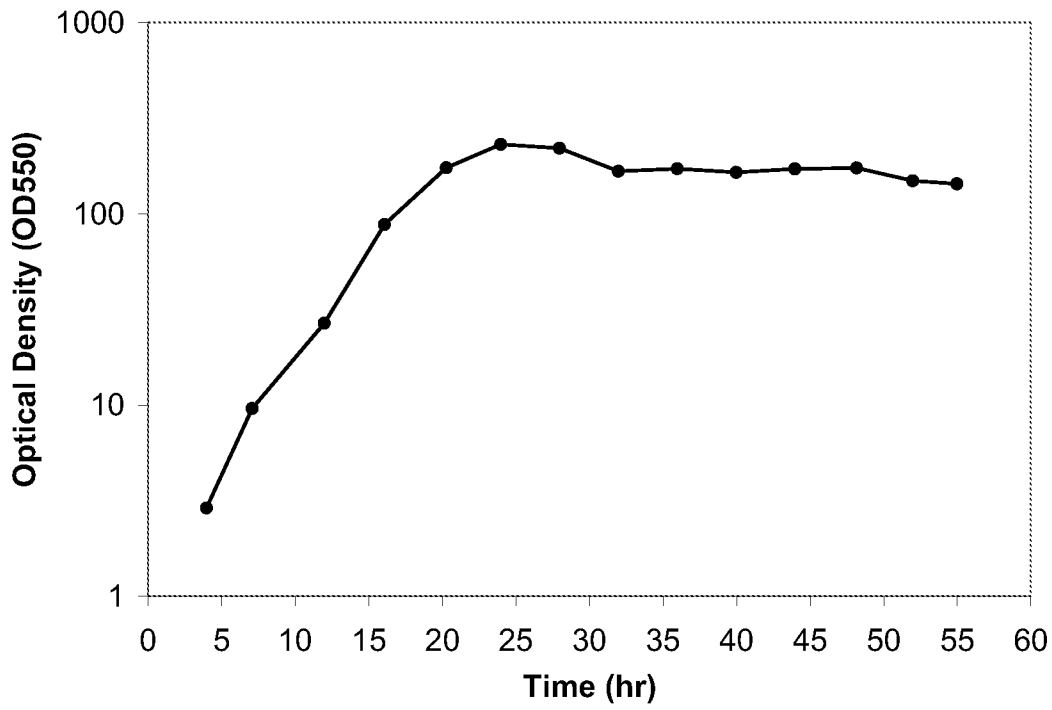
1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 129

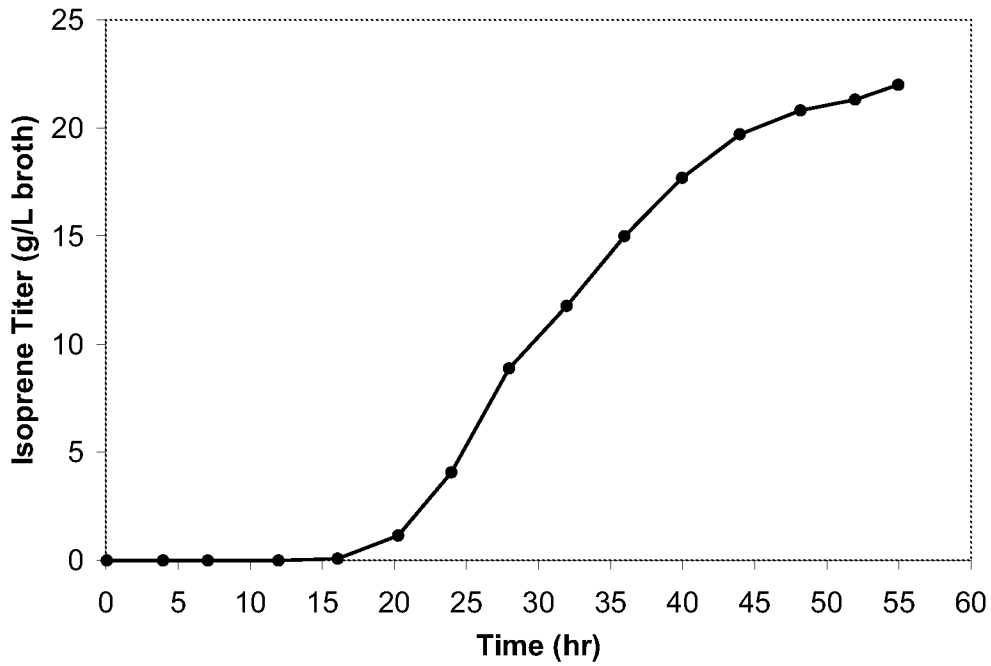
Isoprene Titer (g/L broth)
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 130

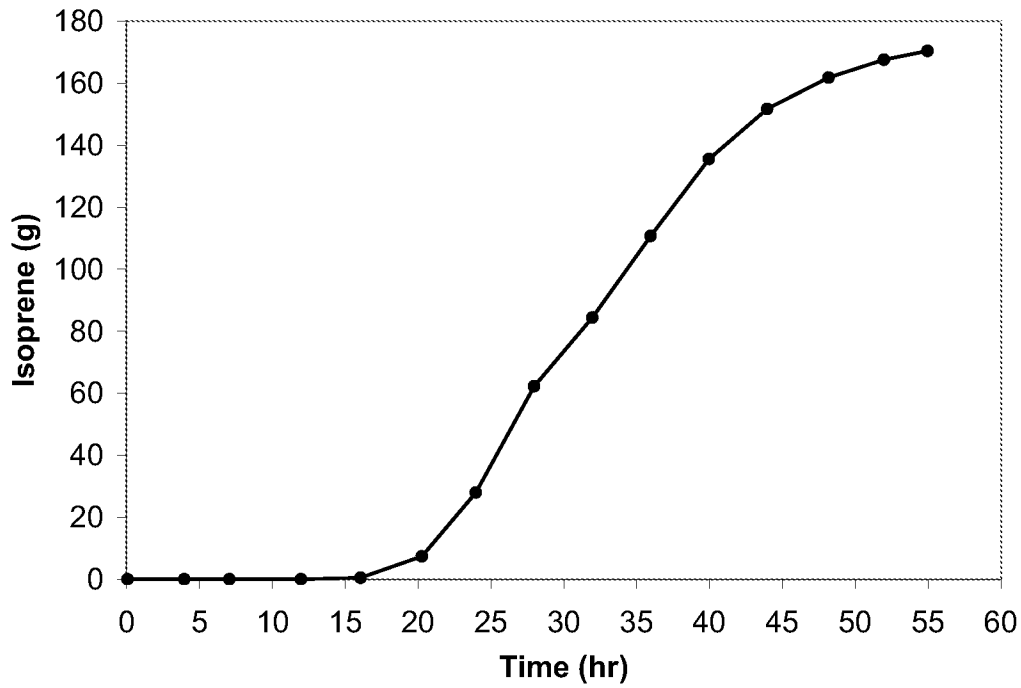
Isoprene (g)
180
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 131

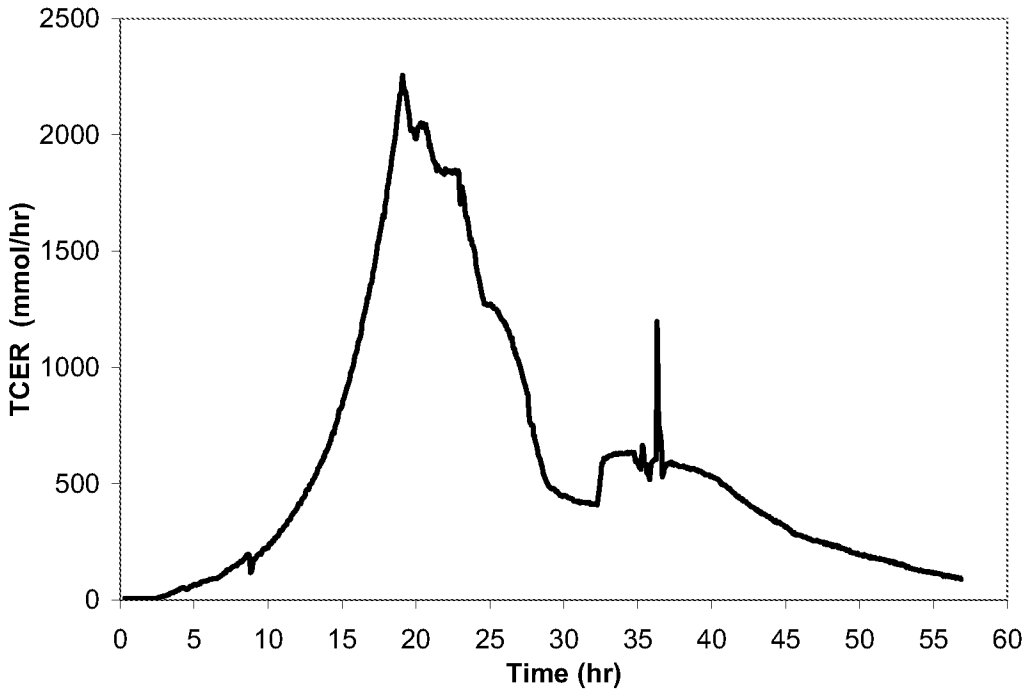
2500
2000
1500
1000
500
0
TCER (mmol/hr)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 132

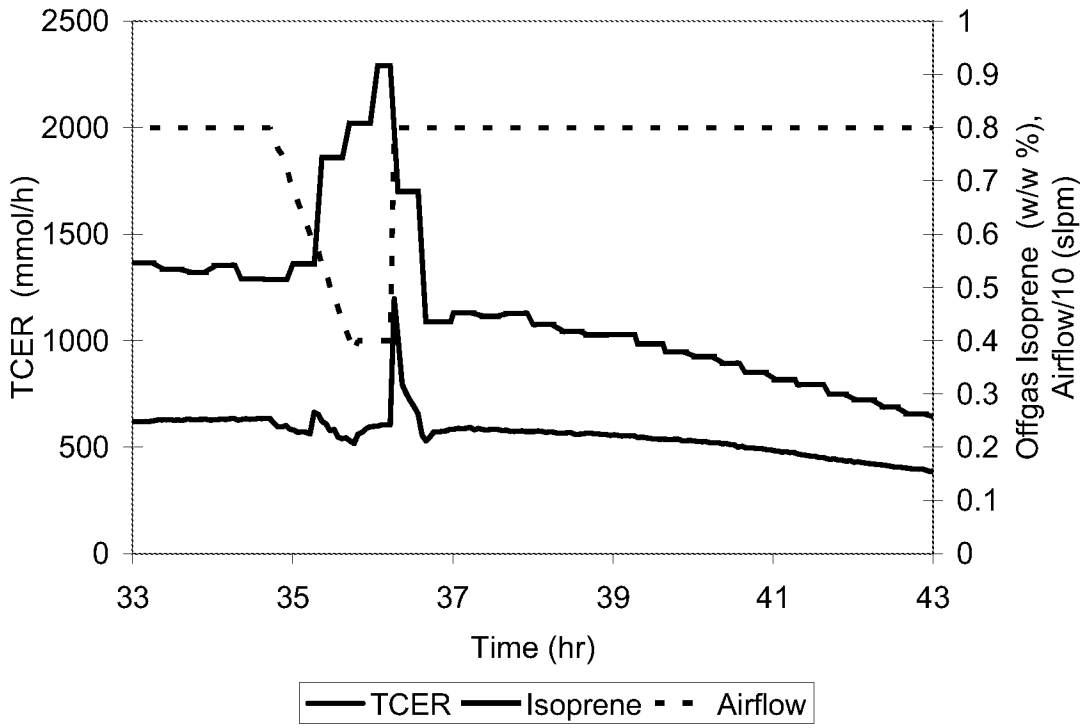
2500
2000
1500
1000
500
0
TCER (mmol/h)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Offgas Isoprene (w/w %), Airflow/10 (slpm)
33
35
37
39
41
43
Time (hr)
TCER
Isoprene
Airflow

Figure 133

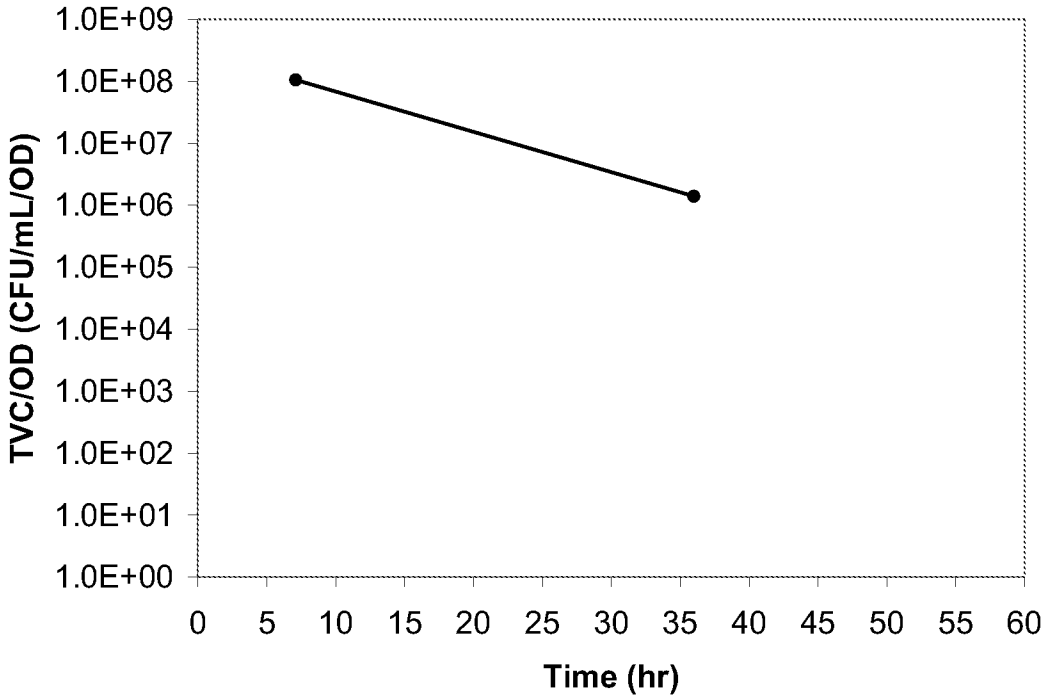
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
TVC/OD (CFU/mL/OD)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 134

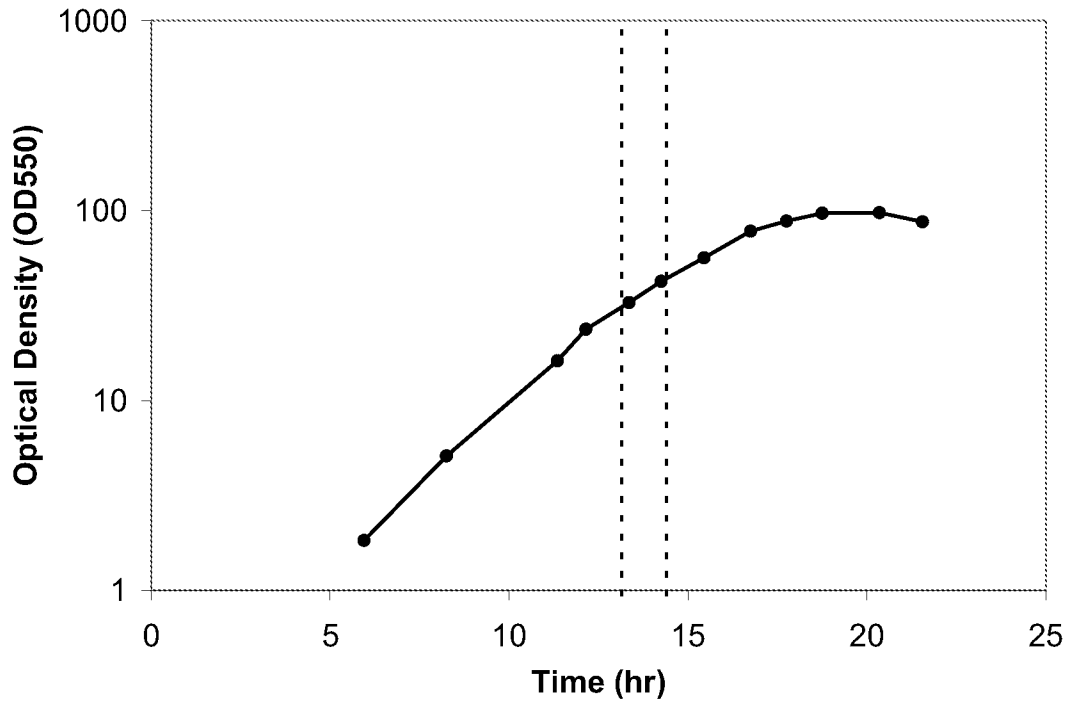

Figure 135

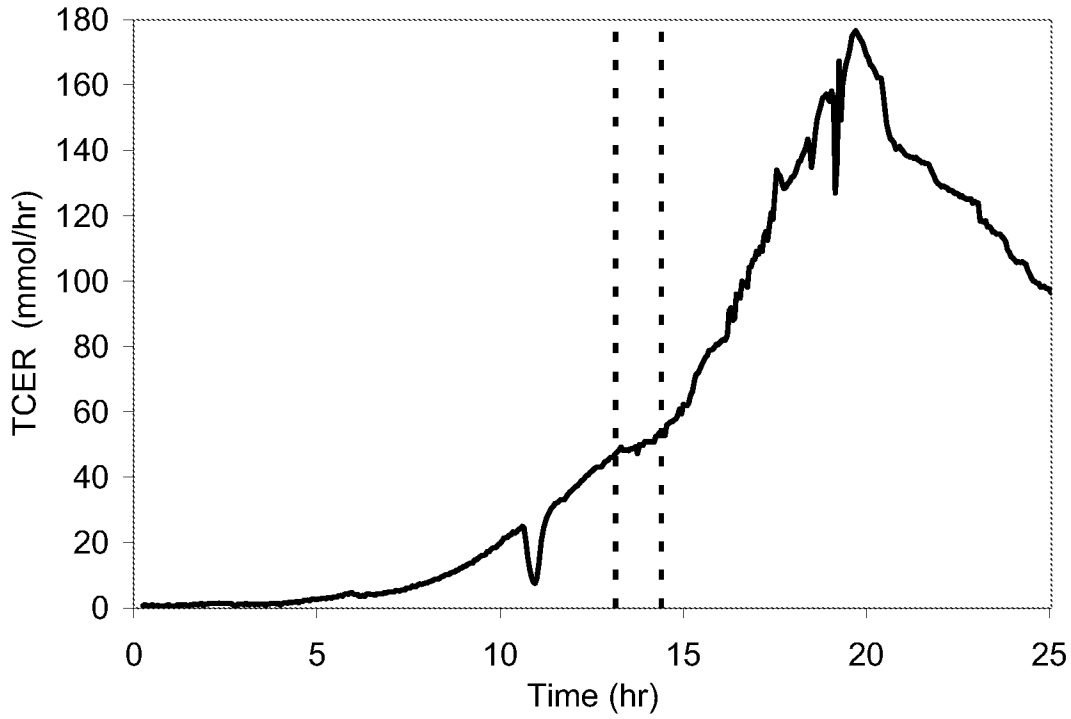
180
160
140
120
100
80
60
40
20
0
TCER (mmol/hr)
0
5
10
15
20
25
Time (hr)

Figure 136

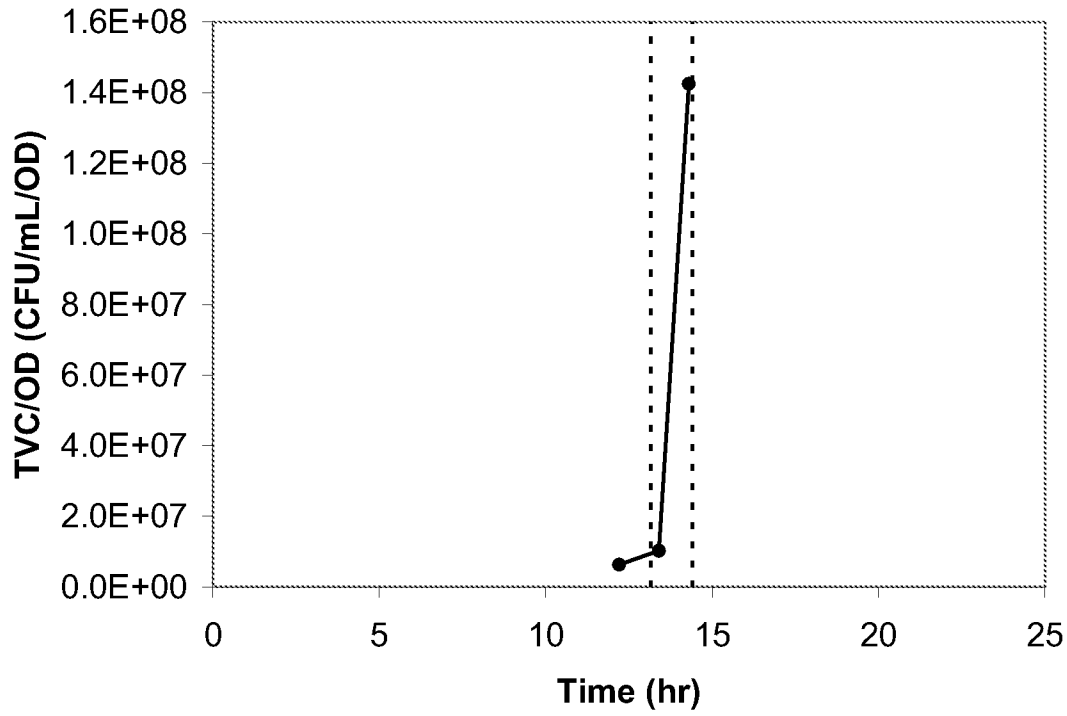
1.6E+08
1.4E+08
1.2E+08
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0.0E+00
TVC/OD (CFU/mL/OD)
0
5
10
15
20
25
Time (hr)

Figure 137A

Figure 137B

gtcgtatcccactaccgagatatccgcaccaacgcgcagcccggactcggtaatggcgcgcattgcgcccagcgccatctgat
cgttggcaaccagcatcgcagtgggaacgatgccctcattcagcatttgcatggtttgttgaaaaccggacatggcactccagtc
gccttcccgttccgctatcggctgaatttgattgcgagtgagatatttatgccagccagccagacgcagacgcgccgagacaga
acttaatgggcccgctaacagcgcgatttgctggtgacccaatgcgaccagatgctccacgcccagtcgcgtaccgtcttcatgg
gagaaaataatactgttgatgggtgtctggtcagagacatcaagaaataacgccggaacattagtgcaggcagcttccacagca
atggcatcctggtcatccagcggatagttaatgatcagcccactgacgcgttgcgcgagaagattgtgcaccgccgctttacagg
cttcgacgccgcttcgttctaccatcgacaccaccacgctggcacccagttgatcggcgcgagatttaatcgccgcgacaatttg
cgacggcgcgtgcagggccagactggaggtggcaacgccaatcagcaacgactgtttgcccgccagttgttgtgccacgcgg
ttgggaatgtaattcagctccgccatcgccgcttccactttttcccgcgttttcgcagaaacgtggctggcctggttcaccacgcgg
gaaacggtctgataagagacaccggcatactctgcgacatcgtataacgttactggtttcacattcaccaccctgaattgactctctt
ccgggcgctatcatgccataccgcgaaaggttttgcgccattcgatggtgtccgggatctcgacgctctcccttatgcgactcctg
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caacagtcccccggccacggggcctgccaccatacccacgccgaaacaagcgctcatgagcccgaagtggcgagcccgatc
ttccccatcggtgatgtcggcgatataggcgccagcaaccgcacctgtggcgccggtgatgccggccacgatgcgtccggcgt
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attttgtttaactttaagaaggagatatacat**atgcgttgtagcgtgtccaccgaaaatgtgtctttccaccgaaactgaaaccga**
agctcgtcgttctgcgaactacgaacctaacagctgggactatgattacctgctgtcctccgacacggacgagtccatcg
aagtatacaaagacaaagcgaaaaagctggaagccgaagttcgtcgcgagattaataacgaaaaagcagaatttctg
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cgcttcgtttcctccggcggcttcgatgcggtaaccaagacttccctgcacggtacggcactgtctttccgtctgctgcgtc
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agatatcaaagctatcctgagcctgtacgaggccagcttcctggctctggaaggcgaaaacatcctggacgaggcgaa
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ctggaactgccactgcatcgccgtactcagcgtctggaagcagtatggtctatcgaggcctaccgtaaaaaggaggacg
cgaatcaggttctgctggagctggcaattctggattacaacatgatccagtctgtataccagcgtgatctgcgtgaaacgt
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tgggtgtagcattcgaaccgcaatactccgactgccgtaactccgtcgcaaaaatgttttctttcgtaaccattatcgacga
tatctacgatgtatacggcaccctggacgaactggagctgtttactgatgcagttgagcgttgggacgtaaacgccatca
acgacctgccggattacatgaaactgtgctttctggctctgtataacactattaacgaaatcgcctacgacaacctgaaag
ataaaggtgagaacatcctgccgtatctgaccaaagcctgggctgacctgtgcaaacgctttcctgcaagaagccaagtg
gctgtacaacaaatctactccgaccttgacgactacttcggcaacgcatggaaatcctcttctggcccgctgcaactggt
gttcgcttacttcgctgtcgtgcagaacattaaaaaggaagagatcgaaaacctgcaaaaatacccatgacaccatctctc
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SEQ ID NO:30

Figure 137C

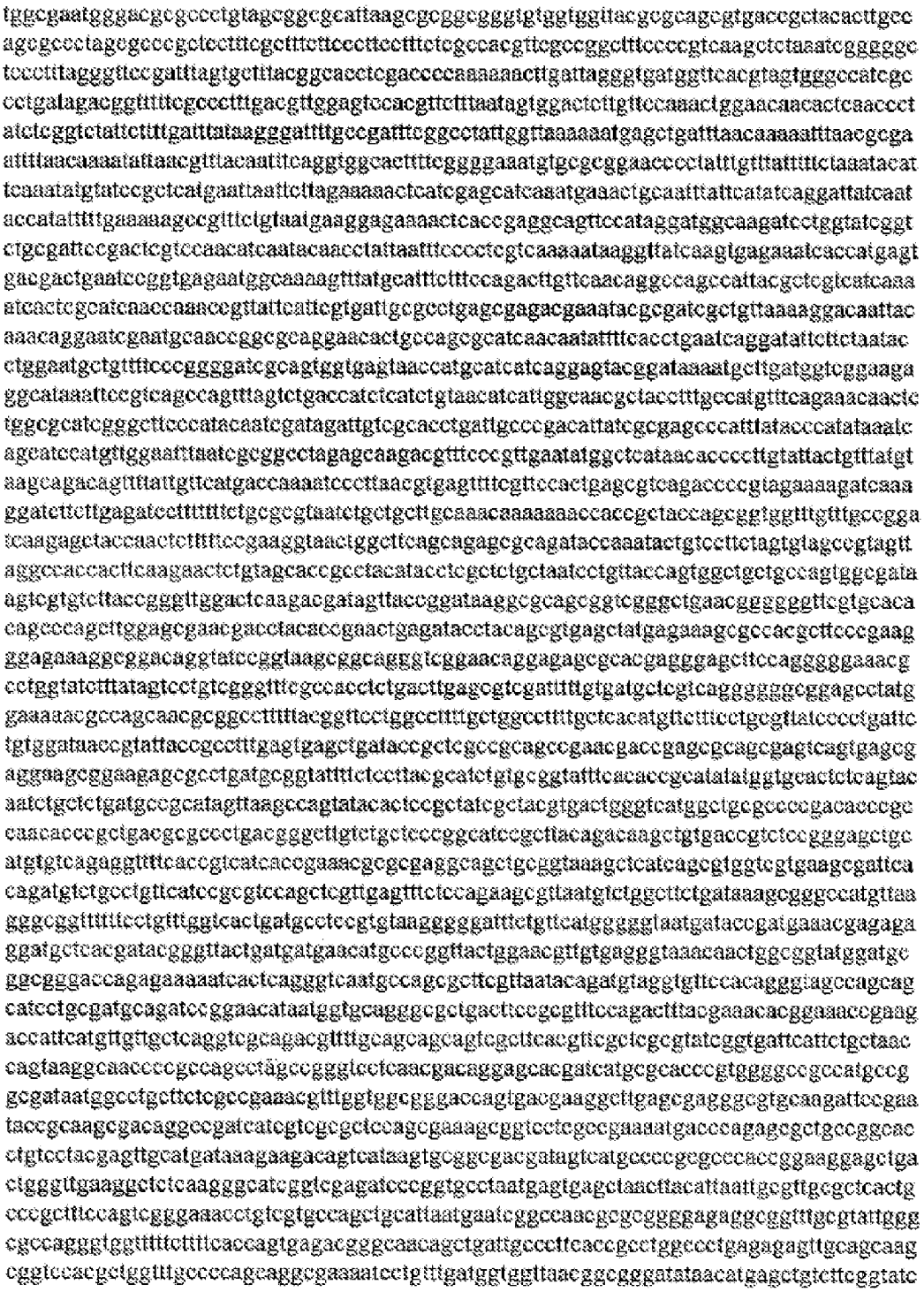

Figure 137D

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acgatctgccggattacatgaaactgtgctttctggctctgtataacactattaacgaaatcgcctacgacaaccctgaaag
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gtccttcccatatcttccgtctgtgcaatgacctggctagcgcgtctgcggaaattgcgcgtggtgaaaccgcaaatagcg
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aaaaagatgaacaaggaaaaactgggtggtagcctgttcgcgaaaccgttcgtggaaaccgcgatcaacctggcacgt
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actgaaccgattctgccgtttgaacgctaaggatccgaattcgagctccgtcgacaagcttgcggccgcactcgagcaccac
caccaccaccactgagatccggctgctaacaaagcccgaaaggaagctgagttggctgctgccaccgctgagcaataactagc
ataaccccttggggcctctaaacgggtcttgaggggttttttgctgaaaggaggaactatatccggat

SEQ ID NO:31

Figure 137E

Figure 137F

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Figure 137G

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Figure 137H

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Figure 137I

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Figure 137J

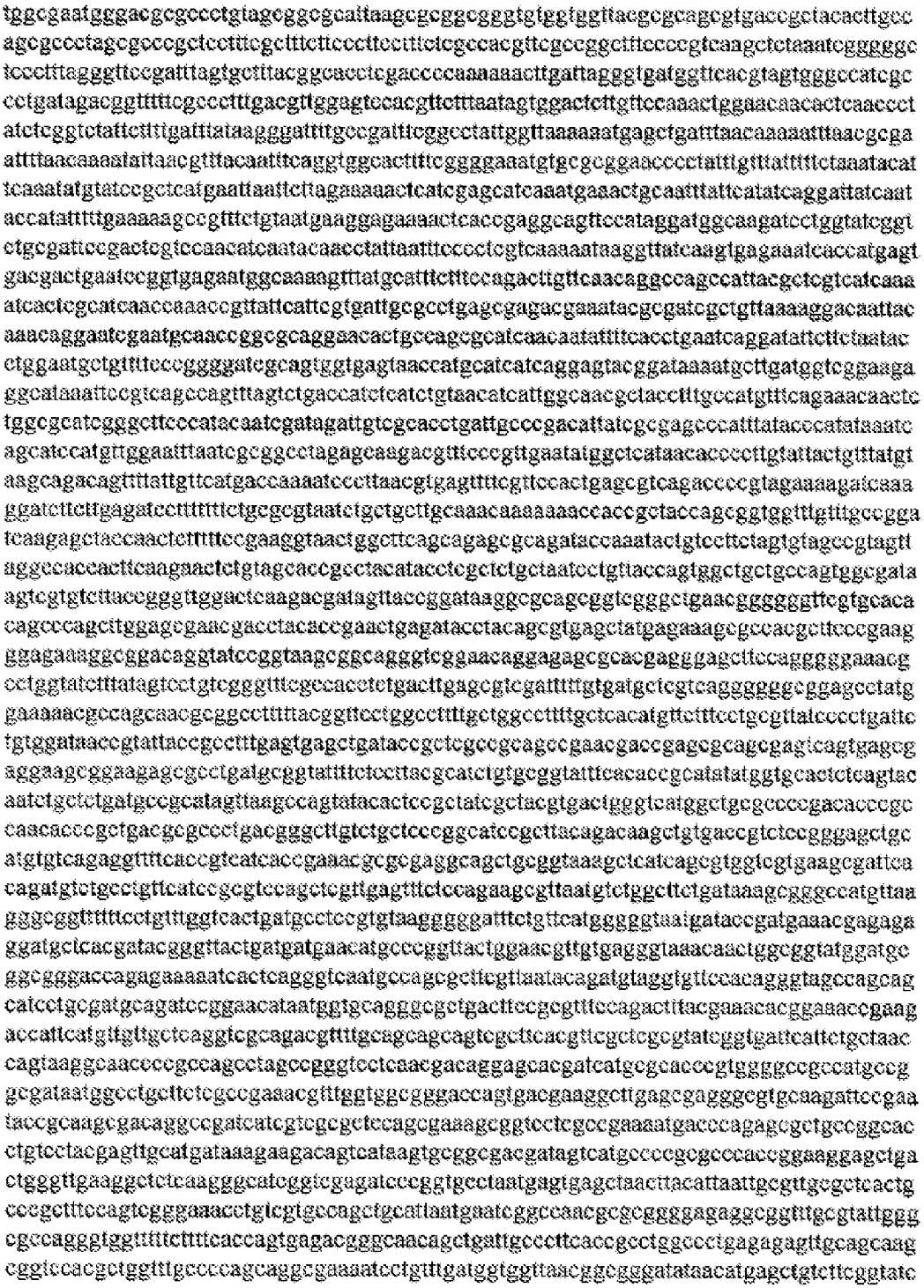

Figure 137K

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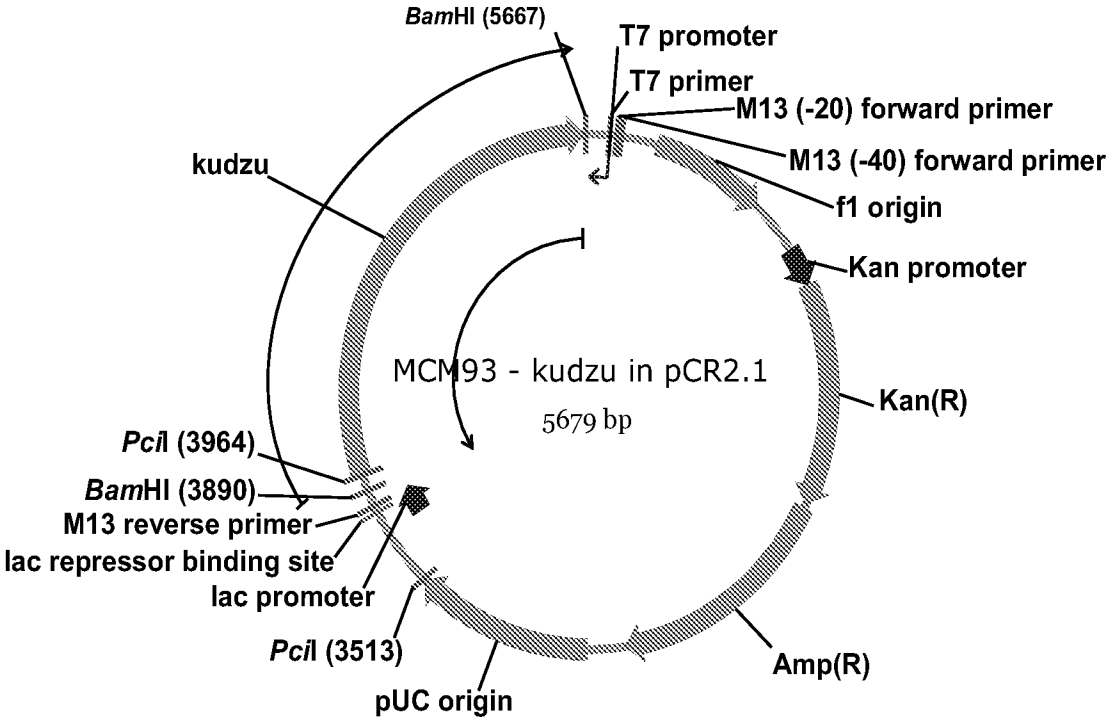
Figure 137L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pUC origin
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Figure 137M

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Figure 137N

SEQ ID NO:36

Figure 137O

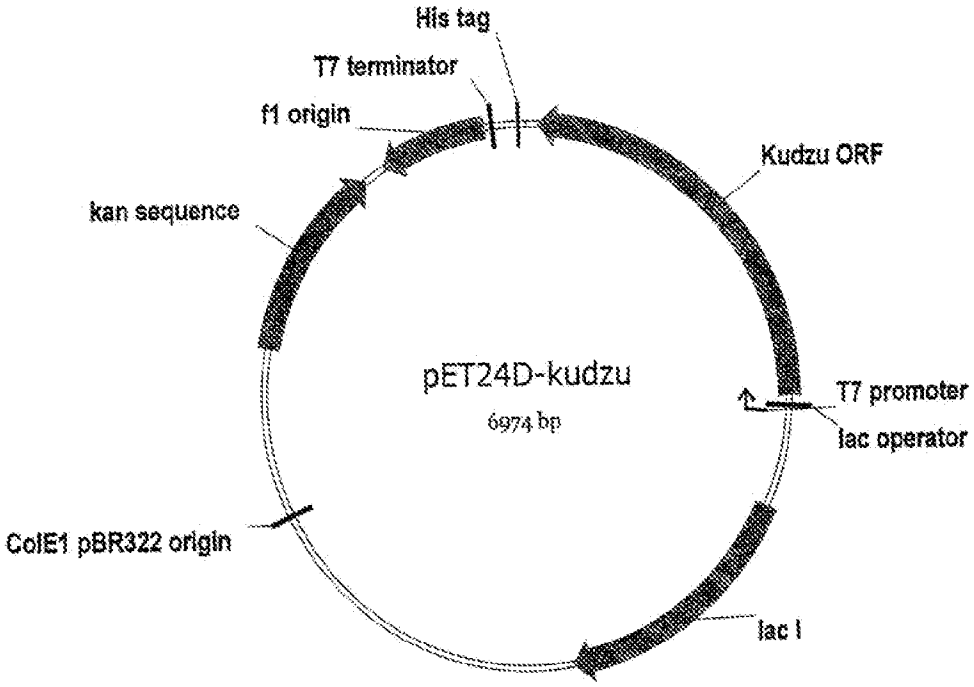

His tag
T7 terminator
f1 origin
Kudzu ORF
kan sequence
pET24D-kudzu
6974 bp
T7 promoter
lac operator
ColE1 pBR322 origin
lac I

Figure 138P

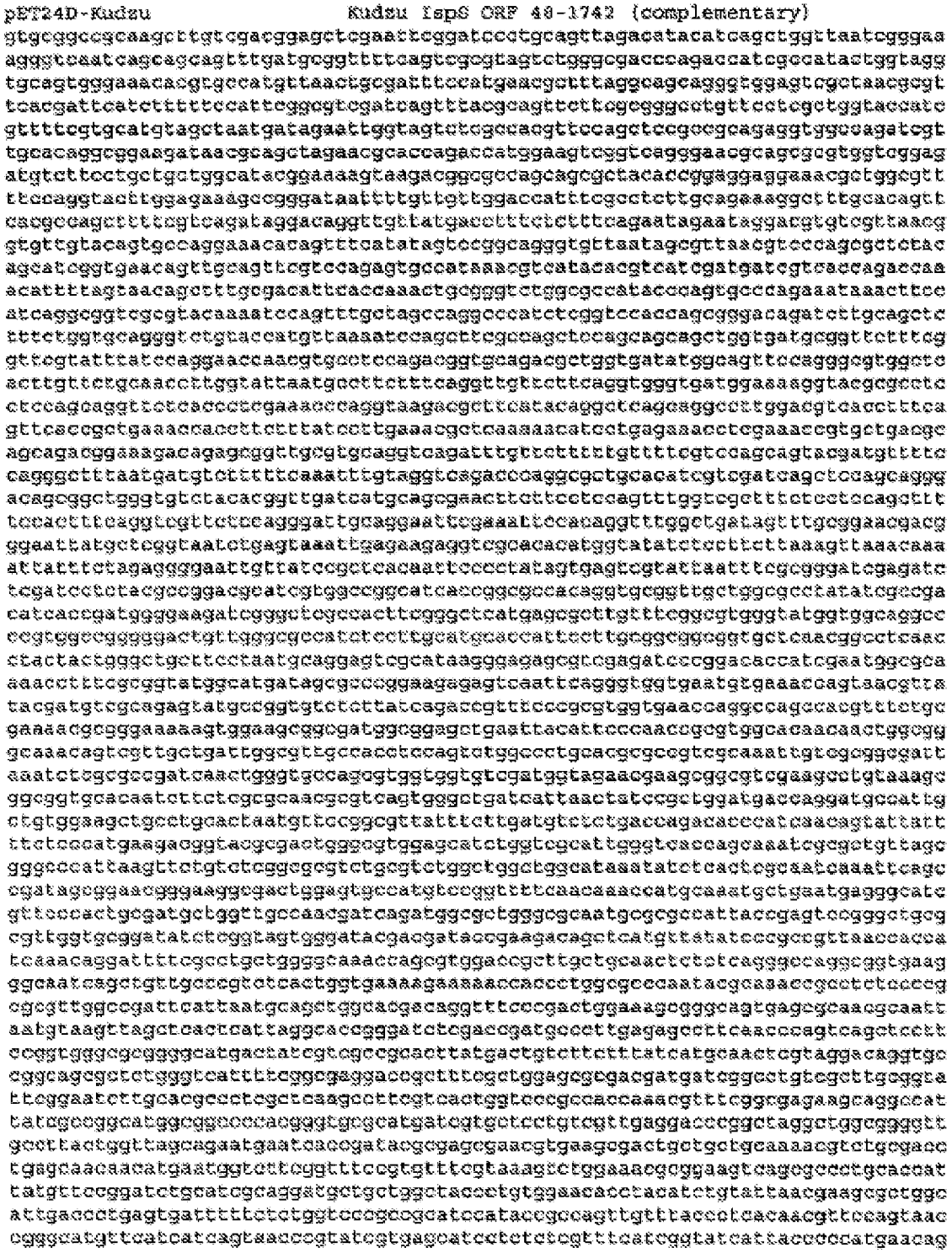
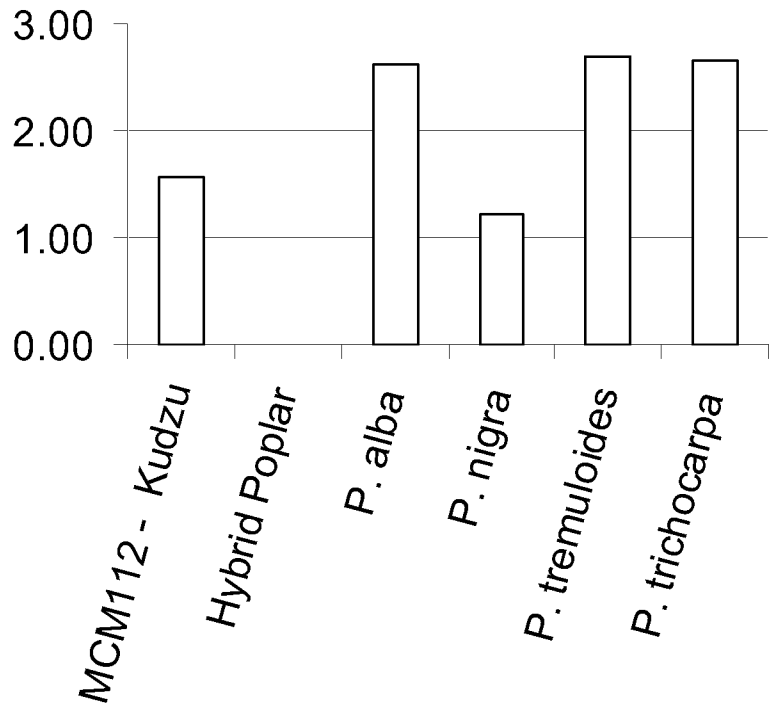


Figure 137Q

SEQ ID NO:37

Figure 138


3.00
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1.00
0.00
MCM112 - Kudzu
Hybrid Poplar
P. alba
P. nigra
P. tremuloides
P. trichocarpa

Figure 139

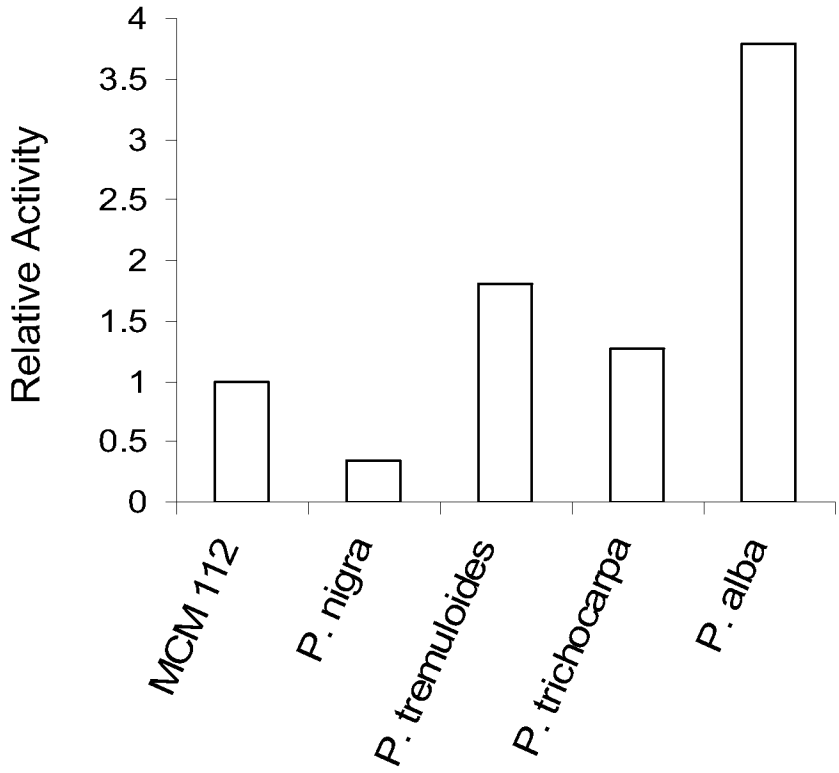
Relative Activity
4
3.5
3
2.5
2
1.5
1
0.5
0
MCM 112
P. nigra
P. tremuloides
P. trichocarpa
P. alba

Figure 140

DNA 2.0
Plasmid Map
pDONR221:19430 - hybrid_HGS
Only single cutters are shown in the map, for a more complete list see table.
pDONR221 is a Gateway® vector
pDONR221:19430
hybrid_HGS
4263 bp
insert:19430
Kan
pUC_ori
Original Author
DNA2.0, Inc.
1430 OBrien Drive, Suite E
Menlo Park, CA 94025-1436
1-877-DNA-TOGO (Toll free) 1-650-853-8351 (Fax)
info@dna20.com
www.dna20.com
Feature Map
insert:19430 - Start:669 End:2387
Kan - Start:2657 End:3466
pUC-ori - Start:3588 End:4268
Restriction Map
Name Sequence Cut Positions
AclI AACGTT 364,1655
AlwNI CAGNNNCTG 911,1130,3848
ApaI GGGCCC 567
ApaLI GTGCAC 3943
AsiSI GCGATCGC 3982
AvaI CYCGRG 562
BamHI GGATCC 2376
BbsI GAAGAC 437(C)
BsmBI CGTCTC 3958
BsrDI GCAATG 632,2039,2425(C),2580(C)
BstBI TTCGAA 931
BstXI CCANNNNNNTGG 754,1157
BtsI GCAGTG 214,3030,2943(C)
ClaI ATCGAT 2158
EcoRI GAATTC 662,2382
EcoRV GATATC 1955,2500
HpaI GTTAAC 561,2240
MluI ACGCGT 330,3561
NheI GCTAGC 239,505
NruI TCGCGA 2739
PvuI CGATCG 3682
PvuII CAGCTG 174,2495
XbaI TCTAGA 723,971
No Cuts: AccB51, AgeI, AscI, AvrII, BglI, BglII, BsaI, BspEI, EagI, FseI, HindIII, KasI, KpnI, MfeI, NcoI, NdeI, NotI, PacI, PstI, SacI, SacII, SalI, SanDI, SfiI, SnaBI, SpeI, SphI, XhoI, XmaI

Figure 141

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SEQ ID NO:38

Figure 142A

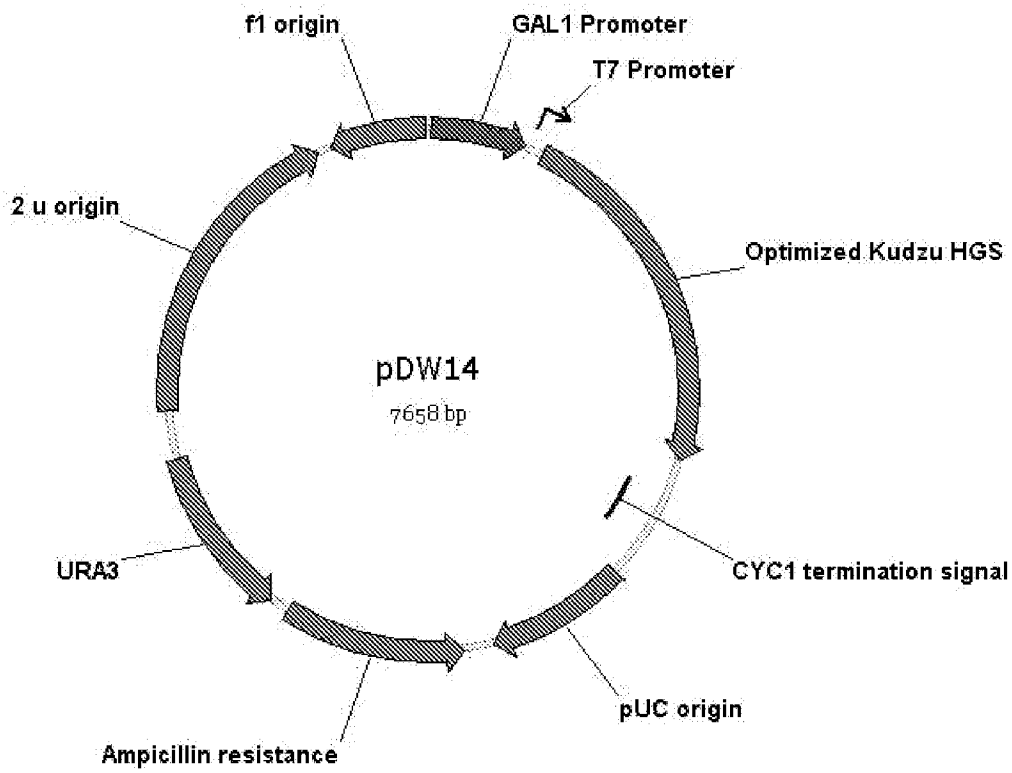
f1 origin
GAL1 Promoter
T7 Promoter
2 u origin
Optimized Kudzu HGS
pDW14
7658 bp
URA3
CYC1 termination signal
pUC origin
Ampicillin resistance

Figure 142B

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Figure 142C

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Figure 143

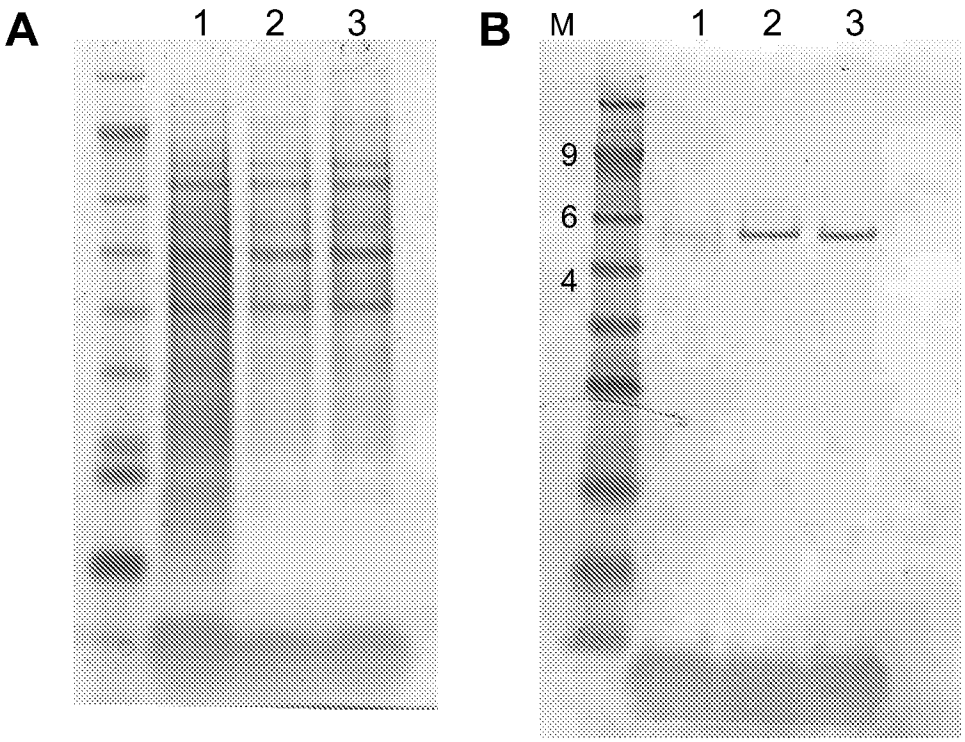
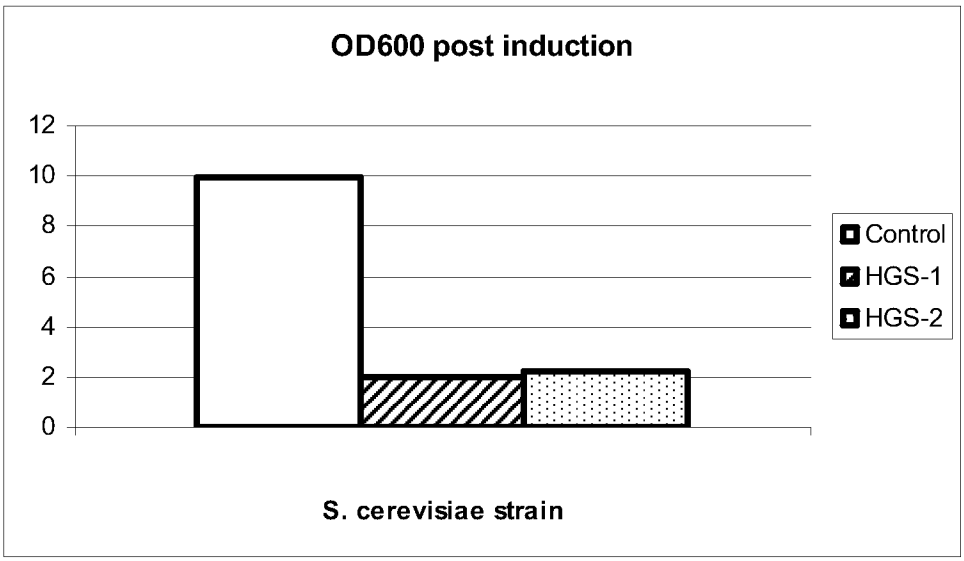


Figure 144A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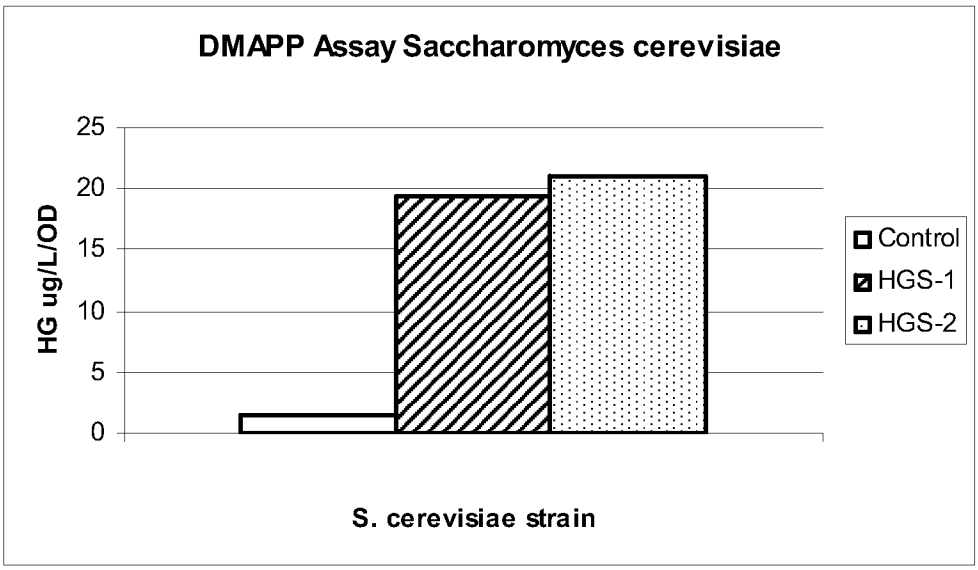
Control
HGS-1
HGS-2
S. cerevisiae strain

Figure 144 B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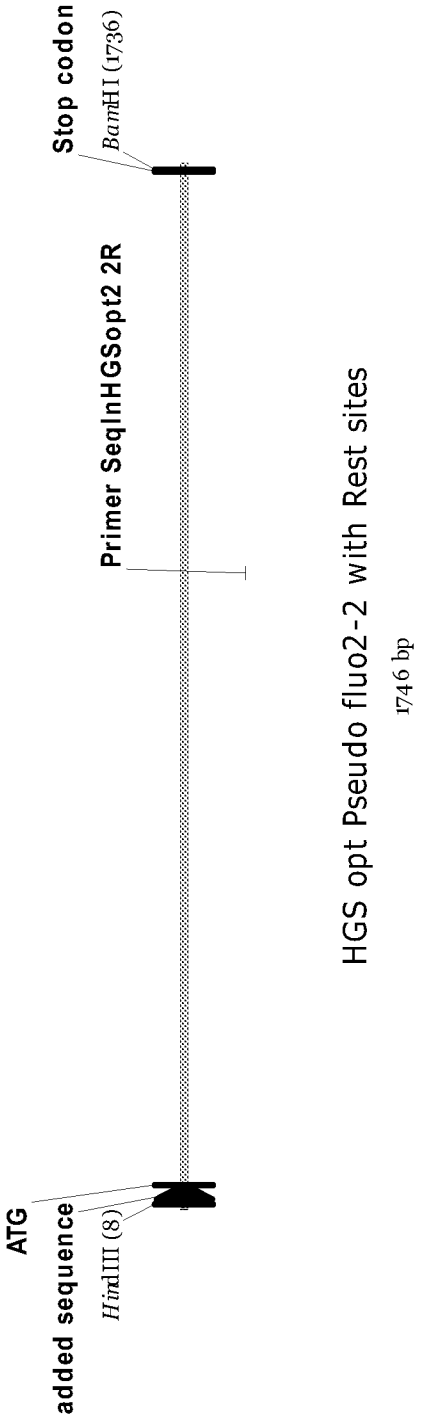
S. cerevisiae strain
Control
HGS-1
HGS-2

Figure 145A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Primer SeqInHGSopt2 2R
HGS opt Pseudo flu02-2 with Rest sites
1746 bp
ATG
added sequence
HindIII (8)

Figure 145B

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SEQ ID NO:40

Figure 146A

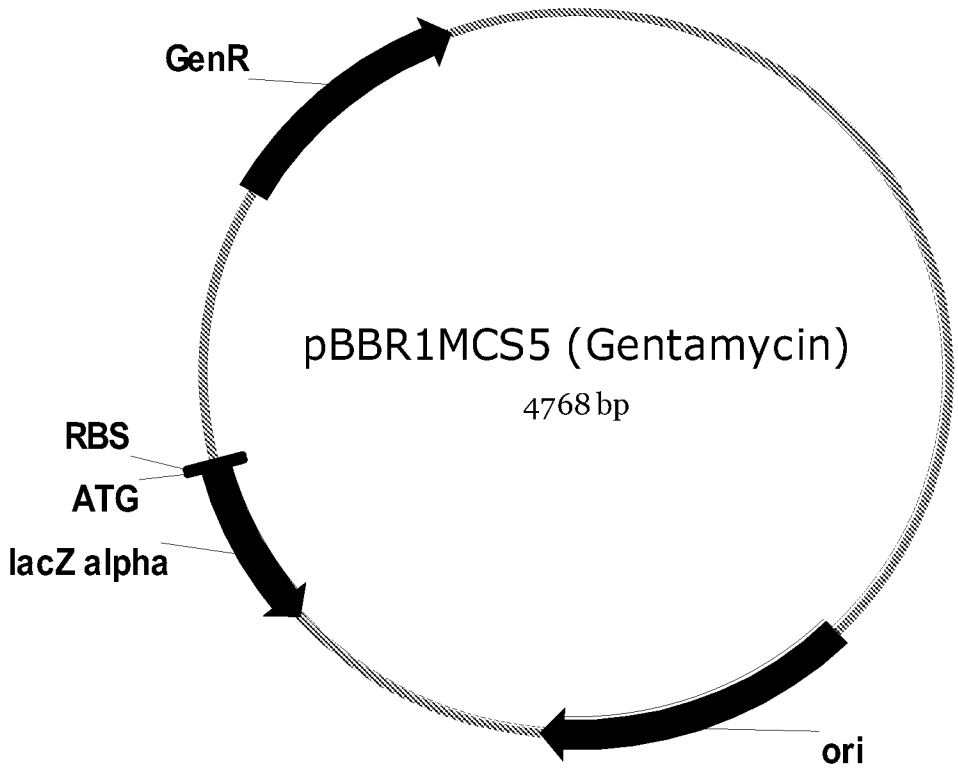

Figure 146B

Figure 146C

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Figure 147A

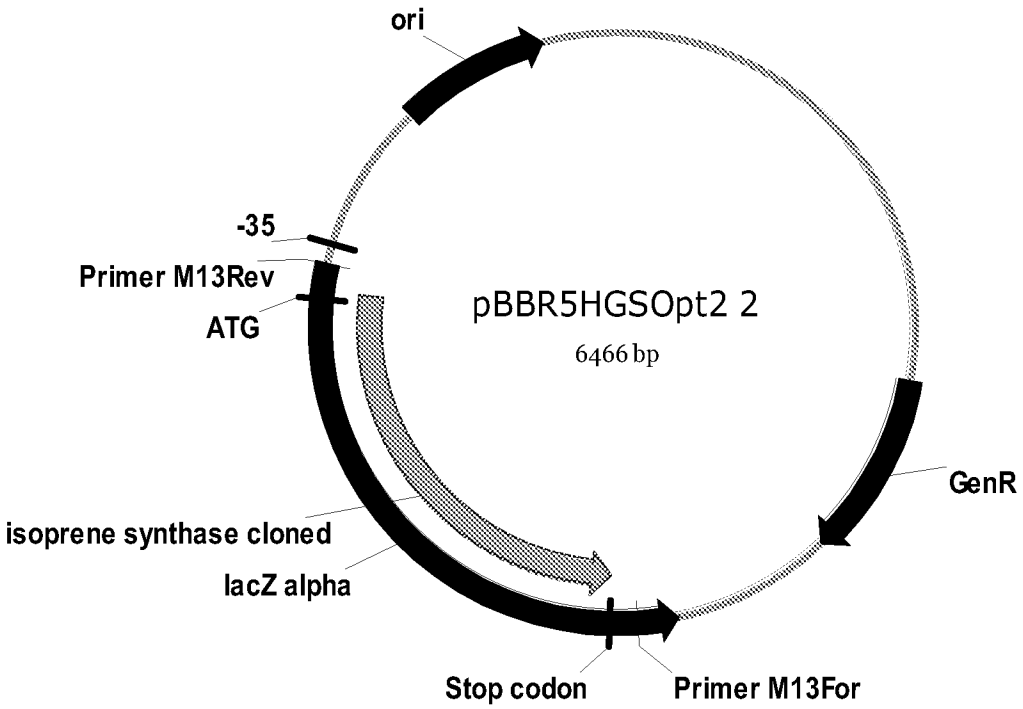
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Figure 147B

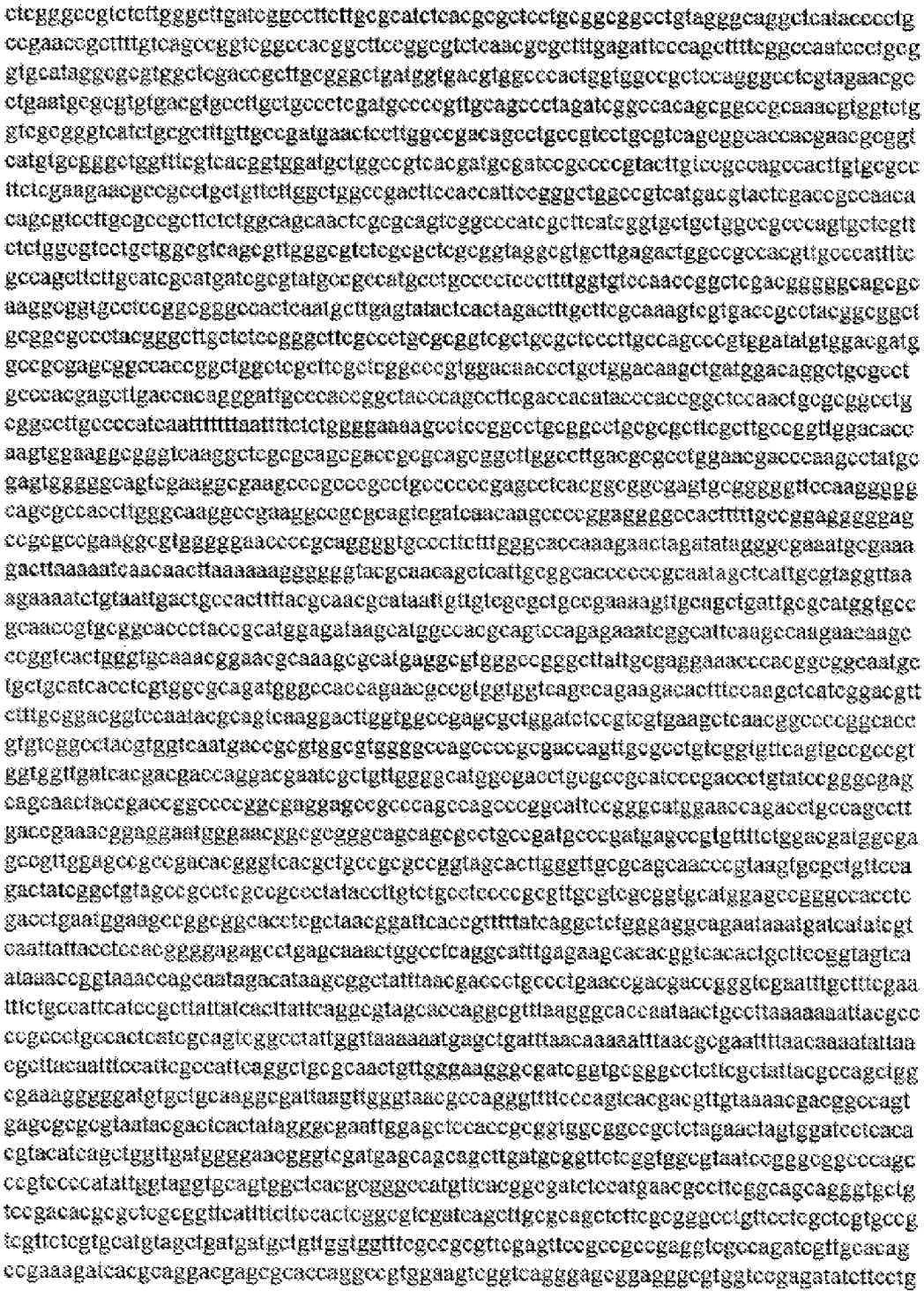

Figure 147C

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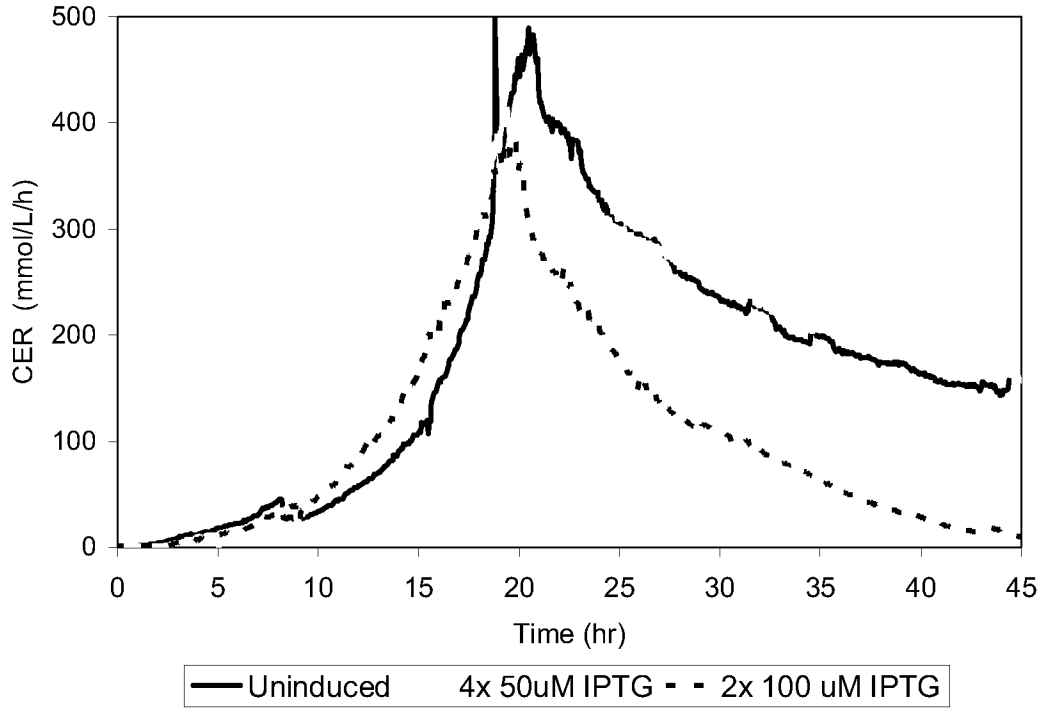
Figure 148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25
30
35
40
45
Time (hr)
Uninduced
4x 50uM IPTG
2x 100 uM IPTG

Figure 149

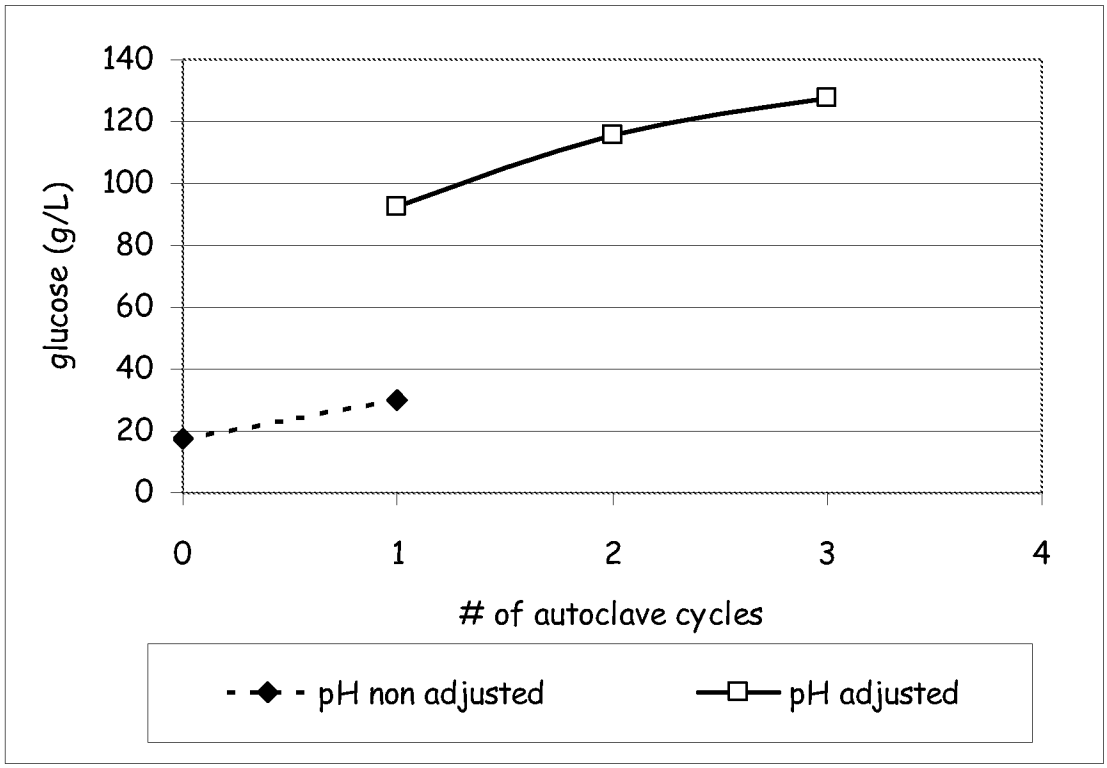
140
120
100
80
60
40
20
0
glucose (g/L)
0
1
2
3
4
# of autoclave cycles
pH non adjusted
pH adjusted

Figure 150

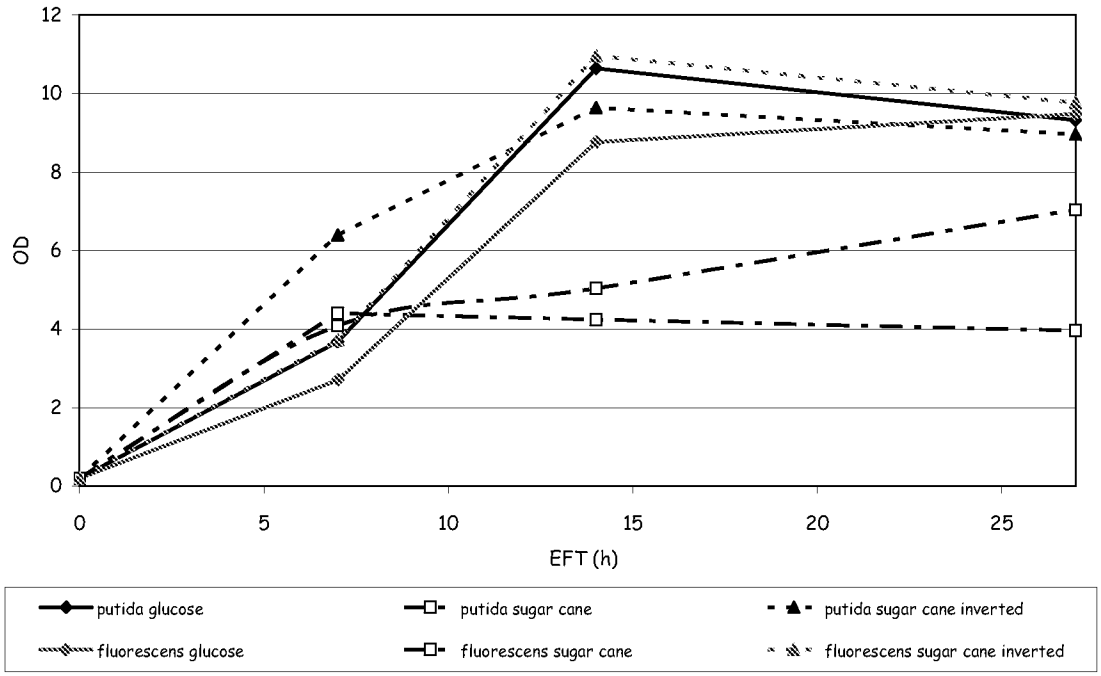
12
10
8
6
4
2
0
OD
0
5
10
15
20
25
EFT (h)
putida glucose
putida sugar cane
putida sugar cane inverted
fluorescens glucose
fluorescens sugar cane
fluorescens sugar cane inverted

Figure 151

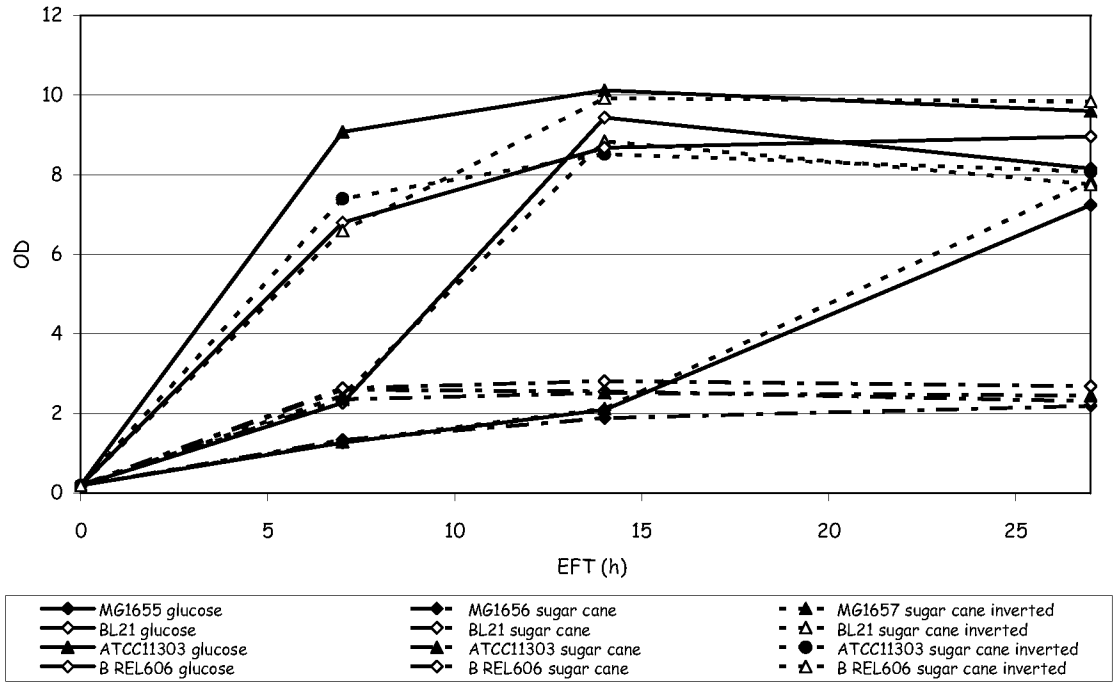
12
10
8
6
4
2
0
OD
0
5
10
15
20
25
EFT (h)
MG1655 glucose
BL21 glucose
ATCC11303 glucose
B REL606 glucose
MG1656 sugar cane
BL21 sugar cane
ATCC11303 sugar cane
B REL606 sugar cane
MG1657 sugar cane inverted
BL21 sugar cane inverted
ATCC11303 sugar cane inverted
B REL606 sugar cane inverted

Figure 152

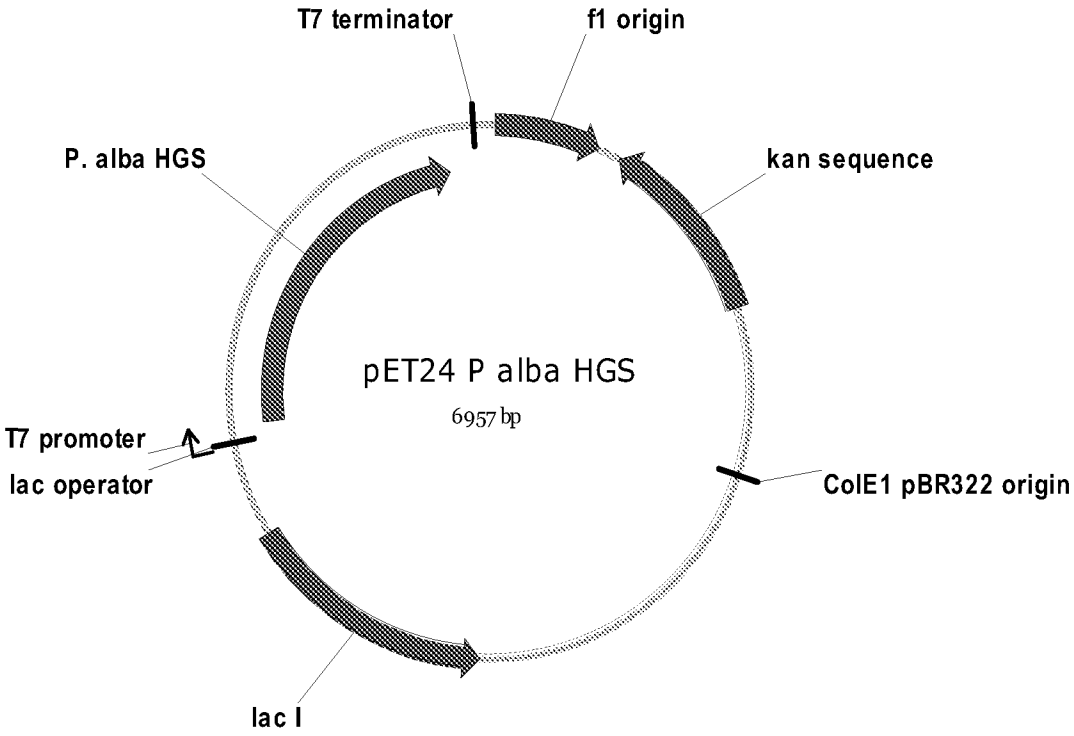
T7 terminator
f1 origin
P. alba HGS
kan sequence
pET24 P alba HGS
6957 bp
T7 promoter
lac operator
ColE1 pBR322 origin
lac I

Figure 153A

1-

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Figure 153B

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Figure 154

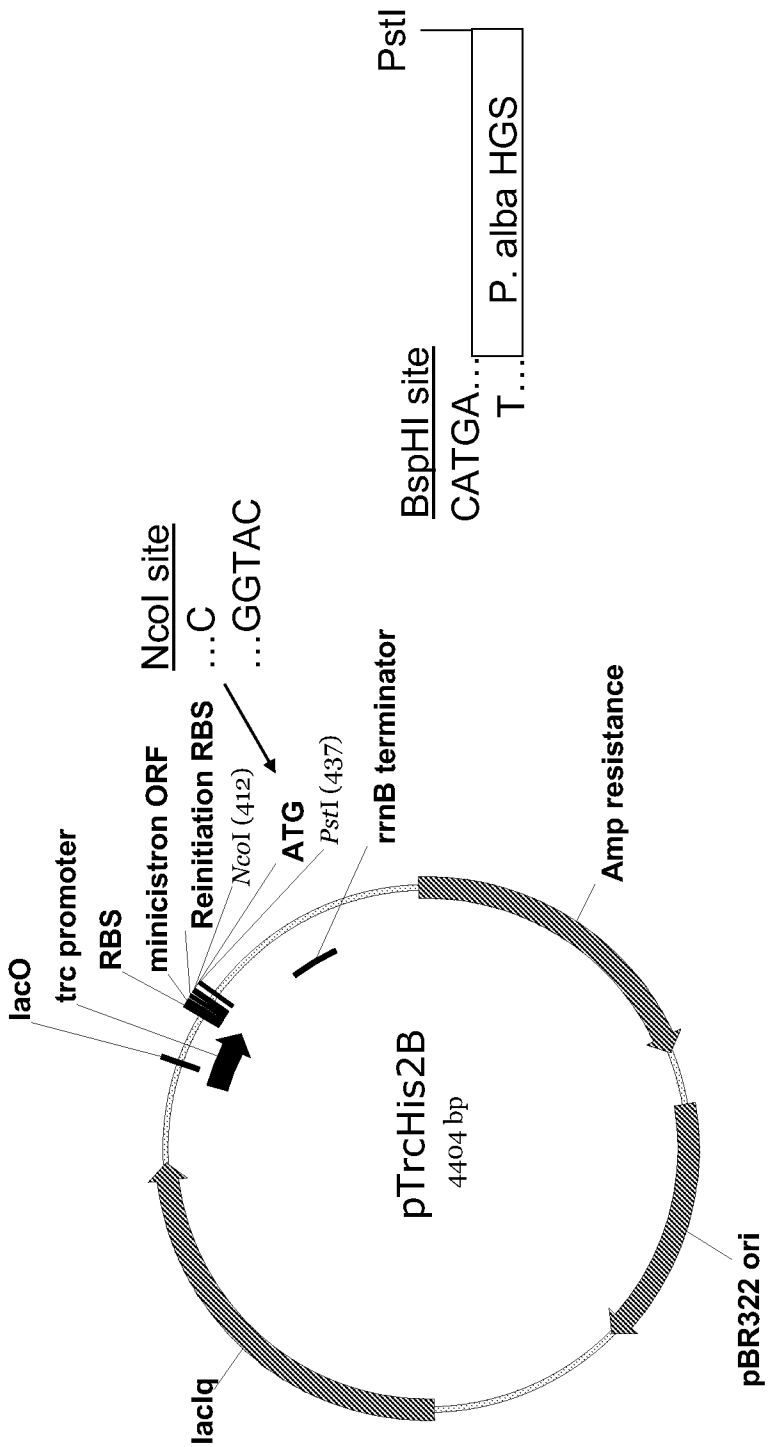
PstI
P. alba HGS
BspHI site
CATGA...
T...
NcoI site
...C
...GGTAC
lacO
trc promoter
RBS
minicistron ORF
Reinitiation RBS
NcoI (412)
ATG
PstI (437)
rrnB terminator
Amp resistance
pTrcHis2B
4404 bp
lacIq
pBR322 ori

Figure 155

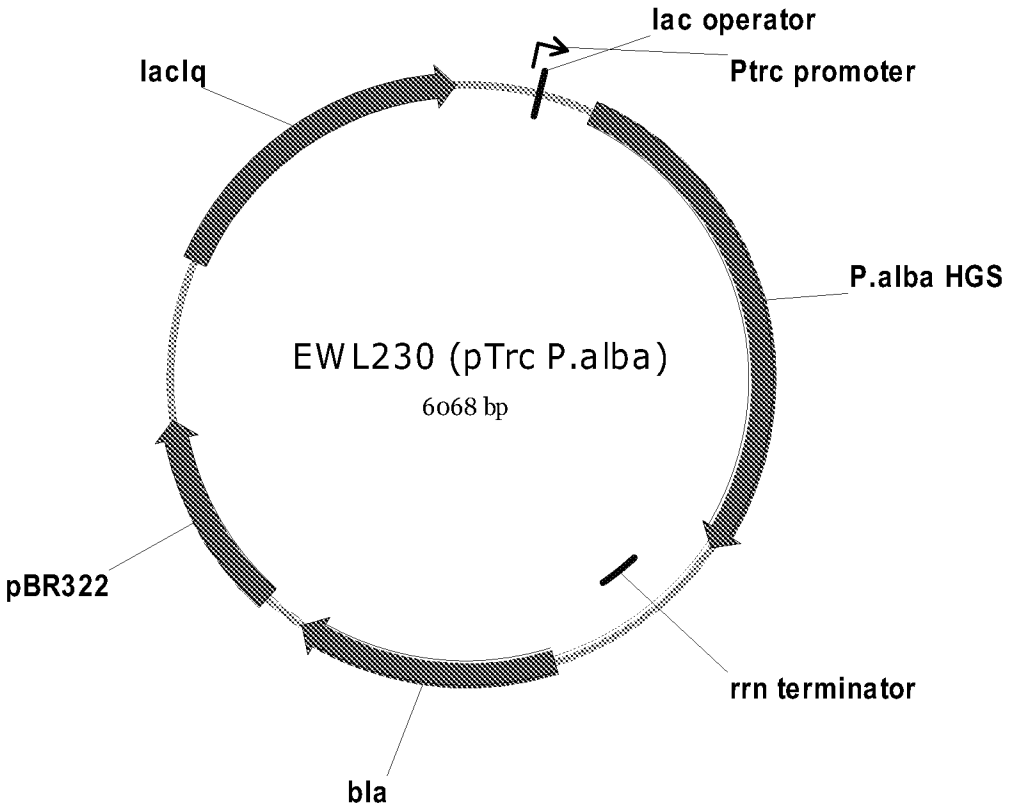
lac operator
Ptrc promoter
laclq
P.alba HGS
EWL230 (pTrc P.alba)
6068 bp
pBR322
rrn terminator
bla

Figure 156A

1

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Figure 156B

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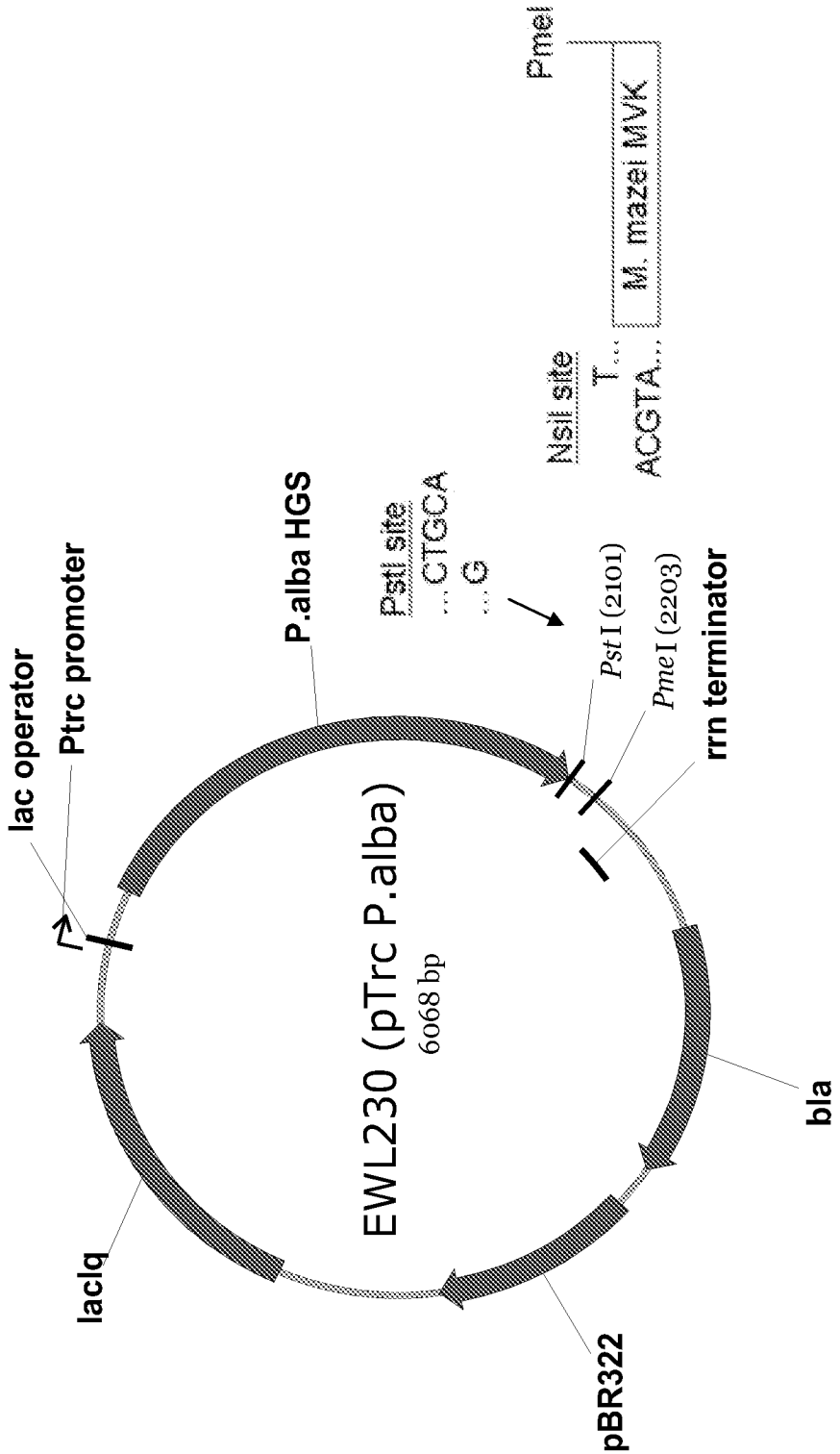
Figure 157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Ptrc promoter
P.alba HGS
PstI site
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...G
PstI (2101)
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NsiI site
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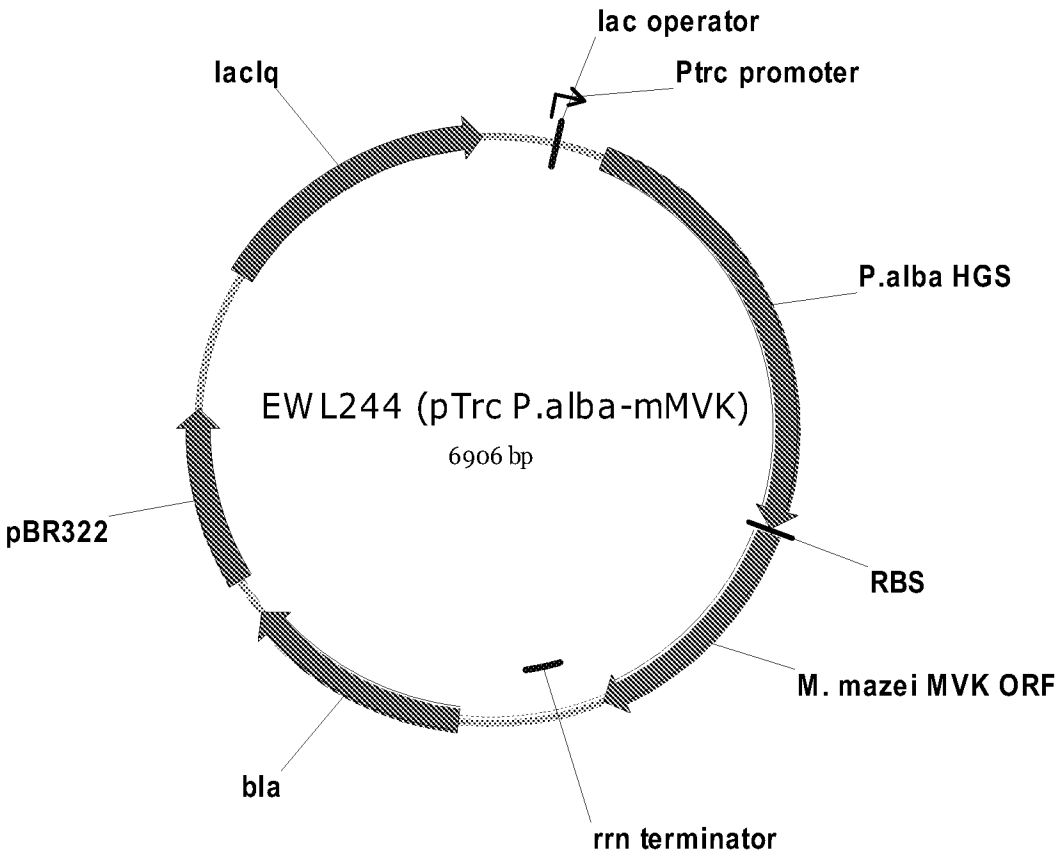
PmeI
M. mazei MVK
rrn terminator
bla
pBR322
laclq

Figure 158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laclq
Ptrc promoter
P.alba HGS
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pBR322
RBS
M. mazei MVK ORF
bla
rrn terminator

Figure 159A

Figure 159B

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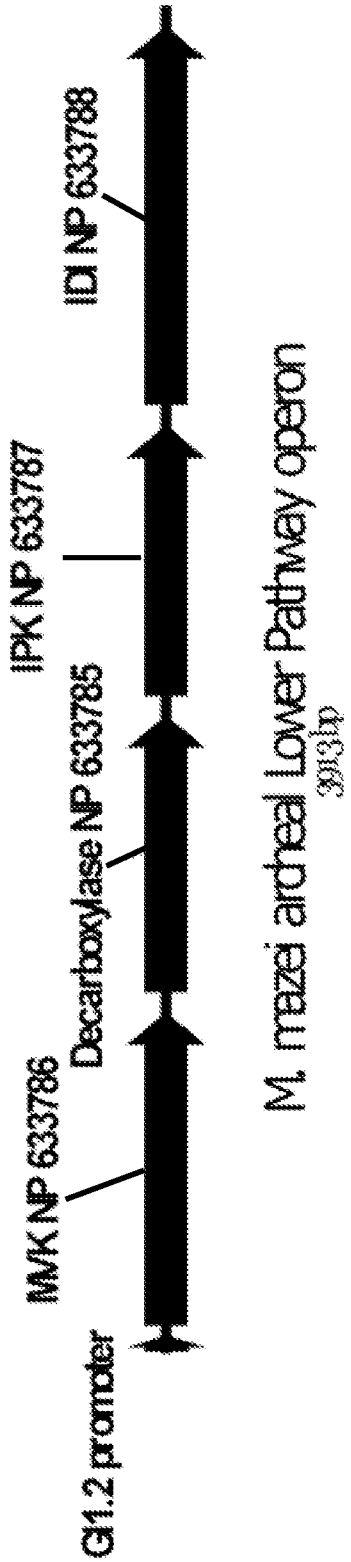
Figure 160A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IPK NP 633787
IDI NP 633788
M. mazei archeal Lower Pathway operon
3913bp

Figure 160B

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Figure 160C

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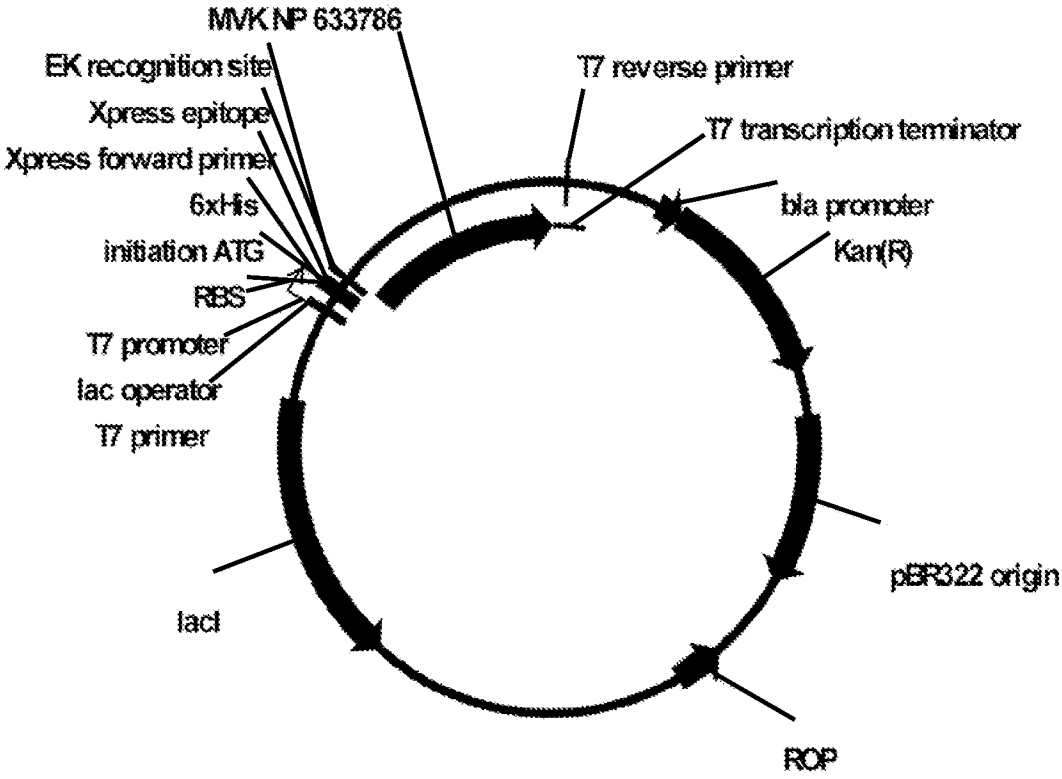
Figure 161A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6xHis
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RBS
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lac operator
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T7 transcription terminator
bla promoter
Kan(R)
pBR322 origin
ROP
MCM376 - MVK from M. mazei archeal Lowerin pET200D
6647 bp

Figure 161B

Figure 161C

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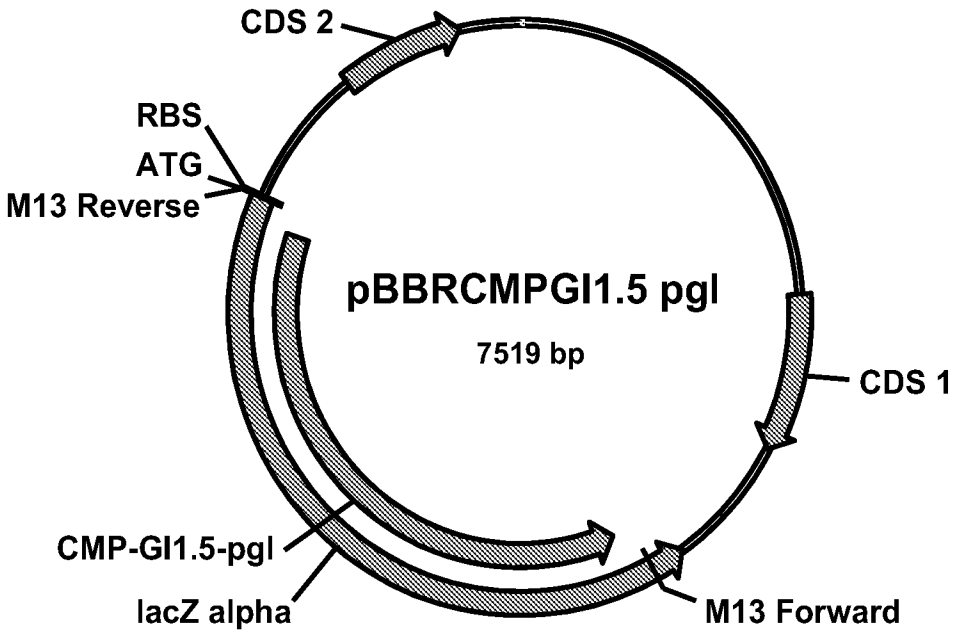

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Figure 163A

1-

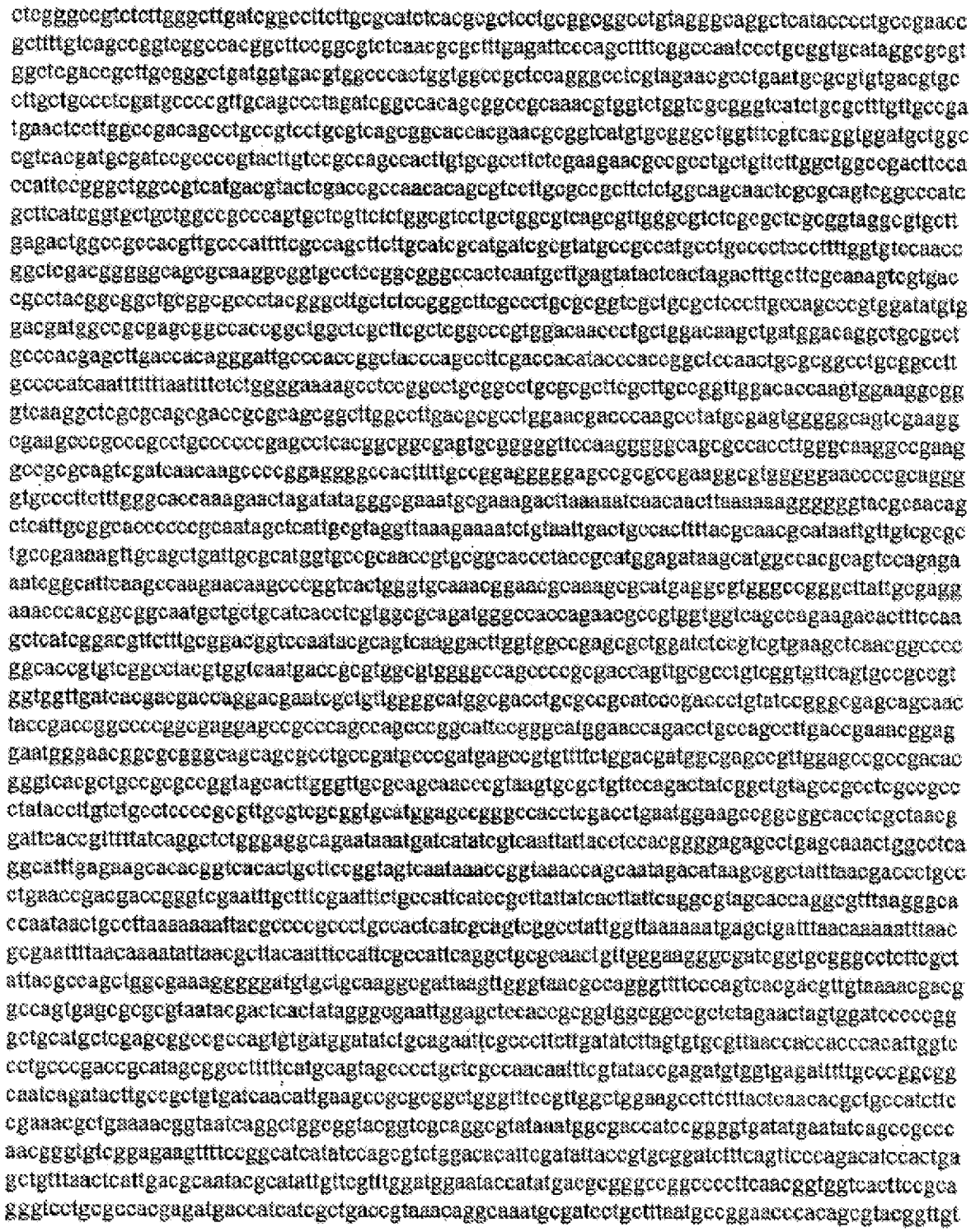

Figure 163B

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gcgcggggagaggcggtttgcgtattgggcgcatgcataaaaactgttgtaattcattaagcattctgccgacatggaagccatcacaaacg
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ggcacgaacccagttgacataagcctgttcggttcgtaaactgtaatgcaagtagcgtatgcgctcacgcaactggtccagaaccttgaccg
aacgcagcggtggtaacggcgcagtggcggttttcatggcttgttatgactgttttttgtacagtctatgcctcgggcatccaagcagcaagc
gcgttacgccgtgggtcgatgtttgatgttatggagcagcaacgatgttacgcagcagcaacgatgttacgcagcagggcagtcgccctaa
aacaaagttaggtggctcaagtatgggcatcattcgcacatgtaggctcggcccttgaccaagtcaaatccatgcgggctgctcttgatcttttc
ggtcgtgagttcggagacgtagccacctactcccaacatcagccggactccgattacctcgggaacttgctccgtagtaagacattcatcgc
gcttgctgccttcgaccaagaagcggttgttggcgctctcgcggcttacgttctgcccaggtttgagcagccgcgtagtgagatctatatctat
gatctcgcagtctccggcgagcaccggaggcagggcattgccaccgcgctcatcaatctcctcaagcatgaggccaacgcgcttggtgct
tatgtgatctacgtgcaagcagattacggtgacgatccccgcagtggctctctatacaaagttgggcatacgggaagaagtgatgcactttgat
atcgacccaagtaccgccacctaacaattcgttcaagccgagatcggcttcccggccgcggagttgttcggtaaattgtcacaacgccgcc
aggtggcacttttcggggaaatgtgcgcgcccgcgttcctgctggcgctgggcctgtttctggcgctggacttcccgctgttccgtcagcag
ctttcgcccacggccttgatgatcgcggcggccttggcctgcatatcccgattcaacggccccagggcgtccagaacgggcttcaggcgc
tcccgaaggt (SEQ ID NO:48).

Figure 164A

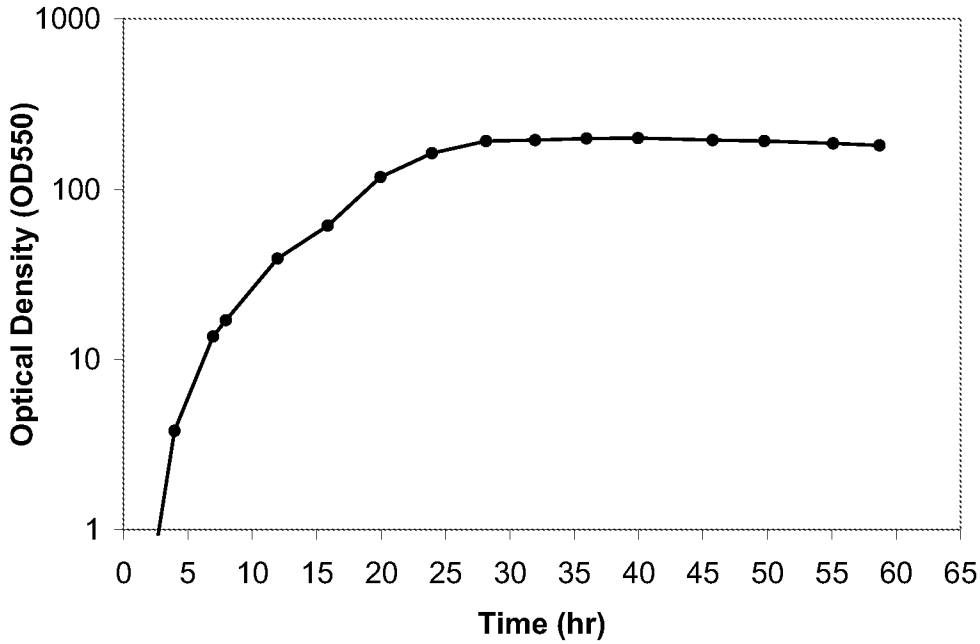

1000
100
10
1
Optical Density (OD550)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time (hr)

Figure 164B

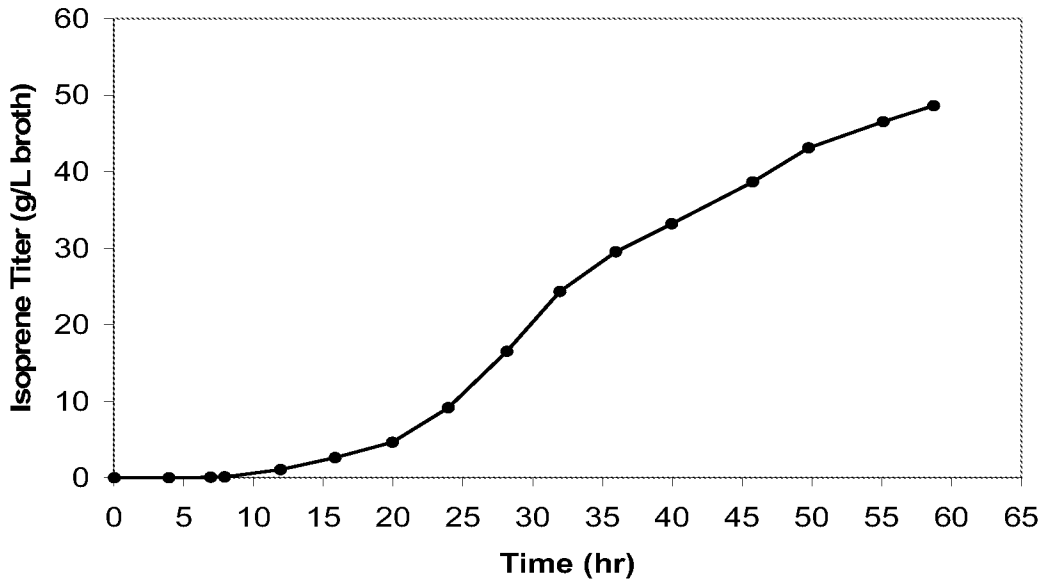

Isoprene Titer (g/L broth)
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Time (hr)

Figure 164C

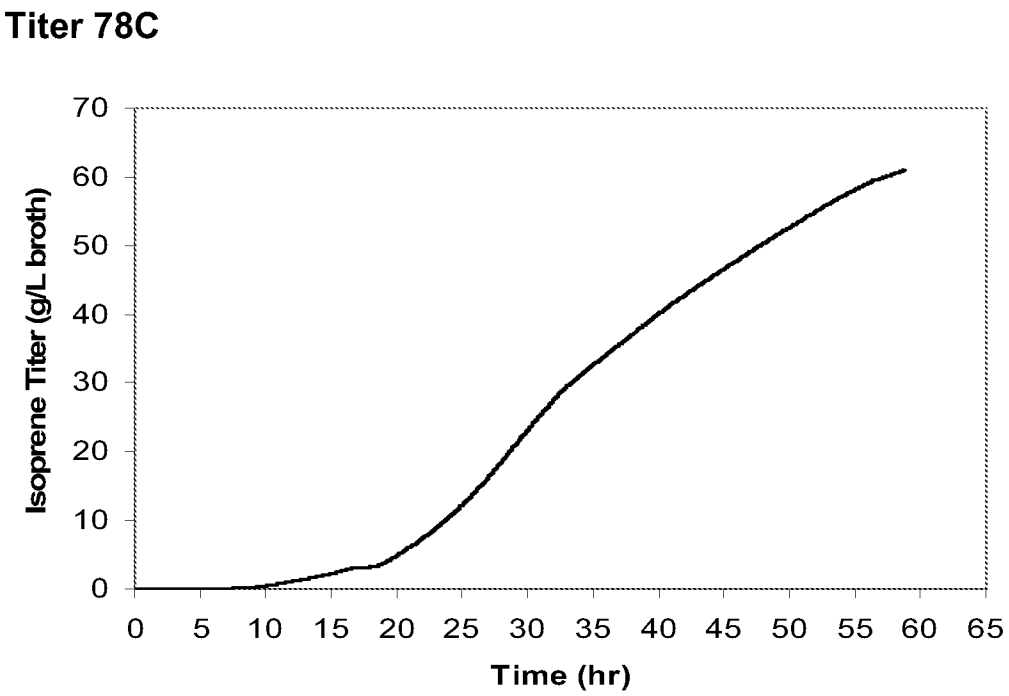

Titer 78C
Isoprene Titer (g/L broth)
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Time (hr)

Figure 164D

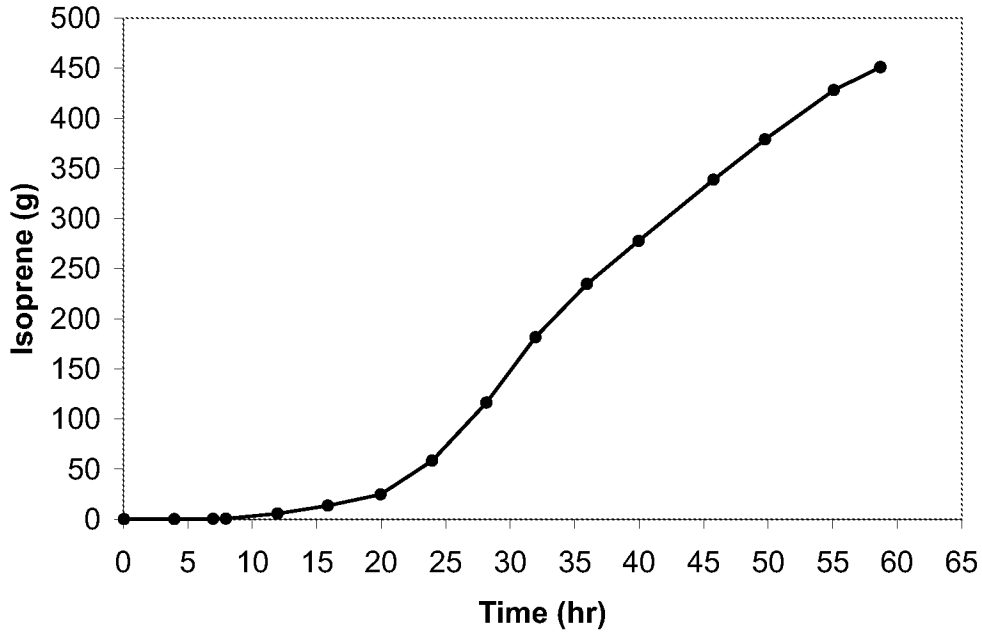

Isoprene (g)
500
450
400
350
300
250
200
150
100
50
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Time (hr)

Figure 164E

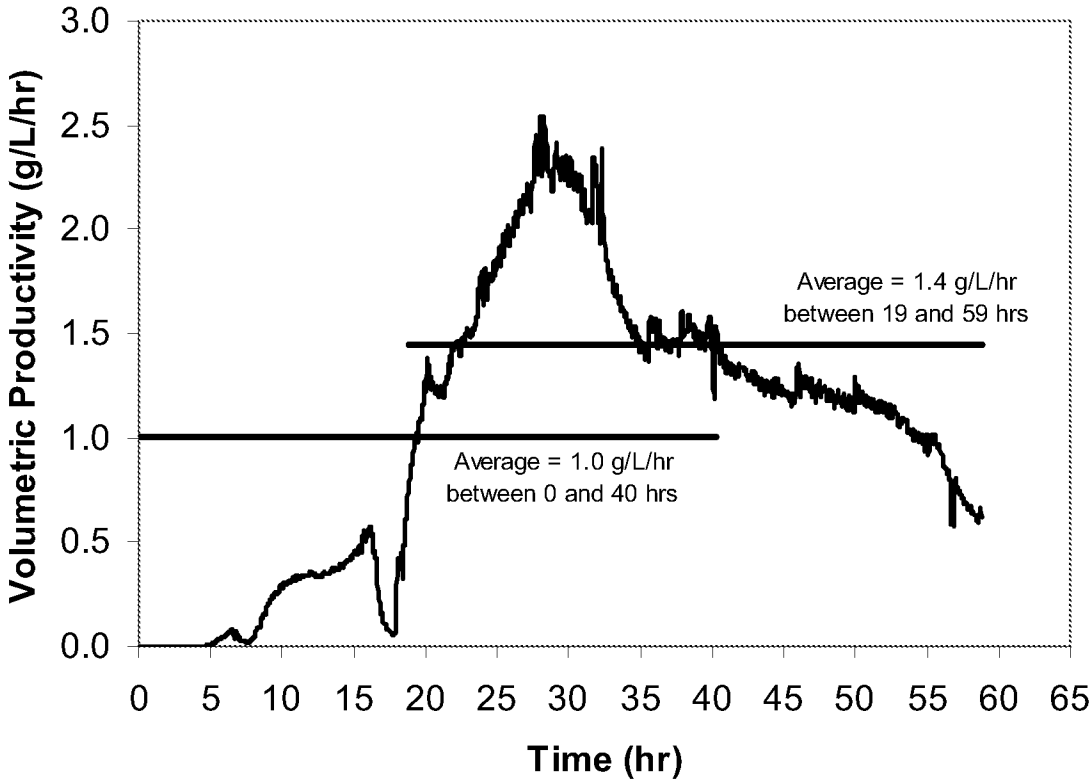

Volumetric Productivity (g/L/hr)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time (hr)
Average = 1.4 g/L/hr
between 19 and 59 hrs
Average = 1.0 g/L/hr
between 0 and 40 hrs

Figure 164F

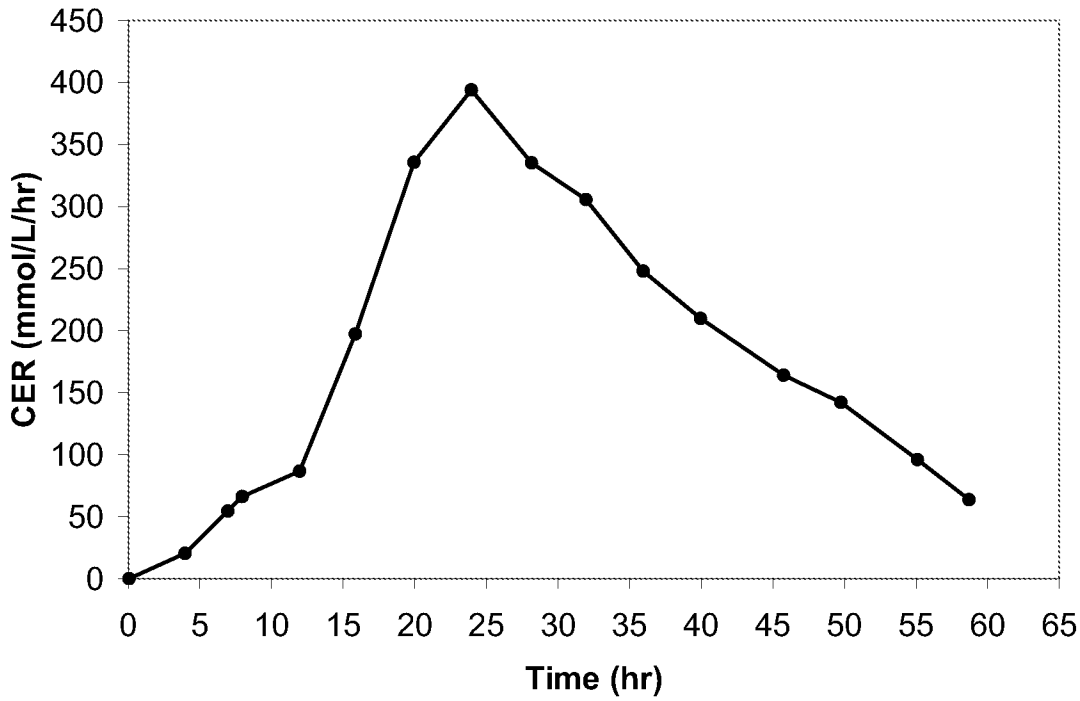

CER (mmol/L/hr)
450
400
350
300
250
200
150
100
50
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Time (hr)

Figure 165A-B

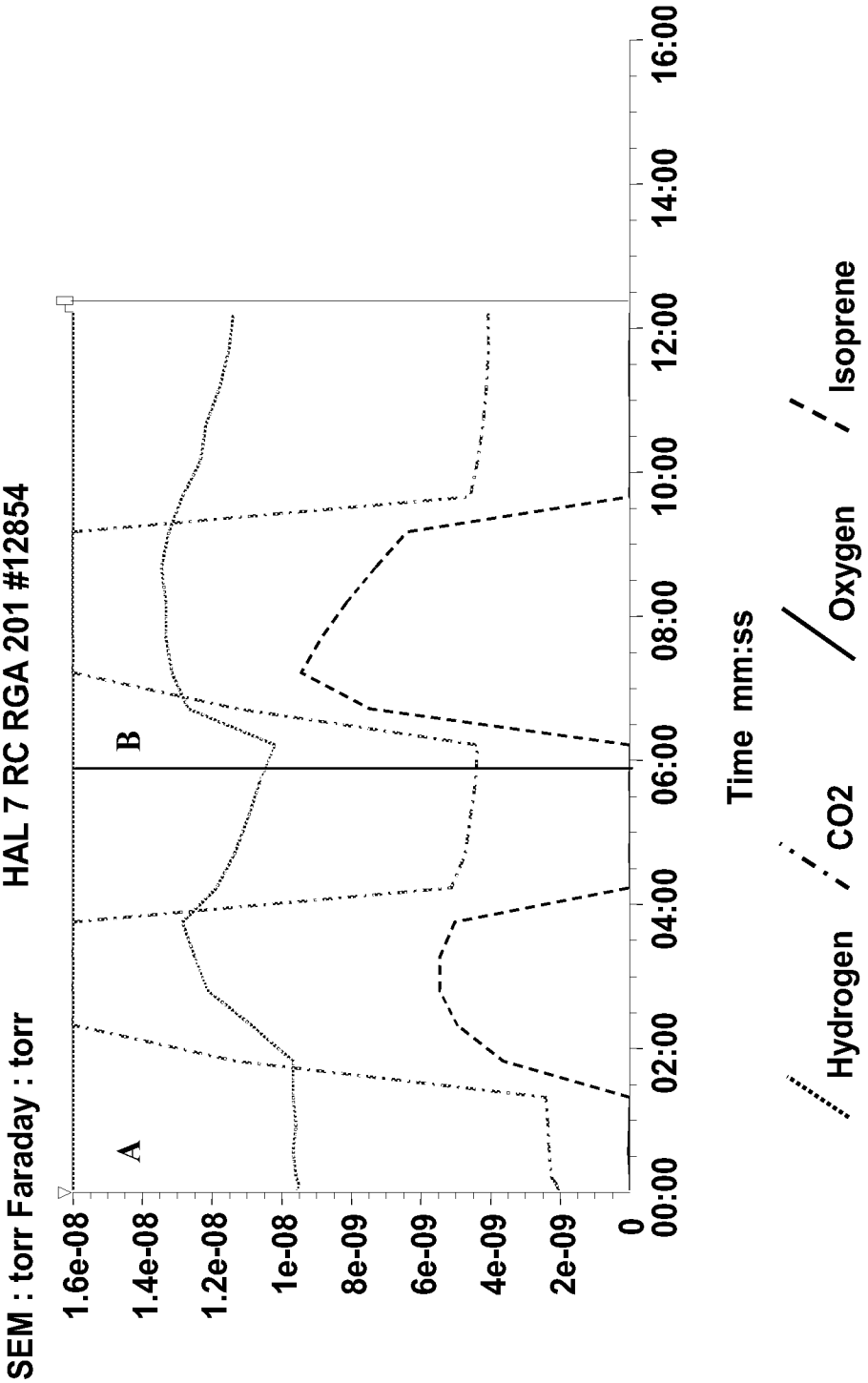


Figure 166A

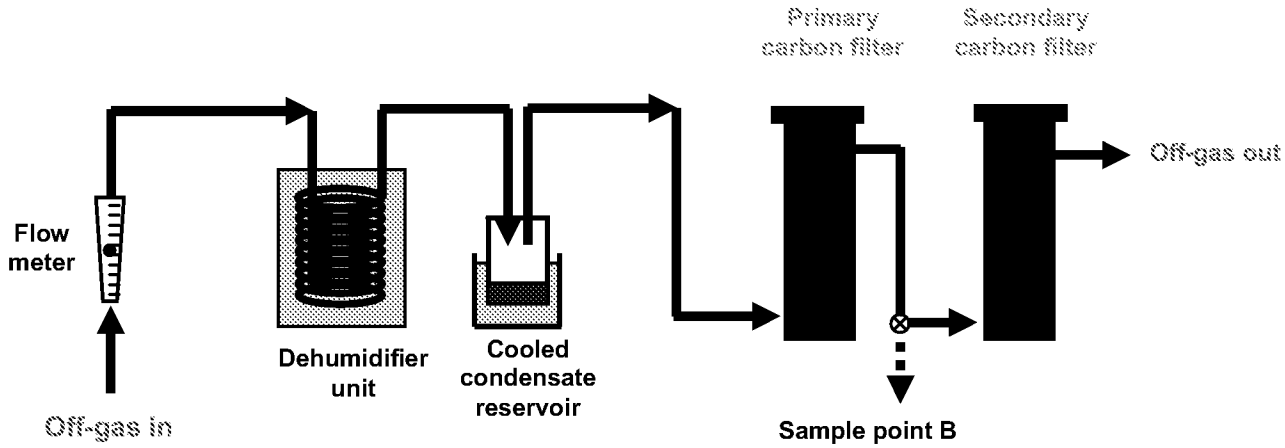

Primary carbon filter
Secondary carbon filter
Off-gas out
Flow meter
Dehumidifier unit
Cooled condensate reservoir
Off-gas in
Sample point B

Figure 166B

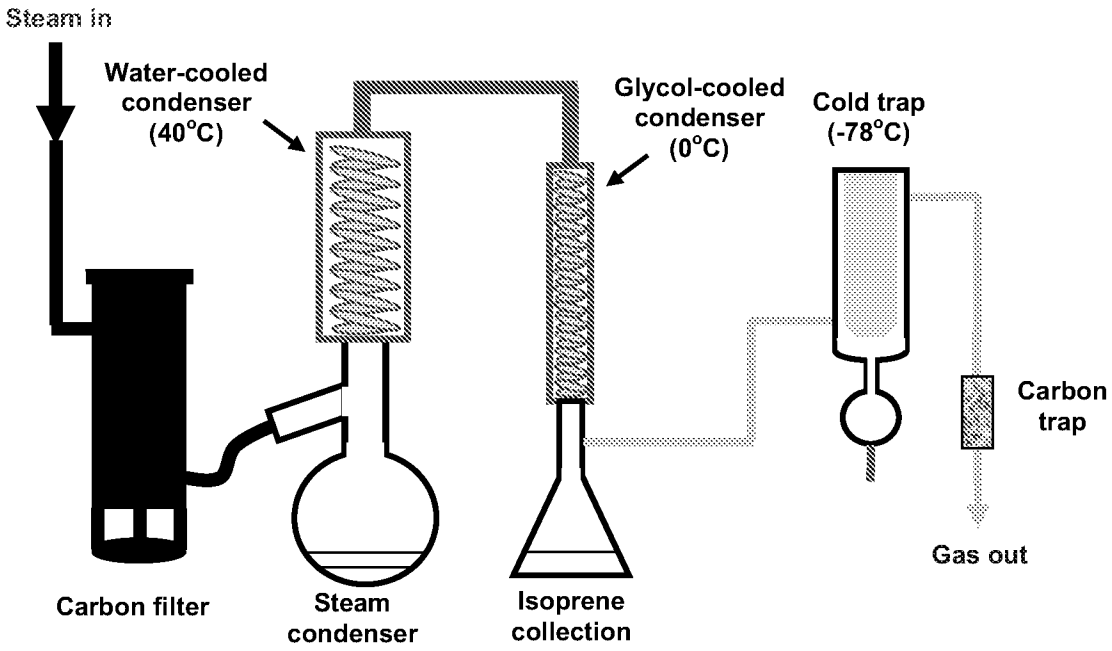

Steam in
Water-cooled condenser (40°C)
Glycol-cooled condenser (0°C)
Cold trap (-78°C)
Carbon trap
Gas out
Carbon filter
Steam condenser
Isoprene collection

Figure 167

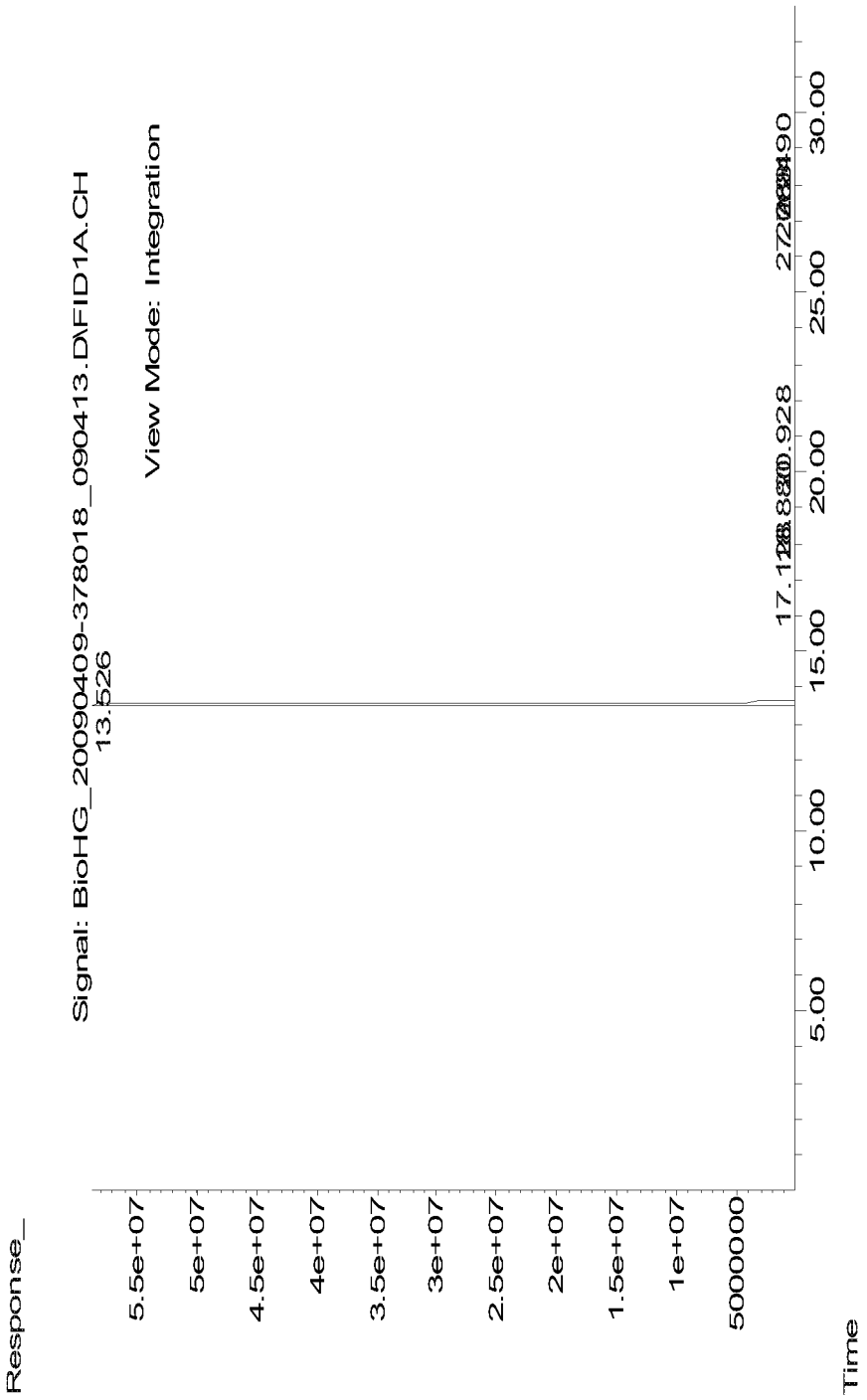

Response_
Signal: BioHG_20090409-378018_090413.DFID1A.CH
View Mode: Integration
13.526
17.128
20.928
27.190
5.5e+07
5e+07
4.5e+07
4e+07
3.5e+07
3e+07
2.5e+07
2e+07
1.5e+07
1e+07
5000000
5.00
10.00
15.00
20.00
25.00
30.00
Time

Figure 168A-C

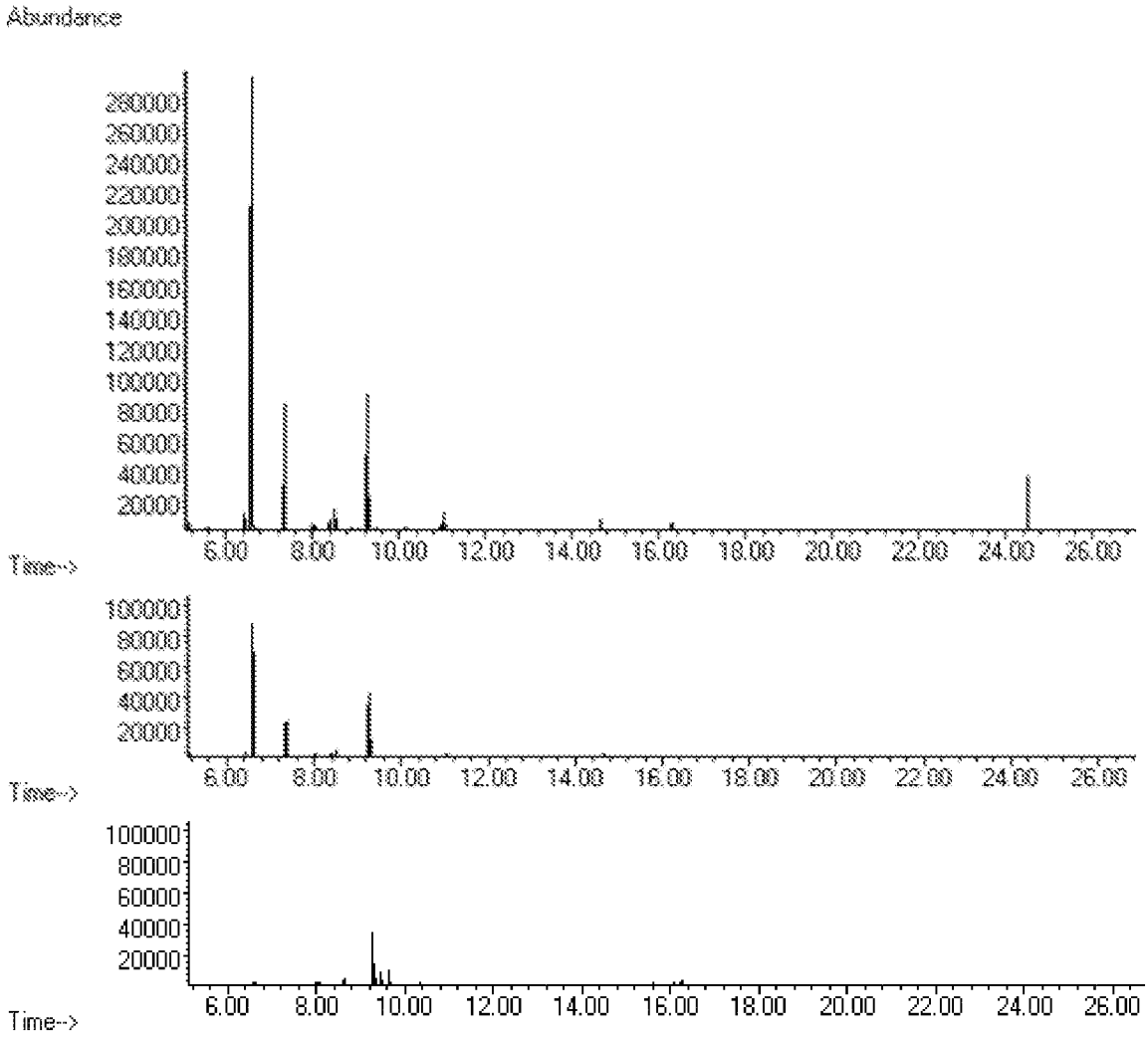


Figure 169

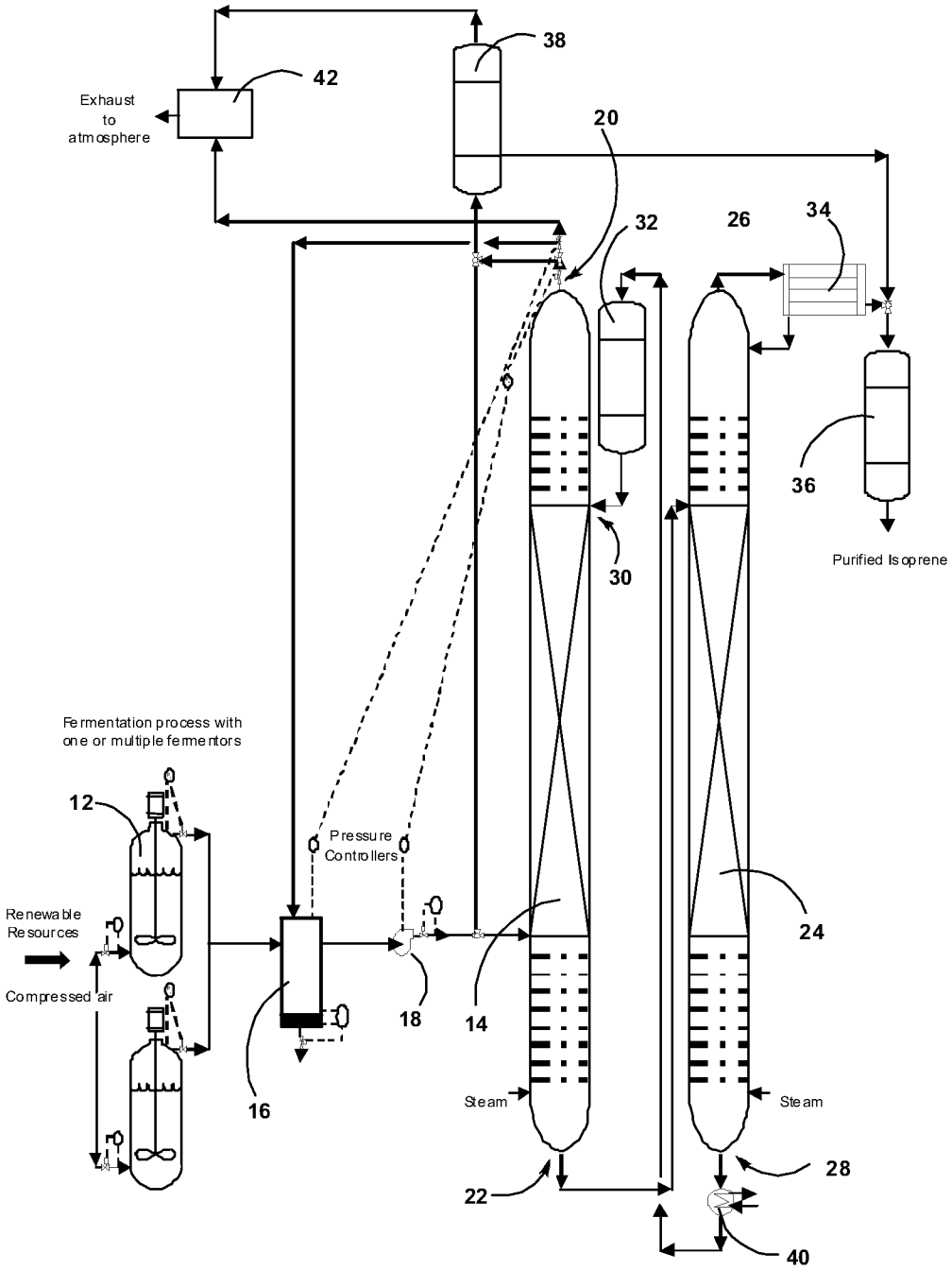

Exhaust to atmosphere
42
38
20
32
26
34
36
Purified Isoprene
30
Fermentation process with one or multiple fermentors
12
Pressure Controllers
Renewable Resources
Compressed air
18
14
16
24
Steam
Steam
22
28
40

Figure 170

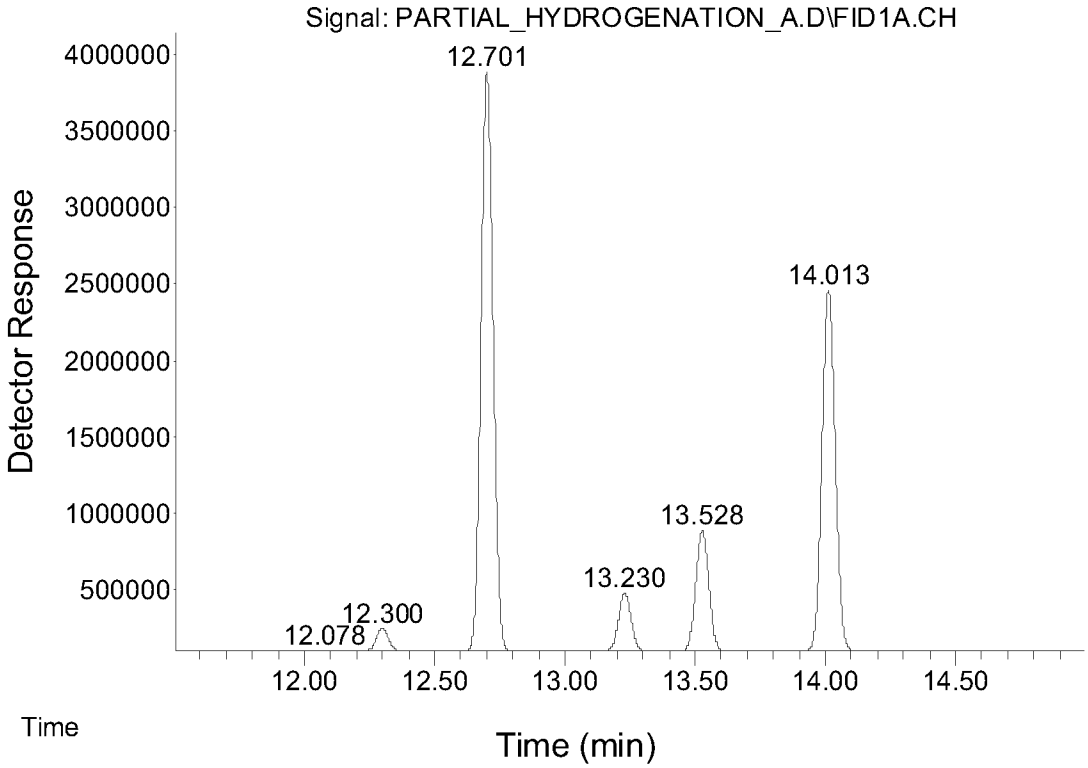

Signal: PARTIAL_HYDROGENATION_A.D\FID1A.CH
Detector Response
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
12.701
14.013
13.528
13.230
12.300
12.078
12.00
12.50
13.00
13.50
14.00
14.50
Time (min)
Time

Figure 171

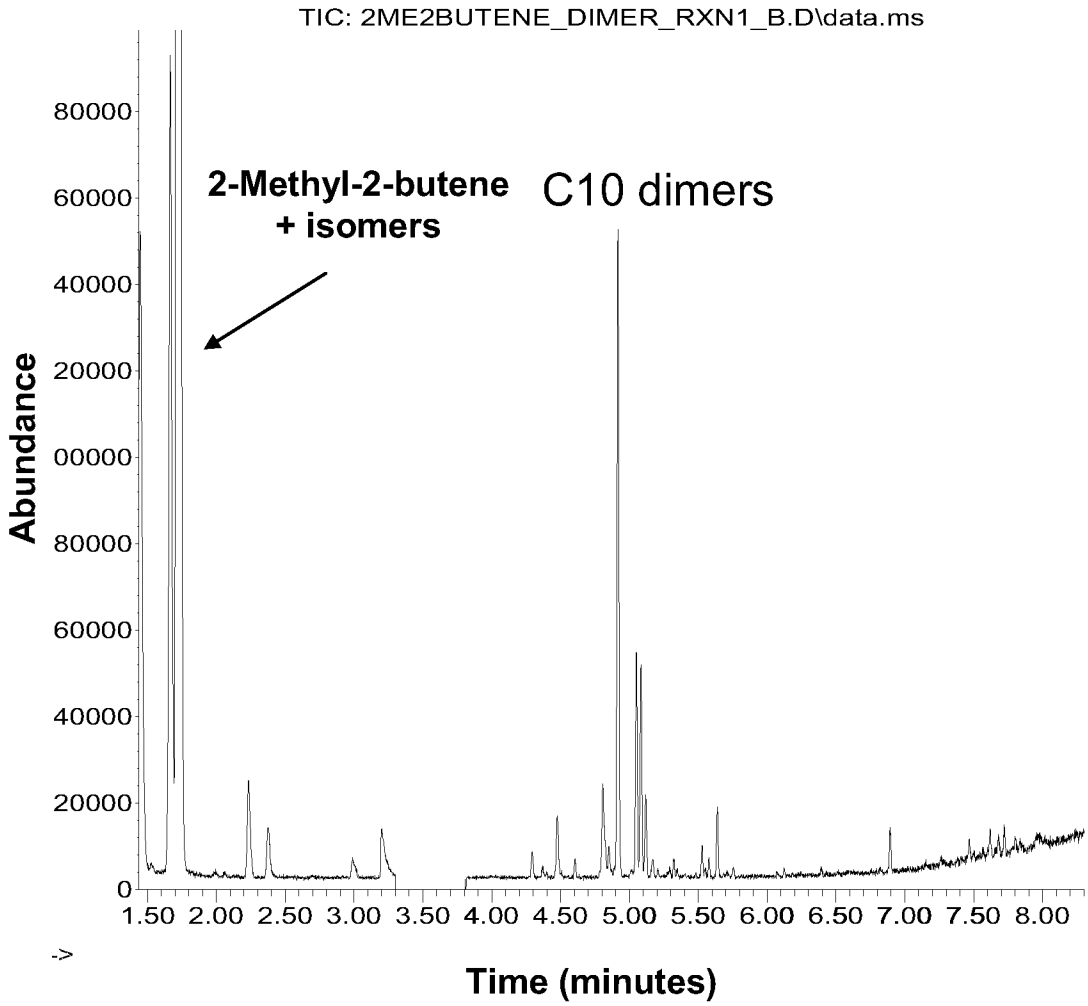

TIC: 2ME2BUTENE_DIMER_RXN1_B.D\data.ms
2-Methyl-2-butene
+ isomers
C10 dimers
Abundance
80000
60000
40000
20000
00000
80000
60000
40000
20000
0
1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50 7.00 7.50 8.00
->
Time (minutes)

Figure 172

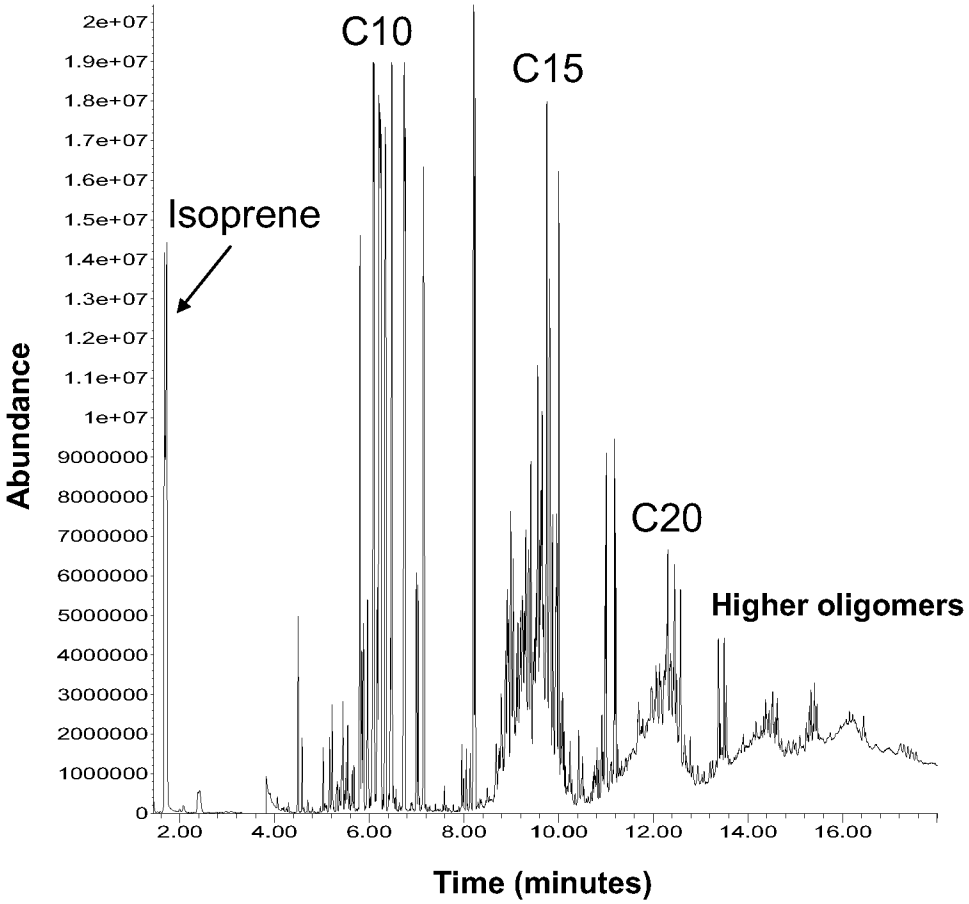
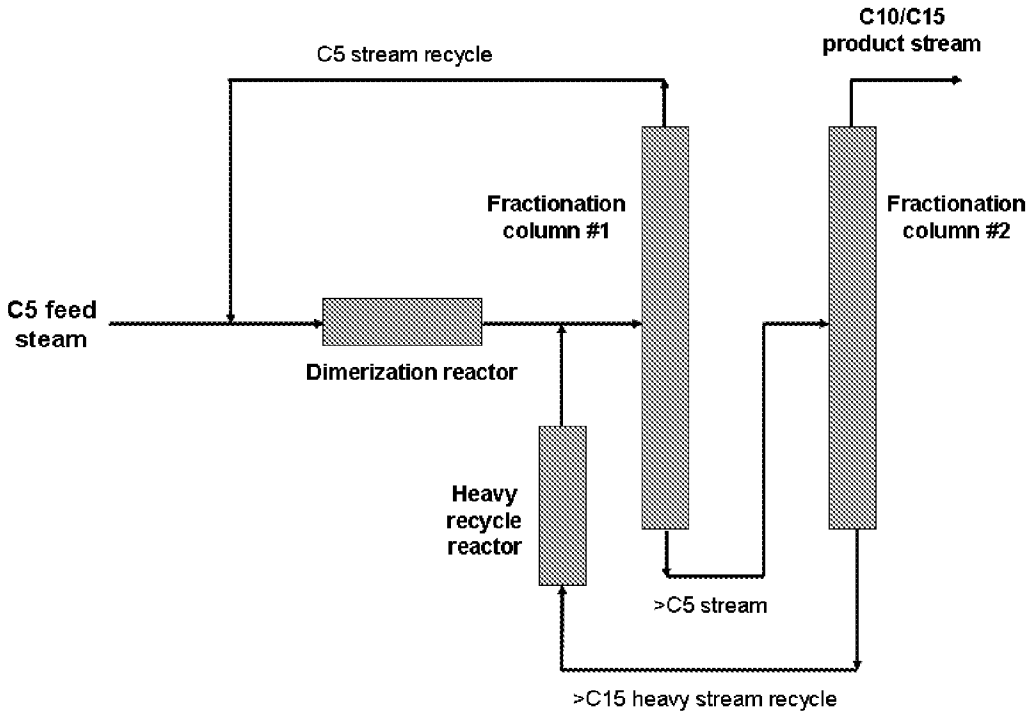

C10
C15
C20
Isoprene
Higher oligomers
Abundance
Time (minutes)
2e+07
1.9e+07
1.8e+07
1.7e+07
1.6e+07
1.5e+07
1.4e+07
1.3e+07
1.2e+07
1.1e+07
1e+07
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00

Figure 173


C10/C15
product stream
C5 stream recycle
Fractionation
column #1
Fractionation
column #2
C5 feed
steam
Dimerization reactor
Heavy
recycle
reactor
>C5 stream
>C15 heavy stream recycle

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/39088

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07C 5/02 (2010.01)
USPC - 585/265; 585/250
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)
IPC(8)-C07C 5/02 (2010.01)
USPC-585/265; 585/250

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents; Google Scholar
bioisoprene, isoprene, 2-methyl-l,3-butadiene, dimer$, oligomer$, dimate, condensation, cataly$, acid catalyst, palladium, iron, ruthenium, nickel, chromium, hydrogen$, isoamylene, conversion, dimersol, cyclooctane, fuel

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,166,076 A (ROOBEEK et al.) 28 August 1979 (28.08.1979) (col 1 ln 50-col 2 ln 20; col 6 ln 30-35; col 6 Table;	1-19
Y	US 3,418,386 A (HAYES) 24 December 1968 (24.12.1968) (col 1 ln 10-20; col 2 ln 50-72; Col 3 ln 20-70)	1-19
Y	US 5,906,662 A (MCCOMBES) 25 May 1999 (25.05.1999) (col 2 ln 50-67).	1-19
Y	US 5,329,057 A (DIESEN et al.) 12 July 1994 (12.07.1994) (Col 3 ln 60-67; ; col 11 ln 25-40;)	3, and 10
Y	US 3,917,730 A (THATCHENKO) 04 November 1975 (04.11.1975) (col 5 ln 25-col 6 ln 5)	5, and 13
Y	US 4,283,305 A (CHAUVIN et al.) 11 August 1981 (11.08.1981) (col 1 ln 5-15)	8, 9, and 16

☐ 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
12 August 2010 (12.08.2010)	23 AUG 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)