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(54) Titre : PRODUCTION D'ISOPRENE AUGMENTEE EN UTILISANT LA MEVALONATE KINASE ET L'ISOPRENE SYNTHASE

(54) Title: INCREASED ISOPRENE PRODUCTION USING MEVALONATE KINASE AND ISOPRENE SYNTHASE

Figure 19A

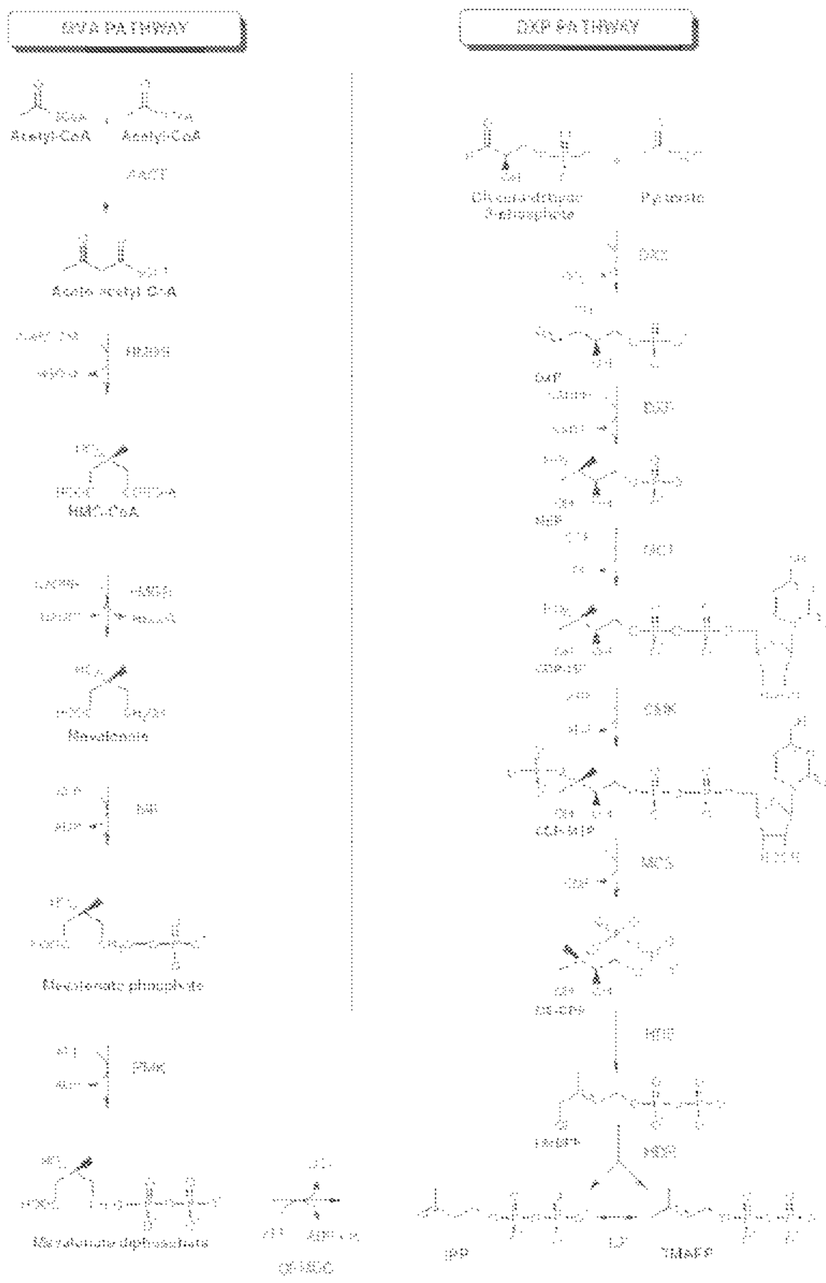
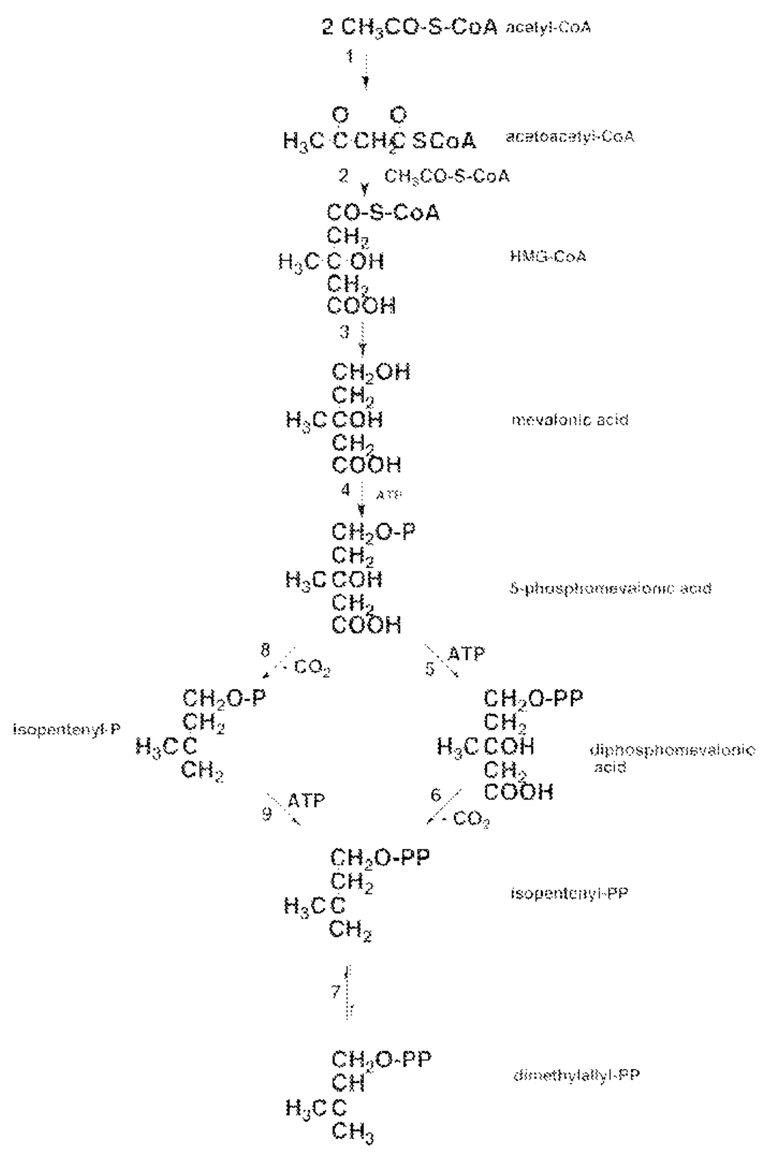


Figure 19B



(57) Abrégé/Abstract:
The invention features methods for producing isoprene from cultured cells having increased expression levels and/or activity levels of a mevalonate kinase polypeptide and an isoprene synthase polypeptide. The invention also provides methods for producing isoprene from cultured cells having reduced accumulation of intermediates (such as mevalonate, isopentenyl diphosphate, 3,3-dimethylallyl diphosphate, geranyl diphosphate, or farnesyl diphosphate) in the biosynthesis of isoprene or isoprenoids that may

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(57) Abrégé(suite)/Abstract(continued):
otherwise cause undesirable amounts of growth inhibition, toxicity, or cell death. The resulting isoprene compositions may have increased yields and/or purity of isoprene.

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(54) Title: INCREASED ISOPRENE PRODUCTION USING MEVALONATE KINASE AND ISOPRENE SYNTHASE

Figure 19A

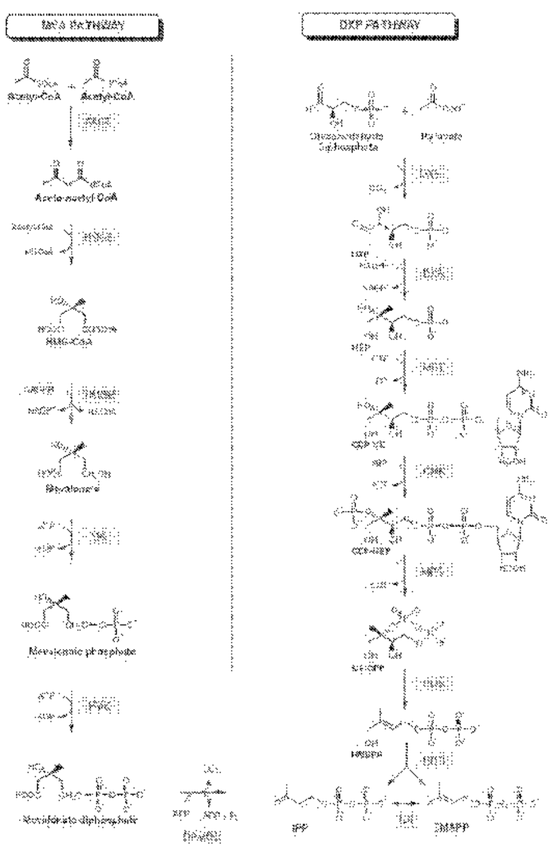
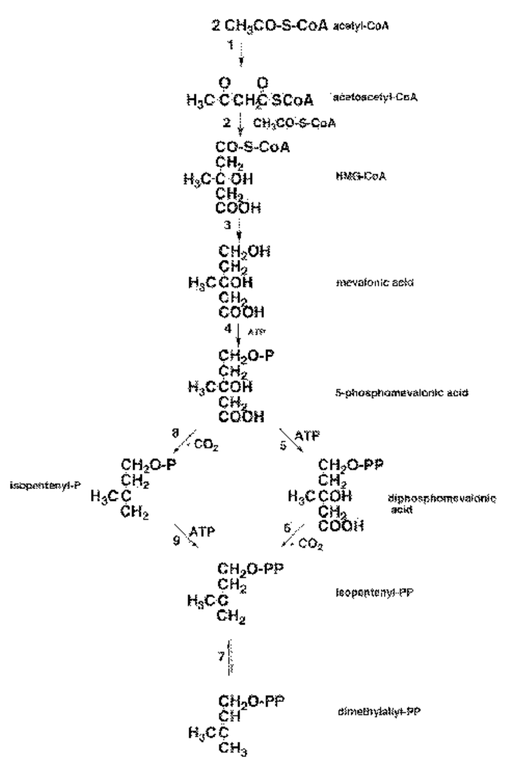


Figure 19B



(57) Abstract: The invention features methods for producing isoprene from cultured cells having increased expression levels and/or activity levels of a mevalonate kinase polypeptide and an isoprene synthase polypeptide. The invention also provides methods for producing isoprene from cultured cells having reduced accumulation of intermediates (such as mevalonate, isopentenyl diphosphate, 3,3-dimethylallyl diphosphate, geranyl diphosphate, or farnesyl diphosphate) in the biosynthesis of isoprene or isoprenoids that may otherwise cause undesirable amounts of growth inhibition, toxicity, or cell death. The resulting isoprene compositions may have increased yields and/or purity of isoprene.

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INCREASED ISOPRENE PRODUCTION USING MEVALONATE KINASE AND ISOPRENE SYNTHASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This applications claims the benefit of U.S. Provisional patent application 61/097,189, filed on September 15, 2008, the contents of which are hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

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

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

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

BRIEF SUMMARY OF THE INVENTION

[0005] The invention provides compositions, methods and systems for isoprene, making isoprene and using isoprene. In one aspect, the invention provides for cells in culture comprising a nucleic acid encoding a heterologous isoprene synthase polypeptide and one or more nucleic acids encoding MVA pathway polypeptides, wherein the cells further comprise i) one or more copies of a nucleic acid encoding a mevalonate kinase polypeptide, or ii) a nucleic acid encoding a mevalonate kinase polypeptide under the control of a strong promoter, and wherein the cells express the mevalonate kinase polypeptide at a level that is at least about 2-fold higher than the level of expression in cells that do not comprise one or more copies of a nucleic acid encoding a mevalonate kinase polypeptide or a nucleic acid encoding a mevalonate kinase polypeptide under the control of a strong promoter. In one embodiment, the cells produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In another embodiment, the mevalonate kinase polypeptide is *M. mazei* mevalonate kinase. In another embodiment, the MVA pathway polypeptide is selected from the group consisting of Lactobacillus mevalonate kinase polypeptide, *Lactobacillus sakei* mevalonate kinase polypeptide, yeast mevalonate kinase polypeptide, *Saccharomyces cerevisiae* mevalonate kinase polypeptide, Streptococcus mevalonate kinase polypeptide, *Streptococcus pneumoniae* mevalonate kinase polypeptide, and Streptomyces mevalonate kinase polypeptide, Streptomyces CL190 mevalonate kinase polypeptide. In another embodiment, the MVA pathway polypeptide is a polypeptide from *Saccharomyces cerevicia* or *Enterococcus faecalis*.

[0006] In another aspect, the invention features cells in culture that produce isoprene. In some embodiments, the cells in culture comprise a nucleic acid (such as a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid) encoding an isoprene synthase polypeptide and a nucleic acid (such as a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid) encoding a mevalonate kinase polypeptide as a first MVA pathway polypeptide. In some embodiments, the cells express the mevalonate kinase polypeptide at a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of a second MVA pathway polypeptide in the cell. In some embodiments, the nucleic acid encoding a mevalonate kinase polypeptide is under the control of a strong promoter. In some embodiments, the nucleic acid encoding a mevalonate kinase polypeptide

is under the control of a strong promoter, and the second MVA pathway polypeptide is not under the control of a strong promoter. In various embodiments, the second MVA pathway polypeptide is an acetyl-CoA acetyltransferase polypeptide, 3-hydroxy-3-methylglutaryl-CoA synthase polypeptide, 3-hydroxy-3-methylglutaryl-CoA reductase polypeptide, phosphomevalonate kinase polypeptide, diphosphomevalonate decarboxylase polypeptide, or isopentenyl-diphosphate delta-isomerase polypeptide. In some embodiments, the cells express an entire MVA pathway. In some embodiments, the mevalonate kinase polypeptide is an archaeal mevalonate kinase polypeptide (*e.g.*, a *Methanosarcina mazei* mevalonate kinase polypeptide), a Lactobacillus mevalonate kinase polypeptide (*e.g.*, a *Lactobacillus sakei* mevalonate kinase polypeptide), a yeast mevalonate kinase polypeptide (*e.g.*, a *Saccharomyces cerevisia* mevalonate kinase polypeptide), a Streptococcus mevalonate kinase polypeptide (*e.g.*, a *Streptococcus pneumoniae* mevalonate kinase polypeptide), or a Streptomyces mevalonate kinase polypeptide (*e.g.*, a Streptomyces CL190 mevalonate kinase polypeptide). In some embodiments, the cells in culture produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the cells in culture convert more than about 0.002% of the carbon in a cell culture medium into isoprene.

[0007] In some embodiments, the cells in culture comprise a nucleic acid (such as a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid) encoding an isoprene synthase polypeptide and a nucleic acid (such as a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid) encoding a mevalonate kinase polypeptide. In some embodiments, (i) the intracellular concentration of 3,3-dimethylallyl diphosphate (DMAPP) is between about 0 to about 25 μmol/g_{dcw}, (ii) the intracellular concentration of isopentenyl diphosphate (IPP) is between about 0 to about 60 μmol/g_{dcw}, (iii) the intracellular concentration of geranyl diphosphate (GPP) is between about 0 to about 8 μmol/g_{dcw}, (iv) the intracellular concentration of farnesyl diphosphate (FPP) is between about 0 to about 6 μmol/g_{dcw}, or (v) any combination of two or more of the foregoing. In some embodiments, the cells express an entire MVA pathway. In some embodiments, the mevalonate kinase polypeptide is an archaeal mevalonate kinase polypeptide (*e.g.*, a *Methanosarcina mazei* mevalonate kinase polypeptide), a Lactobacillus mevalonate kinase polypeptide (*e.g.*, a *Lactobacillus sakei* mevalonate kinase polypeptide), a yeast mevalonate kinase polypeptide (*e.g.*, a *Saccharomyces cerevisiae* mevalonate kinase polypeptide), a Streptococcus mevalonate kinase polypeptide (*e.g.*, a *Streptococcus pneumoniae* mevalonate kinase

polypeptide), or a Streptomyces mevalonate kinase polypeptide (*e.g.*, a Streptomyces CL190 mevalonate kinase polypeptide). In some embodiments, the cells in culture produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the cells in culture convert more than about 0.002% of the carbon in a cell culture medium into isoprene.

[0008] In some embodiments of any of the cells, 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.

[0009] In another aspect, the invention features compositions comprising any one or more of the cells described herein. In one aspect, the invention features compositions comprising cells in culture comprising a nucleic acid encoding a heterologous isoprene synthase polypeptide and one or more nucleic acids encoding MVA pathway polypeptides, wherein the cells further comprise i) one or more copies of a nucleic acid encoding a mevalonate kinase polypeptide, or ii) a nucleic acid encoding a mevalonate kinase polypeptide under the control of a strong promoter, and wherein the cells express the mevalonate kinase polypeptide at a level that is at least about 2-fold higher than the level of expression in cells that do not comprise one or more copies of a nucleic acid encoding a mevalonate kinase polypeptide or a nucleic acid encoding a mevalonate kinase polypeptide under the control of a strong promoter.

[0010] In one aspect, the invention features methods of producing isoprene, such as methods of using any of the cells described herein to produce isoprene. In one aspect, the invention features methods of producing isoprene, the method comprising (a) culturing cells in culture comprising a nucleic acid encoding a heterologous isoprene synthase polypeptide and one or more nucleic acids encoding MVA pathway polypeptides, wherein the cells further comprise i) one or more copies of a nucleic acid encoding a mevalonate kinase polypeptide, or ii) a nucleic acid encoding a mevalonate kinase polypeptide under the control of a strong promoter, and wherein the cells express the mevalonate kinase polypeptide at a level that is at least about 2-fold higher than the level of expression in cells that do not

comprise one or more copies of a nucleic acid encoding a mevalonate kinase polypeptide or a nucleic acid encoding a mevalonate kinase polypeptide under the control of a strong promoter under suitable culture conditions for the production of isoprene, and (b) producing isoprene. In some embodiments, the method involves culturing cells comprising a nucleic acid (such as a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid) encoding an isoprene synthase polypeptide and a nucleic acid (such as a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid) encoding a mevalonate kinase polypeptide as a first MVA pathway polypeptide. In some embodiments, the nucleic acid encoding a mevalonate kinase polypeptide is under the control of a strong promoter. In some embodiments, the nucleic acid encoding a mevalonate kinase polypeptide is under the control of a strong promoter, and the second MVA pathway polypeptide is not under the control of a strong promoter. In some embodiments, the cells are cultured under suitable culture conditions for the production of isoprene, and isoprene is produced. In some embodiments, the cells express the mevalonate kinase polypeptide at a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of a second MVA pathway polypeptide in the cell. In various embodiments, the second MVA pathway polypeptide is an acetyl-CoA acetyltransferase polypeptide, 3-hydroxy-3-methylglutaryl-CoA synthase polypeptide, 3-hydroxy-3-methylglutaryl-CoA reductase polypeptide, phosphomevalonate kinase polypeptide, diphosphomevalonate decarboxylase polypeptide, or isopentenyl-diphosphate delta-isomerase polypeptide. In some embodiments, the cells express an entire MVA pathway. In some embodiments, the mevalonate kinase polypeptide is an archaeal mevalonate kinase polypeptide (*e.g.*, a *Methanosarcina mazei* mevalonate kinase polypeptide), a Lactobacillus mevalonate kinase polypeptide (*e.g.*, a *Lactobacillus sakei* mevalonate kinase polypeptide), a yeast mevalonate kinase polypeptide (*e.g.*, a *Saccharomyces cerevisia* mevalonate kinase polypeptide), a Streptococcus mevalonate kinase polypeptide (*e.g.*, a *Streptococcus pneumoniae* mevalonate kinase polypeptide), or a Streptomyces mevalonate kinase polypeptide (*e.g.*, a Streptomyces CL190 mevalonate kinase polypeptide). In some embodiments, the method involves culturing cells under conditions sufficient to produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the method 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.

[0011] In some embodiments, the method involves culturing cells comprising a nucleic acid (such as a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid) encoding an isoprene synthase polypeptide and a nucleic acid (such as a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid) encoding a mevalonate kinase polypeptide. In some embodiments, (i) the intracellular concentration of DMAPP is between about 0 to about 25 μmol/g_{dcw}, (ii) the intracellular concentration of IPP is between about 0 to about 60 μmol/g_{dcw}, (iii) the intracellular concentration of GPP is between about 0 to about 8 μmol/g_{dcw}, (iv) the intracellular concentration of FPP is between about 0 to about 6 μmol/g_{dcw}, or (v) any combination of two or more of the foregoing. In some embodiments, the cells are cultured under suitable culture conditions for the production of isoprene, and isoprene is produced. In some embodiments, the cells express an entire MVA pathway. In some embodiments, the mevalonate kinase polypeptide is an archaeal mevalonate kinase polypeptide (*e.g.*, a *Methanosarcina mazei* mevalonate kinase polypeptide), a Lactobacillus mevalonate kinase polypeptide (*e.g.*, a *Lactobacillus sakei* mevalonate kinase polypeptide), a yeast mevalonate kinase polypeptide (*e.g.*, a *Saccharomyces cerevisia* mevalonate kinase polypeptide), a Streptococcus mevalonate kinase polypeptide (*e.g.*, a *Streptococcus pneumoniae* mevalonate kinase polypeptide), or a Streptomyces mevalonate kinase polypeptide (*e.g.*, a Streptomyces CL190 mevalonate kinase polypeptide). In some embodiments, the method involves culturing cells under conditions sufficient to produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the method 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.

[0012] In some embodiments of any of the methods, 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 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.

[0013] In some embodiments of any of the compositions, systems, and methods of the invention, a mevalonate kinase polypeptide and/or an isoprene synthase polypeptide is expressed a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold (i) higher than the level of expression of a second MVA pathway polypeptide (such as an acetyl-CoA acetyltransferase polypeptide, 3-hydroxy-3-methylglutaryl-CoA synthase polypeptide, 3-hydroxy-3-methylglutaryl-CoA reductase polypeptide, phosphomevalonate kinase polypeptide, diphosphomevalonate decarboxylase polypeptide, or isopentenyl-diphosphate delta-isomerase polypeptide) or (ii) higher than the level of expression of all other MVA pathway polypeptides in the cell. In particular embodiments, the mevalonate kinase polypeptide and/or an isoprene synthase polypeptide is expressed a level that is at least about any of 2, 5, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of an acetyl-CoA acetyltransferase polypeptide, 3-hydroxy-3-methylglutaryl-CoA synthase polypeptide, and 3-hydroxy-3-methylglutaryl-CoA reductase polypeptide. In particular embodiments, the mevalonate kinase polypeptide and/or an isoprene synthase polypeptide is expressed a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of an phosphomevalonate kinase polypeptide, diphosphomevalonate decarboxylase polypeptide, and isopentenyl-diphosphate delta-isomerase polypeptide. In some embodiments, the total amount of mevalonate kinase polypeptide is similar to the total amount of isoprene synthase polypeptide. For example, in some embodiments, the total amount of mevalonate kinase polypeptide is within about any of 10, 8, 6, 4, 2, 1, or 0.5-fold higher or lower than the total

amount of isoprene synthase polypeptide (*e.g.*, the amount of mevalonate kinase polypeptide may be between about 10-fold lower to about 10-fold higher than the amount of isoprene synthase polypeptide).

[0014] In some embodiments of any of the compositions, systems, and methods of the invention, a mevalonate kinase RNA molecule and/or an isoprene synthase RNA molecule is expressed a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold (i) higher than the level of expression of a second MVA pathway RNA molecule (such as an acetyl-CoA acetyltransferase RNA molecule, 3-hydroxy-3-methylglutaryl-CoA synthase RNA molecule, 3-hydroxy-3-methylglutaryl-CoA reductase RNA molecule, phosphomevalonate kinase RNA molecule, diphosphomevalonate decarboxylase RNA molecule, or isopentenyl-diphosphate delta-isomerase RNA molecule) or (ii) higher than the level of expression of all other MVA pathway RNA molecules in the cell. In particular embodiments, the mevalonate kinase RNA molecule and/or an isoprene synthase RNA molecule is expressed a level that is at least about any of 2, 5, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of an acetyl-CoA acetyltransferase RNA molecule, 3-hydroxy-3-methylglutaryl-CoA synthase RNA molecule, and 3-hydroxy-3-methylglutaryl-CoA reductase RNA molecule. In particular embodiments, the mevalonate kinase RNA molecule and/or an isoprene synthase RNA molecule is expressed a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of an phosphomevalonate kinase RNA molecule, diphosphomevalonate decarboxylase RNA molecule, and isopentenyl-diphosphate delta-isomerase RNA molecule. In some embodiments, the total amount of mevalonate kinase RNA is similar to the total amount of isoprene synthase RNA. For example, in some embodiments, the total amount of mevalonate kinase RNA is within about any of 10, 8, 6, 4, 2, 1, or 0.5-fold higher or lower than the total amount of isoprene synthase RNA (*e.g.*, the amount of mevalonate kinase RNA may be between about 10-fold lower to about 10-fold higher than the amount of isoprene synthase RNA).

[0015] In some embodiments of any of the compositions, systems, and methods of the invention, the number of copies of a mevalonate kinase DNA molecule and/or an isoprene synthase DNA molecule is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100,

125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold (i) higher than the number of copies of a second MVA pathway DNA molecule (such as an acetyl-CoA acetyltransferase DNA molecule, 3-hydroxy-3-methylglutaryl-CoA synthase DNA molecule, 3-hydroxy-3-methylglutaryl-CoA reductase DNA molecule, phosphomevalonate kinase DNA molecule, diphosphomevalonate decarboxylase DNA molecule, or isopentenyl-diphosphate delta-isomerase DNA molecule) or (ii) higher than the number of copies of all other MVA pathway DNA molecules in the cell. In particular embodiments, the number of copies of a mevalonate kinase DNA molecule and/or an isoprene synthase DNA molecule is at least about any of 2, 5, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the number of copies of an acetyl-CoA acetyltransferase DNA molecule, 3-hydroxy-3-methylglutaryl-CoA synthase DNA molecule, and 3-hydroxy-3-methylglutaryl-CoA reductase DNA molecule. In particular embodiments, the number of copies of a mevalonate kinase DNA molecule and/or an isoprene synthase DNA molecule is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the number of copies of an phosphomevalonate kinase DNA molecule, diphosphomevalonate decarboxylase DNA molecule, and isopentenyl-diphosphate delta-isomerase DNA molecule. In some embodiments, the number of copies of a mevalonate kinase DNA molecule is similar to the number of copies of an isoprene synthase DNA molecule. For example, in some embodiments, the number of copies of a mevalonate kinase DNA molecule is within about any of 10, 8, 6, 4, 2, 1, or 0.5-fold higher or lower than the number of copies of an isoprene synthase DNA molecule (*e.g.*, the number of copies of a mevalonate kinase DNA may be between about 10-fold lower to about 10-fold higher than the number of copies of an isoprene synthase DNA molecule).

[0016] In some embodiments of any of the compositions, systems, and methods of the invention, the intracellular concentration of DMAPP is between about 0 to about 25 $\mu mol/g_{dcw}$, such as between about 0.1 to about 20 $\mu mol/g_{dcw}$, about 0. 1 to about 15 $\mu mol/g_{dcw}$, about 0.1 to about 11 $\mu mol/g_{dcw}$, about 0.1 to about 7 $\mu mol/g_{dcw}$, about 0.1 to about 5 $\mu mol/g_{dcw}$, about 0.1 to about 2 $\mu mol/g_{dcw}$, about 0.1 to about 1 $\mu mol/g_{dcw}$, about 0.1 to about 0.8 $\mu mol/g_{dcw}$, about 0.1 to about 0.6 $\mu mol/g_{dcw}$, about 0.2 to about 15 $\mu mol/g_{dcw}$, about 0.2 to about 11 $\mu mol/g_{dcw}$, about 0.2 to about 7 $\mu mol/g_{dcw}$, about 0.2 to about 5 $\mu mol/g_{dcw}$, about 0.2 to about 2 $\mu mol/g_{dcw}$, about 0.3 to about 11 $\mu mol/g_{dcw}$, about 0.3 to about 7

$\mu mol/g_{dcw}$, about 0.3 to about 5 $\mu mol/g_{dcw}$, about 0.3 to about 2 $\mu mol/g_{dcw}$, about 0.3 to about 1 $\mu mol/g_{dcw}$, about 0.4 to about 11 $\mu mol/g_{dcw}$, about 0.4 to about 7 $\mu mol/g_{dcw}$, about 0.4 to about 5 $\mu mol/g_{dcw}$, about 0.4 to about 2 $\mu mol/g_{dcw}$, about 0.5 to about 7 $\mu mol/g_{dcw}$, about 0.5 to about 5 $\mu mol/g_{dcw}$, or about 0.5 to about 2 $\mu mol/g_{dcw}$. In some embodiments, the intracellular concentration of DMAPP is equal to or less than about any of 25, 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 $\mu mol/g_{dcw}$.

[0017] In some embodiments of any of the compositions, systems, and methods of the invention, the intracellular concentration of IPP is between about 0 to about 60 $\mu mol/g_{dcw}$, such as between about 0.1 to about 50 $\mu mol/g_{dcw}$, about 0.1 to about 40 $\mu mol/g_{dcw}$, about 0.1 to about 30 $\mu mol/g_{dcw}$, about 0.1 to about 20 $\mu mol/g_{dcw}$, about 0. 1 to about 15 $\mu mol/g_{dcw}$, about 0.1 to about 11 $\mu mol/g_{dcw}$, about 0.1 to about 7 $\mu mol/g_{dcw}$, about 0.1 to about 5 $\mu mol/g_{dcw}$, about 0.1 to about 2 $\mu mol/g_{dcw}$, about 0.1 to about 1 $\mu mol/g_{dcw}$, about 0.1 to about 0.8 $\mu mol/g_{dcw}$, about 0.1 to about 0.6 $\mu mol/g_{dcw}$, about 0.2 to about 60 $\mu mol/g_{dcw}$, about 0.2 to about 50 $\mu mol/g_{dcw}$, about 0.2 to about 40 $\mu mol/g_{dcw}$, about 0.2 to about 30 $\mu mol/g_{dcw}$, about 0.2 to about 20 $\mu mol/g_{dcw}$, about 0.2 to about 15 $\mu mol/g_{dcw}$, about 0.2 to about 11 $\mu mol/g_{dcw}$, about 0.2 to about 7 $\mu mol/g_{dcw}$, about 0.2 to about 5 $\mu mol/g_{dcw}$, about 0.2 to about 2 $\mu mol/g_{dcw}$, about 0.3 to about 60 $\mu mol/g_{dcw}$, about 0.3 to about 50 $\mu mol/g_{dcw}$, about 0.3 to about 40 $\mu mol/g_{dcw}$, about 0.3 to about 30 $\mu mol/g_{dcw}$, about 0.3 to about 15 $\mu mol/g_{dcw}$, about 0.3 to about 11 $\mu mol/g_{dcw}$, about 0.3 to about 7 $\mu mol/g_{dcw}$, about 0.3 to about 5 $\mu mol/g_{dcw}$, about 0.3 to about 2 $\mu mol/g_{dcw}$, about 0.4 to about 60 $\mu mol/g_{dcw}$, about 0.4 to about 50 $\mu mol/g_{dcw}$, about 0.4 to about 40 $\mu mol/g_{dcw}$, about 0.4 to about 30 $\mu mol/g_{dcw}$, about 0.4 to about 15 $\mu mol/g_{dcw}$, about 0.4 to about 7 $\mu mol/g_{dcw}$, about 0.4 to about 5 $\mu mol/g_{dcw}$, about 0.4 to about 2 $\mu mol/g_{dcw}$, about 0.5 to about 60 $\mu mol/g_{dcw}$, about 0.5 to about 50 $\mu mol/g_{dcw}$, about 0.5 to about 40 $\mu mol/g_{dcw}$, about 0.5 to about 30 $\mu mol/g_{dcw}$, about 0.5 to about 15 $\mu mol/g_{dcw}$, about 0.5 to about 11 $\mu mol/g_{dcw}$, about 0.5 to about 7 $\mu mol/g_{dcw}$, about 0.5 to about 5 $\mu mol/g_{dcw}$, or about 0.5 to about 2 $\mu mol/g_{dcw}$. In some embodiments, the intracellular concentration of IPP is equal to or less than about any of 60, 50, 40, 30, 25, 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 $\mu mol/g_{dcw}$.

[0018] In some embodiments of any of the compositions, systems, and methods of the invention, the intracellular concentration of GPP is between about 0 to about 8 $\mu mol/g_{dcw}$, such as between about 0.1 to about 7 $\mu mol/g_{dcw}$, about 0. 1 to about 6 $\mu mol/g_{dcw}$, about 0.1 to about 5 $\mu mol/g_{dcw}$, about 0.1 to about 4 $\mu mol/g_{dcw}$, about 0.1 to about 3 $\mu mol/g_{dcw}$, about 0.1

to about 2 μmol/g_{dcw}, about 0.1 to about 1 μmol/g_{dcw}, about 0.1 to about 0.8 μmol/g_{dcw}, about 0.1 to about 0.6 μmol/g_{dcw}, about 0.2 to about 7 μmol/g_{dcw}, about 0.2 to about 6 μmol/g_{dcw}, about 0.2 to about 5 μmol/g_{dcw}, about 0.2 to about 4 μmol/g_{dcw}, about 0.2 to about 3 μmol/g_{dcw}, about 0.2 to about 2 μmol/g_{dcw}, about 0.3 to about 7 μmol/g_{dcw}, about 0.3 to about 6 μmol/g_{dcw}, about 0.3 to about 5 μmol/g_{dcw}, about 0.3 to about 4 μmol/g_{dcw}, about 0.3 to about 3 μmol/g_{dcw}, about 0.3 to about 2 μmol/g_{dcw}, about 0.4 to about 7 μmol/g_{dcw}, about 0.4 to about 6 μmol/g_{dcw}, about 0.4 to about 5 μmol/g_{dcw}, about 0.4 to about 2 μmol/g_{dcw}, about 0.5 to about 7 μmol/g_{dcw}, about 0.5 to about 5 μmol/g_{dcw}, about 0.5 to about 2 μmol/g_{dcw}, about 0.7 to about 7 μmol/g_{dcw}, about 0.7 to about 5 μmol/g_{dcw}, or about 0.7 to about 2 μmol/g_{dcw}. In some embodiments, the intracellular concentration of GPP is equal to or less than about any of 8, 6, 4, 2, 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 μmol/g_{dcw}.

[0019] In some embodiments of any of the compositions, systems, and methods of the invention, the intracellular concentration of FPP is between about 0 to about 6 μmol/g_{dcw}, such as between about 0. 1 to about 6 μmol/g_{dcw}, about 0.1 to about 5 μmol/g_{dcw}, about 0.1 to about 4 μmol/g_{dcw}, about 0.1 to about 3 μmol/g_{dcw}, about 0.1 to about 2 μmol/g_{dcw}, about 0.1 to about 1 μmol/g_{dcw}, about 0.1 to about 0.8 μmol/g_{dcw}, about 0.1 to about 0.6 μmol/g_{dcw}, about 0.2 to about 6 μmol/g_{dcw}, about 0.2 to about 5 μmol/g_{dcw}, about 0.2 to about 4 μmol/g_{dcw}, about 0.2 to about 3 μmol/g_{dcw}, about 0.2 to about 2 μmol/g_{dcw}, about 0.3 to about 6 μmol/g_{dcw}, about 0.3 to about 5 μmol/g_{dcw}, about 0.3 to about 4 μmol/g_{dcw}, about 0.3 to about 3 μmol/g_{dcw}, about 0.3 to about 2 μmol/g_{dcw}, about 0.4 to about 6 μmol/g_{dcw}, about 0.4 to about 5 μmol/g_{dcw}, about 0.4 to about 2 μmol/g_{dcw}, about 0.5 to about 6 μmol/g_{dcw}, about 0.5 to about 5 μmol/g_{dcw}, about 0.5 to about 2 μmol/g_{dcw}, about 0.8 to about 6 μmol/g_{dcw}, about 0.8 to about 5 μmol/g_{dcw}, about 0.8 to about 2 μmol/g_{dcw}, about 1 to about 6 μmol/g_{dcw}, about 1 to about 5 μmol/g_{dcw}, about 1 to about 2 μmol/g_{dcw}, about 1.1 to about 6 μmol/g_{dcw}, about 1.1 to about 5 μmol/g_{dcw}, about 1.1 to about 2 μmol/g_{dcw}, about 1.1 to about 1.5 μmol/g_{dcw}, about 1.2 to about 6 μmol/g_{dcw}, about 1.2 to about 5 μmol/g_{dcw}, about 1.2 to about 2 μmol/g_{dcw}, or about 1.2 to about 1.5 μmol/g_{dcw}. In some embodiments, the intracellular concentration of FPP is equal to or less than about any of 6, 4, 2, 1.5, 1.4, 1.3, 1.2, 1.1, 1, 0.9, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 μmol/g_{dcw}.

[0020] In some embodiments of any of the compositions, systems, and methods of the invention, the concentration (*e.g.*, concentration in the cell medium) of MVA is between

about 0 to about 120 g/L, such as between about about 0 to about 110 g/L, such as between about 0.1 to about 100 g/L, about 0.1 to about 75 g/L, about 0.1 to about 60 g/L, about 0.1 to about 50 g/L, about 0.1 to about 40 g/L, about 0.1 to about 30 g/L, about 0.1 to about 20 g/L, about 0. 1 to about 15 g/L, about 0.1 to about 11 g/L, about 0.1 to about 7 g/L, about 0.1 to about 5 g/L, about 0.1 to about 2 g/L, about 0.1 to about 1 g/L, about 0.1 to about 0.8 g/L, about 0.1 to about 0.6 g/L, about 0.2 to about 120 g/L, about 0.2 to about 100 g/L, about 0.2 to about 75 g/L, about 0.2 to about 60 g/L, about 0.2 to about 50 g/L, about 0.2 to about 40 g/L, about 0.2 to about 30 g/L, about 0.2 to about 20 g/L, about 0.2 to about 15 g/L, about 0.2 to about 11 g/L, about 0.2 to about 7 g/L, about 0.2 to about 5 g/L, about 0.2 to about 2 g/L, about 0.3 to about 120 g/L, about 0.3 to about 100 g/L, about 0.3 to about 75 g/L, about 0.3 to about 60 g/L, about 0.3 to about 50 g/L, about 0.3 to about 40 g/L, about 0.3 to about 30 g/L, about 0.3 to about 15 g/L, about 0.3 to about 11 g/L, about 0.3 to about 7 g/L, about 0.3 to about 5 g/L, about 0.3 to about 2 g/L, about 0.4 to about 120 g/L, about 0.4 to about 100 g/L, about 0.4 to about 75 g/L, about 0.4 to about 60 g/L, about 0.4 to about 50 g/L, about 0.4 to about 40 g/L, about 0.4 to about 30 g/L, about 0.4 to about 15 g/L, about 0.4 to about 7 g/L, about 0.4 to about 5 g/L, about 0.4 to about 2 g/L, about 0.5 to about 1200 g/L, about 0.5 to about 100 g/L, about 0.5 to about 75 g/L, about 0.5 to about 60 g/L, about 0.5 to about 50 g/L, about 0.5 to about 40 g/L, about 0.5 to about 30 g/L, about 0.5 to about 15 g/L, about 0.5 to about 11 g/L, about 0.5 to about 7 g/L, about 0.5 to about 5 g/L, about 0.5 to about 2 g/L, about 50 to about 60 g/L, or about 1 g/L. In some embodiments, the concentration (*e.g.*, concentration in the cell medium) of MVA is equal to or less than about any of 120, 100, 80, 70, 60, 50, 40, 30, 25, 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 g/L.

[0021] In some embodiments of any of the compositions, systems, and methods of the invention, the cells comprise a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid encoding a mevalonate kinase polypeptide. In some embodiments, the mevalonate kinase nucleic acid is operably linked to a promoter. In some embodiments, the cells express (i) a heterologous nucleic acid encoding a second mevalonate kinase polypeptide or (ii) a duplicate copy of a nucleic acid encoding a second mevalonate kinase polypeptide that differs from the first mevalonate kinase polypeptide. In some embodiments, the cells comprise a heterologous nucleic acid or a duplicate copy of an endogenous 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.

[0022] 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.

[0023] In some embodiments, 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.

[0024] In some embodiments, the invention also features systems that include any of the cells and/or compositions described herein. In some embodiments, the system includes a reactor that chamber comprises cells in culture that produce greater than about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments, the system is not a closed system. In some embodiments, at least a portion of the isoprene is removed from the system. In some embodiments, the system includes a gas phase comprising isoprene. In various embodiments, the gas phase comprises any of the compositions described herein.

[0025] In one aspect, the invention provides a tire comprising polyisoprene. In some embodiments, the polyisoprene is produced by (i) polymerizing isoprene in any of the compositions described herein or (ii) polymerizing isoprene recovered from any of the compositions described herein. In some embodiments, the polyisoprene comprises *cis*-1,4-polyisoprene. In another aspect, the invention provides methods of manufacturing a tire

wherein the improvement comprises using any one or more the compositions, cells, systems and/or methods described herein to produce isoprene for the manufacture of the tire.

[0026] In some embodiments of any of the compositions, systems, and methods of the invention, a nonflammable concentration of isoprene in the gas phase is produced. In some embodiments, the gas phase comprises less than about 9.5 % (volume) oxygen. In some embodiments, the gas phase comprises greater than or about 9.5 % (volume) oxygen, and the concentration of isoprene in the gas phase is less than the lower flammability limit or greater than the upper flammability limit. In some embodiments, the portion of the gas phase other than isoprene comprises between about 0% to about 100% (volume) oxygen, such as between about 10% to about 100% (volume) oxygen. In some embodiments, the portion of the gas phase other than isoprene comprises between about 0% to about 99% (volume) nitrogen. In some embodiments, the portion of the gas phase other than isoprene comprises between about 1% to about 50% (volume) CO_2.

[0027] In some embodiments of any of the aspects of the invention, the cells in culture produce isoprene at greater than or about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments of any of the aspects of the invention, the cells in culture convert greater than or about 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6%, or more of the carbon in the cell culture medium into isoprene. In some embodiments of any of the aspects of the invention, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 100,000, or more ng of isoprene/gram of cells for the wet weight of the cells /hr (ng/g_{wcm}/h). In some embodiments of any of the aspects of the invention, the cells in culture produce a cumulative titer (total amount) of isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 50,000, 100,000, or more mg of isoprene/L of broth (mg/L_{broth}, wherein the volume of broth includes the volume of the cells and the cell medium). Other exemplary rates of isoprene production and total amounts of isoprene production are disclosed herein.

[0028] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments of

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

[0029] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase, DXS, and MVA pathway nucleic acid. In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase nucleic acid, a DXS nucleic acid, an IDI nucleic acid, and a MVA pathway nucleic (in addition to the IDI nucleic acid).

[0030] In some embodiments of any of the aspects of the invention, the isoprene synthase polypeptide is a polypeptide from a plant such as *Pueraria* (*e.g., Pueraria montana or Pueraria lobata*) or *Populus* (*e.g., Populus tremuloides, Populus alba, Populus nigra, Populus trichocarpa*, or the hybrid, *Populus alba x Populus tremula*).

[0031] 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.

[0032] In some embodiments of any of the aspects of the invention, the cells are bacterial cells, such as gram-positive bacterial cells (*e.g.*, *Bacillus* cells such as *Bacillus subtilis* cells or *Streptomyces* cells such as *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus* cells). In some embodiments of any of the aspects of the invention, the cells are gram-negative bacterial cells (*e.g.*, *Escherichia* cells such as *Escherichia coli* cells or *Pantoea* cells such as *Pantoea citrea* cells). In some embodiments of any of the aspects of the invention, the cells are fungal, cells such as filamentous fungal cells (*e.g.*, *Trichoderma* cells such as *Trichoderma reesei* cells or *Aspergillus* cells such as *Aspergillus oryzae* and *Aspergillus niger*) or yeast cells (*e.g.*, *Yarrowia* cells such as *Yarrowia lipolytica* cells or Saccharomyces cells such as *Saccharomyces cerevisiae*).

[0033] In some embodiments of any of the aspects of the invention, the microbial polypeptide carbon source includes one or more polypeptides from yeast or bacteria. In some embodiments of any of the aspects of the invention, the plant polypeptide carbon source includes one or more polypeptides from soy, corn, canola, jatropha, palm, peanut, sunflower, coconut, mustard, rapeseed, cottonseed, palm kernel, olive, safflower, sesame, or linseed.

[0034] In one aspect, the invention features a product produced by any of the compositions or methods of the invention.

[0035] It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] Figure 1 is the nucleotide sequence of a kudzu isoprene synthase gene codon-optimized for expression in *E. coli* (SEQ ID NO:1). The atg start codon is in italics, the stop codon is in bold and the added *Pst*I site is underlined.

[0037] Figure 2 is a map of pTrcKudzu.

[0038] Figures 3A-3C 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, capitol, italics letters. The vector backbone is pTrcHis2B.

[0039] Figure 4 is a map of pETNHisKudzu.

[0040] Figures 5A-5C are the nucleotide sequence of pETNHisKudzu (SEQ ID NO:5).

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

[0042] Figures 7A-7C are the nucleotide sequence of pCL-lac-Kudzu (SEQ ID NO:7).

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

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

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

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

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

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

[0049] Figure 10A is a graph showing the production of isoprene in *Panteoa citrea.* Control cells without recombinant kudzu isoprene synthase. Grey diamonds represent isoprene synthesis, black squares represent OD_{600}.

[0050] Figure 10B is a graph showing the production of isoprene in *Panteoa citrea* expressing pCL-lac Kudzu. Grey diamonds represent isoprene synthesis, black squares represent OD_{600}.

[0051] Figure 10C is a graph showing the production of isoprene in *Panteoa citrea* expressing pTrcKudzu. Grey diamonds represent isoprene synthesis, black squares represent OD_{600}.

[0052] Figure 11 is a graph showing the production of isoprene in *Bacillus subtilis* expressing recombinant isoprene synthase. BG3594comK is a *B. subtilis* strain without plasmid (native isoprene production). CF443-BG3594comK is a *B. subtilis* strain with pBSKudzu (recombinant isoprene production). IS on the y-axis indicates isoprene.

[0053] Figures 12A-12C are the nucleotide sequence of pBS Kudzu #2 (SEQ ID NO:57).

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

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

[0056] Figures 15A-15C are the nucleotide sequence of vector pSPZ1(MAP29Spb) (SEQ ID NO:11).

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

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

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

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

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

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

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

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

[0065] Figure 19A shows the MVA and DXP metabolic pathways for isoprene (based on F. Bouvier *et al.*, Progress in Lipid Res. 44: 357-429, 2005). The following description includes alternative names for each polypeptide in the pathways and a reference that discloses an assay for measuring the activity of the indicated polypeptide (each of these references are each hereby incorporated by reference in their entireties, particularly with respect to assays for polypeptide activity for polypeptides in the MVA and DXP pathways). **Mevalonate Pathway: AACT**; Acetyl-CoA acetyltransferase, MvaE, EC 2.3.1.9. Assay: J. Bacteriol., 184: 2116–2122, 2002; **HMGS**; Hydroxymethylglutaryl-CoA synthase, MvaS, EC 2.3.3.10. Assay: J. Bacteriol., 184: 4065–4070, 2002; **HMGR**; 3-Hydroxy-3-methylglutaryl-CoA reductase, MvaE, EC 1.1.1.34. Assay: J. Bacteriol., 184: 2116–2122, 2002; **MVK**; Mevalonate kinase, ERG12, EC 2.7.1.36. Assay: Curr Genet 19:9-14, 1991. **PMK**; Phosphomevalonate kinase, ERG8, EC 2.7.4.2, Assay: Mol Cell Biol., 11:620–631, 1991; **DPMDC**; Diphosphomevalonate decarboxylase, MVD1, EC 4.1.1.33. Assay: Biochemistry, 33:13355-13362, 1994; **IDI**; Isopentenyl-diphosphate delta-isomerase, IDI1, EC 5.3.3.2. Assay: J. Biol. Chem. 264:19169-19175, 1989. **DXP Pathway: DXS**; 1-Deoxyxylulose-5-phosphate synthase, dxs, EC 2.2.1.7. Assay: PNAS, 94:12857-62, 1997; **DXR**; 1-Deoxy-D-xylulose 5-phosphate reductoisomerase, dxr, EC 2.2.1.7. Assay: Eur. J. Biochem. 269:4446–4457, 2002; **MCT**; 4-Diphosphocytidyl-2C-methyl-D-erythritol synthase, IspD, EC 2.7.7.60. Assay: PNAS, 97: 6451–6456, 2000; **CMK**; 4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase, IspE, EC 2.7.1.148. Assay: PNAS, 97:1062-1067, 2000; **MCS**; 2C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase, IspF, EC 4.6.1.12. Assay: PNAS, 96:11758-11763, 1999; **HDS**; 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, ispG, EC 1.17.4.3. Assay: J. Org. Chem., 70:9168 -9174, 2005; **HDR**; 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, IspH, EC 1.17.1.2. Assay: JACS, 126:12847-12855, 2004.

[0066] Figure 19B illustrates the classical and modified MVA pathways. 1, acetyl-CoA acetyltransferase (**AACT**); 2, HMG-CoA synthase (**HMGS**); 3, HMG-CoA reductase (**HMGR**); 4, mevalonate kinase (**MVK**); 5, phosphomevalonate kinase (**PMK**); 6, diphosphomevalonate decarboxylase (**MVD or DPMDC**); 7, isopentenyl diphosphate isomerase (**IDI**); 8, phosphomevalonate decarboxylase (**PMDC**); 9, isopentenyl phosphate kinase (**IPK**). The classical MVA pathway proceeds from reaction 1 through reaction 7 via reactions 5 and 6, while a modified MVA pathway goes through reactions 8 and 9. P and PP

in the structural formula are phosphate and pyrophosphate, respectively. This figure was taken from Koga and Morii, *Microbiology and Mol. Biology Reviews*, 71:97-120, 2007, which is incorporated by reference in its entirety, particularly with respect to nucleic acids and polypeptides of the modified MVA pathway. The modified MVA pathway is present, for example, in some archaeal organisms, such as *Methanosarcina mazei.*

[0067] Figure 20 shows graphs representing results of the GC-MS analysis of isoprene production by recombinant *Y. lipolytica* strains without (left) or with (right) a kudzu isoprene synthase gene. The arrows indicate the elution time of the authentic isoprene standard.

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

[0069] Figures 22A-22D are the nucleotide sequence of pTrcKudzu yIDI DXS Kan (SEQ ID NO:20).

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

[0071] Figure 23B is a graph showing production of isoprene from glucose in BL21/pTrcKudzu yIDI kan. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0072] Figure 23C is a graph showing production of isoprene from glucose in BL21/pTrcKudzu DXS kan. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0073] Figure 23D is a graph showing production of isoprene from glucose in BL21/pTrcKudzu yIDI DXS kan. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0074] Figure 23E is a graph showing production of isoprene from glucose in BL21/pCL PtrcKudzu. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0075] Figure 23F is a graph showing production of isoprene from glucose in BL21/pCL PtrcKudzu yIDI. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0076] Figure 23G is a graph showing production of isoprene from glucose in BL21/pCL PtrcKudzu DXS. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0077] Figure 23H is a graph showing production of isoprene from glucose in BL21/pTrcKudzuIDIDXSkan. The arrow indicates the time of induction with IPTG (400 μmol). The x-axis is time after inoculation; the y-axis is OD600 and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD600, triangles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

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

[0079] Figures 25A-25D are the nucleotide sequence of pTrcKKDyIkIS kan (SEQ ID NO:33).

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

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

[0082] Figure 28 shows a map of the cassette containing the lower MVA pathway and yeast idi for integration into the *B.* subtilis chromosome at the nprE locus. nprE upstream/downstream indicates 1 kb each of sequence from the nprE locus for integration. aprE promoter (alkaline serine protease promoter) indicates the promoter (-35, -10, +1 transcription start site, RBS) of the *aprE* gene. MVK1 indicates the yeast mevalonate kinase gene. RBS-PMK indicates the yeast 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.

[0083] Figures 29A-29D are the nucleotide sequence of cassette containing the lower MVA pathway and yeast idi for integration into the *B. subtilis* chromosome at the *nprE* locus (SEQ ID NO:47).

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

[0085] Figures 31A and 31B are the nucleotide sequence of p9796-poplar (SEQ ID NO:48).

[0086] Figure 32 is a map of pTrcPoplar.

[0087] Figures 33A-33C are the nucleotide sequence of pTrcPoplar (SEQ ID NO:49).

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

[0089] Figures 35A-35C are the nucleotide sequence of pTrcKudzu yIDI Kan (SEQ ID NO:50).

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

[0091] Figures 37A-37C are the nucleotide sequence of pTrcKudzuDXS Kan (SEQ ID NO:51).

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

[0093] Figures 39A-39C are the nucleotide sequence of pCL PtrcKudzu (SEQ ID NO:52).

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

[0095] Figures 41A-41C are the nucleotide sequence of pCL PtrcKudzu A3 (SEQ ID NO:53).

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

[0097] Figures 43A-43C are the nucleotide sequence of pCL PtrcKudzu yIDI (SEQ ID NO:54).

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

[0099] Figures 45A-45D are the nucleotide sequence of pCL PtrcKudzu DXS (SEQ ID NO:55).

[0100] Figure 46A is a map of the *M. mazei* archaeal Lower Pathway operon.

[0101] Figures 46B and 46C are the nucleotide sequence of the *M. mazei* archaeal lower Pathway operon (SEQ ID NO:102).

[0102] Figure 47A is a map of MCM382 – pTrcKudzuMVK(mazei).

[0103] Figures 47B and 47C are the nucleotide sequence of MCM382 – pTrcKudzuMVK(mazei) (SEQ ID NO:103).

[0104] Figures 48A-48C are graphs demonstrating the effect of yeast extract of isoprene production. Figure 48A is the time course of optical density within fermentors fed with

varying amounts of yeast extract. Figure 48B is 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. Figure 48C shows the effect of yeast extract on isoprene production in *E. coli* grown in fed-batch culture.

[0105] Figure 49 shows graphs demonstrating isoprene production from a 500 L bioreactor with *E. coli* cells containing the pTrcKudzu + yIDI + DXS plasmid. Panel A shows the time course of optical density within the 500-L bioreactor fed with glucose and yeast extract. Panel B shows the time course of isoprene titer within the 500-L bioreactor fed with glucose and yeast extract. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Panel C shows the time course of total isoprene produced from the 500-L bioreactor fed with glucose and yeast extract.

[0106] Figure 50 is a map of pJMupperpathway2.

[0107] Figures 51A-51C are the nucleotide sequence of pJMupperpathway2 (SEQ ID NO:56).

[0108] Figure 52 is a map of pBS Kudzu #2.

[0109] Figure 53A is a graph showing growth during fermentation time of Bacillus expressing recombinant kudzu isoprene synthase in 14 liter fed batch fermentation. Black diamonds represent a control strain (BG3594comK) without recombinant isoprene synthase (native isoprene production) and grey triangles represent *Bacillus* with pBSKudzu (recombinant isoprene production).

[0110] Figure 53B is a graph showing isoprene production during fermentation time of *Bacillus* expressing recombinant kudzu isoprene synthase in 14 liter fed batch fermentation. Black diamonds represent a control strain (BG3594comK) without recombinant isoprene synthase (native isoprene production) and grey triangles represent *Bacillus* with pBSKudzu (recombinant isoprene production).

[0111] Figure 54 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0112] 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.

[0113] Figure 56 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0114] Figure 57A is a map of MCM376 - MVK from *M. mazei* archaeal Lower in pET200D.

[0115] Figures 57B and 57C are the nucleotide sequence of MCM376 - MVK from *M. mazei* archaeal Lower in pET200D (SEQ ID NO:104).

[0116] Figure 58A is a map of Streptomyces CL190 Lower Pathway Operon.

[0117] Figures 58B and 58C are the nucleotide sequence of Streptomyces CL190 Lower Pathway Operon (SEQ ID NO:105).

[0118] Figure 59A is a map of MCM 383 – pTrcKudzuMVK (*S. cerevisiae*).

[0119] Figures 59B and 59C are the nucleotide sequence of MCM 383 – pTrcKudzuMVK (*S. cerevisiae*) (SEQ ID NO:106).

[0120] Figures 60A-60C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 150-L bioreactor fed with glucose.

[0121] Figures 61A-61C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0122] Figures 62A-62C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0123] Figure 63A-63C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0124] Figures 64A-64C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0125] Figures 65A-65C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0126] Figures 66A-66C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0127] Figure 67A-67C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0128] 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.

[0129] 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.

[0130] 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.

[0131] 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.

[0132] 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.

[0133] 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.

[0134] 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.

[0135] Figure 75A is a table of the conversion of the CAFT Model results from weight percent to volume percent for series A.

[0136] Figure 75B is a graph of the flammability results from the CAFT model for Series A in Figure 68 plotted as volume percent.

[0137] Figure 76A is a table of the conversion of the CAFT Model results from weight percent to volume percent for series B.

[0138] Figure 76B is a graph of the flammability results from the CAFT model for Series B in Figure 69 plotted as volume percent.

[0139] Figure 77 is a figure of the flammability test vessel.

[0140] Figure 78A is a graph of the flammability Curve for Test Series 1: 0% Steam, 0 psig, and 40°C.

[0141] Figure 78B is a table summarizing the explosion and non-explosion data points for Test Series 1.

[0142] Figure 78C is a graph of the flammability curve for Test Series 1 compared with the CAFT Model.

[0143] Figure 79A is a graph of the flammability curve for Test Series 2: 4% Steam, 0 psig, and 40°C.

[0144] Figure 79B is a table summarizing the explosion and non-explosion data points for Test Series 2.

[0145] Figure 79C is a graph of the flammability curve for Test Series 2 compared with the CAFT Model.

[0146] Figures 80A and 80B are a table of the detailed experimental conditions and results for Test Series 1.

[0147] Figure 81 is a table of the detailed experimental conditions and results for Test Series 2.

[0148] 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.

[0149] 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.

[0150] Figure 84 is a graph of the flammability envelope constructed using data from Figure 82 and following the methodology described in Example 24. The experimental data points (circles) are from tests described herein that were conducted at 1 atmosphere initial system pressure.

[0151] Figure 85 is a graph of the flammability envelope constructed using data from Figure 83 and following the methodology described in Example 24. The experimental data points (circles) are from tests described herein that were conducted at 1 atmosphere initial system pressure.

[0152] Figure 86A is a GC/MS chromatogram of fermentation off-gas.

[0153] Figure 86B is an expansion of Fig 86A to show minor volatiles present in fermentation off-gas.

[0154] Figure 87A is a GC/MS chromatogram of trace volatiles present in off-gas following cryo-trapping at -78 °C.

[0155] Figure 87B is a GC/MS chromatogram of trace volatiles present in off-gas following cryo-trapping at -196 °C.

[0156] Figure 87C is an expansion of Figure 87B.

[0157] Figure 87D is an expansion of Figure 87C.

[0158] Figures 88A and 88B 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).

[0159] 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.

[0160] Figure 90 shows the structures of several impurities that are structurally similar to isoprene and may also act as polymerization catalyst poisons.

[0161] Figure 91 is a map of pTrcHis2AUpperPathway (also called pTrcUpperMVA).

[0162] Figures 92A-92C are the nucleotide sequence of pTrcHis2AUpperPathway (also called pTrcUpperMVA) (SEQ ID NO:86).

[0163] Figure 93 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0164] 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.

[0165] Figure 95 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0166] Figure 96A is a map of MCM380 – pTrcKudzuMVK (Lactobacillus sakei).

[0167] Figures 96B and 96C are the nucleotide sequence of MCM380 – pTrcKudzuMVK (Lactobacillus sakei) (SEQ ID NO:107).

[0168] Figure 97A is a map of MCM379 – pTrcKudzuMVK (Streptococcus pneumoniae).

[0169] Figures 97B and 97C are the nucleotide sequence of MCM379 – pTrcKudzuMVK (Streptococcus pneumoniae) (SEQ ID NO:108).

[0170] Figure 98A is a map of MCM381 – pTrcKudzuMVK (Streptomyces CL190).

[0171] Figures 98B and 98C are the nucleotide sequence of MCM381 – pTrcKudzuMVK (Streptomyces CL190) (SEQ ID NO:109).

[0172] Figure 99 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0173] 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.

[0174] Figure 101 is a time course of isoprene specific activity from the 15-L bioreactor fed with glucose.

[0175] Figure 102 is a map of pCLPtrcUpperPathwayHGS2 (also referred to as pCL UpperHGS2).

[0176] Figures 103A-103C are the nucleotide sequence of pCLPtrcUpperPathwayHGS2 (SEQ ID NO:87).

[0177] Figure 104 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0178] 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.

[0179] Figure 106 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0180] Figure 107 is a map of plasmid MCM330.

[0181] Figures 108A-108C are the nucleotide sequence of plasmid MCM330 (SEQ ID NO:90).

[0182] Figure 109 is a map of pET24D-Kudzu.

[0183] Figures 110A and 110B are the nucleotide sequence of pET24D-Kudzu (SEQ ID NO:101).

[0184] Figure 111A is a time course of optical density within the 15-L bioreactor fed with glucose.

[0185] 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.

[0186] Figure 111C is a time course of specific productivity of isoprene in the 15-L bioreactor fed with glucose.

[0187] Figure 112A is a graph of the growth of MCM127 in TM3 media at 30°C measured as optical density (OD600). One culture was induced with 150 μM IPTG 4 hours after inoculation.

[0188] Figure 112B is a graph of the accumulated key metabolic intermediates after induction of MCM127 with 150 μM IPTG. The culture was induced 4 hours after inoculation and samples were analyzed using LCMS.

[0189] Figures 112C-112K are isoprene fermentation expressing genes from the MVA pathway and grown in fed-batch culture at the 15-L scale in different *E. coli* strains (MCM343 strain (Figures 112C-112E); MCM127 strain (Figures 112F-112H); *dxr* knock-out strain (Figures 112I-112K)). Figures 112C, 112F, and 112I show the time course of optical density within the 15-L bioreactor fed with glucose in MCM343 strain, MCM127 strain, and *dxr* knock-out strain, respectively. Figures 112D, 112G, and 112J are the time course of isoprene titer within the 15-L bioreactor fed with glucose in MCM343 strain, MCM127 strain, and *dxr* knock-out strain, respectively. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Figures 112E, 112H, and 112K are the time course of total isoprene produced from the 15-L bioreactor fed with glucose in MCM343 strain, MCM127 strain, and *dxr* knock-out strain, respectively.

[0190] Figures 112L-112N depict the construction and phenotype of the *dxr* mutant in *E. coli*. 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr*) was deleted using the GeneBridges Quick & Easy *E. coli* Gene Deletion Kit. Figure 112L shows the chromosomal location of *dxr* (from EcoCyc) and the approximate primer binding sites for testing the insertion of the GB resistance cassette. Figure 112M is a PCR analysis of *dxr* deletion strains (in MG1655) using primers dxrTest1 and GBprimer2 (GB2), and dxrTest2 and GBprimerDW (GB3). PCR products were run on an Egel (Invitrogen) according to the manufacturer's protocol. Figure 112N shows the inhibition of the growth of *dxr* deletion strains at 10 mM

MVA. DW28 were grown overnight at 37°C on LB medium plates containing spectinomycin 50 μg/ml, chloramphenicol 25 μg/ml, and the indicated concentrations of MVA.

[0191] Figure 112O lists forward and reverse primers for pCL Ptrc(minus lacO) UpperPathway: forward primer MCM63 (SEQ ID NO:139) and reverse primer MCM64 (SEQ ID NO:140).

[0192] Figure 112P is a map of MCM184 – pCL Ptrc(minus lacO) UpperPathway.

[0193] Figure 112Q-112S are the nucleotide sequence of MCM184 (SEQ ID NO:141).

[0194] Figure 112T lists PCR and sequencing primers for pCL Ptrc (ΔlacO)KKDyI: primer EL-976 (SEQ ID NO:142), primer EL-977 (SEQ ID NO:143), and primer EL-978 (SEQ ID NO:144).

[0195] Figure 112U is a map of pCL Ptrc (ΔlacO)KKDyI.

[0196] Figures 112V-112X are the nucleotide sequence of pCL Ptrc (ΔlacO)KKDyI (SEQ ID NO:145).

[0197] Figures 113A-113D demonstrate that over-expression of MVK and isoprene synthase results in increased isoprene production. Accumulated isoprene and CO_2 from MCM401 and MCM343 during growth on glucose in 100 mL bioreactors with 100 and 200 uM IPTG induction of isoprene production was measured over a 22 hour time course. Figure 113A is a graph of the accumulated isoprene (%) from MCM343. Figure 113B is a graph of the accumulated isoprene (%) from MCM401. Figure 113C is a graph of the accumulated CO_2 (%) from MCM343. Figure 113D is a graph of the accumulated CO_2 (%) from MCM401.

[0198] Figure 114 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0199] Figure 115 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.

[0200] Figure 116 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0201] Figure 117 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0202] Figure 118 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.

[0203] Figure 119 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0204] Figure 120 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0205] Figure 121 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.

[0206] Figure 122 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0207] Figure 123 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0208] Figure 124 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.

[0209] Figure 125 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0210] Figures 126A and 126B are the nucleotide sequence of pDU-5 MVK from *S. cerevsiae* in pET-16b (SEQ ID NO:111).

[0211] Figure 127A is a map of pDW01.

[0212] Figures 127B and 127C are the nucleotide sequence of pDW01 (ORF of 6XHis-*Lb. sakei* Mvk is underlined) (SEQ ID NO:112).

[0213] Figure 128A is a map of pDW02.

[0214] Figures 128B and 128C are the nucleotide sequence of pDW02 (ORF of 6XHis-*S. pneumoniae* Mvk is underlined) (SEQ ID NO:113).

[0215] Figure 129 is a picture of a gel showing the induction of *Lb. sakei* and *S. pneumoniae* MVK constructs. This gel shows expression of *Lactobacillus sakei* and *Streptococcus pneumoniae* MVK in BL21 Star (DE3) (Invitrogen). Cells were grown to late exponential phase (OD600 ~ 1) and induced with 1 mM IPTG. After 2 hours of induction (at 37 °C) samples were removed and visualized on a 4-12% Novex SDS gel (Nupage – Invitrogen). The SeeBlue Plus2 standard (Invitrogen) was used to visualize approximate molecular weights. Lane 1 – *Lb. sakei* Mvk (pDW01) and no IPTG; lane 2 – pDW01 and 1mM IPTG; lane 3 – *S. pneumoniae* Mvk (pDW02) and no IPTG; lane 4 – pDW02 and 1mM IPTG; lane 5 – *S. pneumoniae* Mvk (pDW02 isolate #2) and no IPTG; lane 6 – pDW02 (isolate #2) and 1mM IPTG. The arow on the left indicates the induced band from pDW01; the arrow on the right indicates the induced bands from pDW02 and pDW02#2 in lanes 4 and 6.

[0216] Figure 130 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0217] Figure 131 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.

[0218] Figure 132 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0219] Figure 133 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.

[0220] Figure 134 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.

[0221] Figure 135 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0222] Figure 136 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.

[0223] Figure 137 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0224] Figure 138A is a map of plasmid MCM94 – pTrcHis2B kan.

[0225] Figures 138B and 138C are the nucleotide sequence of plasmid MCM94 – pTrcHis2B kan (SEQ ID NO:114).

[0226] Figure 139 is a graph showing that over-expression of both isoprene synthase and MVK results in an increased specific productivity of isoprene compared to over-expression of each of the enzymes alone, or low expression of both enzymes. The specific productivity of isoprene using MCM343, MCM401, MCM437, and MCM438 during growth on glucose in mini-fermentations with 200 μM IPTG induction was measured over time. Error bars represent one standard deviation.

[0227] Figure 140 is a typical elution profile of phosphorylated intermediates in the isoprenoid pathway extracted from the MCM391 strain of *E. coli* after 50 hours of fermentation and detected using LC-ESI-MS/MS.

[0228] Figures 141A-141F are graphs showing the accumulation of isoprenoid pathway intermediates in MCM401 strain of *E. coli* containing MVK from *M. mazei* upon different levels of enzyme expression. Figures 141A-141C show ODs and specific isoprene production of the cultures grown in 14-L fermentors, and Figures 141D-141F show intracellular levels of

isoprenoid metabolites. Arrows on top of the figures indicate the time points when IPTG was added to fermentors (1 – 4 x 50 μM; 2 – 2 x 100 μM and 3 – 1 x 200 μM).

[0229] Figures 142A and 142B are graphs showing the accumulation of isoprenoid pathway intermediates in the MCM402 strain of *E. coli* containing MVK from yeast and grown in 14-L fermentors. Arrows on the top figure indicate the time points when 50 μM IPTG doses were added to fermentors.

[0230] Figures 143A and 143B are graphs showing the accumulation of isoprenoid pathway intermediates in the MCM400 strain of *E. coli* containing MVK from *Streptomyces* and grown in 14-L fermentor. Arrows on the top figure indicate the time points when 50 μM IPTG doses were added to the fermentor.

[0231] Figures 144A and 144B are graphs showing the accumulation of isoprenoid pathway intermediates in the MCM343 strain of *E. coli.* Arrows on the top figure indicate the time point when 100 μM IPTG dose was added to the fermentor.

[0232] Figure 145 is a graph of growth curves for cultures of BL21 expressing MVK, circles; MVK+PMV, triangles; MVK+PMV+MDD, squares. Cultures were either fed 5.8 mM MVA, filled symbols, or grown without addition of MVA, open symbols. Y-axis is OD_{600}. Samples were taken for analysis at times indicated by the arrow. Numbers above the arrows correspond to *E. coli* BL21 cells bearing pTrcK, representing a plasmid expressing MVK (#5), pTrcKK representing a plasmid expressing MVK plus PMK (#7), and pTrcKKD, representing a plasmid expressing MVK plus PMK plus MDD (#6) were grown.

[0233] Figure 146 is a graph of isoprene synthase (IS) activity versus volumetric productivity in strains MCM127, MCM343, and MCM401.

DETAILED DESCRIPTION OF THE INVENTION.

[0234] As illustrated in Figures 19A and 19B, mevalonate kinase (MVK) polypeptides phosphorylate mevalonate (MVA) to form mevalonate-5-phosphate (MVAP), as part of the MVA pathway for the biosynthesis of isoprene. Isoprene synthase polypeptides convert dimethylallyl diphosphate (DMAPP) into isoprene. As used herein, the term "isoprene" or "2-methyl-1,3-butadiene" (CAS# 78-79-5) refers to the direct and final volatile C5 hydrocarbon product from the elimination of pyrophosphate from 3,3-dimethylallyl

pyrophosphate (DMAPP), and does not involve the linking or polymerization of one or more isopentenyl diphosphate (IPP) molecules to one or more DMAPP molecules.

[0235] Both a high flux from central metabolism to DMAPP and a robust enzyme activity to catalyze the conversion of DMAPP to isoprene are desirable for the commercial scale production of isoprene *in vivo*. Increasing MVK polypeptide activity is desirable because it reduces the accumulation of MVA and increases the supply of MVAP for conversion to isoprene using the MVA pathway. Since high concentrations of DMAPP are growth inhibitory, high flux through the MVA pathway is desirably accompanied by high isoprene synthase polypeptide activity to avoid accumulation of toxic amounts of DMAPP. Accordingly, in one aspect, the invention features a method of producing isoprene that involves increasing the expression and/or activity of (i) a MVK polypeptide and (ii) an isoprene synthase polypeptide compared to the expression level and/or activity level normally found in the cell. For example, overexpressing the MVK polypeptide from *M. mazei* and the isoprene synthase from kudzu supports high flux to DMAPP and simultaneous conversion of DMAPP to isoprene. Furthermore, by balancing the activity of the MVK polypeptide and the isoprene synthase polypeptide, we have generated cells which convert acetyl-CoA to isoprene at high flux and titer without the accumulation of DMAPP. The total activity level of an MVK polypeptide is influenced by both the level of protein expressed and the enzymatic characteristics of the specific MVK polypeptide used. Limiting the accumulation of DMAPP is valuable because it prevents DMAPP-associated growth inhibition and loss of metabolic activity.

[0236] As described further in the Examples, overexpression of the *M. mazei* MVK polypeptide and the kudzu isoprene synthase polypeptide resulted in an eight-fold increase in isoprene titer compared to overexpression of isoprene synthase alone. As discussed in Examples 3-5, *E. coli* cells containing the 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*)) were used to produce isoprene in 15-L bioreactors. Example 3 indicates that the total amount of isoprene produced during a 68 hour fermentation was 227.2 g. Instantaneous volumetric productivity levels reached values as high as 1.5 g isoprene/L broth/hr, and the instantaneous yield levels

reached as high as 17.7% w/w (Example 4). Example 5 indicates that the molar yield of utilized carbon that went into producing isoprene during this fermentation was 16.6%, and the weight percent yield of isoprene from glucose over the entire fermentation was 7.7%. Additionally, overexpression of a kudzu isoprene synthase polypeptide and either a Streptomyces MVK polypeptide (Example 9), Lactobacillus MVK polypeptide (Example 10), or Saccharomyces MVK polypeptide (Example 11) also resulted in the production of significant amounts of isoprene. Additionally, Example 12 describes the expression of *Lactobacillus sakei* and *Streptococcus pneumoniae* mevalonate kinase polypeptides. These Examples support the general applicability of overexpressing both an MVK polypeptide and an isoprene synthase polypeptide to increase production of isoprene.

[0237] Example 6 describes the comparison of four strains with different relative levels of isoprene synthase polypeptide activity and MVK polypeptide activity: (i) the MCM343 strain with low MVK polypeptide activity and high isoprene synthase polypeptide activity, (ii) the MCM401 strain with high MVK polypeptide activity and high isoprene synthase polypeptide activity, (iii) the MCM437 with low MVK polypeptide activity and low isoprene synthase, and (iv) the MCM438 strain with high MVK polypeptide activity and low isoprene synthase polypeptide activity. In particular, the specific productivity of isoprene from a strain expressing the full mevalonic acid pathway and kudzu isoprene synthase polypeptide at low levels (MCM437) was compared to a strain that in addition over-expressed MVK polypeptide from *M. mazei* and kudzu isoprene synthase polypeptide (MCM401), as well as strains that either over-expressed just MVK polypeptide (MCM438), or just kudzu isoprene synthase polypeptide (MCM343). The strain over-expressing both MVK polypeptide and isoprene synthase polypeptide (MCM401) had higher specific productivity of isoprene compared to the strain over-expressing just MVK polypeptide (MCM438) or just kudzu isoprene synthase polypeptide (MCM343). The strain with low activities of both MVK polypeptide and kudzu isoprene synthase polypeptide (MCM437) had the lowest specific productivity of isoprene overall.

[0238] Accordingly, in some embodiments, the cells overexpress both an MVK polypeptide and an isoprene synthase polypeptide. In the experiments described in Examples 2-5, *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*) were used to produce isoprene. In these experiments, the *M. mazei* MVK polypeptide and kudzu isoprene synthase polypeptide were overexpressed from a high copy plasmid under the control of a strong promoter. In contrast, the *S. cerevisiae* lower MVA pathway nucleic acids (mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase) were present as a single copy of the nucleic acids integrated in the chromosome under the control of a weak promoter. The *E. faecalis* upper MVA pathway nucleic acids (mvaE encoding a naturally occurring fusion protein that has both acetyl-CoA acetyltransferase and 3-hydroxy-3-methylglutaryl-CoA reductase activities and mvaS encoding a 3-hydroxy-3-methylglutaryl-CoA synthase polypeptide) were overexpressed from a medium copy plasmid under the control of a strong promoter (the same promoter used to express the *M. mazei* MVK polypeptide and kudzu isoprene synthase polypeptide). Thus, the *M. mazei* MVK polypeptide and kudzu isoprene synthase polypeptide were expressed at a much higher level than the other MVA pathway polypeptides. Since the *M. mazei* MVK polypeptide was expressed at a much higher level than the *S. cerevisiae* MVK polypeptide, most of the conversion of MVA to MVAP seems to be due to the *M. mazei* MVK polypeptide rather than the *S. cerevisiae* MVK polypeptide. If desired, the *S. cerevisiae* MVK nucleic acid can be removed from any of the cells disclosed herein using standard methods (such that the only heterologous MVK nucleic acid is the *M. mazei* MVK nucleic acid). If desired, the *S. cerevisiae* MVK nucleic acid can alternatively be replaced by any other MVK nucleic acid in any of the cells described herein.

[0239] Accordingly, in some embodiments, an MVK polypeptide and/or an isoprene synthase polypeptide is expressed a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold (i) higher than the level of expression of a second MVA pathway polypeptide (such as an acetyl-CoA acetyltransferase (AACT) polypeptide, 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) polypeptide, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) polypeptide, phosphomevalonate kinase (PMK) polypeptide, diphosphomevalonate decarboxylase (DPMDC) polypeptide, or isopentenyl-diphosphate delta-isomerase (IDI) polypeptide) or (ii) higher than the level of expression of all other MVA pathway polypeptides in the cell. In particular embodiments, the MVK polypeptide and/or an isoprene synthase polypeptide is

expressed a level that is at least about any of 2, 5, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of an AACT polypeptide, HMGS polypeptide, and HMGR polypeptide. In particular embodiments, the MVK polypeptide and/or an isoprene synthase polypeptide is expressed a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of an PMK polypeptide, DPMDC polypeptide, and IDI polypeptide. In some embodiments, the total amount of MVK polypeptide is similar to the total amount of isoprene synthase polypeptide. For example, in some embodiments, the total amount of MVK polypeptide is within about any of 10, 8, 6, 4, 2, 1, or 0.5-fold higher or lower than the total amount of isoprene synthase polypeptide (*e.g.*, the amount of MVK polypeptide may be between about 10-fold lower to about 10-fold higher than the amount of isoprene synthase polypeptide). Standard methods (such as western blotting) can be used to quantitate the amount of any of these polypeptides. Standard methods can be used to alter the relative amounts of expressed MVA pathway polypeptides, such as by using a stronger promoter or a plasmid with a higher copy number to express an MVK polypeptide and/or an isoprene synthase polypeptide compared to the promoter(s) and plasmid(s) used to express other MVA pathway polypeptides.

[0240] In some embodiments, an MVK RNA molecule and/or an isoprene synthase RNA molecule is expressed a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold (i) higher than the level of expression of a second MVA pathway RNA molecule (such as an AACT RNA molecule, HMGS RNA molecule, HMGR RNA molecule, PMK RNA molecule, DPMDC RNA molecule, or IDI RNA molecule) or (ii) higher than the level of expression of all other MVA pathway RNA molecules in the cell. In particular embodiments, the MVK RNA molecule and/or an isoprene synthase RNA molecule is expressed a level that is at least about any of 2, 5, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of an AACT RNA molecule, HMGS RNA molecule, and HMGR RNA molecule. In particular embodiments, the MVK RNA molecule and/or an isoprene synthase RNA molecule is expressed a level that is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the level of expression of an PMK RNA

molecule, DPMDC RNA molecule, and IDI RNA molecule. In some embodiments, the total amount of MVK RNA is similar to the total amount of isoprene synthase RNA. For example, in some embodiments, the total amount of MVK RNA is within about any of 10, 8, 6, 4, 2, 1, or 0.5-fold higher or lower than the total amount of isoprene synthase RNA (*e.g.*, the amount of MVK RNA may be between about 10-fold lower to about 10-fold higher than the amount of isoprene synthase RNA). Standard methods (such as northern blotting) can be used to quantitate the amount of any of these RNA molecules. Standard methods can be used to alter the relative amounts of expressed MVA pathway RNA molecules, such as by using a stronger promoter or a plasmid with a higher copy number to express an MVK RNA molecule and/or an isoprene synthase RNA molecule compared to the promoter(s) and plasmid(s) used to express other MVA pathway RNA molecules.

[0241] In some embodiments, the number of copies of an MVK DNA molecule and/or an isoprene synthase DNA molecule is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold (i) higher than the number of copies of a second MVA pathway DNA molecule (such as an AACT DNA molecule, HMGS DNA molecule, HMGR DNA molecule, PMK DNA molecule, DPMDC DNA molecule, or IDI DNA molecule) or (ii) higher than the number of copies of all other MVA pathway DNA molecules in the cell. In particular embodiments, the number of copies of an MVK DNA molecule and/or an isoprene synthase DNA molecule is at least about any of 2, 5, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the number of copies of an AACT DNA molecule, HMGS DNA molecule, and HMGR DNA molecule. In particular embodiments, the number of copies of a MVK DNA molecule and/or an isoprene synthase DNA molecule is at least about any of 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 225, 250, 275, 300, 350, 400, 450, or 500-fold higher than the number of copies of an PMK DNA molecule, DPMDC DNA molecule, and IDI DNA molecule. In some embodiments, the number of copies of an MVK DNA molecule is similar to the number of copies of an isoprene synthase DNA molecule. For example, in some embodiments, the number of copies of an MVK DNA molecule is within about any of 10, 8, 6, 4, 2, 1, or 0.5-fold higher or lower than the number of copies of an isoprene synthase DNA molecule (*e.g.*, the number of copies of a MVK DNA may be between about 10-fold lower to about 10-fold higher than the number of copies of an isoprene synthase DNA molecule). Standard methods (such as

southern blotting) can be used to quantitate the amount of any of these DNA molecules. Standard methods can be used to alter the relative amounts of MVA pathway DNA molecules, such as by using a plasmid with a higher copy number to insert an MVK DNA molecule and/or an isoprene synthase DNA molecule compared to the plasmid(s) used to insert other MVA pathway DNA molecules.

[0242] As discussed above, increasing the expression of an MVK polypeptide, decreases that amount of MVA that accumulates in the cell medium since more MVA is converted to MVAP. Increasing the expression of an isoprene synthase polypeptide decreases the accumulation of DMAPP since more DMAPP is converted to isoprene. If desired, the expression of a PMK polypeptide, DPMDC polypeptide, IDI polypeptide, or any combination of two or more of the foregoing can also be increased to reduce the accumulation of MVA pathway or isoprenoid biosynthesis intermediates and/or to increase the flux through the MVA pathway. In some embodiments, the amount of mevalonate (MVA), 3,3-dimethylallyl diphosphate (DMAPP), isopentenyl diphosphate (IPP), geranyl diphosphate (GPP), farnesyl diphosphate (FPP), or any combination of two or more of the foregoing allows production of isoprene without causing undesirable amounts of growth inhibition, toxicity, or cell death. In some embodiments, the amount of MVA, DMAPP, and/or IPP is high enough to allow production of isoprene in any of the amounts or concentrations disclosed below in the "Exemplary Production of Isoprene" section. In some embodiments, a detectable amount of MVA, DMAPP, and/or IPP does not accumulate since the intermediate(s) are being converted to downstream molecules at a rate that does not allow a detectable amount of MVA, DMAPP, and/or IPP to accumulate. Example 8, parts IV, V, and VI indicate that overexpression of either the *M. mazei* MVK polypeptide or the Streptomyces MVK polypeptide is correlated with the accumulation of less DMAPP and IPP than overexpression of the *S. cerevisiae* MVK polypeptide. A goal is therefore to achieve a pathway enzyme balance to minimize the accumulation of these metabolites for the relief of growth inhibition.

[0243] Tables 15A and 15B list exemplary desirable concentrations of DMAPP, IPP, GPP, and FPP as well as examples of relatively high concentrations of these metabolites that have been detected using the cells and methods described herein. Table 15B has the same data as Table 15A that has been normalized to grams of dry cell weight assuming that 1 liter of the culture at OD=1 has 0.33 grams dry cell weight (g_{dcw}). For these experiments, the quantitation limit is below 0.1 mM for the intracellular concentrations of DMAPP, FPP, GPP,

and IPP. In desired, more sensitive equipment can be used to detect even smaller amounts of these compounds. The lowest absolute concentrations that were used as standards for the LCMS calibration of these compounds was 3.4 uM DMAPP, 1.7 uM IPP, 0.9 uM GPP, and 2.3 uM FPP. Thus, absolute amounts that are equal to or greater than these standard amounts can be readily detected.

[0244] In these experiments, there was a negligible amount of DMAPP, FPP, GPP, and IPP in the liquid cell medium (outside of the cells). Thus, the amounts listed in Tables 15A and 15B are representative of the intracellular concentrations of DMAPP, FPP, GPP, and IPP.

Table 15A. Exemplary metabolite concentrations

		Metabolite			
		DMAPP	IPP	GPP	FPP
Intracellular concentration, mM	Exemplary desirable concentrations	0.4 mM [1]	0.3 mM [1]	0.7 mM [2]	1.4 mM [1]
	Exemplary detected concentrations	9.2 mM [3] 15.3 mM [5]	27-40 mM [4] 6.3 mM [5]	2.8 mM [3] 3.3 mM [5]	3.6 mM [3]

[1] Example 3.
[2] Example 8, Part VII.
[3] Example 7, Part III.
[4] Example 8, Part VIII.
[5] Example 7, Part II.

Table 15B. Exemplary metabolite concentrations

		Metabolite			
		DMAPP	IPP	GPP	FPP
Intracellular concentration, $\mu mol/g_{dcw}$ [6]	Exemplary desirable concentrations	0.3 [1]	0.2 [1]	0.5 [2]	1.1 [1]
	Exemplary detected concentrations	7.0 [3] 11.6 [5]	20-30 [4] 4.8 [5]	2.1 [3] 3.3 [5]	2.0 [3]

[1] Example 3.
[2] Example 8, Part VII.
[3] Example 7, Part III.
[4] Example 8, Part VIII.
[5] Example 7, Part II.

[0245] In some embodiments, the intracellular concentration of DMAPP is between about 0 to about 25 μmol/g_{dcw}, such as between about 0.1 to about 20 μmol/g_{dcw}, about 0. 1 to about 15 μmol/g_{dcw}, about 0.1 to about 11 μmol/g_{dcw}, about 0.1 to about 7 μmol/g_{dcw}, about 0.1 to about 5 μmol/g_{dcw}, about 0.1 to about 2 μmol/g_{dcw}, about 0.1 to about 1 μmol/g_{dcw}, about 0.1 to about 0.8 μmol/g_{dcw}, about 0.1 to about 0.6 μmol/g_{dcw}, about 0.2 to about 15 μmol/g_{dcw}, about 0.2 to about 11 μmol/g_{dcw}, about 0.2 to about 7 μmol/g_{dcw}, about 0.2 to about 5 μmol/g_{dcw}, about 0.2 to about 2 μmol/g_{dcw}, about 0.3 to about 11 μmol/g_{dcw}, about 0.3 to about 7 μmol/g_{dcw}, about 0.3 to about 5 μmol/g_{dcw}, about 0.3 to about 2 μmol/g_{dcw}, about 0.3 to about 1 μmol/g_{dcw}, about 0.4 to about 11 μmol/g_{dcw}, about 0.4 to about 7 μmol/g_{dcw}, about 0.4 to about 5 μmol/g_{dcw}, about 0.4 to about 2 μmol/g_{dcw}, about 0.5 to about 7 μmol/g_{dcw}, about 0.5 to about 5 μmol/g_{dcw}, or about 0.5 to about 2 μmol/g_{dcw}. In some embodiments, the intracellular concentration of DMAPP is equal to or less than about any of 25, 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 μmol/g_{dcw}.

[0246] In some embodiments, the intracellular concentration of IPP is between about 0 to about 60 μmol/g_{dcw}, such as between about 0.1 to about 50 μmol/g_{dcw}, about 0.1 to about 40 μmol/g_{dcw}, about 0.1 to about 30 μmol/g_{dcw}, about 0.1 to about 20 μmol/g_{dcw}, about 0. 1 to about 15 μmol/g_{dcw}, about 0.1 to about 11 μmol/g_{dcw}, about 0.1 to about 7 μmol/g_{dcw}, about 0.1 to about 5 μmol/g_{dcw}, about 0.1 to about 2 μmol/g_{dcw}, about 0.1 to about 1 μmol/g_{dcw}, about 0.1 to about 0.8 μmol/g_{dcw}, about 0.1 to about 0.6 μmol/g_{dcw}, about 0.2 to about 60 μmol/g_{dcw}, about 0.2 to about 50 μmol/g_{dcw}, about 0.2 to about 40 μmol/g_{dcw}, about 0.2 to about 30 μmol/g_{dcw}, about 0.2 to about 20 μmol/g_{dcw}, about 0.2 to about 15 μmol/g_{dcw}, about 0.2 to about 11 μmol/g_{dcw}, about 0.2 to about 7 μmol/g_{dcw}, about 0.2 to about 5 μmol/g_{dcw}, about 0.2 to about 2 μmol/g_{dcw}, about 0.3 to about 60 μmol/g_{dcw}, about 0.3 to about 50 μmol/g_{dcw}, about 0.3 to about 40 μmol/g_{dcw}, about 0.3 to about 30 μmol/g_{dcw}, about 0.3 to about 15 μmol/g_{dcw}, about 0.3 to about 11 μmol/g_{dcw}, about 0.3 to about 7 μmol/g_{dcw}, about 0.3 to about 5 μmol/g_{dcw}, about 0.3 to about 2 μmol/g_{dcw}, about 0.4 to about 60 μmol/g_{dcw}, about 0.4 to about 50 μmol/g_{dcw}, about 0.4 to about 40 μmol/g_{dcw}, about 0.4 to about 30 μmol/g_{dcw}, about 0.4 to about 15 μmol/g_{dcw}, about 0.4 to about 7 μmol/g_{dcw}, about 0.4 to about 5 μmol/g_{dcw}, about 0.4 to about 2 μmol/g_{dcw}, about 0.5 to about 60 μmol/g_{dcw}, about 0.5 to about 50 μmol/g_{dcw}, about 0.5 to about 40 μmol/g_{dcw}, about 0.5 to about 30 μmol/g_{dcw}, about 0.5 to about 15 μmol/g_{dcw}, about 0.5 to about 11 μmol/g_{dcw}, about 0.5 to about 7 μmol/g_{dcw}, about 0.5 to about 5 μmol/g_{dcw}, or about 0.5 to about 2 μmol/g_{dcw}. In some embodiments, the

intracellular concentration of IPP is equal to or less than about any of 60, 50, 40, 30, 25, 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 $\mu mol/g_{dcw}$.

[0247] In some embodiments, the intracellular concentration of GPP is between about 0 to about 8 $\mu mol/g_{dcw}$, such as between about 0.1 to about 7 $\mu mol/g_{dcw}$, about 0. 1 to about 6 $\mu mol/g_{dcw}$, about 0.1 to about 5 $\mu mol/g_{dcw}$, about 0.1 to about 4 $\mu mol/g_{dcw}$, about 0.1 to about 3 $\mu mol/g_{dcw}$, about 0.1 to about 2 $\mu mol/g_{dcw}$, about 0.1 to about 1 $\mu mol/g_{dcw}$, about 0.1 to about 0.8 $\mu mol/g_{dcw}$, about 0.1 to about 0.6 $\mu mol/g_{dcw}$, about 0.2 to about 7 $\mu mol/g_{dcw}$, about 0.2 to about 6 $\mu mol/g_{dcw}$, about 0.2 to about 5 $\mu mol/g_{dcw}$, about 0.2 to about 4 $\mu mol/g_{dcw}$, about 0.2 to about 3 $\mu mol/g_{dcw}$, about 0.2 to about 2 $\mu mol/g_{dcw}$, about 0.3 to about 7 $\mu mol/g_{dcw}$, about 0.3 to about 6 $\mu mol/g_{dcw}$, about 0.3 to about 5 $\mu mol/g_{dcw}$, about 0.3 to about 4 $\mu mol/g_{dcw}$, about 0.3 to about 3 $\mu mol/g_{dcw}$, about 0.3 to about 2 $\mu mol/g_{dcw}$, about 0.4 to about 7 $\mu mol/g_{dcw}$, about 0.4 to about 6 $\mu mol/g_{dcw}$, about 0.4 to about 5 $\mu mol/g_{dcw}$, about 0.4 to about 2 $\mu mol/g_{dcw}$, about 0.5 to about 7 $\mu mol/g_{dcw}$, about 0.5 to about 5 $\mu mol/g_{dcw}$, about 0.5 to about 2 $\mu mol/g_{dcw}$, about 0.6 to about 7 $\mu mol/g_{dcw}$, about 0.6 to about 5 $\mu mol/g_{dcw}$, about 0.6 to about 2 $\mu mol/g_{dcw}$, about 0.7 to about 7 $\mu mol/g_{dcw}$, about 0.7 to about 5 $\mu mol/g_{dcw}$, or about 0.7 to about 2 $\mu mol/g_{dcw}$. In some embodiments, the intracellular concentration of GPP is equal to or less than about any of 8, 6, 4, 2, 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 $\mu mol/g_{dcw}$.

[0248] In some embodiments, the intracellular concentration of FPP is between about 0 to about 6 $\mu mol/g_{dcw}$, such as between about 0. 1 to about 6 $\mu mol/g_{dcw}$, about 0.1 to about 5 $\mu mol/g_{dcw}$, about 0.1 to about 4 $\mu mol/g_{dcw}$, about 0.1 to about 3 $\mu mol/g_{dcw}$, about 0.1 to about 2 $\mu mol/g_{dcw}$, about 0.1 to about 1 $\mu mol/g_{dcw}$, about 0.1 to about 0.8 $\mu mol/g_{dcw}$, about 0.1 to about 0.6 $\mu mol/g_{dcw}$, about 0.2 to about 6 $\mu mol/g_{dcw}$, about 0.2 to about 5 $\mu mol/g_{dcw}$, about 0.2 to about 4 $\mu mol/g_{dcw}$, about 0.2 to about 3 $\mu mol/g_{dcw}$, about 0.2 to about 2 $\mu mol/g_{dcw}$, about 0.3 to about 6 $\mu mol/g_{dcw}$, about 0.3 to about 5 $\mu mol/g_{dcw}$, about 0.3 to about 4 $\mu mol/g_{dcw}$, about 0.3 to about 3 $\mu mol/g_{dcw}$, about 0.3 to about 2 $\mu mol/g_{dcw}$, about 0.4 to about 6 $\mu mol/g_{dcw}$, about 0.4 to about 5 $\mu mol/g_{dcw}$, about 0.4 to about 2 $\mu mol/g_{dcw}$, about 0.5 to about 6 $\mu mol/g_{dcw}$, about 0.5 to about 5 $\mu mol/g_{dcw}$, about 0.5 to about 2 $\mu mol/g_{dcw}$, about 0.8 to about 6 $\mu mol/g_{dcw}$, about 0.8 to about 5 $\mu mol/g_{dcw}$, about 0.8 to about 2 $\mu mol/g_{dcw}$, about 1 to about 6 $\mu mol/g_{dcw}$, about 1 to about 5 $\mu mol/g_{dcw}$, about 1 to about 2 $\mu mol/g_{dcw}$, about 1.1 to about 6 $\mu mol/g_{dcw}$, about 1.1 to about 5 $\mu mol/g_{dcw}$, about 1.1 to about 2 $\mu mol/g_{dcw}$, about 1.1 to about 1.5 $\mu mol/g_{dcw}$, about 1.2 to about 6 $\mu mol/g_{dcw}$, about 1.2 to about 5 $\mu mol/g_{dcw}$, about

1.2 to about 2 μmol/g_{dcw}, or about 1.2 to about 1.5 μmol/g_{dcw}. In some embodiments, the intracellular concentration of FPP is equal to or less than about any of 6, 4, 2, 1.5, 1.4, 1.3, 1.2, 1.1, 1, 0.9, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 μmol/g_{dcw}.

[0249] In some embodiments, the concentration (*e.g.*, concentration in the cell medium) of MVA is between about 0 to about 120 g/L, such as between about about 0 to about 110 g/L, such as between about 0.1 to about 100 g/L, about 0.1 to about 75 g/L, about 0.1 to about 60 g/L, about 0.1 to about 50 g/L, about 0.1 to about 40 g/L, about 0.1 to about 30 g/L, about 0.1 to about 20 g/L, about 0. 1 to about 15 g/L, about 0.1 to about 11 g/L, about 0.1 to about 7 g/L, about 0.1 to about 5 g/L, about 0.1 to about 2 g/L, about 0.1 to about 1 g/L, about 0.1 to about 0.8 g/L, about 0.1 to about 0.6 g/L, about 0.2 to about 120 g/L, about 0.2 to about 100 g/L, about 0.2 to about 75 g/L, about 0.2 to about 60 g/L, about 0.2 to about 50 g/L, about 0.2 to about 40 g/L, about 0.2 to about 30 g/L, about 0.2 to about 20 g/L, about 0.2 to about 15 g/L, about 0.2 to about 11 g/L, about 0.2 to about 7 g/L, about 0.2 to about 5 g/L, about 0.2 to about 2 g/L, about 0.3 to about 120 g/L, about 0.3 to about 100 g/L, about 0.3 to about 75 g/L, about 0.3 to about 60 g/L, about 0.3 to about 50 g/L, about 0.3 to about 40 g/L, about 0.3 to about 30 g/L, about 0.3 to about 15 g/L, about 0.3 to about 11 g/L, about 0.3 to about 7 g/L, about 0.3 to about 5 g/L, about 0.3 to about 2 g/L, about 0.4 to about 120 g/L, about 0.4 to about 100 g/L, about 0.4 to about 75 g/L, about 0.4 to about 60 g/L, about 0.4 to about 50 g/L, about 0.4 to about 40 g/L, about 0.4 to about 30 g/L, about 0.4 to about 15 g/L, about 0.4 to about 7 g/L, about 0.4 to about 5 g/L, about 0.4 to about 2 g/L, about 0.5 to about 1200 g/L, about 0.5 to about 100 g/L, about 0.5 to about 75 g/L, about 0.5 to about 60 g/L, about 0.5 to about 50 g/L, about 0.5 to about 40 g/L, about 0.5 to about 30 g/L, about 0.5 to about 15 g/L, about 0.5 to about 11 g/L, about 0.5 to about 7 g/L, about 0.5 to about 5 g/L, about 0.5 to about 2 g/L, about 50 to about 60 g/L, or about 1 g/L. In some embodiments, the concentration (*e.g.*, concentration in the cell medium) of MVA is equal to or less than about any of 120, 100, 80, 70, 60, 50, 40, 30, 25, 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 g/L.

[0250] Examples 13-24 also support the use of the compositions and methods disclosed herein to produce large amounts of isoprene. The methods described herein can be used to modify any of the cells and methods of Examples 13-24 to increase the expression level and/or activity level of a mevalonate kinase polypeptide and/or an isoprene synthase polypeptide. Additionally, methods described herein can be used to modify any of the cells

and methods of U.S.S.N. 61/134,094, filed July 2, 2008 (which is hereby incorporated by reference in its entirety, particularly with respect to methods of making isoprene and isoprene compositions) to increase the expression level and/or activity level of a mevalonate kinase polypeptide and/or an isoprene synthase polypeptide. As discussed above, increasing the expression level and/or activity level of a mevalonate kinase polypeptide and/or an isoprene synthase polypeptide may further increase the production of isoprene.

Summary of Exemplary Compositions and Methods for Producing Isoprene

[0251] This section summaries exemplary compositions and methods for producing isoprene that can be used with cells having increased expression levels and/or activity levels of a mevalonate kinase polypeptide and an isoprene synthase polypeptide. In one aspect, the invention features compositions and methods for the production of isoprene in increased amounts and/or purity. In one aspect, compositions and methods of the invention increase the rate of isoprene production and increase the total amount of isoprene that is produced. For example, cell culture systems that generate 4.8×10^4 nmole/g_{wcm}/hr of isoprene have been produced (Table 1). The efficiency of these systems is demonstrated by the conversion of about 2.2% of the carbon that the cells consume from a cell culture medium into isoprene. As shown in the Examples and Table 2, approximately 3 g of isoprene per liter of broth was generated. If desired, even greater amounts of isoprene can be obtained using other conditions, such as those described herein. In some embodiments, a renewable carbon source is used for the production of isoprene. In some embodiments, the production of isoprene is decoupled from the growth of the cells. In some embodiments, the concentrations of isoprene and any oxidants are within the nonflammable ranges to reduce or eliminate the risk that a fire may occur during production or recovery of isoprene. The compositions and methods of the present invention are desirable because they allow high isoprene yield per cell, high carbon yield, high isoprene purity, high productivity, low energy usage, low production cost and investment, and minimal side reactions. This efficient, large scale, biosynthetic process for isoprene production provides an isoprene source for synthetic isoprene-based rubber and provides a desirable, low-cost alternative to using natural rubber.

[0252] As discussed further herein, the amount of isoprene produced by cells can be greatly increased by introducing a heterologous nucleic acid encoding an isoprene synthase polypeptide (*e.g.*, a plant isoprene synthase polypeptide) into the cells. Isoprene synthase

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

Table 1: Exemplary yields of isoprene from a shake flask using the cell cultures and methods of the invention. The assay for measuring isoprene production is described in Example 13, part II. For this assay, a sample was removed at one or more time points from the shake flask and cultured for 30 minutes. The amount of isoprene produced in this sample was then measured. The headspace concentration and specific rate of isoprene production are listed in Table 1 and described further herein.

Strain	**Isoprene Production in a Headspace vial***	
	Headspace concentration $\mu g/L_{gas}$	**Specific Rate** $\mu g/L_{broth}/hr/OD$ **(**$nmol/g_{wcm}/hr$**)**
E. coli BL21/ pTrcKudzu IS	1.40	53.2 (781.2)
E. coli BL21/ pCL DXS yidi Kudzu IS	7.61	289.1 (4.25×10^3)

E. coli BL21/MCM127 with kudzu IS and entire MVA pathway	23.0	874.1 (12.8×10^3)
E. coli BL21/ pET N-HisKudzu IS	1.49	56.6 (831.1)
Pantoea citrea /pTrcKudzu IS	0.66	25.1 (368.6)
E. coli w/ Poplar IS [Miller (2001)]	-	5.6 (82.2)
Bacillis licheniformis Fall US 5849970	-	4.2 (61.4)
Yarrowia lipolytica with kudzu isoprene synthase	~0.05 μg/L	~2 (~30)
Trichoderma reesei with kudzu isoprene synthase	~0.05 μg/L	~2 (~30)
E. coli BL21/ pTrcKKD$_y$I$_k$IS with kudzu IS and lower MVA pathway	85.9	3.2×10^3 (4.8×10^4)

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

Table 2: Exemplary yields of isoprene in a fermentor using the cell cultures and methods of the invention. The assay for measuring isoprene production is described in Example 13, part II. For this assay, a sample of the off-gas of the fermentor was taken and analyzed for the amount of isoprene. The peak headspace concentration (which is the highest headspace concentration during the fermentation), titer (which is the cumulative, total amount of isoprene produced per liter of broth), and peak specific rate of isoprene production (which is the highest specific rate during the fermentation) are listed in Table 2 and described further herein.

Strain	Isoprene Production in Fermentors		
	Peak Headspace concentration (ug/L_{gas})**	**Titer (mg/L_{broth})**	**Peak Specific rate $\mu g/L_{broth}/hr/OD$ ($nmol/g_{wcm}/hr$)**
E. coli BL21 /pTrcKudzu with Kudzu IS	52	41.2	37 (543.3)
E. coli FM5/pTrcKudzu IS	3	3.5	21.4 (308.1)
E. coli BL21/ triple strain (DXS, yidi, IS)	285	300	240 (3.52×10^3)
E. coli FM5/ triple strain (DXS, yidi, IS)	50.8	29	180.8 (2.65×10^3)
E. coli/MCM127 with Kudzu IS and entire MVA pathway	3815	3044	992.5 (1.46×10^4)
E. coli BL21/pCLPtrc UpperPathway gi1.2 integrated lower pathway pTrcKudzu	2418	1640	1248 (1.83×10^4)
E. coli BL21/MCM401 with 4 x 50uM IPTG	13991	23805	3733 (5.49×10^4)
E. coli BL21/MCM401 with 2 x 100uM IPTG	22375	19541	5839.5 (8.59×10^4)
E. coli BL21/pCLPtrc UpperPathwayHGS2 - pTrcKKDyIkIS	3500	3300	1088 (1.60×10^4)
Bacillus subtilis wild-type	1.5	2.5	0.8 (11.7)

Bacillus pBS Kudzu IS	16.6	~30 (over 100 hrs)	5 (73.4)
Bacillus Marburg 6051 [Wagner and Fall (1999)]	2.04	0.61	24.5 (359.8)
Bacillus Marburg 6051 Fall US 5849970	0.7	0.15	6.8 (100)

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

[0253] 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.

[0254] The encoded DXS and IDI polypeptides are part of the DXP pathway for the biosynthesis of isoprene (Figure 19A). DXS polypeptides convert pyruvate and D-glyceraldehyde-3-phosphate into 1-deoxy-D-xylulose-5-phosphate. While not intending to be bound by any particular theory, it is believed that increasing the amount of DXS polypeptide increases the flow of carbon through the DXP pathway, leading to greater isoprene production. IDI polypeptides catalyze the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). While not intending to be bound by any particular theory, it is believed that increasing the amount of IDI polypeptide in cells increases the amount (and conversion rate) of IPP that is converted into DMAPP, which in turn is converted into isoprene.

[0255] 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 19, part VII).

[0256] 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.

[0257] In some embodiments, the production of isoprene by cells by cells that contain a heterologous isoprene synthase nucleic acid is augmented by increasing the amount of a MVA pathway 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.

[0258] For example, *E. coli* cells containing a nucleic acid encoding a kudzu isoprene synthase polypeptide and nucleic acids encoding *Saccharomyces cerevisia* MVK, PMK, MVD, and IDI polypeptides generated isoprene at a rate of 6.67×10^{-4} $mol/L_{broth}/OD_{600}/hr$ (*see* Example 20). Additionally, a 14 liter fermentation of *E. coli* cells with nucleic acids encoding *Enterococcus faecalis* AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase polypeptides produced 22 grams of mevalonic acid (an intermediate of the MVA pathway). A shake flask of these cells produced 2-4 grams of mevalonic acid per liter. These results indicate that heterologous MVA pathways nucleic acids are active in *E. coli*. *E. coli* cells that contain nucleic acids for both the upper MVA pathway and the lower MVA pathway as well as a kudzu isoprene synthase (strain MCM 127) produced significantly more isoprene (874 ug/L) compared to *E. coli* cells with nucleic acids for only the lower MVA pathway and the kudzu isoprene synthase (strain MCM 131) (*see* Table 10 and Example 20, part VIII).

[0259] In some embodiments, at least a portion of the cells maintain the heterologous isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid for at least about 5, 10, 20, 50, 75, 100, 200, 300, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects of the invention, the nucleic acid comprising the heterologous or duplicate copy of an endogenous isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid also comprises a selective marker,

such as a kanamycin, ampicillin, carbenicillin, gentamicin, hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol antibiotic resistance nucleic acid.

[0260] As indicated in Example 19, 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 19, 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.

[0261] Isoprene production was also demonstrated using three types of hydrolyzed biomass (bagasse, corn stover, and soft wood pulp) as the carbon source. *E. coli* cells with kudzu isoprene synthase, *S. cerevisia* IDI, and *E. coli* DXS nucleic acids produced as much isoprene from these hydrolyzed biomass carbon sources as from the equivalent amount of glucose (*e.g.*, 1% glucose, w/v). If desired, any other biomass carbon source can be used in the compositions and methods of the invention. Biomass carbon sources are desirable because they are cheaper than many conventional cell mediums, thereby facilitating the economical production of isoprene.

[0262] Additionally, invert sugar was shown to function as a carbon source for the generation of isoprene. For example, 2.4 g/L of isoprene was produced from cells expressing MVA pathway polypeptides and a Kudzu isoprene synthase. 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. 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.

[0263] 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 16, part III). In some embodiments, more than one oil (such as 2, 3, 4, 5, or more oils) is included in the cell medium. While not intending to be bound to any particular theory, it is believed that (i) the oil may increase the amount of carbon in the cells that is available for conversion to isoprene, (ii) the oil may increase the amount of acetyl-CoA in the cells, thereby increasing the carbon flow through the MVA pathway, and/or (ii) the oil may provide extra nutrients to the cells, which is desirable since a lot of the carbon in the cells is converted to isoprene rather than other products. In some embodiments, cells that are cultured in a cell medium containing oil naturally use the MVA pathway to produce isoprene or are genetically modified to contain nucleic acids for the entire MVA pathway. In some embodiments, the oil is partially or completely hydrolyzed before being added to the cell culture medium to facilitate the use of the oil by the host cells.

[0264] 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 22, *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.

[0265] The vast majority of isoprene is derived from petrochemical sources as an impure C5 hydrocarbon fraction which requires extensive purification before the material is suitable for polymerization. Several impurities are particularly problematic given their structural similarity to isoprene and the fact that they can act as polymerization catalyst poisons. Such compounds include 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol).

[0266] (Figure 90). In some embodiments, the isoprene composition of the invention is substantially free of any contaminating unsaturated C5 hydrocarbons. No detectable amount of unsaturated C5 hydrocarbons other than isoprene (such as 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol)) was found in isoprene compositions produced using the methods described herein. Some isoprene compositions produced using the methods described herein contain ethanol, acetone, and C5 prenyl alcohols as determined by GC/MS analysis. All of these components are far more readily removed from the isoprene stream than the isomeric C5 hydrocarbon fractions that are present in isoprene compositions derived from petrochemical sources. Accordingly, in some embodiments, the isoprene compositions of the invention require minimal treatment in order to be of polymerization grade.

Exemplary Polypeptides and Nucleic Acids

[0267] Various isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids can be used in the compositions and methods of the invention.

[0268] As used herein, "polypeptides" includes polypeptides, proteins, peptides, fragments of polypeptides, and fusion polypeptides. In some embodiments, the fusion polypeptide includes part or all of a first polypeptide (*e.g.*, an isoprene synthase, DXS, IDI, or MVA pathway polypeptide or catalytically active fragment thereof) and may optionally include part

or all of a second polypeptide (*e.g.*, a peptide that facilitates purification or detection of the fusion polypeptide, such as a His-tag). In some embodiments, the fusion polypeptide has an activity of two or more MVA pathway polypeptides (such as AA-CoA thiolase and HMG-CoA reductase polypeptides). In some embodiments, the polypeptide is a naturally-occurring polypeptide (such as the polypeptide encoded by an *Enterococcus faecalis mvaE* nucleic acid) that has an activity of two or more MVA pathway polypeptides.

[0269] In various embodiments, a polypeptide has at least or about 50, 100, 150, 175, 200, 250, 300, 350, 400, or more amino acids. In some embodiments, the polypeptide fragment contains at least or about 25, 50, 75, 100, 150, 200, 300, or more contiguous amino acids from a full-length polypeptide and has at least or about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% of an activity of a corresponding full-length polypeptide. In particular embodiments, the polypeptide includes a segment of or the entire amino acid sequence of any naturally-occurring isoprene synthase, DXS, IDI, or MVA pathway polypeptide. In some embodiments, the polypeptide has one or more mutations compared to the sequence of a wild-type (*i.e.*, a sequence occurring in nature) isoprene synthase, DXS, IDI, or MVA pathway polypeptide.

[0270] In some embodiments, the polypeptide is an isolated polypeptide. As used herein, an "isolated polypeptide" is not part of a library of polypeptides, such as a library of 2, 5, 10, 20, 50 or more different polypeptides and is separated from at least one component with which it occurs in nature. An isolated polypeptide can be obtained, for example, by expression of a recombinant nucleic acid encoding the polypeptide.

[0271] In some embodiments, the polypeptide is a heterologous polypeptide. By "heterologous polypeptide" is meant a polypeptide whose amino acid sequence is not identical to that of another polypeptide naturally expressed in the same host cell. In particular, a heterologous polypeptide is not identical to a wild-type nucleic acid that is found in the same host cell in nature.

[0272] As used herein, a "nucleic acid" refers to two or more deoxyribonucleotides and/or ribonucleotides in either single or double-stranded form. In some embodiments, the nucleic acid is a recombinant nucleic acid. By "recombinant nucleic acid" means a nucleic acid of interest that is free of one or more nucleic acids (*e.g.*, genes) which, in the genome occurring in nature of the organism from which the nucleic acid of interest is derived, flank the nucleic

acid of interest. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA, a genomic DNA fragment, or a cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In various embodiments, a nucleic acid is a recombinant nucleic acid. In some embodiments, an isoprene synthase, DXS, IDI, or MVA pathway nucleic acid is operably linked to another nucleic acid encoding all or a portion of another polypeptide such that the recombinant nucleic acid encodes a fusion polypeptide that includes an isoprene synthase, DXS, IDI, or MVA pathway polypeptide and all or part of another polypeptide (*e.g.*, a peptide that facilitates purification or detection of the fusion polypeptide, such as a His-tag). In some embodiments, part or all of a recombinant nucleic acid is chemically synthesized. It is to be understood that mutations, including single nucleotide mutations, can occur within a nucleic acid as defined herein.

[0273] In some embodiments, the nucleic acid is a heterologous nucleic acid. By "heterologous nucleic acid" is meant a nucleic acid whose nucleic acid sequence is not identical to that of another nucleic acid naturally found in the same host cell.

[0274] In particular embodiments, the nucleic acid includes a segment of or the entire nucleic acid sequence of any naturally-occurring isoprene synthase, DXS, IDI, or MVA pathway nucleic acid. In some embodiments, the nucleic acid includes at least or about 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, or more contiguous nucleotides from a naturally-occurring isoprene synthase nucleic acid DXS, IDI, or MVA pathway nucleic acid. In some embodiments, the nucleic acid has one or more mutations compared to the sequence of a wild-type (*i.e.*, a sequence occurring in nature) isoprene synthase, DXS, IDI, or MVA pathway nucleic acid. In some embodiments, the nucleic acid has one or more mutations (*e.g.*, a silent mutation) that increase the transcription or translation of isoprene synthase, DXS, IDI, or MVA pathway nucleic acid. In some embodiments, the nucleic acid is a degenerate variant of any nucleic acid encoding an isoprene synthase, DXS, IDI, or MVA pathway polypeptide.

[0275] "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.

[0276] 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

[0277] As noted above, isoprene synthase polypeptides convert dimethylallyl diphosphate (DMAPP) into isoprene. Exemplary isoprene synthase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of an isoprene synthase polypeptide. Standard methods can be used to determine whether a polypeptide has isoprene synthase polypeptide activity by measuring the ability of the polypeptide to convert DMAPP into isoprene *in vitro*, in a cell extract, or *in vivo*. In an exemplary assay, cell extracts are prepared by growing a strain (*e.g.*, the *E. coli*/pTrcKudzu strain described herein) in the shake flask method as described in Example 13. After induction is complete, approximately 10 mL of cells are pelleted by centrifugation at 7000 x

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

[0278] Isoprene synthase polypeptide activity in the cell extract can be measured, for example, as described in Silver *et al.*, J. Biol. Chem. 270:13010-13016, 1995 and references therein, which are each hereby incorporated by reference in their entireties, particularly with respect to assays for isoprene synthase polypeptide activity. DMAPP (Sigma) is evaporated to dryness under a stream of nitrogen and rehydrated to a concentration of 100 mM in 100 mM potassium phosphate buffer pH 8.2 and stored at -20 °C. To perform the assay, a solution of 5 μL of 1M $MgCl_2$, 1 mM (250 μg/ml) DMAPP, 65 μL of Plant Extract Buffer (PEB) (50 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 5% glycerol, and 2 mM DTT) is added to 25 μL of cell extract in a 20 ml Headspace vial with a metal screw cap and teflon coated silicon septum (Agilent Technologies) and cultured at 37 °C for 15 minutes with shaking. The reaction is quenched by adding 200 μL of 250 mM EDTA and quantified by GC/MS as described in Example 13, part II.

[0279] 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.

[0280] 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

[0281] 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

[0282] Isopentenyl diphosphate isomerase polypeptides (isopentenyl-diphosphate delta-isomerase or IDI) catalyses the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (*e.g.*, converting IPP into DMAPP and/or converting DMAPP into IPP). Exemplary IDI polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of an IDI polypeptide. Standard methods (such as those described herein) can be used to determine

whether a polypeptide has IDI polypeptide activity by measuring the ability of the polypeptide to interconvert IPP and DMAPP *in vitro*, in a cell extract, or *in vivo*. Exemplary IDI nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of an IDI polypeptide. Exemplary IDI polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

Exemplary MVA Pathway Polypeptides and Nucleic Acids

[0283] 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.

[0284] 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*.

[0285] 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*.

[0286] 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*.

[0287] 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*.

[0288] 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*.

[0289] 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*.

[0290] 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*.

[0291] 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*.

[0292] Exemplary IDI polypeptides and nucleic acids are described above.

Exemplary Methods for Isolating Nucleic Acids

[0293] Isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acids can be isolated using standard methods. Methods of obtaining desired nucleic acids from a source organism of interest (such as a bacterial genome) are common and well known in the art of molecular biology (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to the isolation of nucleic acids of interest). For example, if the sequence of the nucleic acid is known (such as any of the known nucleic acids described herein), suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes complementary to the desired nucleic acid sequence. Once the sequence is isolated, the DNA may be amplified using standard primer directed amplification methods such as polymerase chain reaction (PCR) (U.S. Patent No. 4,683,202, which is incorporated by reference in its entirety, particularly with respect to PCR methods) to obtain amounts of DNA suitable for transformation using appropriate vectors.

[0294] Alternatively, isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acids (such as any isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acids with a known nucleic acid sequence) can be chemically synthesized using standard methods.

[0295] Additional isoprene synthase, DXS, IDI, or MVA pathway polypeptides and nucleic acids which may be suitable for use in the compositions and methods described herein can be identified using standard methods. For example, cosmid libraries of the chromosomal DNA of organisms known to produce isoprene naturally can be constructed in organisms such as *E. coli*, and then screened for isoprene production. In particular, cosmid libraries may be created where large segments of genomic DNA (35-45 kb) are packaged into vectors and used to transform appropriate hosts. Cosmid vectors are unique in being able to

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

[0296] 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.

[0297] Additional methods for obtaining isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acids include screening a metagenomic library by assay (such as the headspace assay described herein) or by PCR using primers directed against nucleotides encoding for a length of conserved amino acids (for example, at least 3 conserved amino acids). Conserved amino acids can be identified by aligning amino acid sequences of known isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides. Conserved amino acids for isoprene synthase polypeptides can be identified based on aligned sequences of known isoprene synthase polypeptides. An organism found to produce isoprene naturally can be subjected to standard protein purification methods (which are well known in the art) and the resulting purified polypeptide can be sequenced using standard methods. Other methods are found in the literature (*see*, for example, Julsing *et al.*, *Applied. Microbiol. Biotechnol.* 75: 1377-84, 2007; Withers *et al.*, *Appl Environ Microbiol.* 73(19):6277-83, 2007, which are each hereby incorporated by reference in their entireties, particularly with respect to identification of nucleic acids involved in the synthesis of isoprene).

[0298] Additionally, standard sequence alignment and/or structure prediction programs can be used to identify additional DXS, IDI, or MVA pathway polypeptides and nucleic acids based on the similarity of their primary and/or predicted polypeptide secondary structure with that of known DXS, IDI, or MVA pathway polypeptides and nucleic acids. Standard databases such as the swissprot-trembl database (world-wide web at "expasy.org", Swiss Institute of Bioinformatics Swiss-Prot group CMU - 1 rue Michel Servet CH-1211 Geneva 4, Switzerland) can also be used to identify isoprene synthase, DXS, IDI, or MVA pathway polypeptides and nucleic acids. The secondary and/or tertiary structure of an isoprene synthase, DXS, IDI, or MVA pathway polypeptide can be predicted using the default settings of standard structure prediction programs, such as PredictProtein (630 West, 168 Street, BB217, New York, N.Y. 10032, USA). Alternatively, the actual secondary and/or tertiary structure of an isoprene synthase, DXS, IDI, or MVA pathway polypeptide can be determined using standard methods. Additional isoprene synthase, DXS, IDI, or MVA pathway nucleic acids can also be identified by hybridization to probes generated from known isoprene synthase, DXS, IDI, or MVA pathway nucleic acids.

Exemplary Promoters and Vectors

[0299] Any of the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid described herein can be included in one or more vectors. Accordingly, the invention also features vectors with one more nucleic acids encoding any of the isoprene synthase, DXS, IDI, or MVA pathway polypeptides that are described herein. As used herein, a "vector" means a construct that is capable of delivering, and desirably expressing one or more nucleic acids of interest in a host cell. Examples of vectors include, but are not limited to, plasmids, viral vectors, DNA or RNA expression vectors, cosmids, and phage vectors. In some embodiments, the vector contains a nucleic acid under the control of an expression control sequence.

[0300] 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.

[0301] In some embodiments, the vector contains a selective marker. The term "selective marker" refers to a nucleic acid capable of expression in a host cell that allows for ease of selection of those host cells containing an introduced nucleic acid or vector. Examples of selectable markers include, but are not limited to, antibiotic resistance nucleic acids (*e.g.*, kanamycin, ampicillin, carbenicillin, gentamicin, hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol) and/or nucleic acids that confer a metabolic advantage, such as a nutritional advantage on the host cell. Exemplary nutritional selective markers include those markers known in the art as *amd*S, *arg*B, and *pyr4*. Markers useful in vector systems for transformation of *Trichoderma* are known in the art (*see*, *e.g.*, Finkelstein, Chapter 6 in Biotechnology of Filamentous Fungi, Finkelstein *et al., Eds.* Butterworth-Heinemann, Boston, MA, Chap. 6., 1992; and Kinghorn *et al.*, Applied Molecular Genetics of Filamentous Fungi, Blackie Academic and Professional, Chapman and Hall, London, 1992, which are each hereby incorporated by reference in their entireties, particularly with respect to selective markers). In some embodiments, the selective marker is the *amd*S nucleic acid, which encodes the enzyme acetamidase, allowing transformed cells to grow on acetamide as a nitrogen source. The use of an *A. nidulans amd*S nucleic acid as a selective marker is described in Kelley *et al.*, *EMBO J.* 4:475 – 479, 1985 and Penttila *et al.*, *Gene* 61:155-164, 1987 (which are each hereby incorporated by reference in their entireties, particularly with respect to selective markers). In some embodiments, an isoprene synthase, DXS, IDI, or MVA pathway nucleic acid integrates into a chromosome of the cells without a selective marker.

[0302] 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).

[0303] Promoters are well known in the art. Any promoter that functions in the host cell can be used for expression of an isoprene synthase, DXS, IDI, or MVA pathway nucleic acid in the host cell. Initiation control regions or promoters, which are useful to drive expression of isoprene synthase, DXS, IDI, or MVA pathway nucleic acids in various host cells are

numerous and familiar to those skilled in the art (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to vectors for the expression of nucleic acids of interest). Virtually any promoter capable of driving these nucleic acids is suitable for the present invention including, but not limited to, CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADCI, TRP1, URA3, LEU2, ENO, and TPI (useful for expression in *Saccharomyces*); AOX1 (useful for expression in *Pichia*); and lac, trp, λP_L, λP_R, T7, tac, and trc (useful for expression in *E. coli*).

[0304] 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).

[0305] In various embodiments, an isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid is contained in a low copy plasmid (*e.g.*, a plasmid that is maintained at about 1 to about 4 copies per cell), medium copy plasmid (*e.g.*, a plasmid that is maintained at about 10 to about 15 copies per cell), or high copy plasmid (*e.g.*, a plasmid that is maintained at about 50 or more copies per cell). In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway nucleic acid is operably linked to a T7 promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway nucleic acid operably linked to a T7 promoter is contained in a medium or high copy plasmid. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway nucleic acid is operably linked to a Trc promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway nucleic acid operably linked to a Trc promoter is contained in a medium or high copy plasmid. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway nucleic acid is operably linked to a Lac promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, or MVA pathway nucleic acid operably linked to a

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

[0306] 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.

[0307] In some embodiments, an isoprene synthase, DXS, IDI, or MVA pathway nucleic acid is operably linked to a suitable promoter that shows transcriptional activity in a fungal host cell. The promoter may be derived from one or more nucleic acids encoding a polypeptide that is either endogenous or heterologous to the host cell. In some embodiments, the promoter is useful in a *Trichoderma* host. Suitable non-limiting examples of promoters include *cbh*1, *cbh*2, *egl*1, *egl*2, *pepA*, *hfb*1, *hfb*2, *xyn*1, and *amy*. In some embodiments, the promoter is one that is native to the host cell. For example, in some embodiments when *T. reesei* is the host, the promoter is a native *T. reesei* promoter. In some embodiments, the promoter is *T. reesei cbh1*, which is an inducible promoter and has been deposited in GenBank under Accession No. D86235, which is incorporated by reference in its entirety,

particularly with respect to promoters. In some embodiments, the promoter is one that is heterologous to the fungal host cell. Other examples of useful promoters include promoters from the genes of *A. awamori* and *A. niger* glucoamylase (glaA) (Nunberg *et al.*, *Mol. Cell Biol.* 4:2306-2315, 1984 and Boel *et al.*, *EMBO J.* 3:1581-1585, 1984, which are each hereby incorporated by reference in their entireties, particularly with respect to promoters); *Aspergillus niger* alpha amylases, *Aspergillus oryzae* TAKA amylase, *T. reesei xln*1, and the *T. reesei cellobiohydrolase* 1 (EP 137280, which is incorporated by reference in its entirety, particularly with respect to promoters).

[0308] 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.

[0309] In some embodiments, the promoter, coding, region, and terminator all originate from the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid to be expressed. In some embodiments, the coding region for an isoprene synthase, DXS, IDI, or MVA pathway nucleic acid is inserted into a general-purpose expression vector such that it is under the transcriptional control of the expression construct promoter and terminator sequences. In some embodiments, genes or part thereof are inserted downstream of the strong *cbh*1 promoter.

[0310] An isoprene synthase, DXS, IDI, or MVA pathway nucleic acid can be incorporated into a vector, such as an expression vector, using standard techniques (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982, which is hereby

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

[0311] In some embodiments, it may be desirable to over-express isoprene synthase, DXS, IDI, or MVA pathway nucleic acids at levels far higher than currently found in naturally-occurring cells. This result may be accomplished by the selective cloning of the nucleic acids encoding those polypeptides into multicopy plasmids or placing those nucleic acids under a strong inducible or constitutive promoter. Methods for over-expressing desired polypeptides are common and well known in the art of molecular biology and examples may be found in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to cloning techniques.

[0312] The following resources include descriptions of additional general methodology useful in accordance with the invention: Kreigler, Gene Transfer and Expression; A Laboratory Manual,1990 and Ausubel *et al.*, Eds. Current Protocols in Molecular Biology, 1994, which are each hereby incorporated by reference in their entireties, particularly with respect to molecular biology and cloning techniques.

Exemplary Source Organisms

[0313] Isoprene synthase, DXS, IDI, or MVA pathway nucleic acids (and their encoded polypeptides) can be obtained from any organism that naturally contains isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acids. As noted above, isoprene is formed naturally by a variety of organisms, such as bacteria, yeast, plants, and animals. Organisms contain the MVA pathway, DXP pathway, or both the MVA and DXP pathways for producing isoprene (Figures 19A and 19B). Thus, DXS nucleic acids can be obtained, *e.g.*, from any organism that contains the DXP pathway or contains both the MVA and DXP pathways. IDI and isoprene synthase nucleic acids can be obtained, *e.g.*, from any organism that contains the MVA pathway, DXP pathway, or both the MVA and DXP pathways. MVA pathway nucleic acids can be obtained, *e.g.*, from any organism that contains the MVA pathway or contains both the MVA and DXP pathways.

[0314] In some embodiments, the nucleic acid sequence of the isoprene synthase, DXS, IDI, or MVA pathway nucleic is identical to the sequence of a nucleic acid that is produced by any of the following organisms in nature. In some embodiments, the amino acid sequence of the isoprene synthase, DXS, IDI, or MVA pathway polypeptide is identical to the sequence of a polypeptide that is produced by any of the following organisms in nature. In some embodiments, the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid or polypeptide is a mutant nucleic acid or polypeptide derived from any of the organisms described herein. As used herein, "derived from" refers to the source of the nucleic acid or polypeptide into which one or more mutations is introduced. For example, a polypeptide that is "derived from a plant polypeptide" refers to polypeptide of interest that results from introducing one or more mutations into the sequence of a wild-type (*i.e.*, a sequence occurring in nature) plant polypeptide.

[0315] 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.*

[0316] 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.

[0317] In some embodiments, the source organism is a yeast, such as *Saccharomyces sp., Schizosaccharomyces sp., Pichia sp.,* or *Candida sp.*

[0318] In some embodiments, the source organism is a bacterium, such as strains of *Bacillus* such as *B. lichenformis* or *B. subtilis,* strains of *Pantoea* such as *P. citrea*, strains of *Pseudomonas* such as *P. alcaligenes,* strains of *Streptomyces* such as *S. lividans* or *S. rubiginosus,* or strains of *Escherichia* such as *E. coli*.

[0319] 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*.

[0320] In some embodiments, the source organism is a gram-positive bacterium. Non-limiting examples include strains of *Streptomyces* (*e.g.*, *S. lividans*, *S. coelicolor*, or *S. griseus*) and *Bacillus*. In some embodiments, the source organism is a gram–negative bacterium, such as *E. coli* or *Pseudomonas sp.*

[0321] 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*.

[0322] In some embodiments, the source organism is an algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates.

[0323] 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*.

[0324] In some embodiments, the source organism is an archaeon, such as *Methanosarcina mazei*. Exemplary archaea include those disclosed by Koga and Morii (*Microbiology & Mol. Biology Reviews*, 71:97-120, 2007, which is hereby incorporated by reference in its entirety, particularly with respect to archaea (see Table 3)). Other exemplary archaea are hyperthermophilic archaea, such as *Methanococcus jannaschii* (Huang *et al.*, *Protein Expression and Purification* 17(1):33-40, 1999) and halophilic archaea (such as *Halobacterium salanarium*).

Table 3. Exemplary archaea

Original name	Exemplary Strain	Name most recently proposed
Caldariella acidophila		*Sulfolobus solfataricus*
Halobacterium cutirubrum		*Halobacterium salinarum*
Halobacterium halobium		*Halobacterium salinarum*
Halobacterium mediterranei		*Haloferax mediterranei*
Halobacterium vallismortis		*Haloarcula vallismortis*
Methanobacterium thermoautotrophicum	ΔH	*Methanothermobacter thermautotrophicus*
Methanobacterium thermoautotrophicum	Marburg	*Methanothermobacter marburgensis*
Methanobacterium thermoformicicum	SF-4	*Methanothermobacter wolfeii*
Methanococcus igneus		*Methanotorris igneus*
Natronobacterium pharaonis		*Natronomonas pharaonis*
Pseudomonas salinaria		*Halobacterium salinarum*

Exemplary Host Cells

[0325] A variety of host cells can be used to express isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and to produce isoprene in the methods of the invention. Exemplary host cells include cells from any of the organisms listed in the prior section under the heading "*Exemplary Source Organisms.*" The host cell may be a cell that naturally produces isoprene or a cell that does not naturally produce isoprene. In some embodiments, the host cell naturally produces isoprene using the DXP pathway, and an isoprene synthase, DXS, and/or IDI nucleic acid is added to enhance production of isoprene using this pathway. In some embodiments, the host cell naturally produces isoprene using the MVA pathway, and an isoprene synthase and/or one or more MVA pathway nucleic acids are added to enhance production of isoprene using this pathway. In some embodiments, the host cell naturally produces isoprene using the DXP pathway and one or more MVA pathway nucleic acids are

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

Exemplary Transformation Methods

[0326] Isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acids or vectors containing them can be inserted into a host cell (*e.g.*, a plant cell, a fungal cell, a yeast cell, or a bacterial cell described herein) using standard techniques for expression of the encoded isoprene synthase, DXS, IDI, and/or MVA pathway polypeptide. Introduction of a DNA construct or vector into a host cell can be performed using techniques such as transformation, electroporation, nuclear microinjection, transduction, transfection (*e.g.*, lipofection mediated or DEAE-Dextrin mediated transfection or transfection using a recombinant phage virus), incubation with calcium phosphate DNA precipitate, high velocity bombardment with DNA-coated microprojectiles, and protoplast fusion. General transformation techniques are known in the art (*see*, *e.g.*, Current Protocols in Molecular Biology (F. M. Ausubel *et al.* (eds) Chapter 9, 1987; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989; and Campbell *et al.*, *Curr. Genet.* 16:53-56, 1989, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods). The expression of heterologous polypeptide in *Trichoderma* is described in U.S. Patent No. 6,022,725; U.S. Patent No. 6,268,328; U.S. Patent No. 7,262,041;WO 2005/001036; Harkki *et al.*; *Enzyme Microb. Technol.* 13:227-233, 1991; Harkki *et al.*, *Bio Technol.* 7:596-603, 1989; EP 244,234; EP 215,594; and Nevalainen *et al.*, "*The Molecular Biology of Trichoderma and its Application to the Expression of Both Homologous and Heterologous Genes*," in Molecular Industrial Mycology, *Eds.* Leong and Berka, Marcel Dekker Inc., NY pp. 129 – 148, 1992, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation and expression methods). Reference is also made to Cao *et al.*, (*Sci.* 9:991–1001, 2000; EP 238023; and Yelton *et al.*, *Proceedings. Natl. Acad. Sci.* USA 81:1470-1474, 1984 (which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods) for transformation of *Aspergillus* strains. The introduced nucleic acids may be integrated into chromosomal DNA or maintained as extrachromosomal replicating sequences.

[0327] 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.

[0328] 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.

[0329] 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.

[0330] 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).

[0331] 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.

[0332] 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

[0333] The invention also includes a cell or a population of cells in culture that produce isoprene. By "cells in culture" is meant two or more cells in a solution (*e.g.*, a cell medium) that allows the cells to undergo one or more cell divisions. "Cells in culture" do not include plant cells that are part of a living, multicellular plant containing cells that have differentiated

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

[0334] 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.

[0335] In some embodiments, the carbon source is a carbohydrate (such as monosaccharide, disaccharide, oligosaccharide, or polysaccharide), 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.

[0336] 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.

[0337] 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.

[0338] 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..

[0339] 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.

[0340] Exemplary lipids are any substance containing one or more fatty acids that are C4 and above fatty acids that are saturated, unsaturated, or branched.

[0341] 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.

[0342] Exemplary fatty acids include compounds of the formula RCOOH, where "R" is a hydrocarbon. Exemplary unsaturated fatty acids include compounds where "R" includes at least one carbon-carbon double bond. Exemplary unsaturated fatty acids include, but are not limited to, oleic acid, vaccenic acid, linoleic acid, palmitelaidic acid, and arachidonic acid. Exemplary polyunsaturated fatty acids include compounds where "R" includes a plurality of carbon-carbon double bonds. Exemplary saturated fatty acids include compounds where "R" is a saturated aliphatic group. In some embodiments, the carbon source includes one or more C_{12}-C_{22} fatty acids, such as a C_{12} saturated fatty acid, a C_{14} saturated fatty acid, a C_{16} saturated fatty acid, a C_{18} saturated fatty acid, a C_{20} saturated fatty acid, or a C_{22} saturated fatty acid. In an exemplary embodiment, the fatty acid is palmitic acid. In some embodiments, the carbon source is a salt of a fatty acid (*e.g.*, an unsaturated fatty acid), a derivative of a fatty acid (*e.g.*, an unsaturated fatty acid), or a salt of a derivative of fatty acid (*e.g.*, an unsaturated fatty acid). Suitable salts include, but are not limited to, lithium salts, potassium salts, sodium salts, and the like. Di- and triglycerols are fatty acid esters of glycerol.

[0343] 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.

[0344] 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.

[0345] 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.

[0346] 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.

[0347] 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.

[0348] 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.

[0349] 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).

[0350] 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.

[0351] 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).

[0352] 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.

[0353] 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

[0354] 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.

[0355] Standard cell culture conditions can be used to culture the cells (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to cell culture and fermentation conditions). Cells are grown and maintained at an appropriate temperature, gas mixture, and pH (such as at about 20 to about 37°C, at about 6% to about 84% CO_2, and at a pH between about 5 to about 9). In some embodiments, cells are grown at 35 °C in an appropriate cell medium. In some embodiments, *e.g.*, cultures are cultured at approximately 28 °C in appropriate medium in shake cultures or fermentors until desired amount of isoprene production is achieved. In some embodiments, the pH ranges for fermentation are between about pH 5.0 to about pH 9.0 (such as about pH 6.0 to about pH 8.0 or about 6.5 to about 7.0). Reactions may be performed under aerobic, anoxic, or anaerobic conditions based on the requirements of the host cells. Exemplary culture conditions for a given filamentous fungus are known in the art and may be found in the scientific literature and/or from the source of the fungi such as the American Type Culture Collection and Fungal Genetics Stock Center.

[0356] 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.

[0357] 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.

[0358] 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.

[0359] 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.

[0360] In some embodiments, cells are immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isoprene production.

[0361] 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.

[0362] 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).

[0363] 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

[0364] 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.

[0365] 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, Figures 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.

[0366] 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}.

[0367] 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%.

[0368] 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.

[0369] 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

[0370] The production of isoprene within safe operating levels according to its flammability characteristics simplifies the design and construction of commercial facilities, vastly improves the ability to operate safely, and limits the potential for fires to occur. In particular, the optimal ranges for the production of isoprene are within the safe zone, *i.e.*, the nonflammable range of isoprene concentrations. In one such aspect, the invention features a method for the production of isoprene within the nonflammable range of isoprene concentrations (outside the flammability envelope of isoprene).

[0371] 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.

[0372] 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

[0373] 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.

[0374] 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.

[0375] 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.

[0376] 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.

[0377] 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).

[0378] 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.

[0379] 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.

[0380] In some embodiments, an isoprene composition also contains ethanol. For example, ethanol may be used for extractive distillation of isoprene, resulting in compositions (such as intermediate product streams) that include both ethanol and isoprene. Desirably, the amount of ethanol is outside the flammability envelope for ethanol. The LOC of ethanol is about 8.7 vol%, and the LFL for ethanol is about 3.3 vol% at standard conditions, such as about 1 atmosphere and about 60 °F (NFPA 69 *Standard on Explosion Prevention Systems*, 2008 edition, which is hereby incorporated by reference in its entirety, particularly with respect to LOC, LFL, and UFL values). In some embodiments, compositions that include isoprene and ethanol are produced in the presence of less than the LOC required to have a flammable mixture of ethanol (such as less than about 8.7% vol%). In some embodiments in which compositions that include isoprene and ethanol are produced in the presence of greater than or about the LOC required to have a flammable mixture of ethanol, the ethanol concentration is below the LFL (such as less than about 3.3 vol.%).

[0381] In various embodiments, the amount of oxidant (such as oxygen) is below the LOC of any fuel in the system (such as isoprene or ethanol). In various embodiments, the amount

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

Exemplary Production of Isoprene

[0382] In some embodiments, the cells are cultured in a culture medium under conditions permitting the production of isoprene by the cells. By "peak absolute productivity" is meant the maximum absolute amount of isoprene in the off-gas during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak absolute productivity time point" is meant the time point during a fermentation run when the absolute amount of isoprene in the off-gas is at a maximum during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). In some embodiments, the isoprene amount is measured at the peak absolute productivity time point. In some embodiments, the peak absolute productivity for the cells is about any of the isoprene amounts disclosed herein.

[0383] By "peak specific productivity" is meant the maximum amount of isoprene produced per cell during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak specific productivity time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per cell is at a maximum. The specific productivity is determined by dividing the total productivity by the amount of cells, as determined by optical density at 600nm (OD600). In some embodiments, the isoprene amount is measured at the peak specific productivity time point. In some embodiments, the peak specific productivity for the cells is about any of the isoprene amounts per cell disclosed herein.

[0384] 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.

[0385] By "relative detector response" refers to the ratio between the detector response (such as the GC/MS area) for one compound (such as isoprene) to the detector response (such as the GC/MS area) of one or more compounds (such as all C5 hydrocarbons). The detector response may be measured as described herein, such as the GC/MS analysis performed with an Agilent 6890 GC/MS system fitted with an Agilent HP-5MS GC/MS column (30 m x 250 μm; 0.25 μm film thickness). If desired, the relative detector response can be converted to a weight percentage using the response factors for each of the compounds. This response factor is a measure of how much signal is generated for a given amount of a particular compound (that is, how sensitive the detector is to a particular compound). This response factor can be used as a correction factor to convert the relative detector response to a weight percentage when the detector has different sensitivities to the compounds being compared. Alternatively, the weight percentage can be approximated by assuming that the response factors are the same for the compounds being compared. Thus, the weight percentage can be assumed to be approximately the same as the relative detector response.

[0386] In some embodiments, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr). In some embodiments, the amount of isoprene is between about 2 to about 5,000 nmole/g_{wcm}/hr, such as between about 2 to about 100 nmole/g_{wcm}/hr, about 100 to about 500 nmole/g_{wcm}/hr, about 150 to about 500 nmole/g_{wcm} /hr, about 500 to about 1,000 nmole/g_{wcm}/hr, about 1,000 to about 2,000 nmole/g_{wcm}/hr, or about 2,000 to about 5,000 nmole/g_{wcm}/hr. In some embodiments, the amount of isoprene is between about 20 to about 5,000 nmole/g_{wcm}/hr, about 100 to about 5,000 nmole/g_{wcm}/hr, about 200 to about 2,000 nmole/g_{wcm}/hr, about 200 to about 1,000 nmole/g_{wcm}/hr, about 300 to about 1,000 nmole/g_{wcm}/hr, or about 400 to about 1,000 nmole/g_{wcm}/hr.

[0387] 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.

[0388] 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).

[0389] 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}.

[0390] 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 13, 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.

[0391] 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 13, 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}.

[0392] In some embodiments, the cells in culture convert greater than or about 0.0015, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0,

1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, or 8.0% of the carbon in the cell culture medium into isoprene. In some embodiments, the percent conversion of carbon into isoprene is between such as about 0.002 to about 4.0%, about 0.002 to about 3.0%, about 0.002 to about 2.0%, about 0.002 to about 1.6%, about 0.002 to about 0.005%, about 0.005 to about 0.01%, about 0.01 to about 0.05%, about 0.05 to about 0.15%, 0.15 to about 0.2%, about 0.2 to about 0.3%, about 0.3 to about 0.5%, about 0.5 to about 0.8%, about 0.8 to about 1.0%, or about 1.0 to about 1.6%. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about 0.4%, 0.002 to about 0.16%, 0.04 to about 0.16%, about 0.005 to about 0.3%, about 0.01 to about 0.3%, or about 0.05 to about 0.3%.

[0393] 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

[0394] For this calculation, yeast extract can be assumed to contain 50% w/w carbon. As an example, for the 500 liter described in Example 19, 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%

[0395] For the two 500 liter fermentations described herein (Example 19, 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 22, part V describes the 1.53% conversion of carbon to isoprene using the methods described herein.

[0396] One skilled in the art can readily convert the rates of isoprene production or amount of isoprene produced into any other units. Exemplary equations are listed below for interconverting between units.

Units for Rate of Isoprene production (total and specific)

Equation 3

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

Equation 4

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

Equation 5

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

Equation 6

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

Equation 7

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

Units for Titer (total and specific)

Equation 8

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

Equation 9

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

[0397] 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

[0398] If desired, Equation 11 can be used to convert between units of ppm and ug/L. In particular, "ppm" means parts per million defined in terms of ug/g (w/w). Concentrations of gases can also be expressed on a volumetric basis using "ppmv" (parts per million by volume), defined in terms of uL/L (vol/vol). Conversion of ug/L to ppm (*e.g.*, ug of analyte per g of gas) can be performed by determining the mass per L of off-gas (*i.e.*, the density of the gas). For example, a liter of air at standard temperature and pressure (STP; 101.3 kPa (1 bar) and 273.15K) has a density of approximately 1.29 g/L. Thus, a concentration of 1 ppm (ug/g) equals 1.29 ug/L at STP (equation 11). The conversion of ppm (ug/g) to ug/L is a function of both pressure, temperature, and overall composition of the off-gas.

Equation 11

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

[0399] Conversion of ug/L to ppmv (*e.g.*, uL of analyte per L of gas) can be performed using the Universal Gas Law (equation 12). For example, an off-gas concentration of 1000 ug/L_{gas} corresponds to 14.7 $umol/L_{gas}$. The universal gas constant is 0.082057 $L.atm\ K^{-1}mol^{-1}$, so using equation 12, the volume occupied by 14.7 umol of HG at STP is equal to 0.329 mL. Therefore, the concentration of 1000 ug/L HG is equal to 329 ppmv or 0.0329% (v/v) at STP.

Equation 12

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

[0400] The amount of impurities in isoprene compositions are typically measured herein on a weight per volume (w/v) basis in units such as ug/L. If desired, measurements in units of ug/L can be converted to units of mg/m^3 using equation 13.

Equation 13

$1\ ug/L = 1\ mg/m^3$

[0401] In some embodiments encompassed by the invention, a cell comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide produces an amount of isoprene that is at least or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 150-fold, 200-fold, 400-fold, or greater than the amount of isoprene produced from a corresponding cell grown under essentially the same conditions without the heterologous nucleic acid encoding the isoprene synthase polypeptide.

[0402] In some embodiments encompassed by the invention, a cell comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide and one or more heterologous nucleic acids encoding a DXS, IDI, and/or MVA pathway polypeptide produces an amount of isoprene that is at least or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 150-fold, 200-fold, 400-fold, or greater than the amount of isoprene produced from a corresponding cell grown under essentially the same conditions without the heterologous nucleic acids.

[0403] 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.

[0404] In some embodiments, the isoprene composition comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *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, *cis*-pent-3-ene-1-yne, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol))

by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has 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, 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, *cis*-pent-3-ene-1-yne, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol) compared to the detector response for all C5 hydrocarbons in the composition. In some embodiments, the isoprene composition comprises between about 0.02 to about 0.04%, about 0.04 to about 0.06%, about 0.06 to 0.08%, about 0.08 to 0.10%, or about 0.10 to about 0.12% C5 hydrocarbons other than isoprene (such as 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol)) by weight compared to the total weight of all C5 hydrocarbons in the composition. .

[0405] In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In some embodiments, the isoprene composition comprises between about 0.005 to about 50, such as about 0.01 to about 10, about 0.01 to about 5, about 0.01 to about 1, about 0.01 to about 0.5, or about 0.01 to about 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a hydrocarbon other than isoprene (such as 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol)). In some

embodiments, the isoprene composition comprises between about 0.005 to about 50, such as about 0.01 to about 10, about 0.01 to about 5, about 0.01 to about 1, about 0.01 to about 0.5, or about 0.01 to about 0.005 ug/L of a hydrocarbon other than isoprene. In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a protein or fatty acid (such as a protein or fatty acid that is naturally associated with natural rubber).

[0406] In some embodiments, the isoprene composition comprises less than or about 10, 5, 1, 0.8, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of alpha acetylenes, piperylenes, acetonitrile, or 1,3-cyclopentadiene. In some embodiments, the isoprene composition comprises less than or about 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of sulfur or allenes. In some embodiments, the isoprene composition comprises less than or about 30, 20, 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of all acetylenes (such as pentyne-1, butyne-2, 2MB1-3yne, and 1-pentyne-4yne). In some embodiments, the isoprene composition comprises less than or about 2000, 1000, 500, 200, 100, 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of isoprene dimers, such as cyclic isoprene dimmers (*e.g.*, cyclic C10 compounds derived from the dimerization of two isoprene units).

[0407] 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.

[0408] In some embodiments, the composition includes ethanol. In some embodiments, the composition includes between about 75 to about 90% by weight of ethanol, such as between about 75 to about 80%, about 80 to about 85%, or about 85 to about 90% by weight of

ethanol. In some embodiments in which the composition includes ethanol, the composition also includes between about 4 to about 15% by weight of isoprene, such as between about 4 to about 8%, about 8 to about 12%, or about 12 to about 15% by weight of isoprene.

Exemplary Isoprene Purification Methods

[0409] In some embodiments, any of the methods described herein further include recovering the isoprene. For example, the isoprene produced using the compositions and methods of the invention can be recovered using standard techniques. such as gas stripping, membrane enhanced separation, fractionation, adsorption/desorption, pervaporation, thermal or vacuum desorption of isoprene from a solid phase, or extraction of isoprene immobilized or absorbed to a solid phase with a solvent (see, for example, U.S. Patent Nos. 4,703,007 and 4,570,029, which are each hereby incorporated by reference in their entireties, particularly with respect to isoprene recovery and purification methods). In particular, embodiments, extractive distillation with an alcohol (such as ethanol, methanol, propanol, or a combination thereof) is used to recover the isoprene. In some embodiments, the recovery of isoprene involves the isolation of isoprene in a liquid form (such as a neat solution of isoprene or a solution of isoprene in a solvent). Gas stripping involves the removal of isoprene vapor from the fermentation off-gas stream in a continuous manner. Such removal can be achieved in several different ways including, but not limited to, adsorption to a solid phase, partition into a liquid phase, or direct condensation (such as condensation due to exposure to a condensation coil or do to an increase in pressure). In some embodiments, membrane enrichment of a dilute isoprene vapor stream above the dew point of the vapor resulting in the condensation of liquid isoprene. In some embodiments, the isoprene is compressed and condensed.

[0410] 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.

[0411] In some embodiments, any of the methods described herein further include purifying the isoprene. For example, the isoprene produced using the compositions and methods of the invention can be purified using standard techniques. Purification refers to a process through which isoprene is separated from one or more components that are present when the isoprene is produced. In some embodiments, the isoprene is obtained as a substantially pure liquid. Examples of purification methods include (i) distillation from a solution in a liquid extractant and (ii) chromatography. As used herein, "purified isoprene" means isoprene that has been separated from one or more components that are present when the isoprene is produced. In some embodiments, the isoprene is at least about 20%, by weight, free from other components that are present when the isoprene is produced. In various embodiments, the isoprene is at least or about 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, or 99%, by weight, pure. Purity can be assayed by any appropriate method, *e.g.*, by column chromatography, HPLC analysis, or GC-MS analysis.

[0412] 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.

[0413] In some embodiments, any of the methods described herein further include polymerizing the isoprene. For example, standard methods can be used to polymerize the purified isoprene to form *cis*-polyisoprene or other down stream products using standard methods. Accordingly, the invention also features a tire comprising polyisoprene, such as *cis*-1,4- polyisoprene and/or *trans*-1,4- polyisoprene made from any of the isoprene compositions disclosed herein.

[0414] The following Examples are provided to illustrate but not limit the invention.

EXAMPLES

[0415] The examples, which are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way, also describe and detail aspects and embodiments of the invention discussed above. Unless indicated otherwise, temperature is in degrees Centigrade and pressure is at or near atmospheric. The foregoing examples and detailed description are offered by way of illustration and not by way of limitation. All publications, patent applications, and patents cited in this specification are

herein incorporated by reference as if each individual publication, patent application, or patent were specifically and individually indicated to be incorporated by reference. In particular, all publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Example 1. Expression Constructs and Strains

I. Construction of plasmids encoding mevalonate kinase.

[0416] 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* archaeal Lower Pathway operon (Figures 46A-46C) 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 4) 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 uL 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 in an 11uL reaction 5uL Roche Quick Ligase buffer 1, 1 uL buffer 2, 1 uL plasmid, 3 uL amplicon, and 1 uL ligase (1 hour at room temperature). 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 47A-47C).

[0417] Using the method described above for plasmid MCM382, pTrcKudzu-MVK(mazei), four additional plasmids were constructed with MVK genes from different

source organisms (Table 5 and Figures 58A-58C, 59A-59C, 96A-96C, 97A-97C, and 98A-98C).

Table 5. Plasmids encoding MVK from different source organisms.

Source Organism	PCR Template	Forward Primer	Reverse Primer	Final Plasmid	MVK Protein Accession
Streptococcus penumoniae	pDW02	MCM166	MCM167	MCM379	
Lactobacillus sakei	pDW01	MCM168	MCM169	MCM380	
Streptomyces CL190	Streptomyces CL190 Lower Pathway operon	MCM164	MCM176	MCM381	BAB07790.1
Saccharomyces cerevisiae	pTrcKK (described herein)	MCM170	MCM171	MCM383	

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

[0418] Plasmid MCM382 was transformed into MCM331 cells (which contain chromosomal construct gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase) that had been grown to midlog in LB medium and washed three times in iced, sterile water. 1 uL of DNA was added to 50 uL 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 uL 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).

[0419] Production strains analogous to MCM401 were generated for each of the four plasmids detailed in Table 5 using the methods described above for MCM401. MCM331 was transformed with plasmid MCM379, 380, 381, or 383, and then selected on LA+kan50. The resulting strains were transformed with MCM82 and selected on LA+kan50+spec50.

Table 6. Strains overexpressing mevalonate kinase and isoprene synthase

MVK Source	Plasmid pTrcKudzu-MVK	Strain MCM331 transformed with pTrcKudzuMVK	Strain MCM331 transformed with pTrcKudzuMVK then transformed with MCM82
Streptococcus pneumoniae	MCM379	MCM388	MCM398
Lactobacillus sakei	MCM380	MCM389	MCM399
Streptomyces CL190	MCM381	MCM390	MCM400
Methanosarcina mazei	MCM382	MCM391	MCM401
Saccharomyces cerevisiae	MCM383	MCM392	MCM402

MVK Source	Plasmid pTrcKudzu-MVK	Strain MCM333 transformed with pTrcKudzuMVK	Strain MCM333 transformed with pTrcKudzuMVK then transformed with MCM82
Streptococcus pneumoniae	MCM379	MCM393	MCM403
Lactobacillus sakei	MCM380	MCM394	MCM404
Streptomyces CL190	MCM381	MCM395	
Methanosarcina mazei	MCM382	MCM396	MCM406
Saccharomyces cerevisiae	MCM383	MCM397	MCM407

[0420] Additional strain information is provided below.

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* archaeal Lower in pET200D.

[0421] The MVK ORF from the *M. mazei* archaeal Lower Pathway operon (Figures 46A-46C) was PCR amplified using primers MCM161 and MCM162 (Table 4) using the Invitrogen Platinum HiFi PCR mix. 45 uL of PCR mix was combined with 1 uL template, 1 uL of each primer at 10 uM, and 2 uL water. The reaction was cycled as follows: 94 °C for 2:00; 30 cycles of 94 °C for 0:30, 55 °C for 0:30. and 68 °C for 1:15; and then 72 °C for 7:00, and 4 °C until cool. 3 uL of this PCR reaction was ligated to Invitrogen pET200D plasmid

according to the manufacturer's protocol. 3 uL of this ligation was introduced into Invitrogen TOP10 cells, and transformants were selected on LA/kan50. A plasmid from a transformant was isolated and the insert sequenced, resulting in MCM376 (Figures 57A-57C).

IV. Construction of MCM420 expressing Streptomyces CL190 MVK

[0422] The Streptomyces CL190 MVK was cloned into pET200D as described above for plasmid MCM376 (Table 7).

V. Construction of pDu5 expressing S. cerevisiae MVK

[0423] The S. cerevisiae MVK was cloned into pET16b from Invitrogen as follows (Table 7). The MVK enzyme from *S. cerevisiae* was PCR amplified with *Hg-MVK-F2-NdeI and Hg-MVK-R2-NdeI* primers using Stratagene *Pfu UltraII* Fusion DNA Polymerase Kit according to manufacturer's protocol, and pMVK1 (described herein) as the template DNA. The following cycle parameter was used for the reaction (95 °C for 2 minutes, 29cycles (95 °C for 20 seconds, 55 °C for 20 seconds, 72 °C for 21sececonds), 72 °C for 3 minutes, and 4 °C until cool) using an Eppendorf Mastercycler Gradient Machine).

[0424] As a result, a 1.352 kb MVK PCR fragment was obtained and was gel purified using Qiagen's gel purification kit. The purified PCR product was digested with NdeI restriction enzyme. The digested DNA was purified over Qiagen PCR column. 5uL of purified PCR product was ligated to 1 uL of pET-16b vector that was previously digested with NdeI and then treated with SAP (Shrimp Alkaline Phosphatase). A New England BioLab (NEB) T4 ligase kit was used for ligation at approximately 16 °C overnight according to manufacturer's protocol.

[0425] 5 uL of overnight ligation mixture was transformed into Invitrogen TOP10 cells. The transformation was carried on ice for a 30 minute incubation followed by a 30 second heat shock at approximately 42 °C and a 1 hour recovery in 1ml LB at approximately 37 °C. The transformation was selected on LA/Carb50 incubated at approximately 37 °C overnight. Plasmids from transformants were isolated and the insert sequenced with T7 promoter and T7 terminator using Quintara Bio Sequencing Service. The resulting plasmid for S. cerevisiae MVK in pET-16b vector is called pDu5 (Figures 126A and 126B).

[0426] Once the sequence is verified, 1ul of plasmid (pDu5) is then transformed into BL21 *pLysS* host strain. Transformants are selected on LA/Carb50 plates and incubated at approximately 37 °C. The resulting expression strain is called MD08-MVK.

Table 7. Plasmids and Strains overexpressing mevalonate kinase

	Template	For. Primer	Rev. Primer	Plasmid	Expression Strain
S. cerevisiae	pMVK1	Hg-MVK-F2-NdeI	Hg-MVK-R2-NdeI	pDu5	MD08-MVK
Streptomyces CL190	Streptomyces CL190 Lower Pathway operon	MCM159	MCM160	MCM420	MCM429

V. Creation of expression strain MCM378.

[0427] Plasmid MCM376 was transformed into Invitrogen BL21 Star (DE3) cells according to the manufacturer's protocol. Transformant MCM378 was selected on LA/kan50. Additional strains were created using the same protocol and are listed in the Table 7. Invitrogen OneShot BL21(DE3) pLysS transformed with the indicatd plasmid and selected on LA and carb50 cmp35 (for MD08-MVK) or selected on LA and kan50 cmp35 (for MCM429) were used.

VI. Construction of plasmid pCLPtrcUpperPathway HGS2

[0428] The gene encoding isoprene synthase from Pueraria lobata was PCR-amplified using primers NsiI-RBS-HGS F (cttgATGCATCCTGCATTCGCCCTTAGGAGG, SEQ ID NO:115) and pTrcR (CCAGGCAAATTCTGTTTTATCAG, SEQ ID NO:116), and pTrcKKDyIkIS (MCM118) as a template. The resulting PCR product was restriction-digested with *Nsi*I and *Pst*I and gel-purified using the Qiagen QIAquick Gel Extraction kit using standard methods. MCM82 (pCL PtrcUpperPathway) was restriction-digested with *Pst*I and dephosphorylated using rAPid alkaline phosphatase (Roche). These DNA pieces were ligated together using T4 ligase and the ligation reaction was transformed in *E. coli* Top10 electrocompetent cells (Invitrogen). Plasmid was prepared from six clones using the Qiagen QiaPrep Spin MiniPrep kit. The plasmids were digested with restriction enzymes EcoRV and MluI, and a clone in which the insert had the right orientation (*i.e.*, gene oriented in the same way as the pTrc promoter) was identified. The resulting plasmid

pCLPtrcUpperPathwayHGS2 (Figures 112A-112D) was found to produce isoprene in *E. coli* Top10, using a headspace assay described herein, thus validating the functionality of the expression construct.

Table 4. Oligonucleotides

	Hg-MVK-F2-NdeI	cagcagcagCATATGtcattaccgttcttaacttc (SEQ ID NO:117)
	Hg-MVK-R2-NdeI	cagcagcagCATATGgcctatcgcaaattagcttatg (SEQ ID NO:118)
MCM159	Strep CL190 MVK for	CACCATGCAAAAACGCCAACGTGA (SEQ ID NO:119)
MCM160	Strep CL190 MVK rev	TTACTGCGCATGGTTATCAAGGC (SEQ ID NO:120)
MCM161	*M. mazei* MVK for	CACCATGGTATCCTGTTCTGCG (SEQ ID NO:121)
MCM162	*M. mazei* MVK rev	TTAATCTACTTTCAGACCTTGC (SEQ ID NO:122)
MCM164	Strep CL190 MVK for w/ RBS	gcgaacgATGCATaaaggaggtaaaaaaacATGCAAAAACGCCAACGTGA (SEQ ID NO:123)
MCM165	*M. mazei* MVK for w/ RBS	gcgaacgATGCATaaaggaggtaaaaaaacATGGTATCCTGTTCTGCGCCGGGTAAGAT TTACCTG (SEQ ID NO:124)
MCM166	*S. pneumoniae* MVK for w/ RBS	gcgaacgATGCATaaaggaggtaaaaaaacATGACAAAAAAAGTTGGTGTCGGT (SEQ ID NO:125)
MCM167	*S. pneumoniae* MVK rev	gggcccgtttaaactttaactagactCTGCAGTCACAGGCTCTCTATCCATGTCTGAA (SEQ ID NO:126)
MCM168	*L. sakei* MVK for w/ RBS	gcgaacgATGCATaaaggaggtaaaaaaacATGCAAACGAGTGTGGGAAACA (SEQ ID NO:127)
MCM169	*L. sakei* MVK rev	gggcccgtttaaactttaactagactCTGCAGTTAATTAGTGTGTAGTGCGTGTAATGGTTG (SEQ ID NO:128)
MCM170	*S. cerevisiae* MVK for w/ RBS	gcgaacgATGCATaaaggaggtaaaaaaacATGTCATTACCGTTCTTAACTTCTGCA (SEQ ID NO:129)
MCM171	*S. cerevisiae* MVK rev	gggcccgtttaaactttaactagactCTGCAGTTATGAAGTCCATGGTAAATTCGTGT (SEQ ID NO:130)
MCM176	Strep CL190 MVK rev Pst	gggcccgtttaaactttaactagactTTACTGCGCATGGTTATCAAGGC (SEQ ID NO:131)
MCM177	*M. mazei* MVK rev Pst	gggcccgtttaaactttaactagactTTAATCTACTTTCAGACCTTGC (SEQ ID NO:132)

Example 2. 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):

[0429] 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:

[0430] 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:

[0431] 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). The *S. cerevisiae* MVK gene is present only as one copy on the chromosome of the MCM343 cells and is controlled by a weak promoter. The expression level of isoprene synthase may not be limiting in the MCM343 cells. The isoprene synthase gene has the same plasmid backbone and promoter as in the MCM401 cells.

[0432] MCM401 cells are BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL Upper), the integrated lower MVA pathway (gi1.2KKDyI), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(*M. mazei*)). The *M. mazei* MVK gene is present in multiple copies on a

plasmid in the MCM401 cells (~ 30-50 copies/cell) and is under a stronger promoter than the *S. cerevisiae* MVK gene. Based on this information, the MVK protein level in the MCM401 cells is expected to be at least about 30 to 50 fold higher than the level in the MCM343 cells. The expression level of isoprene synthase may not be limiting in the MCM401 cells. The isoprene synthase gene shares the same plasmid backbone and promoter as the MCM343 cells. In addition, the amount of isoprene synthase made is higher in the MCM401 cells, and the protein level of the isoprene synthase was not dependent upon the inhibition of MVK.

[0433] Isoprene production was analyzed by growing the strains in 100 mL bioreactors with a 20mL 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 8mL/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.

[0434] 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 100mL bioreactors. The bacteria were grown under identical conditions in defined media with glucose as carbon source. Induction of isoprene production was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG) to a final concentration of either 100 uM or 200 uM. Off-gas measurements revealed that the strain over-expressing both MVK and isoprene synthase (MCM401) produced significantly more isoprene compared to the strain expressing only the mevalonic acid pathway and Kudzu isoprene synthase (MCM343) as shown in Figures 113A-113D. At 100 uM induction, the MCM401 strain produced 2-fold more isoprene compared to the MCM343 strain. At 200 uM IPTG induction, the MCM401 strain produced 3.4-fold more isoprene when compared to the MCM343 strain. Analysis of

CO_2 in the off-gas from the bioreactors, which is a measure of metabolic activity, indicates that metabolic activity was independent from IPTG induction and isoprene production.

Example 3. 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):

[0435] 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:

[0436] 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.

[0437] 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 innoculate 5-L of cell medium in the 15-L bioreactor. In particular, the 15-L bioreactor had an initial working volume of 5 L. The liquid volume increases throughout the fermentation (such as to approximately 10 liters).

[0438] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 68 hour fermentation was 3.8 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 51 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 88 uM when OD_{550} reached 149. Additional IPTG additions raised the concentration to 119 uM at OD_{550} = 195 and 152 uM at OD_{550} = 210. The OD_{550} profile within the bioreactor over time is shown in Figure 114. 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 115). 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 116. 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 4. 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):

[0439] 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:

[0440] 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.

[0441] 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 innoculate 5-L of cell medium in the 15-L bioreactor. The liquid volume increases throughout the fermentation (such as to approximately 10 liters).

[0442] 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 IPTG. The IPTG concentration was brought to 111 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 193 uM when OD_{550} reached 155. The OD_{550} profile within the bioreactor over time is shown in Figure 130. 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 131). 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 132. Instantaneous volumetric productivity levels reached values as high as 1.5 g isoprene/L broth/hr (Figure 133). Instantaneous yield levels reached as high as 17.7% w/w (Figure 134). 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%.

Example 5. 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):

[0443] 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:

[0444] 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.

[0445] 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 innoculate 5-L of cell medium in the 15-L bioreactor. The liquid volume increases throughout the fermentation (such as to approximately 10 liters).

[0446] 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 IPTG. The IPTG concentration was brought to 51 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. In addition to the IPTG spike, at OD_{550} = 10 a constant feed began and delivered 164 mg of IPTG over 18 hours. The OD_{550} profile within the bioreactor over time is shown in Figure 135. 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 136). 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 137. 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 6. Over-expression of mevalonate kinase and isoprene synthase in *E. coli* harboring the MVA pathway

[0447] Over-expression of both mevalonate kinase and isoprene synthase results in high specific productivity of isoprene production by *E. coli* harboring the MVA pathway

I. Construction of Plasmid MCM94

[0448] Plasmid pTrcHis2B (Invitrogen) was digested for 2 hours at 30 °C in 10 uL containing ApaI (Roche) and Roche BufferA. The reaction was brought to a total of 30 uL containing 1x Roche Buffer H and 2uL PstI (Roche) and incubated for 1 hour at 37 °C. The 996 bp fragment containing the pTrc promoter region was gel purified from an Invitrogen E-gel (1.2%) using a Qiagen Gel Purification spin column according to the manufacturer's protocol.

[0449] Plasmid MCM29 was digested as described above, and the 3338bp fragment containing the origin and kanR genes was gel purified as described above. The two fragments (3 uL pTrcHis2B fragment, 1 uL MCM29 fragment) were ligated for 1 hour at room temperature in a 20 uL reaction following the Roche Rapid DNA Ligation kit protocol. 5 uL of this ligation reaction was used to transform Invitrogen TOP10 chemically competent cells according to the manufacturer's protocol. Transformants were selected on LA and

kanamycin50ppm. Plasmids were isolated by Qiagen Spin Miniprep from several colonies which had been grown overnight in 5 mL LB and kan50. A clone with the pTrc promoter but no kudzu isoprene synthase gene was frozen as MCM94.

II. Construction of Strains MCM433, 437, and 438

[0450] Plasmid pCL PtrcUpperHGS2 (Construction of this plasmid is described in Example 1, part VI) was transformed into MCM331 by electroporation as described herein for expression strain MCM401. Transformant MCM433 was selected on LA and spectinomycin 50ppm. Strain MCM433 was subsequently transformed with either plasmid MCM94 (described above) or MCM376 and selected on LA, spectinomycin 50ppm, and kanamycin 50ppm.

Table 8. Strains MCM433, 437, and 438

Strain	Parent	Host Origin	Integrated	Plasmid(s)	Markers
MCM433	MCM331	BL21(DE3)	gi1.2KKDyI	pCLUpperHGS2	cmp5, spec50
MCM437	MCM433	BL21(DE3)	gi1.2KKDyI	pCLUpperHGS2 pTrcHis2B kan (MCM94)	cmp5, spec50. kan50
MCM438	MCM433	BL21(DE3)	gi1.2KKDyI	pCLUpperHGS2 pTrcKudzuMVK(mazei) MCM376	cmp5, spec50. kan50

III. Cell fermentation

Medium Recipe (per liter fermentation medium):

[0451] 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 5.0 g and antibiotics were added after sterilization and pH adjustment.

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

[0452] 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:

[0453] The MCM343 strain is 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 isoprene synthase from Kudzu (pTrcKudzu). This strain has low MVK polypeptide activity and high isoprene synthase polypeptide activity.

[0454] The MCM401 strain is BL21 (DE3) *E. coli* cells containing the upper MVA pathway (pCL PtrcUpperPathway), the integrated lower MVA pathway (gi1.2KKDyI), and high expression of MVK from *M. mazei* and IS from Kudzu (pTrcKudzuMVK(*M. mazei*). This strain has high MVK polypeptide activity and high isoprene synthase polypeptide activity.

[0455] The MCM437 strain is BL21 (DE3) *E. coli* cells containing the upper MVA pathway and low expression of IS from Kudzu (pCLPtrcUpperPathwayHGS2), the integrated lower MVA pathway (gi1.2KKDyI), and a control plasmid conferring kanamycin resistance (so that the growth media was identical in all cases). This strain has low MVK polypeptide activity and low isoprene synthase.

[0456] The MCM438 strain is BL21 (DE3) *E. coli* cells containing the upper MVA pathway and low expression of IS from Kudzu (pCLPtrcUpperPathwayHGS2), the integrated lower MVA pathway (gi1.2KKDyI), and strong expression of *M. mazei* MVK (*M. mazei* MVK in pET200). This strain has high MVK polypeptide activity and low isoprene synthase polypeptide activity.

[0457] Isoprene production was analyzed by growing the strains in a Cellerator^{TM} 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.

[0458] 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.

[0459] 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. Samples were taken at three time points for each of the 24-wells over the course of the mini-fermentations. There were six replicates for each strain (4 strains x 6 wells/strain).

[0460] Specific productivity of isoprene from a strain expressing the full mevalonic acid pathway and Kudzu isoprene synthase at low levels (MCM437) was compared to a strain that in addition over-expressed MVK from *M. mazei* and Kudzu isoprene synthase (MCM401), as well as strains that either over-expressed just MVK (MCM438), or just Kudzu isoprene synthase (MCM343). The bacteria were grown under identical conditions in defined media with glucose as a carbon source in mini-fermentations. Induction of isoprene production was achieved by adding IPTG to a final concentration of 200 μM at the start of the run. Headspace measurements over time (Figure 139) revealed that the strain over-expressing both MVK and isoprene synthase (MCM401) had higher specific productivity of isoprene compared to the strain over-expressing just MVK (MCM438) or just Kudzu isoprene synthase (MCM343). The strain with low activities of both MVK and Kudzu isoprene synthase (MCM437) had the lowest specific productivity of isoprene overall.

IV. Determination of Isoprene synthase activity and volumetric productivity in fermentation runs.

[0461] Strain MCM401 that overexpresses both *M. mazei* MVK and isoprene synthase had a greater maximum volumetric productivity for isoprene than either strain MC343 or strain MCM127 that do not express *M. mazei* MVK.

(i). Isoprene synthase DMAPP Activity from lysate protocol

[0462] For this assay, the following reagents were used: 50% glycerol in PEB containing 1 mg/mL lysozyme (Sigma) and 0.1 mg/mL DNAaseI (Sigma). 1 mL of fermentation broth was mixed with 1 mL of 50% glycerol in PEB containing 1 mg lysozyme and 0.1 mg DNAaseI. The mixture is passed through the french press one time. 25 µL of the mixture is then used for the DMAPP assay. The DMAPP assay contained the following components:

DMAPP Assay

25 µL lysate mixture

5 µL $MgCl_2$ (1 M)

5 µL DMAPP (100mM)

65 µL 50 mM Tris pH 8

Total volume: 100 µL

[0463] The reaction is performed at 30° C for 15 minutes in a gas tight 1.8 mL GC tube. Reactions are terminated by the addition of 100 µL 250 mM EDTA (pH 8).

[0464] The active protein concentration was measured using Equation 14.

Equation 14

mg/mL active isoprene synthase = (Dilution factor)* X ug/L (DMAPP Assay reading)*0.0705/294(specific activity from 14-L) or 0.0002397 * X ug/L

[0465] The volumetric productivity was measured using Equation 15.

Equation 15

mg/L/h isoprene = (dilution factor)*0.288*X ug/L (DMAPP Assay reading)

[0466] The maximum *in vitro* isoprene synthase polypeptide activity was compared with the maximum volumetric productivity for strains MCM401, MC343, and MCM127 (Figure 146).

Example 7. Exemplary methods for producing isoprene: 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):

[0467] 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, 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 brought to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0468] 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 * $7H_2O$ 1 g, CuSO4 * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in Di H2O, pH to 3.0 with HCl/NaOH, then brought to volume and filter sterilized with 0.22 micron filter.

I. MCM343 High Titer: Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale

[0469] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the gi1.2 integrated lower MVA pathway 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.

[0470] 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 4.5 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 98 uM when the carbon dioxide evolution rate reached 25 mmol/L/hr (OD_{550} = 9). The OD_{550} profile within the bioreactor over time is shown in Figure 112C. 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 1.6 g/L (Figure 112D). The total amount of isoprene produced during the 58 hour fermentation was 17.9 g and the time course of production is shown in Figure 112E. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.8%. The weight percent yield of isoprene from glucose was 0.4%.

II. MCM127: Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale

[0471] 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.

[0472] 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 43 hour fermentation was 1.4 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 23 uM when the carbon dioxide evolution rate reached 25 mmol/L/hr (OD_{550} = 129). The OD_{550} profile within the bioreactor over time is shown in Figure 112F. The isoprene level in the off gas from the bioreactor was determined as previously described by measuring isoprene concentrations in the offgas by GC. The isoprene titer increased over the course of the fermentation to a final value of 0.4 g/L (Figure 112G). The total amount of isoprene produced during the 43 hour fermentation was 3.0 g and

the time course of production is shown in Figure 112H. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.5%. The weight percent yield of isoprene from glucose was 0.3%.

III. *dxr* knock-out strain: Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale.

[0473] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells (*Δdxr*) 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 15-L bioreactor containing an initial volume of 5-L

[0474] 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 43 hour fermentation was 1.7 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 8. The IPTG concentration was raised to 40 uM when OD_{550} reached 140. The OD_{550} profile within the bioreactor over time is shown in Figure 112I. The isoprene level in the off gas from the bioreactor was determined as previously described (GC of offgas samples). The isoprene titer increased over the course of the fermentation to a final value of 0.9 g/L (Figure 112J). The total amount of isoprene produced during the 43 hour fermentation was 6.0 g and the time course of production is shown in Figure 112K. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.8 %. The weight percent yield of isoprene from glucose was 0.4 %.

(i) Construction of the *dxr* mutant in *E. coli*

[0475] To generate a deletion of *dxr* (1-deoxy-D-xylulose 5-phosphate reductoisomerase), the enzyme that encodes the first committed step in the deoxy-xylulose-phosphate (DXP)

pathway in *Escherichia coli*, the GeneBridges Quick & Easy *E. coli* Gene Deletion Kit (GB) was used according to the manufacturer's recommended protocol. Briefly, GB insertion cassettes encoding either kanamycin (FRT-PGK-gb2-neo-FRT) or chloramphenicol (FRT-cm-FRT) resistance were PCR amplified using primers GBdxr1 and GBdxr2 (see below for primer sequences and cycling parameters). PCR products of the correct size (for the respective GB insertion cassette) were pooled, purified (Qiagen) and diluted to a concentration of approximately 300 ng/µl. The deletion of *dxr* was then carried out according to the protocol described in the GB manual. All replicating plasmids were introduced into *E. coli* strains via electroporation using standard molecular biology techniques (see Table 16 below for a complete strain list). LB medium containing ampicillin (50 µg/ml) and spectinomycin (50 µg/ml) was inoculated with *E. coli* strains (DW13 or DW38) harboring the pRed/ET plasmid (encoding ampicillin/carbenicillin resistance) and pCL Ptrc(minus lacO) KKDyI (from Edwin Lee, encoding spectinomycin resistance). These strains carried pCL Ptrc(minus lacO) KKDyI (see (iv) below) so that *E. coli*, in the absence of a functional DXP pathway, could convert mevalonic acid (MVA) through the MVA lower pathway to IPP/DMAPP as a source for all lower isoprenoid molecules. Cultures were grown overnight at 30°C and diluted to an OD_{600} of approximately 0.2 in 5 ml total volume with antibiotics the next morning. After several hours of growth at 30°C, strains were shifted to 37°C and L-arabinose was added at a concentration of 0.4%. After 1 hour of induction, cells were washed multiple times in ice cold H_2O, and approximately 700 ng of the purified PCR product (described above) for each GB insertion template was introduced via electroporation (using standard techniques). Cells were recovered for 3 hours at 37°C in LB with 1 mM MVA with no antibiotics, and then plated onto selective LB medium (MVA 1 mM and spectinomycin 50 µg/ml, with either kanamycin 15 µg/ml or chloramphenicol 25 µg/ml). The next day, positive colonies were tested by PCR, using the dxrTest1 and dxrTest2 primers, with either GBprimer2 or GBprimerDW (*i.e.* GB3, *see* Figure 112M), respectively (*see* Table 16). Colonies that tested positive with these primer combinations were then tested for sensitivity to MVA at varying concentrations. Figure 112J shows that in the absence of MVA, *dxr* deletion strains are unable to grow, whereas in the presence of 1 mM MVA, growth is robust. Figure 112N also shows that at a concentration of 10 mM MVA, growth of *dxr* deletion strains appears to be inhibited, most likely because of the accumulation of isoprenoid molecules. To generate strain DW48, strain DW43 was electroporated with plasmids MCM82 (Sp) and MCM118 (Kan), which harbor the entire MVA pathway and

HGS. Since MVA was omitted from recovery and on the selective plate (LB with Sp μg/ml and Kan μg/ml), strain DW48 was forced to lose plasmid pCL Ptrc(minus lacO) KKDyI and gain MCM82, which contains the MVA upper pathway. Thus, only cells harboring the entire MVA pathway to convert acetyl-CoA to IPP/DMAPP and lower isoprenoids were able to grow without exogenous MVA.

(ii) PCR Cycling Parameters

[0476] The Herculase II (Stratagene) DNA polymerase enzyme was used for amplification of all GB templates with oligonucleotide primer pairs at a concentration of 0.4 μM each in 50 μl total volume/reaction according to the manufacturer's protocol. All PCR products for generating *dxr* deletion strains via GB were of the expected size: approximately 1.6 kb (kanamycin), and 1.5 kb (chloramphenicol).

[0477] To test GB insertions at the *dxr* locus, 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.

1) 95°C – 4 min
2) 95°C – 20 sec
3) 55°C – 20 sec (52°C for Beads)
4) 72°C – 2 min (30 sec for Beads)
5 cycles of steps 2 through 4
5) 95°C – 20 sec
6) 58°C – 20 sec (55°C for Beads)
7) 72°C – 2 min (30 sec for Beads)
25 cycles of steps 5 through 7
72°C – 10 min
4°C – end

Table 16 – PCR primers, plasmids, and Strains

Primer Name	Sequence (5′ to 3′)	Purpose
GBdxr1	GGCTGGCGGCGTTTTGCTTTTTATTCTGTCTCAACTCTGGATGTTTCATGAATTAACCCTCACTAAAGGGCG (SEQ ID NO:146)	*dxr* knock out GB – Forward primer for all templates
GBdxr2	AAGCCCTACGCTAACAAATAGCGCGACTCTCTGTAGCCGGATTATCCTCATAATACGACTCACTATAGGGCTC (SEQ ID NO:147)	*dxr* knock out GB – Reverse primer for all GB templates
dxrTest1	ACGCCGCTCAGTAGATCCTTGCGGAT (SEQ ID NO:148)	5′ of 50 bp homology region (in GBdxr1) used for GB knock-out

dxrTest2	CTACTTACGATCAGATGGCGCAGA CTA (SEQ ID NO:149)	3′ of 50 bp homology region (in GBdxr2) used for GB knock-out
GBprimer2	CGAGACTAGTGAGACGTGCTAC (SEQ ID NO:150)	GB test primer all cassettes – amplifies towards 5' end
GBprimerDW	AAAGACCGACCAAGCGACGTCTGA (SEQ ID NO:151)	GB test primer all cassettes - amplifies towards 3' end
Plasmid	Resistance	purpose
pCL Ptrc(minus lacO) KKDyI	Spectinomycin (sp)	Lower MVA pathway for conversion of MVA to IPP/DMAPP – lower isoprenoids
FRT-cm-FRT	Chloramphenicol (GBchlor)	GB template - chloramphenicol
FRT-PGK-gb2-neo-FRT	Kanamycin (GBkan)	GB template - kanamycin
pRedET	Ampicillin (amp)	GB L-arabinose inducible expression of Red/ET proteins
MCM82	Spectinomycin (sp)	Upper MVA pathway
MCM118	Kanamycin (kan)	Lower MVA pathway + HGS
Strain	Genotype	purpose
DW13	MG1655 with pCL Ptrc(minus lacO) KKDyI and pRedET, sp, amp	Parent strain of dxr deletion – has entire MVA lower pathway
DW23	MG1655 Δ*dxr*::GBkan with pCL Ptrc(minus lacO) KKDyI, kan, sp	*dxr* delection (kan) in MG1655
DW28	MG1655 Δ*dxr*::GBchlor with pCL Ptrc(minus lacO) KKDyI, chlor, sp	*dxr* delection (chlor) in MG1655
DW38	BL21 DE3 (Invitrogen) with pCL Ptrc(minus lacO) KKDyI and pRedET, sp, amp	Parent strain of *dxr* deletion – has entire MVA lower pathway
DW43	BL21 DE3 Δ*dxr*::GBchlor with pCL Ptrc(minus lacO) KKDyI, chlor, sp	*dxr* delection (chlor) in BL21 DE3
DW48	BL21 DE3 Δ*dxr*::GBchlor with MCM82 and MCM118, sp, kan	*dxr* delection (chlor) in BL21 DE3 with entire MVA pathway – requires no MVA

(iii) Construction of MCM184 - pCL Ptrc(minus lacO) UpperPathway

[0478] Plasmid MCM82 was mutagenized using the Stratagene QuikChange XL II kit. A reaction consisting of 10uL buffer, 1uL 100ng/uL MCM82 DNA, 2.5uL 10uM primer MCM63 (SEQ ID NO:139), 2.5uL 10uM primer MCM64 (SEQ ID NO:140), 2uL dNTP mix, 6uL QuikSolution, 76uL ddH2O and 2uL polymerase was combined and aliquotted to four PCR tubes. Tubes were cycled in columns 1, 4, 7 and 12 of a BioRad 96-well gradient block using 1x 95C for 1 minute, 18x95˚C for 50 seconds, 60-65˚C for 50 seconds, 68˚C for 10 minute, 1x 68˚C for 7 minutes, 1x 4˚C until cool. 1uL DpnI was added and reactions were incubated at 37˚C for 2hr and then frozen overnight at -20˚C. 5uL was transformed into Invitrogen TOP10 OneShot cells according to the manufacturer's protocol. Transformants were selected on LA + 50ppm Spectinomycin. Several colonies were cultured in LB +

spectinomycin50 and then used for plasmid purification. Clone 2 from reaction 3 (column 7 from gradient block PCR) had the expected sequence and was frozen as MCM184.

(iv) Construction of pCL Ptrc(ΔlacO) KKDyI
(as referred to as pCL Ptrc (minus lacO) KKDyI or pCL Ptrc (minus lacO) Lower Pathway)

[0479] Plasmid MCM184 (pCL Ptrc(minus lacO) UpperPathway) was digested sequentially with SacI and PstI restriction endonucleases to remove the Upper MVA Pathway. A reaction consisting of 8uL MCM184 (80ng/uL), 3ul Roche 10X Buffer A, 2uL SacI restriction endonuclease, and 17uL ddH_2O was prepared and incubated at 37°C for 2 hours. The SacI restriction endonuclease was then inactivated by heating at 65°C for 20 minutes. The DNA fragment was then purified by using a Qiagen PCR Purification column per manufacturer's protocol. The DNA fragment was then eluted from the column with a volume of 34uL ddH_2O. The next (sequential) restriction digest reaction consisted of the 34uL SacI digested eluant, 4uL Roche 10X Buffer H, and 2uL PstI restriction endonuclease. The reaction was incubated at 37°C for 2 hours before being heat inactivated at 65°C for 20 minutes. A dephosphorylation step was then performed by addition of 4.7uL Roche 10X Shrimp Alkaline Phosphatase (SAP) buffer), and 2uL SAP enzyme. The reaction was then incubated at 37°C for 1 hour. The digested MCM184 vector backbone was then separated from the Upper MVA Pathway DNA fragment by electrophoresis on a 1.2% E-gel (Invitrogen).

[0480] The Lower MVA Pathway fragment (KKDyI) was digested sequentially with SacI and PstI restriction endonucleases from plasmid MCM107. A reaction consisting of 2uL MCM107 (375ng/uL), 3uL Roche 10X Buffer A, 2uL SacI restriction endonuclease, and 23uL ddH_2O was prepared and incubated at 37°C for 3 hours. The SacI restriction endonuclease was then inactivated by heating at 65°C for 20 minutes. The DNA fragment was then purified by using a Qiagen PCR Purification column per manufacturer's protocol. The DNA fragment was then eluted from the column with a volume of 34uL ddH_2O. The sequential digest reaction consisted of the 34uL SacI digested eluant, 4uL Roche 10X Buffer H, and 2uL PstI restriction endonuclease. The reaction was incubated at 37°C for 2 hours before being heat inactivated at 65°C for 20 minutes. The digested KKDyI fragment was then separated from the MCM107 vector backbone by electrophoresis on a 1.2% E-gel (Invitrogen).

[0481] A ligation reaction consisting of 3uL MCM184 vector backbone, 6uL KKDyI DNA fragment, 2uL New England Biolabs (NEB) 10X T4 DNA Ligase Buffer, 1ul T4 DNA ligase, and 8uL ddH_2O were incubated at room temperature for 20 minutes. The ligation reaction was then transformed into TOP10 chemically competent *E. coli* cells (Invitrogen) per manufacturer's protocol and plated on LA + 50ppm spectinomycin plates. To confirm that transformants had correct sized insert fragment, a PCR screen was performed. 50uL ddH_2O was inoculated with individual colonies from the transformation, boiled at 95°C for 5 minutes, and microcentrifuged for 5 minutes to pellet cellular debri. PCR was performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare). Individual reaction tubes contained 1uL of boiled cell lysate, 1uL 10uM primer EL-976 (SEQ ID NO:142), 1uL 10uM primer EL-977 (SEQ ID NO:143), and 22uL ddH_2O. PCR tubes were cycled 1X 95°C for 1 minute, 30X (95°C for 30 seconds, 53°C for 30 seconds, 72°C for 45 seconds), 1X 72°C for 2 minutes. The PCR products were then analyzed on a 1.2% E-gel for an 840bp fragment. Clones #2, #3, and #4 were contained the correct sized fragments and were DNA sequenced using primers EL-976 (SEQ ID NO:142) and EL-978 (SEQ ID NO:144). DNA sequencing confirmation showed that all 3 were correct.

Example 8. Metabolite Analysis and Growth Inhibition

I. Metabolite extraction from *E. coli*. sampled from 14-L fermentors.

[0482] The metabolism of bacterial cells grown in fermentors was rapidly inactivated by withdrawing approximately 4 mL of culture into a tube filled with 8 mL of dry ice-cold methanol. The resulting samples were weighed to calculate the amount of sampled broth and then put into -80 °C for storage until further analysis. For metabolite extraction and concentration, 1.5 to 4.0 mL aliquots of cell suspension were diluted with methanol/ammonium acetate buffer (5 mM, pH=8.0) mixture (6:1, v/v) to a final volume of 6 mL, and cell debris was pelleted by a 5 minute centrifugation. The supernatant was collected and loaded onto a Strata-X-AW column (Phenomenex) containing 30 mg of sorbent that selectively retains strong organic acids. The pellet was extracted two more times, first with 3 mL of the methanol/ammonium acetate buffer (5 mM, pH=8.0) mixture (6:1 v/v), and then with 6 mL of methanol/ammonium acetate buffer (5 mM, pH=8.0) mixture (1:1 v/v). Both times the cells were pelleted by centrifugation, and the resulting supernatants were consecutively loaded onto the same Strata-X-AW column. During the extraction-

centrifugation, samples with cells were kept below 4 °C to minimize degradation of metabolites. After washing the columns with 1 mL of water and 1 mL of methanol, metabolites of interest were eluted from the columns first with 0.3 mL of concentrated NH_4OH/methanol (1:14, v/v) mixture and then with 0.3 mL of concentrated NH_4OH/methanol/water (1:12:2, v/v) mixture. The resulting eluant was neutralized by adding 20 µL of glacial acetic acid, and then cleared by centrifugation in a microcentrifuge.

II. Metabolite extraction from *E. coli*. grown in shake flasks.

[0483] To extract metabolites from shake flask-grown *E. coli*, methanol-quenched cells were pelleted by centrifugation, and the resulting supernatant was loaded onto Strata-X-AW anion exchange column (Phenomenex) containing 30 mg of sorbent. The pellet was re-extracted twice with several milliliters of 50%, v/v, aqueous methanol containing 20% ammonium bicarbonate buffer (pH=8.0) and then with 75%, v/v, aqueous bicarbonate-buffered methanol. After each extraction, cell debris was pelleted by centrifugation, and the supernatant was consecutively loaded onto the same anion exchange columns. During the extraction and centrifugation steps, the samples were kept at below +4 °C. Prior to metabolite elution, the columns were washed with water and methanol (1 mL of each), and the analytes were eluted by adding 0.3 mL of concentrated NH_4OH/methanol (1:14, v/v) and then 0.3 mL of concentrated NH_4OH/water/methanol (1:2:12) mixtures. The eluant was neutralized with 40 µL of glacial acetic acid and then cleared by centrifugation in a microcentrifuge.

III. Metabolite Quantification

[0484] Analysis of metabolites was carried out using a Thermo Finnigan TSQ system (Thermo Electron Corporation, San Jose, CA). All system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Electron Corp). For the LC-ESI –MS/MS method, a chiral Nucleodex ß-OH 5µM HPLC column (200 x 4 mm, Macherey-Nagel, Germany) was used with a CC 8/4 Nucleodex beta-OH guard cartridge. A mobile phase gradient (Table 9) was applied at a flow rate of 0.8 mL/min in which mobile phase A was MilliQ –grade water, mobile phase B was 100 mM ammonium acetate (SigmaUltra grade, Sigma) buffer (pH adjusted to 8.0 by ammonium hydroxide) in MilliQ –grade water and mobile phase C was LC-MS grade acetonitrile (Chromasolv, Riedel-de Haën). The column and sample tray temperatures were reduced to 5

°C and 4 °C, respectively. The injection volume was 10 or 20 μL. Figure 140 shows typical elution profiles of selected metabolites extracted from an isoprene-producing *E. coli* strain.

Table 9. HPLC gradient used to elute metabolites in the MVA pathway.

Time, min	Mobile phase, %		
	A (water)	B (100 mM ammonium acetate, pH=8.0)	C (acetonitrile)
0.0	0.0	20.0	80.0
1.0	0.0	20.0	80.0
8.0	0.0	50.0	50.0
11.0	0.0	50.0	50.0
13.0	46.0	4.0	50.0
19.0	49.6	0.4	50.0
22.5	49.6	0.4	50.0
23.0	0.0	20.0	80.0
25.0	0.0	20.0	80.0

[0485] Mass detection was carried out using electrospray ionization in the negative mode (ESI spray voltage of 2.5-3.0 kV and ion transfer tube temperature of 390 °C). The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 245.0 for IPP and DMAPP, 313.1 for GPP, 381.1 for FPP, 227.0 for MVP, and 307.1 for MVPP. Concentrations of metabolites were determined based on the integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0). Calibration curves obtained by injection of standards (IPP, DMAPP, and GPP purchased from Sigma-Aldrich, and FPP purchased from Echelon Biosciences Inc.) were used to calculate concentrations of metabolites in cell extracts. Concentrations of MVP and MVPP were expressed in arbitrary units because of the absence of commercially available standards. Intracellular concentrations of metabolites were determined based on the assumption that in 1 mL of the culture at OD=200 the integrated volume of all cells is 50 μL.

IV. Intracellular concentrations of metabolites in the MCM401 strain of *E. coli* containing MVK from *M. mazei* under different levels of enzyme expression induced by adding IPTG to the fermentors.

[0486] Figure 141A-141F provide an example of intracellular concentrations of metabolites in the MCM401 strain of *E. coli* containing MVK from *M. mazei* under different levels of

enzyme expression induced by adding IPTG to the fermentors. Even though the final IPTG concentrations in all three fermentors were similar (~ 200 μM), cell response was very different depending on the IPTG feeding scheme. A single-shot addition of a high dose of IPTG (Figures 114C and 114F) caused an instant increase in isoprene production and early accumulation of a significant level of MVPP. In contrast, concentrations of DMAPP, the immediate precursor of isoprene, as well as GPP and FPP, the products of IPP and DMAPP condensation, were low (below ~ 0.2 mM). Intracellular concentrations of IPP remained higher than the concentration of DMAPP during the analyzed fermentation period, indicating that DMAPP is synthesized from IPP slower than it is consumed in the isoprene biosynthesis reaction.

[0487] Although the maximum specific productivity of MCM401 cells reached about the same level upon adding IPTG in two steps (~ 100 μM each time; Figures 141B and 141E), the amount of MVPP accumulated in cells by the end of the production period was lower than in the single IPTG shot experiment and the buildup of MVPP pool started only after the second portion of IPTG was added to the fermentor. In both cases a decline in the isoprene production correlated with accumulation of MVP, which pool reached much higher concentrations in cells that had received two doses of IPTG. Moderate levels of IPP and DMAPP (~0.4 mM) were detected in the latter case around 30 hours of fermentation, which correlated in time with the maximum rate of isoprene biosynthesis by these cells. Notably, intracellular concentrations of GPP and FPP were low presumably due to a very high activity of the isoprene synthase.

[0488] Four IPTG shots of about 50 μM each resulted in the lowest specific productivity of the MCM401 strain; however, under these conditions the culture continued to synthesize isoprene at a significant rate for a longer period of time (Figures 141A and 141D). The maximum intracellular levels of IPP and DMAPP generally remained in the range of 0.2 – 0.4 mM during the production period, and FPP raised to 1.0-1.5 mM in response to the second 50 μM dose of IPTG. Notably, DMAPP concentration was slightly higher than the concentration of IPP likely due to the fact that DMAPP conversion into isoprene occurred slower in this case compared to the fermentations illustrated in Figures 141B, 141C, 141E, and 141F, and FPP biosynthesis did not consume significant amounts of DMAPP.

V. Intracellular concentrations of metabolites in the MCM402 strain of *E. coli* overexpressing MVK from *Saccharomyces cerevisiae*

[0489] Figures 142A and 142B illustrate the experiment with the MCM402 strain of *E. coli*, containing overexpressed MVK from *Saccharomyces cerevisiae*. As in the case with the MCM401 strain having MVK from *M. mazei* and grown under similar IPTG induction conditions (4 x 50 μM shots), isoprene production started after the second dose of IPTG has been added to the fermentor, which coincided in time with rapid accumulation of DMAPP and IPP to relatively high levels (up to 1.8 mM of DMAPP) in the MCM402 cells. However, in the MCM402 cells, the isoprene production period remained very short correlating with the drop in DMAPP and IPP pools. In contrast, FPP continued to accumulate up to the level of 2.6 – 3.5 mM even when DMAPP and IPP concentrations dropped to below 1 mM.

VI. Intracellular concentrations of metabolites in the MCM402 strain of *E. coli* overexpressing MVK from *Streptomyces*

[0490] Figures 143A and 143B illustrate the experiment with the MCM400 strain of *E. coli*, containing overexpressed MVK from *Streptomyces*. In terms of accumulation of isoprenoid intermediates/precursors and isoprene production results of this experiment are very similar to the experiment performed with the MCM401 strain containing MVK from *M. mazei* and induced with IPTG using the same scheme (4 x 50 μM shots; see Figures 141A and 141D). Indeed, the isoprene specific productivity in the MCM400 strain reached values slightly above 3 mg/(OD h), and the high rate of production was maintained for a long time. Moreover, MCM400 cells accumulated up to 2 mM of FPP with the FPP accumulation started after the second IPTG shot; DMAPP, IPP, and GPP concentrations remained within the range of 0.2-0.5 mM during the production period, and MVP and MVPP were below the detection limit. Therefore, parts IV to VI of this example emphasize superior properties of MVK from *Streptomyces* and *M. mazei* as compared to yeast MVK.

VII. Safe and maximal metabolite concentrations during isoprene production

Shake flask experiment with MCM127

[0491] A shake flask experiment with MCM127 was performed to investigate the accumulation of key intermediates during strong induction of isoprene production. Strong

induction of this strain resulted in growth inhibition most likely due to accumulation of toxic metabolic intermediates.

Medium Recipe (per liter fermentation medium):

[0492] 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, $(NH_4)_2SO_4$ 3.2g, yeast extract 1 g, 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. Medium was filter-sterilized with a 0.22 micron vacuum filter. Glucose was added to the medium to a final concentration of 0.5%. Antibiotics were added after sterilization and pH adjustment.

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

[0493] 1000X trace metal solution contained citric Acids * 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 was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, and then brought to volume and filter sterilized with 0.22 micron filter.

Strain:

[0494] The MCM127 strain is BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA pathway (pCL Upper) and the lower MVA pathway including isoprene synthase from kudzu (pTrcKKDyIkIS)

[0495] An inoculum of *E. coli* strain MCM127 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 containing glucose as carbon source and grown overnight at 30°C. The bacteria were diluted into fermentation media to reach an optical density of 0.05 measured at 550 nm. A total of 150 mL of culture was dispensed into two 500 mL flasks that were then shaken at 170 rpm in a 30°C incubator. When the cultures reached an optical density (OD_{600}) of 0.5, one of the flasks was induced with 150 μM isopropyl-beta-D-1-thiogalactopyranoside (IPTG). Samples of 20mL from both the induced and non-induced culture were taken approximately every half hour for metabolite analysis after induction. The samples were

quickly quenched in equal volume of methanol cooled on dry ice. After centrifugation, supernatant was loaded on Stata X-AW columns. The pellet was resuspended in 5 mL of Methanol-water (6:1, water contained 5 mM NH4Ac at pH=8.0), cell debris were separated by centrifugation, and the supernatant was loaded on the Stata X-AW columns. Metabolites were eluted with 0.30 mL ethanol:conc NH4OH (14:1 vol/vol), then with 0.3 mL methanol:water:conc NH4OH (12:2:1 vol/vol/vol), finally pH was adjusted by adding 40 uL of glacial acetic acid. Extracted metabolites were analyzed by LCMS using a standard cyclodextrin column protocol. T o increase sensitivity, only ions corresponding to IPP, DMAPP, GPP, and FPP were detected. Injection volume was 20 uL/sample. Standards of all metabolites were used for calibration.

[0496] Upon induction of the MCM127 with 150 μM IPTG, the bacteria continued to grow identical to the un-induced strain for approximately one and a half hour. After this, the induced culture began to show signs of growth inhibition (Figure 112A). Key metabolites were measured during the experiment and showed an increasing accumulation of FPP, GPP, DMAPP and IPP after induction. DMAPP and IPP only began to accumulate when the induced bacteria first showed signs of growth inhibition (Figure 112B). None of the mentioned intermediates were detected in measurable amount in the un-induced culture. The experiment demonstrates that *E. coli* can tolerate significant intracellular concentrations of GPP and FPP (Tables 15A and 15B), while accumulation of DMAPP and IPP coincides with growth inhibition when cultures are grown in shake flasks. Data in Tables 15A and 15B were from the 5.5 hr time point, where growth was still normal in the induced culture.

VIII. Intracellular concentrations of metabolites in the MCM343 strain of *E. coli* expressing the full mevalonic acid pathway and Kudzu isoprene synthase (without overexpression of a second mevalonate kinase)

[0497] Figures 144A and 144B depict changes in concentrations of selected intermediates in the isoprenoid pathway in the course of fermentation of MCM343 *E. coli* strain. This fermentation run was characterized by very low specific productivity and barely detectable concentrations of most of isoprenoid intermediates except for FPP, which intracellular level reached 0.7 mM, after 100 μM IPTG was added to the cells. IPP and DMAPP were detected shortly after the IPTG addition and then their level dropped below the detection limit. No MVP or MVPP were detected during the fermentation.

IX. Growth Inhibition

i) Recovery of Mevalonic acid from fermentation broth.

[0498] Mevalonic acid was obtained by a fed batch fermentation of *Escherichia coli* strain, BL21 harboring an expression plasmid bearing the genes *mvaS* and *mvaE* from *Enterococcus faecalis* (U.S. Appl. Pub. No. 2005/0287655, which is incorporated by reference in its entirety, particularly with respect to genes *mvaS* and *mvaE*). Fermentation of the strains was carried out in fed batch fermentation mode in a minimal medium with a glucose feed for 40 hours. Broth was harvested, mixed with diatomaceous earth (DE; Catalog # Celatom FW-12, American Tartaric Products Inc.), and filtered under vacuum through a Buchner funnel fitted with a filter pad. The filtrate was sterile filtered through a 10,000 MWCO membrane. Mevalonic acid was converted to the lactone by acidification and recovered by continuous organic solvent extraction; NMR analysis indicated a purity of 84%. All recovery steps are well known to those skilled in the art. When the free acid was required for experiments, the MVA lactone was hydrolyzed by the addition of 1 equivalent of base to a solution of lactone and allowed to stand for 1 hour prior to use. The sterile filtered solution can be stored for extended time at 4 °C.

ii) Growth inhibition of *Escherichia coli* BL21 by the accumulation of mevalonate diphosphate, isopentenyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP).

[0499] The purpose of this experiment was to determine the effect of the expression of the proteins mevalonate kinase (MVK), phophomevalonate kinase (PMK), and diphosphomevalonate decarboxylase (MDD) of *Escherichia coli* cultures.

[0500] *E. coli* BL21 cells bearing pTrcK, representing a plasmid expressing MVK, pTrcKK representing a plasmid expressing MVK plus PMK, and pTrcKKD, representing a plasmid expressing MVK plus PMK plus MDD were grown at approximately 30 °C and 250 rpm in 250 mL flasks containing 25 mL of TM3 medium (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$) supplemented with 1% glucose and 0.8g/L Biospringer yeast extract (1% Yeast extract final). When OD600 reached 0.8 to 0.9, 5.8 mM mevalonic acid was added to the cultures and incubation was continues for an additional 5 hours. OD_{600} measurements were taken, and the cultures were sampled for metabolite analysis at 2 hours post MVA addition. Samples were collected into 100% MeOH prechilled in dry ice in a ratio of 1:1.

Samples were stored at -80 °C until analyzed as follows. The methanol-quenched cells were pelleted by centrifugation and the resulting supernatant was loaded onto Strata-X-AW anion exchange column (Phenomenex) containing 30 mg of sorbent. The pellet was reextracted twice with several milliliters of 50%, v/v, aqueous methanol containing 20% ammonium bicarbonate buffer (pH=8.0) and then with 75%, v/v, aqueous bicarbonate-buffered methanol. After each extraction, cell debris were pelleted by centrifugation and the supernatant was consecutively loaded onto the same anion exchange columns. During the extraction and centrifugation steps, the samples were kept at below +4 °C. Prior to metabolite elution, the columns were washed with water and methanol (1 mL of each) and the analytes were eluted by adding 0.3 mL of concentrated NH_4OH/methanol (1:14, v/v) and then 0.3 mL of concentrated NH_4OH/water/methanol (1:2:12) mixtures. The eluant was neutralized with 40 µL of glacial acetic acid and then cleared by centrifugation in microcentrifuge. Analysis of metabolites in these samples is as described above.

[0501] As is shown in Figure 145, inhibition of growth was evident when the enzymes MVK and PMK are expressed (strain #7); additional inhibition is observed when MDD is added to the cloned pathway (strain #6). No growth inhibition was observed when MVK was the only enzyme expressed (strain #5). Analysis of MVA concentration at the time of collection of samples suggests that strain with MVK plus PMK plus MDD consumed 2.9 mM MVA while the other two strains consume lower quantities. Measurement of phosphomevalonate from the culture of the strains carrying only MVK was not successful; however, the culture carrying MVK and PMV showed about 30 and 60 – fold higher levels, respectively, of phosphomevalonate and diphosphomevalonate compared to the strain carrying MVK, PMK, and MDD. The latter strain accumulated surprisingly high levels of IPP and DMAPP on the order of 40 mM IPP and 320 uM DMAPP when calculated as an intracellular concentration. These measurements were conducted on whole cell broth; thus, some of the metabolites may have been excreted by the cells. While not intending to be bound by any particular theory, it is believed that the observed growth inhibition is due to the accumulation of one or more of these metabolites. A goal is therefore to achieve a pathway enzyme balance to minimize the accumulation of these metabolites for the relief of growth inhibition.

[0502] Example 9. Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate

kinase from Streptomyces, and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium):

[0503] 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:

[0504] 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.

[0505] 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 Streptomyces CL190 and isoprene synthase from Kudzu (pTrcKudzuMVK(StreptomycesCL190)). 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 innoculate 5-L of cell medium in the 15-L bioreactor. The liquid volume increases throughout the fermentation (such as to approximately 10 liters).

[0506] 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 3.5 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 50 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 88 uM when OD_{550} reached 165. Additional IPTG additions raised the concentration to 114 uM at OD_{550} = 215 and 147 uM at OD_{550} = 230. The OD_{550} profile within the bioreactor over time is shown in Figure 117. 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 21.1 g/L (Figure 118). The total amount of isoprene produced during the 67 hour fermentation was 193.2 g and the time course of production is shown in Figure 119. The molar yield of utilized carbon that went into producing isoprene during fermentation was 12.0%. The weight percent yield of isoprene from glucose was 6.2%.

Example 10. Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from Lactobacillus, and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium):

[0507] 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:

[0508] 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.

[0509] 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 Lactobacillus and isoprene synthase from Kudzu (pTrcKudzuMVK(Lactobacillus)). 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 innoculate 5-L of cell medium in the 15-L bioreactor. The liquid volume increases throughout the fermentation (such as to approximately 10 liters).

[0510] 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 33 hour fermentation was 1.0 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 58 uM when the optical density at 550 nm (OD_{550}) reached a value of 16. The IPTG concentration was raised to 108 uM when OD_{550} reached 30. Additional IPTG additions raised the concentration to 174 uM at OD_{550} = 56 and 222 uM at OD_{550} = 86. The OD_{550} profile within the bioreactor over time is shown in Figure 120. 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 6.4 g/L (Figure 121). The total amount of isoprene produced during the 33 hour fermentation was 35.2 g and the time course of production is shown in Figure 122. The molar yield of utilized carbon that went into producing isoprene during fermentation was 7.2 %. The weight percent yield of isoprene from glucose was 3.4%.

Example 11. Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from yeast, and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium):

[0511] 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:

[0512] 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.

[0513] 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 yeast and isoprene synthase from Kudzu (pTrcKudzuMVK(yeast)). 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 innoculate 5-L of cell medium in the 15-L bioreactor. The liquid volume increases throughout the fermentation (such as to approximately 10 liters).

[0514] 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 1.6 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 54 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 87 uM when OD_{550} reached 175. Additional IPTG additions raised the concentration to 122 uM at OD_{550} = 180 and 157 uM at OD_{550} = 185. The OD_{550} profile within the bioreactor over time is shown in Figure 123. 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 6.4 g/L (Figure 124). The total amount of isoprene produced during the 54 hour fermentation was 44.6 g and the time course of production is shown in Figure 125. The molar yield of utilized carbon that went into producing isoprene during fermentation was 6.1%. The weight percent yield of isoprene from glucose was 2.8%.

Example 12. Construction and Expression of *Lactobacillus sakei* and *Streptococcus pneumoniae* mevalonate kinase constructs

[0515] The *mvk* genes from both *Lactobacillus sakei* (Danisco strain L110) and *Streptococcus pneumoniae* R6 (ATCC # BAA-255D-5) were PCR amplified (Table 10 for primer pairs) from genomic DNA, TOPO-cloned into the pET200D-TOPO (Invitrogen) expression vector, and transformed into chemically competent *E. coli* TOP10 (Invitrogen) cells according to the manufacturer's recommended protocol. Inserts of *mvk* into pET200D-TOPO, which generates a translational fusion between a 6XHis tag and the gene of interest, were verified by PCR using the T7 Forward primer (Table 10) and either of the reverse primers (Lsmvk2 or Spmvk2), respectively. Positive plasmids, which confer kanamycin resistance to *E. coli*, were purified via miniprep (Qiagen), and the complete *mvk* insertions were sequenced (Quintara Biosciences) using T7 Forward and T7 Reverse primers (Table 10). The complete sequences for pDW01 (harboring the *Lb. sakei mvk* gene) and pDW02 (harboring the *S. pneumoniae mvk* gene) are listed in Figures 127B, 127C, 128B, and 128C, respectively. Figures 127A and 128A show plasmid maps. The DNA sequence of *mvk* from *Lb. sakei* Danisco strain L110 diverged from the sequence of *mvk* from *Lb. sakei* strain 23K (NCBI accession # CR936503). The *mvk* from L110 shared only 92% DNA identity with the *mvk* of strain 23K, and only 97% amino acid identity. pDW01 and pDW02 were transformed into chemically competent *E. coli* BL21 Star (DE3) (Invitrogen) cells for expression analysis.

Individual strains containing pDW01 and pDW02 were grown at 37 °C overnight in LB medium. The following day, strains were diluted to an OD_{600} of 0.05 and grown at 37 °C to an OD_{600} of approximately 1.0. Cultures were split (to generate both uninduced and induced samples) and IPTG was added to one member of each pair at a concentration of 1mM. Strains were returned to the incubator and grown for another 2 hours at 37 °C. Samples of each culture (approximately 10 µl) were removed for SDS-PAGE analysis using the NuPage system (Invitrogen) according to manufacturer's instructions. Figure 129 shows that after induction, proteins of approximately 37.8 kDa (for *Lb. sakei mvk* with the N-terminal 6XHis tag, lane 2) and 35.6 kDa (for *S. pneumoniae mvk* with the N-terminal 6XHis tag, lanes 4 and 6) were produced, in comparison to the uninduced control.

Table 10. Oligonucleotides

Primer Name	**Sequence (5′ to 3′)**	**Purpose**
Lsmvk1	CACCATGCAAACGAGTGTGGGAAACAGTCA CGCT (SEQ ID NO:133)	Forward primer for *Lb. sakei mvk*
Lsmvk2	TGTTTAATTAGTGTGTAGTGCGTGTAATGG (SEQ ID NO:134)	Reverse primer for *Lb. sakei mvk*
Spmvk1	CACCATGACAAAAAAAGTTGGTGTCGGTCA GGCAC (SEQ ID NO:135)	Forward primer for *S. pneumoniae mvk*
Spmvk2	CTGTCACAGGCTCTCTATCCATGTCTGAAC (SEQ ID NO:136)	Reverse primer for *S. pneumoniae mvk*
T7 Forward	TAATACGACTCACTATAGGG (SEQ ID NO:137)	PCR and sequencing primer
T7 Reverse	GCTAGTTATTGCTCAGCGG (SEQ ID NO:138)	PCR and sequencing primer

Example 13. 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*

[0516] 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).

[0517] The isoprene synthase gene was also cloned into pET16b (Novagen). In this case, the isoprene synthase gene was inserted into pET16b such that the recombinant isoprene synthase protein contained the N-terminal His tag. The isoprene synthase gene was amplified from pTrcKudzu by PCR using the primer set pET-His-Kudzu-2F: 5'-CGTGAGATCATATGTGTGCGACCTCTTCTCAATTTAC (SEQ ID NO:3) and pET-His-Kudzu-R: 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:4). These primers added an *Nde*I site at the 5'-end and a *Bam*H1 site at the 3' end of the gene respectively. The plasmid pTrcKudzu, described above, was used as template DNA, Herculase polymerase (Stratagene) was used according to manufacture's directions, and primers were added at a concentration of 10 pMols. The PCR was carried out in a total volume of 25 µl. The PCR product was digested with *Nde*I/*Bam*H1 and cloned into pET16b digested with the same enzymes. The ligation mix was transformed into *E. coli* Top10 (Invitrogen) and the correct clone selected by sequencing. The resulting plasmid, in which the kudzu isoprene synthase gene was expressed from the T7 promoter, was designated pETNHisKudzu (Figures 4 and 5).

[0518] The kudzu isoprene synthase gene was also cloned into the low copy number plasmid pCL1920. Primers were used to amplify the kudzu isoprene synthase gene from pTrcKudzu described above. The forward primer added a *Hin*dIII site and an *E. coli* consensus RBS to the 5' end. The *Pst*I cloning site was already present in pTrcKudzu just 3' of the stop codon so the reverse primer was constructed such that the final PCR product includes the *Pst*I site. The sequences of the primers were: HindIII-rbs-Kudzu F: 5'-CATATGAAAGCTTGTATCGATTAAATAAGGAGGAATAAACC (SEQ ID NO:6) and BamH1-Kudzu R:

[0519] 5'- CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:4). The PCR product was amplified using Herculase polymerase with primers at a concentration of 10 pmol and with 1 ng of template DNA (pTrcKudzu). The amplification protocol included 30 cycles of (95° C for 1 minute, 60° C for 1 minute, 72° C for 2 minutes). The product was digested with *Hin*dIII and *Pst*I and ligated into pCL1920 which had also

been digested with *Hin*dIII and *Pst*I. The ligation mix was transformed into *E. coli* Top10. Several transformants were checked by sequencing. The resulting plasmid was designated pCL-lac-Kudzu (Figures 6 and 7A-7C).

II. Determination of isoprene production

[0520] 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).

[0521] 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).

[0522] The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (30 m x 0.25 mm; 0.25 µm film thickness) was used for separation of analytes. The sampler was set up to inject 500 µL of headspace gas. The GC/MS method utilized helium as the carrier gas at a flow of 1 ml/min. The injection port was held at 250° C with a split ratio of 50:1. The oven temperature was held at 37° C for the 2 minute duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 67. The detector was switched off from 1.4 to 1.7 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at 1.78 minutes. A calibration table was used to quantify the absolute amount of isoprene and was found to be linear from 1 µg/L to 2000 µg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method.

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

[0523] 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 Figures 8A-8D.

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

[0524] Large scale production of isoprene from *E. coli* containing the recombinant kudzu isoprene synthase gene was determined from a fed-batch culture. The recipe for the fermentation media (TM2) per liter of fermentation medium was as follows: K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, MgSO4 * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with potassium hydroxide (KOH) and q.s. to volume. The final product was filter sterilized with 0.22 µ filter (only, do not autoclave). The recipe for 1000X Modified Trace Metal Solution was as follows: Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, $ZnSO_4$ * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 µ filter.

[0525] 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 Figures 9A and 9B.

Example 14. Production of isoprene in *E. coli* expressing recombinant poplar isoprene synthase

[0526] 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 31A and 31B). 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 33A-33C), was verified by sequencing.

Example 15. Production of isoprene in *Panteoa citrea* expressing recombinant kudzu isoprene synthase

[0527] The pTrcKudzu and pCL-lac Kudzu plasmids described in Example 13 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 13 for *E. coli* strains expressing recombinant kudzu isoprene synthase. Results are shown in Figures 10A-10C.

Example 16. 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

[0528] 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

[0529] The *aprE* promoter was amplified from chromosomal DNA from *Bacillus subtilis* using the following primers:

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

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

b) Amplification of the isoprene synthase gene

[0530] The kudzu isoprene synthase gene was amplified from plasmid pTrcKudzu (SEQ ID NO:2). The gene had been codon optimized for *E. coli* and synthesized by DNA 2.0. The following primers were used:

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

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

c) Amplification of the transcription terminator

[0531] The terminator from the alkaline serine protease of *Bacillus amyliquefaciens* was amplified from a previously sequenced plasmid pJHPms382 using the following primers:

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

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

[0532] The kudzu fragment was fused to the terminator fragment using PCR with the following primers:

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

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

[0533] The kudzu-terminator fragment was fused to the promoter fragment using PCR with the following primers:

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

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

[0534] 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.

[0535] 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:65)

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

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

5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:61)

CF 07-48 (+) Sequencing primer for kudzu isoprene synthase

5'- CTTTTCCATCACCCACCTGAAG (SEQ ID NO:67)

CF 07-49 (+) Sequencing in kudzu isoprene synthase

5'- GGCGAAATGGTCCAACAACAAAATTATC (SEQ ID NO:68)

[0536] The plasmid designated pBS Kudzu #2 (Figures 52 and 12A-12C) 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

[0537] Overnight cultures were inoculated with a single colony of CF 443 from a LA + Chloramphenicol (Cm, 25 μg/ml). Cultures were grown in LB + Cm at 37° C with shaking at 200 rpm. These overnight cultures (1 ml) were used to inoculate 250 ml baffled shake flasks containing 25 ml Grants II media and chloramphenicol at a final concentration of 25 μg/ml. Grants II Media recipe was 10 g soytone, 3 ml 1M K_2HPO_4, 75 g glucose, 3.6 g urea, 100 ml 10X MOPS, q.s. to 1 L with H_2O, pH 7.2; 10X MOPS recipe was 83.72 g MOPS, 7.17 g tricine, 12 g KOH pellets, 10 ml 0.276M K_2SO_4 solution, 10 ml 0.528M $MgCl_2$ solution, 29.22 g NaCl, 100 ml 100X micronutrients, q.s. to 1 L with H_2O; and 100X micronutrients recipe was 1.47 g $CaCl_2*2H_2O$, 0.4 g $FeSO_4*7H_20$, 0.1 g $MnSO_4*H_20$, 0.1 g $ZnSO_4*H_2O$, 0.05 g $CuCl_2*2H_2O$, 0.1 g $CoCl_2*6H_2O$, 0.1 g $Na_2MoO_4*2H_2O$, q.s. to 1 L with H_2O. Shake flasks were incubated at 37° C and samples were taken at 18, 24, and 44 hours. At 18 hours the headspaces of CF443 and the control strain were sampled. This represented 18 hours of accumulation of isoprene. The amount of isoprene was determined by gas chromatography as described in Example 13. Production of isoprene was enhanced significantly by expressing recombinant isoprene synthase (Figure 11).

III. Production of isoprene by CF443 in 14 L fermentation

[0538] 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*.

[0539] 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 17. Production of isoprene in Trichoderma

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

[0540] The *Yarrowia lipolytica* codon-optimized kudzu IS gene was synthesized by DNA 2.0 (SEQ ID NO:8) (Figure 13). This plasmid served as the template for the following PCR

amplification reaction: 1 µl plasmid template (20 ng/ul), 1 µl Primer EL-945 (10 uM) 5'-GCTTATGGATCCTCTAGACTATTACACGTACATCAATTGG (SEQ ID NO:9), 1 µl Primer EL-965 (10uM) 5'-CACCATGTGTGCAACCTCCTCCCAGTTTAC (SEQ ID NO:10), 1 µl dNTP (10mM), 5 µl 10x PfuUltra II Fusion HS DNA Polymerase Buffer, 1 µl PfuUltra II Fusion HS DNA Polymerase, 40 µl water in a total reaction volume of 50 µl. The forward primer contained an additional 4 nucleotides at the 5'-end that did not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but was required for cloning into the pENTR/D-TOPO vector. The reverse primer contained an additional 21 nucleotides at the 5'-end that did not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but were inserted for cloning into other vector backbones. Using the MJ Research PTC-200 Thermocycler, the PCR reaction was performed as follows: 95° C for 2 minutes (first cycle only), 95° C for 30 seconds, 55° C for 30 seconds, 72° C for 30 seconds (repeat for 27 cycles), 72° C for 1 minute after the last cycle. The PCR product was analyzed on a 1.2% E-gel to confirm successful amplification of the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene.

[0541] 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.

[0542] 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.

[0543] 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*

[0544] 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 13. 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 18. Production of isoprene in *Yarrowia*

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

[0545] The starting point for the construction of vectors for the expression of the kudzu isoprene synthase gene in *Yarrowia lipolytica* was the vector pSPZ1(MAP29Spb). The complete sequence of this vector (SEQ ID No:11) is shown in Figures 15A-15C.

[0546] The following fragments were amplified by PCR using chromosomal DNA of a *Y. lipolytica* strain GICC 120285 as the template: a promotorless form of the URA3 gene, a fragment of 18S ribosomal RNA gene, a transcription terminator of the *Y. lipolytica* XPR2 gene and two DNA fragments containing the promoters of XPR2 and ICL1 genes. The following PCR primers were used:

ICL1 3

5'- GGTGAATTCAGTCTACTGGGGATTCCCAAATCTATATATACTGCAGGTGAC (SEQ ID NO:69)

ICL1 5

5'- GCAGGTGGGAAACTATGCACTCC (SEQ ID NO:70)

XPR 3

5'- CCTGAATTCTGTTGGATTGGAGGATTGGATAGTGGG (SEQ ID NO:71)

XPR 5

5'- GGTGTCGACGTACGGTCGAGCTTATTGACC (SEQ ID NO:72)

XPRT3

5'- GGTGGGCCCGCATTTTGCCACCTACAAGCCAG (SEQ ID NO:73)

XPRT 5

5'- GGTGAATTCTAGAGGATCCCAACGCTGTTGCCTACAACGG (SEQ ID NO:74)

Y18S3

5'- GGTGCGGCCGCTGTCTGGACCTGGTGAGTTTCCCCG (SEQ ID NO:75)

Y18S 5

5'- GGTGGGCCCATTAAATCAGTTATCGTTTATTTGATAG (SEQ ID NO:76)

YURA3

5'- GGTGACCAGCAAGTCCATGGGTGGTTTGATCATGG (SEQ ID NO:77)

YURA 50

5'- GGTGCGGCCGCCTTTGGAGTACGACTCCAACTATG (SEQ ID NO:78)

YURA 51

5'- GCGGCCGCAGACTAAATTTATTTCAGTCTCC (SEQ ID NO:79)

[0547] 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.

[0548] Synthetic DNA molecules encoding the kudzu isoprene synthase gene, codon-optimized for expression in *Yarrowia,* was obtained from DNA 2.0 (Figure 16; SEQ ID NO:12). Full detail of the construction scheme of the plasmids pYLA(KZ1) and pYLI(KZ1) carrying the synthetic kudzu isoprene synthase gene under control of XPR2 and ICL1 promoters respectively is presented in Figures 18A-18F. 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).

[0549] A similar cloning procedure can be used to express a poplar (*Populus alba* x *Populus tremula*) isoprene synthase gene. The sequence of the poplar isoprene is described in Miller B. *et al.* (2001) *Planta* 213, 483-487 and shown in Figure 17 (SEQ ID NO:13). A construction scheme for the generation the plasmids pYLA(POP1) and pYLI(POP1) carrying synthetic poplar isoprene synthase gene under control of XPR2 and ICL1 promoters respectively is presented in Figure 18A and B.

II. Production of isoprene by recombinant strains of *Y. lipolytica*.

[0550] 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.

[0551] 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 13. 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 19. 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

[0552] The *bla* gene of pTrcKudzu (described in Example 13) was replaced with the gene conferring kanamycin resistance. To remove the *bla* gene, pTrcKudzu was digested with *Bsp*HI, treated with Shrimp Alkaline Phosphatase (SAP), heat killed at 65° C, then end-filled with Klenow fragment and dNTPs. The 5 kbp large fragment was purified from an agarose gel and ligated to the kan^r gene which had been PCR amplified from pCR-Blunt-II-TOPO using primers MCM22 5'- GATCAAGCTTAACCGGAATTGCCAGCTG (SEQ ID NO:14) and MCM23 5'- GATCCGATCGTCAGAAGAACTCGTCAAGAAGGC (SEQ ID NO:15),

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

ii) Construction of pTrcKudzu yIDI Kan

[0553] pTrcKudzuKan was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *idi* from *S. cerevisiae* with a synthetic RBS. The primers for PCR were NsiI-YIDI 1 F 5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC (SEQ ID NO:16) and PstI-YIDI 1 R 5'- CCTTCTGCAGGACGCGTTGTTATAGC (SEQ ID NO:17); and the template was *S. cerevisiae* genomic DNA. The PCR product was digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The ligation mixture was transformed into chemically competent TOP10 cells and selected on LA containing 50 µg/ml kanamycin. Several transformants were isolated and sequenced and the resulting plasmid was called pTrcKudzu-yIDI(kan) (Figures 34 and 35A-35C).

iii) Construction of pTrcKudzu DXS Kan

[0554] 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'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAAT ACCCG (SEQ ID NO:18) and MCM14 5'-CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:19); and the template was *E. coli* genomic DNA. The PCR product was digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The resulting transformation reaction was transformed into TOP10 cells and selected on LA with kanamycin 50 µg/ml. Several transformants were isolated and sequenced and the resulting plasmid was called pTrcKudzu-DXS(kan) (Figures 36 and 37A-37C).

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

[0555] 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'-

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

v) Construction of pCL PtrcKudzu

[0556] A fragment of DNA containing the promoter, structural gene and terminator from Example 13 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 38-41A-41C).

vi) Construction of pCL PtrcKudzu yIDI

[0557] 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 43A-43C).

vii) Construction of pCL PtrcKudzu DXS

[0558] 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 45A-45D).

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

[0559] 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 13). Results are shown in Figure 23A-23G.

[0560] Plasmid pTrcKudzu-yIDI-dxs (kan) was introduced into *E. coli* strain BL21 by transformation. The resulting strain BL21/pTrc Kudzu IDI DXS was grown overnight in LB containing kanamycin (50 μg/ml) at 20° C and used to inoculate shake flasks of TM3 (13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) containing 1% glucose. Flasks were incubated at 30° C until an OD_{600} of 0.8 was reached, and then induced with 400 μM IPTG. Samples were taken at various times after induction and the amount of isoprene in the head space was measured as described in Example 13. Results are shown in Figure 23H.

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

[0561] 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).

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

[0562] 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.

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

Medium Recipe (per liter fermentation medium):

[0563] 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:

[0564] Citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in DI H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0565] 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.

[0566] 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 20. Production of isoprene in *E. coli* expressing kudzu isoprene synthase and recombinant mevalonic acid pathway genes

I. Cloning the lower MVA pathway

[0567] 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), diphosphomevalonate decarboxylase (MVD) and isopentenyl diphosphate isomerase genes were amplified by PCR from *S. cerevisiae* chromosomal DNA and cloned individually into the pCR BluntII TOPO plasmid (Invitrogen). In some cases, the *idi* gene was amplified from *E. coli* chromosomal DNA. The primers were designed such that an *E. coli* consensus RBS (AGGAGGT (SEQ ID NO:80) or AAGGAGG (SEQ ID NO:81)) was inserted at the 5' end, 8 bp upstream of the start codon and a *Pst*I site was added at the 3' end. The genes were then cloned one by one into the pTrcHis2B vector until the entire pathway was assembled.

[0568] Chromosomal DNA from *S. cerevisiae* S288C was obtained from ATCC (ATCC 204508D). The MVK gene was amplified from the chromosome of *S. cerevisiae* using primers MVKF (5'-AGGAGGTAAAAAAACATGTCATTACCGTTCTTAACTTCTGC, SEQ ID NO:21) and MVK-Pst1-R (5'-ATGGCTGCAGGCCTATCGCAAATTAGCTTATGAAGTCCATGGTAAATTCGTG, SEQ ID NO:22) using PfuTurbo as per manufacturer's instructions. The correct sized PCR product (1370 bp) was identified by electrophoresis through a 1.2% E-gel (Invitrogen) and cloned into pZeroBLUNT TOPO. The resulting plasmid was designated pMVK1. The plasmid pMVK1 was digested with *Sac*I and *Taq*1 restriction endonucleases and the fragment

was gel purified and ligated into pTrcHis2B digested with *Sac*I and *Bst*BI. The resulting plasmid was named pTrcMVK1 (also refered to as pTrcK).

[0569] The second gene in the mevalonic acid biosynthesis pathway, PMK, was amplified by PCR using primers: PstI-PMK1 R (5'-GAATTCGCCCTTCTGCAGCTACC, SEQ ID NO:23) and BsiHKA I-PMK1 F (5'-CGACTGGTGCACCCTTAAGGAGGAAAAAAACATGTCAG, SEQ ID NO:24). The PCR reaction was performed using Pfu Turbo polymerase (Stratagene) as per manufacturer's instructions. The correct sized product (1387 bp) was digested with *Pst*I and *Bsi*HKI and ligated into pTrcMVK1 digested with *Pst*I. The resulting plasmid was named pTrcKK.

[0570] The MVD and the *idi* genes were cloned in the same manner. PCR was carried out using the primer pairs PstI-MVD 1 R (5'-GTGCTGGAATTCGCCCTTCTGCAGC, SEQ ID NO:25) and NsiI-MVD 1 F (5'-GTAGATGCATGCAGAATTCGCCCTTAAGGAGG, SEQ ID NO:26) to amplify the MVD gene and PstI-YIDI 1 R (5'-CCTTCTGCAGGACGCGTTGTTATAGC, SEQ ID NO:27) and NsiI-YIDI 1 F (5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC, SEQ ID NO:28) to amplify the yIDI gene. The plasmid with the MVK, PMK, and MVD genes inserted is named pTrcKKD. In some cases the IPP isomerase gene, *idi* from *E. coli* was used. To amplify *idi* from *E. coli* chromosomal DNA, the following primer set was used: PstI-CIDI 1 R (5'-GTGTGATGGATATCTGCAGAATTCG, SEQ ID NO:29) and NsiI-CIDI 1 F (5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAACATG, SEQ ID NO:30). Template DNA was chromosomal DNA isolated by standard methods from *E. coli* FM5 (WO 96/35796 and WO 2004/033646, which are each hereby 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.

[0571] 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 19, 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

[0572] The kudzu isoprene synthase gene was amplified by PCR from pTrcKudzu, described in Example 13, using primers MCM50 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGTGTGCGACCTCTTCTCAATTTACT (SEQ ID NO:31) and MCM53 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:32). The resulting PCR fragment was cloned into pCR2.1 and transformed into *E. coli* TOP10. This fragment contains the coding sequence for kudzu isoprene synthase and an upstream region containing a RBS from *E. coli*. Transformants were incubated overnight at 37° C with selection on LA containing carbenicillin (50 μg/ml). The correct insertion of the fragment was verified by sequencing and this strain was designated MCM93.

[0573] 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 25A-25D). 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.

[0574] 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 13. Maximum production of isoprene was 6.67×10^{-4} $mol/L_{broth}/OD_{600}/hr$ where L_{broth} is the volume of broth and includes both the volume of the cell medium and the volume of the cells. The control culture not supplemented with mevalonic acid did not produce measurable isoprene.

IV. Cloning the upper MVA pathway

[0575] The upper mevalonate biosynthetic pathway, comprising two genes encoding three enzymatic activities, was cloned from *Enterococcus faecalis*. The *mvaE* gene encodes a protein with the enzymatic activities of both acetyl-CoA acetyltransferase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the first and third proteins in the pathway, and the *mvaS* gene encodes second enzyme in the pathway, HMG-CoA synthase. The *mvaE* gene was amplified from *E. faecalis* genomic DNA (ATCC 700802D-5) with an *E. coli* ribosome binding site and a spacer in front using the following primers:

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

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

[0576] The *mvaS* gene was amplified from *E. faecalis* genomic DNA (ATCC 700802D-5) with a RBS and spacer from *E. coli* in front using the following primers:

CF 07-61 (+) Fuse *mvaE* to *mvaS* with RBS in between

5' -

GATTTAAGAAAACAATAAAGGAGGTAAAAAAACATGACAATTGGGATTGATAAA

(SEQ ID NO:36)

CF 07-102 (-) End of *mvaS* gene *Bgl*II

5' -GACATGACATAGATCTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:37)

[0577] The PCR fragments were fused together with PCR using the following primers:

CF 07-60 (+) Start of *mvaE* w/ RBS + ATG start codon *Sac*I

5' -GAGACATGAGCTCAGGAGGTAAAAAAACATGAAAACAGTAGTTATTATTG

(SEQ ID NO:34)

CF 07-102 (-) End of *mvaS* gene *Bgl*II

5'-GACATGACATAGATCTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:37)

[0578] 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.

[0579] 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:38)

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

5' – ATGTTATTGTTTTCTTAAATCATTTAAAATAGC (SEQ ID NO:39)

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

5' – ATGACAATTGGGATTGATAAAATTAG (SEQ ID NO:40)

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

5' – TTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:41)

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

5' – GAAATAGCCCCATTAGAAGTATC (SEQ ID NO:42)

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

5' – TTGCCAATCATATGATTGAAAATC (SEQ ID NO:43)

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

5' – GCTATGCTTCATTAGATCCTTATCG (SEQ ID NO:44)

CF 07-89 (+) Sequence *mvaS*

5' – GAAACCTACATCCAATCTTTTGCCC (SEQ ID NO:45)

[0580] 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

[0581] 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).

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

[0582] 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

[0583] Single colonies of the BL21/pTrcHis2A-*mvaE/mvaS* or FM5/p pTrcHis2A-*mvaE/mvaS* are inoculated into LB + carbenicillin (100 μg/ml) and are grown overnight at 37° C with shaking at 200 rpm. These cultures were diluted into 50 ml medium in 250 ml baffled flasks to an OD_{600} of 0.1. The medium was TM3 + 1 or 2% glucose + carbenicillin (100 ug/ml) or TM3 + 1% glucose + hydrolyzed soy oil + carbenicillin (100 ug/ml) or TM3 + biomass (prepared bagasse, corn stover or switchgrass). Cultures were grown at 30° C with shaking at 200 rpm for approximately 2-3 hours until an OD_{600} of 0.4 was reached. At this point the expression from the *mvaE mvaS* construct was induced by the addition of IPTG (400 μM). Cultures were incubated for a further 20 or 40 hours with samples taken at 2 hour intervals to 6 hour post induction and then at 24, 36 and 48 hours as needed. Sampling was done by removing 1 ml of culture, measuring the OD_{600}, pelleting the cells in a microfuge, removing the supernatant and analyzing it for mevalonic acid.

[0584] 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.

[0585] 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 19. 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)

[0586] 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 13. Results are presented in Table 10. The amount of isoprene made is presented as the amount at the peak production for the particular strain.

Table 10. Production of isoprene in *E. coli* strains

Strain	Isoprene (μg/liter/OD/hr
MCM50	23.8
MCM64	289
MCM125	ND
MCM131	Trace
MCM127	874

ND: not detected

Trace: peak present but not integrable.

IX. Analysis of mevalonic acid

[0587] Mevalonolactone (1.0 g, 7.7 mmol) (CAS# 503-48-0) was supplied from Sigma-Aldrich (WI, USA) as a syrup that was dissolved in water (7.7 mL) and was treated with potassium hydroxide (7.7 mmol) in order to generate the potassium salt of mevalonic acid. The conversion to mevalonic acid was confirmed by ^{1}H NMR analysis. Samples for HPLC analysis were prepared by centrifugation at 14,000 rpm for 5 minutes to remove cells, followed by the addition of a 300 μl aliquot of supernatant to 900 μl of H_2O. Perchloric acid (36 μl of a 70% solution) was then added followed by mixing and cooling on ice for 5 minutes. The samples were then centrifuged again (14,000 rpm for 5 min) and the supernatant transferred to HPLC. Mevalonic acid standards (20, 10, 5, 1 and 0.5 g/L) were prepared in the same fashion. Analysis of mevalonic acid (20 uL injection volume) was performed by HPLC using a BioRad Aminex 87-H+ column (300 mm by 7.0 mm) eluted with 5 mM sulfuric acid at 0.6 mL/min with refractive index (RI) detection. Under these conditions mevalonic acid eluted as the lactone form at 18.5 minutes.

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

[0588] 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):

[0589] 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:

[0590] 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, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0591] 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 15-L bioreactor containing an initial working volume of 5 L.

[0592] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 54 hour fermentation was 3.7 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 190. IPTG

concentration was raised to 100 uM at 38 hours of fermentation. The OD_{550} profile within the bioreactor over time is shown in Figure 54. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 2.2 g/L (Figure 55). The total amount of isoprene produced during the 54 hour fermentation was 15.9 g, and the time course of production is shown in Figure 56.

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

[0593] 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):

[0594] 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:

[0595] 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, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0596] 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 15-L bioreactor containing an initial working volume of 5 L.

[0597] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time, the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 59 hour fermentation was 2.2 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 190. The OD_{550} profile within the bioreactor over time is shown in Figure 93. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 3.0 g/L (Figure 94). The total amount of isoprene produced during the 59 hour fermentation was 22.8 g, and the time course of production is shown in Figure 95. The molar yield of utilized carbon that went into producing isoprene during fermentation was 2.2%. The weight percent yield of isoprene from glucose was 1.0%.

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

[0598] 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

[0599] The gene encoding isoprene synthase from *Pueraria lobata* was PCR-amplified using primers NsiI-RBS-HGS F (CTTGATGCATCCTGCATTCGCCCTTAGGAGG, SEQ ID NO:88) and pTrcR (CCAGGCAAATTCTGTTTTATCAG, SEQ ID NO:89), and pTrcKKDyIkIS as a template. The PCR product thus obtained 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.

[0600] These DNA fragments were ligated together and the ligation reaction was transformed into *E. coli* Top10 chemically competent cells (Invitrogen), plated on L agar containing spectinomycin (50 ug/ml) and incubated overnight at 37°C. Plasmid DNA was prepared from 6 clones using the Qiaquick Spin Mini-prep kit. The plasmid DNA was digested with restriction enzymes *Eco*RV and *Mlu*I to identify a clone in which the insert had the right orientation (*i.e.*, the gene oriented in the same way as the pTrc promoter).

[0601] 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 20, 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 20, 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):

[0602] 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:

[0603] 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, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$

* $2H_2O$ 100 mg. Each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0604] 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 15-L bioreactor containing an initial working volume of 5 L.

[0605] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 58 hour fermentation was 2.1 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 50 uM when OD_{550} reached 170. The OD_{550} profile within the bioreactor over time is shown in Figure 104. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 3.3 g/L (Figure 105). The total amount of isoprene produced during the 58 hour fermentation was 24.5 g and the time course of production is shown in Figure 106. The molar yield of utilized carbon that went into producing isoprene during fermentation was 2.5%. The weight percent yield of isoprene from glucose was 1.2%. Analysis showed that the activity of the isoprene synthase was increased by approximately 3-4 times that compared to BL21 expressing CL PtrcUpperMVA and pTrc KKDyIkIS plasmids (data not shown).

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

[0606] 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.

[0607] Table 11 lists primers used for this experiment.

Table 11. Primers

MCM78	attTn7 up rev for integration construct	gcatgctcgagcggccgcTTTTAATCAAACATCCTGCCAACTC (SEQ ID NO:91)
MCM79	attTn7 down rev for integration construct	gatcgaagggcgatcgTGTCACAGTCTGGCGAAACCG (SEQ ID NO:92)
MCM88	attTn7 up forw for integration construct	ctgaattctgcagatatcTGTTTTTCCACTCTTCGTTCACTTT (SEQ ID NO:93)
MCM89	attTn7 down forw for integration construct	tctagagggcccAAGAAAAATGCCCCGCTTACG (SEQ ID NO:94)
MCM104	GI1.2 promoter – MVK	Gatcgcggccgcgcccttgacgatgccacatcctgagcaaataattcaaccactaa ttgtgagcggataacacaaggaggaaacagctatgtcattaccgttcttaacttc (SEQ ID NO:95)
MCM105	aspA terminator – yIDI	Gatcgggccccaagaaaaaaggcacgtcatctgacgtgccttttttatttgtagacgc gttgttatagcattcta (SEQ ID NO:96)
MCM120	Forward of attTn7: attTn7 homology, GB marker homology	aaagtagccgaagatgacggtttgtcacatggagttggcaggatgtttgattaaaagc AATTAACCCTCACTAAAGGGCGG (SEQ ID NO:97)
MCM127	Rev complement of 1.2 GI: GB marker homology(extra long), promoter, RBS, ATG	AGAGTGTTCACCAAAAATAATAACCTTTCCCGGTGCAgaag ttaagaacggtaatgacatagctgtttcctccttgtgttatccgctcacaattagtggttga attatttgctcaggatgtggcatcgtcaagggcTAATACGACTCACTATAG GGCTCG (SEQ ID NO:98)

i) Target vector construction

[0608] The attTn7 site was selected for integration. Regions of homology upstream (attTn7 up) (primers MCM78 and MCM79) and downstream (attTn7 down) (primers MCM88 and MCM89) were amplified by PCR from MG1655 cells. A 50 uL reaction with 1uL 10uM primers, 3uL ddH2O, 45uL Invitrogen Platinum PCR Supermix High Fidelity, and a scraped colony of MG1655 was denatured for 2:00 at 94°C, cycled 25 times (2:00 at 94°C, 0:30 at 50°C, and 1:00 at 68°C), extended for 7:00 at 72°C, and cooled to 4°C. This resulting DNA was cloned into pCR2.1 (Invitrogen) according to the manufacturer's instructions, resulting in plasmids MCM278 (attTn7 up) and MCM252 (attTn7 down). The 832bp *Apa*I-*Pvu*I fragment digested and gel purified from MCM252 was cloned into *Apa*I-*Pvu*I digested and gel purified plasmid pR6K, creating plasmid MCM276. The 825bp *Pst*I-*Not*I fragment digested and gel purified from MCM278 was cloned into *Pst*I-*Not*I digested and gel purified MCM276, creating plasmid MCM281.

ii) Cloning of lower pathway and promoter

[0609] 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 50uL PCR reactions containing 1uL ~10ng/uL template, 1uL each primer, 1.25 uL 10mM dNTPs, 5uL 10x buffer, 1uL enzyme, and 39.75uL ddH20. Reactions 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).

iii) Integration into *E. coli* chromosome

[0610] 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 2uL of DNA. Integrants were selected on L agar with containing chloramphenicol (5 ug/ml) and subsequently confirmed to not grow on L agar + Kanamycin (50 ug/ml). BL21 integrant MCM331 and MG1655 integrant MCM333 were frozen.

iv) Construction of pET24D-Kudzu encoding Kudzu isoprene synthase

[0611] 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:99) and MCM53 5'-CGGTCGACGG ATCCCTGCAG TTAGACATAC ATCAGCTG (SEQ ID NO:100). 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.

[0612] 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:101) is shown in Figures 110A and 110B. Isoprene synthase polypeptide activity was confirmed using a headspace assay.

v) Production strains

[0613] Strains MCM331 and MCM333 were cotransformed with plasmids pCLPtrcupperpathway and either pTrcKudzu or pETKudzu, resulting in the strains shown in Table 12.

Table 12. 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):

[0614] 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:

[0615] 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, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in Di H_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0616] 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 15-L bioreactor containing an initial working volume of 5 L.

[0617] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time, the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 57 hour fermentation was 3.9 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 100 uM when the carbon dioxide evolution rate reached 100 mmol/L/hr. The OD_{550} profile within the bioreactor over time is shown in Figure 111A. The isoprene level in the off gas from the

bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 1.6 g/L (Figure 111B). The specific productivity of isoprene over the course of the fermentation is shown in Figure 111C and peaked at 1.2 mg/OD/hr. The total amount of isoprene produced during the 57 hour fermentation was 16.2 g. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.9%. The weight percent yield of isoprene from glucose was 0.4%.

Example 21. Construction of the upper and lower MVA pathway for integration into *Bacillus subtilis*

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

[0618] 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.

[0619] 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:82)

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

5'- CAATAATAACTACTGTTTTCACTCTTTACCCTCTCCTTTTAA (SEQ ID NO:83)

Template: *Bacillus subtilis* chromosomal DNA

2. *mvaE*

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

5'- TTAAAAGGAGAGGGTAAAGAGTGAAAACAGTAGTTATTATTG (SEQ ID NO:84)

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

5'- TTTATCAATCCCAATTGTCATGTTTTTTTACCTCCTTTATTGTTTTCTTAAATC

(SEQ ID NO:35)

Template: *Enterococcus faecalis* chromosomal DNA

3. *mvaS*

CF 07-61 (+) Fuse *mvaE* to *mvaS* with RBS in between

5'-

GATTTAAGAAAACAATAAAGGAGGTAAAAAAACATGACAATTGGGATTGATAAA

(SEQ ID NO:36)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator

5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:85)

Template: *Enterococcus faecalis* chromosomal DNA

4. *B. amyliquefaciens* alkaline serine protease terminator

CF 07-123 (+) Fuse the end of mvaS to the terminator

5'- ACCGTTCGTTCTTATCGAAACTAAAAAAAACCGGCCTTGGCCCCG (SEQ ID NO:86)

CF 07-46 (-) End of *B. amyliquefaciens* terminator BamHI

5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:63)

Template: *Bacillus amyliquefaciens* chromosomal DNA

PCR Fusion Reactions

5. Fuse *mvaE* to *mvaS*

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

5'- TTAAAAGGAGAGGGTAAAGAGTGAAAACAGTAGTTATTATTG (SEQ ID NO:84)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator

5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:85)

Template: #2 and 3 from above

6. Fuse *mvaE-mvaS* to *aprE* promoter

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:82)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator

5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:85)

Template #1 and #4 from above

7. Fuse PaprE-*mvaE-mvaS* to terminator

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:82)

CF 07-46 (-) End of *B. amyliquefaciens* terminator BamHI

5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:63)

Template: #4 and #6

[0620] 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 51A-51C). 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.

[0621] 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.

[0622] This strain is used subsequently as a host for the integration of the lower mevalonic acid pathway.

[0623] The following primers are used to sequence the various constructs above.

Sequencing primers:

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:82)

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

5'- ATGAAAACAGTAGTTATTATTGATGC (SEQ ID NO:38)

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

5'- ATGTTATTGTTTTCTTAAATCATTTAAAATAGC (SEQ ID NO:39)

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

5'- ATGACAATTGGGATTGATAAAATTAG (SEQ ID NO:40)

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

5'- TTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:41)

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

5'- GAAATAGCCCCATTAGAAGTATC (SEQ ID NO:42)

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

5'- TTGCCAATCATATGATTGAAAATC (SEQ ID NO:43)

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

5'- GCTATGCTTCATTAGATCCTTATCG (SEQ ID NO:44)

CF 07-89 (+) Sequence *mvaS*

5'- GAAACCTACATCCAATCTTTTGCCC (SEQ ID NO:45)

[0624] 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*

[0625] 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 29A-29D). 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 16 and is transformed into the strain with both upper and lower pathways integrated.

Example 22. 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

[0626] This example illustrates the de-coupling of cell growth from mevalonic acid and isoprene production.

I. Fermentation Conditions

Medium Recipe (per liter fermentation medium):

[0627] 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:

[0628] 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, $ZnSO_4 * 7H_2O$ 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.

[0629] Fermentation was performed with *E. coli* cells containing the pTrcHis2AUpperPathway (also called pTrcUpperMVA, Figures 91 and 92A-92C) (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.

[0630] 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

[0631] BL21 (DE3) cells that were grown on a plate as explained above in Example 22, 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

[0632] BL21 (DE3) cells that were grown on a plate as explained above in Example 22, 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

[0633] FM5 cells that were grown on a plate as explained above in Example 22, 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

[0634] BL21 (DE3) cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 22, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 25 μM when the OD_{550} reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 190. The IPTG concentration was raised to 100 uM at 38 hours of fermentation. The OD_{550} profile within the bioreactor over time is shown in Figure 63A. The isoprene titer increased over the course of the fermentation to a final value of 2.2 g/L broth (Figure 63B). The specific productivity profile throughout the fermentation is shown in Figure 63C and a comparison to

Figure 63A illustrates the 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

[0635] BL21 (DE3) tuner cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 22, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 26 μM when the OD_{550} reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 175. The OD_{550} profile within the bioreactor over time is shown in Figure 64A. The isoprene titer increased over the course of the fermentation to a final value of 1.3 g/L broth (Figure 64B). The specific productivity profile throughout the fermentation is shown in Figure 64C and a comparison to Figure 64A illustrates the 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

[0636] MG1655 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 22, 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

[0637] MG1655ack-pta cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 22, 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

[0638] FM5 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 22, 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 23. 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

[0639] This example illustrates the production of isoprene during the exponential growth phase of cells.

Medium Recipe (per liter fermentation medium):

[0640] 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:

[0641] 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, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in Di H2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0642] 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 15-L bioreactor containing an initial working volume of 5 L.

[0643] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 50 hour fermentation was 2.0 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 190. The OD_{550} profile within the bioreactor over time is shown in Figure 99. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 1.4 g/L (Figure 100). The total amount of isoprene produced during the 50 hour fermentation was 10.0 g. The profile of the isoprene specific productivity over time within the bioreactor is shown in Figure 101. The molar yield of utilized carbon that contributed to producing isoprene during fermentation was 1.1%. The weight percent yield of isoprene from glucose was 0.5%.

Example 24. Flammability modeling and testing of isoprene

I. Summary of flammability modeling and testing of isoprene

[0644] 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 13, and a matrix of the experiments performed is shown in Table 14.

Table 13. 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 14. 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

[0645] 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.

[0646] 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.

[0647] 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.

[0648] 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.

[0649] The limit flame temperature for combustion can be either selected based on theoretical principles or determined experimentally. Each method has its own limitations.

[0650] 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.

[0651] 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.

[0652] 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

[0653] Plotted in Figures 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 Figures 68 through 74. Additionally, the current form is

not conducive to comparison with experimental data which is generally presented in terms of volume percent.

[0654] 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.

[0655] 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. Figures 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 Figures 70-74.

IV. Flammability testing experimental equipment and procedure

[0656] 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.

[0657] 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.

[0658] 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.

[0659] 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

[0660] 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 Figures 80A and 80B.

[0661] 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.

[0662] 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.

[0663] 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.

[0664] Figure 79B summarizes the explosibility data points shown in Figure 79A. Figure 79C is a comparison of the experimental data with the CAFT model predicted flammability envelope. The model agrees very well with the experimental data. Discrepancies may be due to the same factors described in Series 1

V. Calculation of Flammability Limits of Isoprene in Air at 3 Atmospheres of Pressure

[0665] The methods described in Example 24, 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 24, 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).

[0666] 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 Figures 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

[0667] 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.

[0668] 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.

[0669] 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.

[0670] For use herein, unless clearly indicated otherwise, use of the terms “a”, “an,” and the like refers to one or more.

[0671] Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description

referring to "about X" includes description of "X." Numeric ranges are inclusive of the numbers defining the range.

[0672] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments.

Appendix 1

Exemplary 1-deoxy-D-xylulose-5-phosphate synthase nucleic acids and polypeptides

ATH: AT3G21500(DXPS1) AT4G15560(CLA1) AT5G11380(DXPS3)

OSA: 4338768 4340090 4342614

CME: CMF089C

PFA: MAL13P1.186

TAN: TA20470

TPV: TP01_0516

ECO: b0420(dxs)

ECJ: JW0410(dxs)

ECE: Z0523(dxs)

ECS: ECs0474

ECC: c0531(dxs)

ECI: UTI89_C0443(dxs)

ECP: ECP_0479

ECV: APECO1_1590(dxs)

ECW: EcE24377A_0451(dxs)

ECX: EcHS_A0491

STY: STY0461(dxs)

STT: t2441(dxs)

SPT: SPA2301(dxs)

SEC: SC0463(dxs)

STM: STM0422(dxs)

YPE: YPO3177(dxs)

YPK: y1008(dxs)

YPM: YP_0754(dxs)

YPA: YPA_2671

YPN: YPN_0911

YPP: YPDSF_2812

YPS: YPTB0939(dxs)

YPI: YpsIP31758_3112(dxs)

SFL: SF0357(dxs)

SFX: S0365(dxs)

SFV: SFV_0385(dxs)

SSN: SSON_0397(dxs)

SBO: SBO_0314(dxs)

SDY: SDY_0310(dxs)

ECA: ECA1131(dxs)

PLU: plu3887(dxs)

BUC: BU464(dxs)

BAS: BUsg448(dxs)

WBR: WGLp144(dxs)

SGL: SG0656

KPN: KPN_00372(dxs)

BFL: Bfl238(dxs)

BPN: BPEN_244(dxs)

HIN: HI1439(dxs)

HIT: NTHI1691(dxs)

HIP: CGSHiEE_04795

HIQ: CGSHiGG_01080

HDU: HD0441(dxs)

HSO: HS_0905(dxs)

PMU: PM0532(dxs)

MSU: MS1059(dxs)

APL: APL_0207(dxs)

XFA: XF2249

XFT: PD1293(dxs)

XCC: XCC2434(dxs)

XCB: XC_1678

XCV: XCV2764(dxs)

XAC: XAC2565(dxs)

XOO: XOO2017(dxs)

XOM: XOO_1900(XOO1900)

VCH: VC0889

VVU: VV1_0315

VVY: VV0868

VPA: VP0686

VFI: VF0711

PPR: PBPRA0805

PAE: PA4044(dxs)

PAU: PA14_11550(dxs)

PAP: PSPA7_1057(dxs)

PPU: PP_0527(dxs)

PST: PSPTO_0698(dxs)

PSB: Psyr_0604

PSP: PSPPH_0599(dxs)

PFL: PFL_5510(dxs)

PFO: Pfl_5007

PEN: PSEEN0600(dxs)

PMY: Pmen_3844

PAR: Psyc_0221(dxs)

PCR: Pcryo_0245

ACI: ACIAD3247(dxs)

SON: SO_1525(dxs)

SDN: Sden_2571

SFR: Sfri_2790

SAZ: Sama_2436

SBL: Sbal_1357

SLO: Shew_2771

SHE: Shewmr4_2731

SHM: Shewmr7_2804

SHN: Shewana3_2901

SHW: Sputw3181_2831

ILO: IL2138(dxs)

CPS: CPS_1088(dxs)

PHA: PSHAa2366(dxs)

PAT: Patl_1319

SDE: Sde_3381

PIN: Ping_2240

MAQ: Maqu_2438

MCA: MCA0817(dxs)

FTU: FTT1018c(dxs)

FTF: FTF1018c(dxs)

FTW: FTW_0925(dxs)

FTL: FTL_1072

FTH: FTH_1047(dxs)

FTA: FTA_1131(dxs)

FTN: FTN_0896(dxs)

NOC: Noc_1743

AEH: Mlg_1381

HCH: HCH_05866(dxs)

CSA: Csal_0099

ABO: ABO_2166(dxs)

AHA: AHA_3321(dxs)

BCI: BCI_0275(dxs)

RMA: Rmag_0386

VOK: COSY_0360(dxs)

NME: NMB1867

NMA: NMA0589(dxs)

NMC: NMC0352(dxs)

NGO: NGO0036

CVI: CV_2692(dxs)

RSO: RSc2221(dxs)

REU: Reut_A0882

REH: H16_A2732(dxs)

RME: Rmet_2615

BMA: BMAA0330(dxs)

BMV: BMASAVP1_1512(dxs)

BML: BMA10299_1706(dxs)

BMN: BMA10247_A0364(dxs)

BXE: Bxe_B2827

BUR: Bcep18194_B2211

BCN: Bcen_4486

BCH: Bcen2424_3879

BAM: Bamb_3250

BPS: BPSS1762(dxs)

BPM: BURPS1710b_A0842(dxs)

BPL: BURPS1106A_A2392(dxs)

BPD: BURPS668_A2534(dxs)

BTE: BTH_II0614(dxs)

BPE: BP2798(dxs)

BPA: BPP2464(dxs)

BBR: BB1912(dxs)

RFR: Rfer_2875

POL: Bpro_1747

PNA: Pnap_1501

AJS: Ajs_1038

MPT: Mpe_A2631

HAR: HEAR0279(dxs)

MMS: mma_0331

NEU: NE1161(dxs)

NET: Neut_1501

NMU: Nmul_A0236

EBA: ebA4439(dxs)

AZO: azo1198(dxs)

DAR: Daro_3061

TBD: Tbd_0879

MFA: Mfla_2133

HPY: HP0354(dxs)

HPJ: jhp0328(dxs)

HPA: HPAG1_0349

HHE: HH0608(dxs)

HAC: Hac_0968(dxs)

WSU: WS1996

TDN: Tmden_0475

CJE: Cj0321(dxs)

CJR: CJE0366(dxs)

CJJ: CJJ81176_0343(dxs)

CJU: C8J_0298(dxs)

CJD: JJD26997_1642(dxs)

CFF: CFF8240_0264(dxs)

CCV: CCV52592_1671(dxs) CCV52592_1722

CHA: CHAB381_1297(dxs)

CCO: CCC13826_1594(dxs)

ABU: Abu_2139(dxs)

NIS: NIS_0391(dxs)

SUN: SUN_2055(dxs)

GSU: GSU0686(dxs-1) GSU1764(dxs-2)

GME: Gmet_1934 Gmet_2822

PCA: Pcar_1667

PPD: Ppro_1191 Ppro_2403

DVU: DVU1350(dxs)

DVL: Dvul_1718

DDE: Dde_2200

LIP: LI0408(dsx)

DPS: DP2700

ADE: Adeh_1097

MXA: MXAN_4643(dxs)

SAT: SYN_02456

SFU: Sfum_1418

PUB: SAR11_0611(dxs)

MLO: mlr7474

MES: Meso_0735

SME: SMc00972(dxs)

ATU: Atu0745(dxs)

ATC: AGR_C_1351

RET: RHE_CH00913(dxs)

RLE: RL0973(dxs)

BME: BMEI1498

BMF: BAB1_0462(dxs)

BMS: BR0436(dxs)

BMB: BruAb1_0458(dxs)

BOV: BOV_0443(dxs)

BJA: bll2651(dxs)

BRA: BRADO2161(dxs)

BBT: BBta_2479(dxs)

RPA: RPA0952(dxs)

RPB: RPB_4460

RPC: RPC_1149

RPD: RPD_4305

RPE: RPE_1067

NWI: Nwi_0633

NHA: Nham_0778

BHE: BH04350(dxs)

BQU: BQ03540(dxs)

BBK: BARBAKC583_0400(dxs)

CCR: CC_2068

SIL: SPO0247(dxs)

SIT: TM1040_2920

RSP: RSP_0254(dxsA) RSP_1134(dxs)

JAN: Jann_0088 Jann_0170

RDE: RD1_0101(dxs) RD1_0548(dxs)

MMR: Mmar10_0849

HNE: HNE_1838(dxs)

ZMO: ZMO1234(dxs) ZMO1598(dxs)

NAR: Saro_0161

SAL: Sala_2354

ELI: ELI_12520

GOX: GOX0252

GBE: GbCGDNIH1_0221 GbCGDNIH1_2404

RRU: Rru_A0054 Rru_A2619

MAG: amb2904

MGM: Mmc1_1048

SUS: Acid_1783

BSU: BG11715(dxs)

BHA: BH2779

BAN: BA4400(dxs)

BAR: GBAA4400(dxs)

BAA: BA_4853

BAT: BAS4081

BCE: BC4176(dxs)

BCA: BCE_4249(dxs)

BCZ: BCZK3930(dxs)

BTK: BT9727_3919(dxs)

BTL: BALH_3785(dxs)

BLI: BL01523(dxs)

BLD: BLi02598(dxs)

BCL: ABC2462(dxs)

BAY: RBAM_022600

BPU: BPUM_2159

GKA: GK2392

GTN: GTNG_2322

LMO: lmo1365(tktB)

LMF: LMOf2365_1382(dxs)

LIN: lin1402(tktB)

LWE: lwe1380(tktB)

LLA: L108911(dxsA) L123365(dxsB)

LLC: LACR_1572 LACR_1843

LLM: llmg_0749(dxsB)

SAK: SAK_0263

LPL: lp_2610(dxs)

LJO: LJ0406

LAC: LBA0356

LSL: LSL_0209(dxs)

LGA: LGAS_0350

STH: STH1842

CAC: CAC2077 CA_P0106(dxs)

CPE: CPE1819

CPF: CPF_2073(dxs)

CPR: CPR_1787(dxs)

CTC: CTC01575

CNO: NT01CX_1983

CTH: Cthe_0828

CDF: CD1207(dxs)

CBO: CBO1881(dxs)

CBA: CLB_1818(dxs)

CBH: CLC_1825(dxs)

CBF: CLI_1945(dxs)

CKL: CKL_1231(dxs)

CHY: CHY_1985(dxs)

DSY: DSY2348

DRM: Dred_1078

PTH: PTH_1196(dxs)

SWO: Swol_0582

CSC: Csac_1853

TTE: TTE1298(dxs)

MTA: Moth_1511

MPE: MYPE730

MGA: MGA_1268(dxs)

MTU: Rv2682c(dxs1) Rv3379c(dxs2)

MTC: MT2756(dxs)

MBO: Mb2701c(dxs1) Mb3413c(dxs2)

MLE: ML1038(dxs)

MPA: MAP2803c(dxs)

MAV: MAV_3577(dxs)

MSM: MSMEG_2776(dxs)

MMC: Mmcs_2208

CGL: NCgl1827(cgl1902)

CGB: cg2083(dxs)

CEF: CE1796

CDI: DIP1397(dxs)

CJK: jk1078(dxs)

NFA: nfa37410(dxs)

RHA: RHA1_ro06843

SCO: SCO6013(SC1C3.01) SCO6768(SC6A5.17)

SMA: SAV1646(dxs1) SAV2244(dxs2)

TWH: TWT484

TWS: TW280(Dxs)

LXX: Lxx10450(dxs)

CMI: CMM_1660(dxsA)

AAU: AAur_1790(dxs)

PAC: PPA1062

TFU: Tfu_1917

FRA: Francci3_1326

FAL: FRAAL2088(dxs)

ACE: Acel_1393

SEN: SACE_1815(dxs) SACE_4351

BLO: BL1132(dxs)

BAD: BAD_0513(dxs)

FNU: FN1208 FN1464

RBA: RB2143(dxs)

CTR: CT331(dxs)

CTA: CTA_0359(dxs)

CMU: TC0608

CPN: CPn1060(tktB_2)

CPA: CP0790

CPJ: CPj1060(tktB_2)

CPT: CpB1102

CCA: CCA00304(dxs)

CAB: CAB301(dxs)

CFE: CF0699(dxs)

PCU: pc0619(dxs)

TPA: TP0824

TDE: TDE1910(dxs)

LIL: LA3285(dxs)

LIC: LIC10863(dxs)

LBJ: LBJ_0917(dxs)

LBL: LBL_0932(dxs)

SYN: sll1945(dxs)

SYW: SYNW1292(Dxs)

SYC: syc1087_c(dxs)

SYF: Synpcc7942_0430

SYD: Syncc9605_1430

SYE: Syncc9902_1069

SYG: sync_1410(dxs)

SYR: SynRCC307_1390(dxs)

SYX: SynWH7803_1223(dxs)

CYA: CYA_1701(dxs)

CYB: CYB_1983(dxs)

TEL: tll0623

GVI: gll0194

ANA: alr0599

AVA: Ava_4532

PMA: Pro0928(dxs)

PMM: PMM0907(Dxs)

PMT: PMT0685(dxs)

PMN: PMN2A_0300

PMI: PMT9312_0893

PMB: A9601_09541(dxs)

PMC: P9515_09901(dxs)

PMF: P9303_15371(dxs)

PMG: P9301_09521(dxs)

PMH: P9215_09851

PMJ: P9211_08521

PME: NATL1_09721(dxs)

TER: Tery_3042

BTH: BT_1403 BT_4099

BFR: BF0873 BF4306

BFS: BF0796(dxs) BF4114

PGI: PG2217(dxs)

CHU: CHU_3643(dxs)

GFO: GFO_3470(dxs)

FPS: FP0279(dxs)

CTE: CT0337(dxs)

CPH: Cpha266_0671

PVI: Cvib_0498

PLT: Plut_0450

DET: DET0745(dxs)

DEH: cbdb_A720(dxs)

DRA: DR_1475

DGE: Dgeo_0994

TTH: TTC1614

TTJ: TTHA0006

AAE: aq_881

TMA: TM1770

PMO: Pmob_1001

Exemplary acetyl-CoA-acetyltransferase nucleic acids and polypeptides

HSA: 38(ACAT1) 39(ACAT2)

PTR: 451528(ACAT1)

MCC: 707653(ACAT1) 708750(ACAT2)

MMU: 110446(Acat1) 110460(Acat2)

RNO: 25014(Acat1)

CFA: 484063(ACAT2) 489421(ACAT1)

GGA: 418968(ACAT1) 421587(RCJMB04_34i5)

XLA: 379569(MGC69098) 414622(MGC81403) 414639(MGC81256)
444457(MGC83664)

XTR: 394562(acat2)

DRE: 30643(acat2)

SPU: 759502(LOC759502)

DME: Dmel_CG10932 Dmel_CG9149

CEL: T02G5.4 T02G5.7 T02G5.8(kat-1)

ATH: AT5G48230(ACAT2/EMB1276)

OSA: 4326136 4346520

CME: CMA042C CME087C

SCE: YPL028W(ERG10)

AGO: AGOS_ADR165C

PIC: PICST_31707(ERG10)

CAL: CaO19.1591(erg10)

CGR: CAGL0L12364g

SPO: SPBC215.09c

MGR: MGG_01755 MGG_13499

ANI: AN1409.2

AFM: AFUA_6G14200 AFUA_8G04000

AOR: AO090103000012 AO090103000406

CNE: CNC05280

UMA: UM03571.1

DDI: DDB_0231621

PFA: PF14_0484

TET: TTHERM_00091590 TTHERM_00277470 TTHERM_00926980

TCR: 511003.60

ECO: b2224(atoB)

ECJ: JW2218(atoB) JW5453(yqeF)

ECE: Z4164(yqeF)

ECS: ECs3701

ECC: c2767(atoB) c3441(yqeF)

ECI: UTI89_C2506(atoB) UTI89_C3247(yqeF)

ECP: ECP_2268 ECP_2857

ECV: APECO1_3662(yqeF) APECO1_4335(atoB) APECO1_43352(atoB)

ECX: EcHS_A2365

STY: STY3164(yqeF)

STT: t2929(yqeF)

SPT: SPA2886(yqeF)

SEC: SC2958(yqeF)

STM: STM3019(yqeF)

SFL: SF2854(yqeF)

SFX: S3052(yqeF)

SFV: SFV_2922(yqeF)

SSN: SSON_2283(atoB) SSON_3004(yqeF)

SBO: SBO_2736(yqeF)

ECA: ECA1282(atoB)

ENT: Ent638_3299

SPE: Spro_0592

HIT: NTHI0932(atoB)

XCC: XCC1297(atoB)

XCB: XC_2943

XCV: XCV1401(thlA)

XAC: XAC1348(atoB)

XOO: XOO1881(atoB)

XOM: XOO_1778(XOO1778)

VCH: VCA0690

VCO: VC0395_0630

VVU: VV2_0494 VV2_0741

VVY: VVA1043 VVA1210

VPA: VPA0620 VPA1123 VPA1204

PPR: PBPRB1112 PBPRB1840

PAE: PA2001(atoB) PA2553 PA3454 PA3589 PA3925

PAU: PA14_38630(atoB)

PPU: PP_2051(atoB) PP_2215(fadAx) PP_3754 PP_4636

PPF: Pput_2009 Pput_2403 Pput_3523 Pput_4498

PST: PSPTO_0957(phbA-1) PSPTO_3164(phbA-2)

PSB: Psyr_0824 Psyr_3031

PSP: PSPPH_0850(phbA1) PSPPH_2209(phbA2)

PFL: PFL_1478(atoB-2) PFL_2321 PFL_3066 PFL_4330(atoB-2) PFL_5283

PFO: Pfl_1269 Pfl_1739 Pfl_2074 Pfl_2868

PEN: PSEEN3197 PSEEN3547(fadAx) PSEEN4635(phbA)

PMY: Pmen_1138 Pmen_2036 Pmen_3597 Pmen_3662 Pmen_3820

PAR: Psyc_0252 Psyc_1169

PCR: Pcryo_0278 Pcryo_1236 Pcryo_1260

PRW: PsycPRwf_2011

ACI: ACIAD0694 ACIAD1612 ACIAD2516(atoB)

SON: SO_1677(atoB)

SDN: Sden_1943

SFR: Sfri_1338 Sfri_2063

SAZ: Sama_1375

SBL: Sbal_1495

SBM: Shew185_1489

SBN: Sbal195_1525

SLO: Shew_1667 Shew_2858

SPC: Sputcn32_1397

SSE: Ssed_1473 Ssed_3533

SPL: Spea_2783

SHE: Shewmr4_2597

SHM: Shewmr7_2664

SHN: Shewana3_2771

SHW: Sputw3181_2704

ILO: IL0872

CPS: CPS_1605 CPS_2626

PHA: PSHAa0908 PSHAa1454(atoB) PSHAa1586(atoB)

PAT: Patl_2923

SDE: Sde_3149

PIN: Ping_0659 Ping_2401

MAQ: Maqu_2117 Maqu_2489 Maqu_2696 Maqu_3162

CBU: CBU_0974

LPN: lpg1825(atoB)

LPF: lpl1789

LPP: lpp1788

NOC: Noc_1891

AEH: Mlg_0688 Mlg_2706

HHA: Hhal_1685

HCH: HCH_05299

CSA: Csal_0301 Csal_3068

ABO: ABO_0648(fadAx)

MMW: Mmwyl1_0073 Mmwyl1_3021 Mmwyl1_3053 Mmwyl1_3097 Mmwyl1_4182

AHA: AHA_2143(atoB)

CVI: CV_2088(atoB) CV_2790(phaA)

RSO: RSc0276(atoB) RSc1632(phbA) RSc1637(bktB) RSc1761(RS02948)

REU: Reut_A0138 Reut_A1348 Reut_A1353 Reut_B4561 Reut_B4738
Reut_B5587 Reut_C5943 Reut_C6062

REH: H16_A0170 H16_A0867 H16_A0868 H16_A0872 H16_A1297
H16_A1438(phaA) H16_A1445(bktB) H16_A1528 H16_A1713 H16_A1720
H16_A1887 H16_A2148 H16_B0380 H16_B0381 H16_B0406 H16_B0662
H16_B0668 H16_B0759 H16_B1369 H16_B1771

RME: Rmet_0106 Rmet_1357 Rmet_1362 Rmet_5156

BMA: BMA1316 BMA1321(phbA) BMA1436

BMV: BMASAVP1_A1805(bktB) BMASAVP1_A1810(phbA)

BML: BMA10299_A0086(phbA) BMA10299_A0091

BMN: BMA10247_1076(bktB) BMA10247_1081(phbA)

BXE: Bxe_A2273 Bxe_A2335 Bxe_A2342 Bxe_A4255 Bxe_B0377 Bxe_B0739 Bxe_C0332 Bxe_C0574 Bxe_C0915

BVI: Bcep1808_0519 Bcep1808_1717 Bcep1808_2877 Bcep1808_3594 Bcep1808_4015 Bcep1808_5507 Bcep1808_5644

BUR: Bcep18194_A3629 Bcep18194_A5080 Bcep18194_A5091 Bcep18194_A6102 Bcep18194_B0263 Bcep18194_B1439 Bcep18194_C6652 Bcep18194_C6802 Bcep18194_C6874 Bcep18194_C7118 Bcep18194_C7151 Bcep18194_C7332

BCN: Bcen_1553 Bcen_1599 Bcen_2158 Bcen_2563 Bcen_2998 Bcen_6289

BCH: Bcen2424_0542 Bcen2424_1790 Bcen2424_2772 Bcen2424_5368 Bcen2424_6232 Bcen2424_6276

BAM: Bamb_0447 Bamb_1728 Bamb_2824 Bamb_4717 Bamb_5771 Bamb_5969

BPS: BPSL1426 BPSL1535(phbA) BPSL1540

BPM: BURPS1710b_2325(bktB) BURPS1710b_2330(phbA) BURPS1710b_2453(atoB-2)

BPL: BURPS1106A_2197(bktB) BURPS1106A_2202(phbA)

BPD: BURPS668_2160(bktB) BURPS668_2165(phbA)

BTE: BTH_I2144 BTH_I2256 BTH_I2261

PNU: Pnuc_0927

BPE: BP0447 BP0668 BP2059

BPA: BPP0608 BPP1744 BPP3805 BPP4216 BPP4361

BBR: BB0614 BB3364 BB4250 BB4804 BB4947

RFR: Rfer_0272 Rfer_1000 Rfer_1871 Rfer_2273 Rfer_2561 Rfer_2594 Rfer_3839

POL: Bpro_1577 Bpro_2140 Bpro_3113 Bpro_4187

PNA: Pnap_0060 Pnap_0458 Pnap_0867 Pnap_1159 Pnap_2136 Pnap_2804

AAV: Aave_0031 Aave_2478 Aave_3944 Aave_4368

AJS: Ajs_0014 Ajs_0124 Ajs_1931 Ajs_2073 Ajs_2317 Ajs_3548 Ajs_3738 Ajs_3776

VEI: Veis_1331 Veis_3818 Veis_4193

DAC: Daci_0025 Daci_0192 Daci_3601 Daci_5988

MPT: Mpe_A1536 Mpe_A1776 Mpe_A1869 Mpe_A3367

HAR: HEAR0577(phbA)

MMS: mma_0555

NEU: NE2262(bktB)

NET: Neut_0610

EBA: ebA5202 p2A409(tioL)

AZO: azo0464(fadA1) azo0469(fadA2) azo2172(thlA)

DAR: Daro_0098 Daro_3022

HPA: HPAG1_0675

HAC: Hac_0958(atoB)

GME: Gmet_1719 Gmet_2074 Gmet_2213 Gmet_2268 Gmet_3302

GUR: Gura_3043

BBA: Bd0404(atoB) Bd2095

DOL: Dole_0671 Dole_1778 Dole_2160 Dole_2187

ADE: Adeh_0062 Adeh_2365

AFW: Anae109_0064 Anae109_1504

MXA: MXAN_3791

SAT: SYN_02642

SFU: Sfum_2280 Sfum_3582

RPR: RP737

RCO: RC1134 RC1135

RFE: RF_0163(paaJ)

RBE: RBE_0139(paaJ)

RAK: A1C_05820

RBO: A1I_07215

RCM: A1E_04760

PUB: SAR11_0428(thlA)

MLO: mlr3847

MES: Meso_3374

PLA: Plav_1573 Plav_2783

SME: SMa1450 SMc03879(phbA)

SMD: Smed_0499 Smed_3117 Smed_5094 Smed_5096

ATU: Atu2769(atoB) Atu3475

ATC: AGR_C_5022(phbA) AGR_L_2713

RET: RHE_CH04018(phbAch) RHE_PC00068(ypc00040) RHE_PF00014(phbAf)

RLE: RL4621(phaA) pRL100301 pRL120369

BME: BMEI0274 BMEII0817

BMF: BAB1_1783(phbA-1) BAB2_0790(phbA-2)

BMS: BR1772(phbA-1) BRA0448(phbA-2)

BMB: BruAb1_1756(phbA-1) BruAb2_0774(phbA-2)

BOV: BOV_1707(phbA-1)

OAN: Oant_1130 Oant_3107 Oant_3718 Oant_4020

BJA: bll0226(atoB) bll3949 bll7400 bll7819 blr3724(phbA)

BRA: BRADO0562(phbA) BRADO0983(pimB) BRADO3110 BRADO3134(atoB)

BBT: BBta_3558 BBta_3575(atoB) BBta_5147(pimB) BBta_7072(pimB)
BBta_7614(phbA)

RPA: RPA0513(pcaF) RPA0531 RPA3715(pimB)

RPB: RPB_0509 RPB_0525 RPB_1748

RPC: RPC_0504 RPC_0636 RPC_0641 RPC_0832 RPC_1050 RPC_2005
RPC_2194 RPC_2228

RPD: RPD_0306 RPD_0320 RPD_3105 RPD_3306

RPE: RPE_0168 RPE_0248 RPE_3827

NWI: Nwi_3060

XAU: Xaut_3108 Xaut_4665

CCR: CC_0510 CC_0894 CC_3462

SIL: SPO0142(bktB) SPO0326(phbA) SPO0773 SPO3408

SIT: TM1040_0067 TM1040_2790 TM1040_3026 TM1040_3735

RSP: RSP_0745 RSP_1354 RSP_3184

RSH: Rsph17029_0022 Rsph17029_2401 Rsph17029_3179 Rsph17029_3921

RSQ: Rsph17025_0012 Rsph17025_2466 Rsph17025_2833

JAN: Jann_0262 Jann_0493 Jann_4050

RDE: RD1_0025 RD1_0201(bktB) RD1_3394(phbA)

PDE: Pden_2026 Pden_2663 Pden_2870 Pden_2907 Pden_4811 Pden_5022

DSH: Dshi_0074 Dshi_3066 Dshi_3331

MMR: Mmar10_0697

HNE: HNE_2706 HNE_3065 HNE_3133

NAR: Saro_0809 Saro_1069 Saro_1222 Saro_2306 Saro_2349

SAL: Sala_0781 Sala_1244 Sala_2896 Sala_3158

SWI: Swit_0632 Swit_0752 Swit_2893 Swit_3602 Swit_4887 Swit_5019 Swit_5309

ELI: ELI_01475 ELI_06705 ELI_12035

GBE: GbCGDNIH1_0447

ACR: Acry_1847 Acry_2256

RRU: Rru_A0274 Rru_A1380 Rru_A1469 Rru_A1946 Rru_A3387

MAG: amb0842

MGM: Mmc1_1165

ABA: Acid345_3239

BSU: BG11319(mmgA) BG13063(yhfS)

BHA: BH1997 BH2029 BH3801(mmgA)

BAN: BA3687 BA4240 BA5589

BAR: GBAA3687 GBAA4240 GBAA5589

BAA: BA_0445 BA_4172 BA_4700

BAT: BAS3418 BAS3932 BAS5193

BCE: BC3627 BC4023 BC5344

BCA: BCE_3646 BCE_4076 BCE_5475

BCZ: BCZK3329(mmgA) BCZK3780(thl) BCZK5044(atoB)

BCY: Bcer98_2722 Bcer98_3865

BTK: BT9727_3379(mmgA) BT9727_3765(thl) BT9727_5028(atoB)

BTL: BALH_3262(mmgA) BALH_3642(fadA) BALH_4843(atoB)

BLI: BL03925(mmgA)

BLD: BLi03968(mmgA)

BCL: ABC0345 ABC2989 ABC3617 ABC3891(mmgA)

BAY: RBAM_022450

BPU: BPUM_2374(yhfS) BPUM_2941 BPUM_3373

OIH: OB0676 OB0689 OB2632 OB3013

GKA: GK1658 GK3397

SAU: SA0342 SA0534(vraB)

SAV: SAV0354 SAV0576(vraB)

SAM: MW0330 MW0531(vraB)

SAR: SAR0351(thl) SAR0581

SAS: SAS0330 SAS0534

SAC: SACOL0426 SACOL0622(atoB)

SAB: SAB0304(th1) SAB0526

SAA: SAUSA300_0355 SAUSA300_0560(vraB)

SAO: SAOUHSC_00336 SAOUHSC_00558

SAJ: SaurJH9_0402

SAH: SaurJH1_0412

SEP: SE0346 SE2384

SER: SERP0032 SERP0220

SHA: SH0510(mvaC) SH2417

SSP: SSP0325 SSP2145

LMO: lmo1414

LMF: LMOf2365_1433

LIN: lin1453

LWE: lwe1431

LLA: L11745(thiL) L25946(fadA)

LLC: LACR_1665 LACR_1956

LLM: llmg_0930(thiL)

SPY: SPy_0140 SPy_1637(atoB)

SPZ: M5005_Spy_0119 M5005_Spy_0432 M5005_Spy_1344(atoB)

SPM: spyM18_0136 spyM18_1645(atoB)

SPG: SpyM3_0108 SpyM3_1378(atoB)

SPS: SPs0110 SPs0484

SPH: MGAS10270_Spy0121 MGAS10270_Spy0433 MGAS10270_Spy1461(atoB)

SPI: MGAS10750_Spy0124 MGAS10750_Spy0452 MGAS10750_Spy1453(atoB)

SPJ: MGAS2096_Spy0123 MGAS2096_Spy0451 MGAS2096_Spy1365(atoB)

SPK: MGAS9429_Spy0121 MGAS9429_Spy0431 MGAS9429_Spy1339(atoB)

SPF: SpyM50447(atoB2)

SPA: M6_Spy0166 M6_Spy0466 M6_Spy1390

SPB: M28_Spy0117 M28_Spy0420 M28_Spy1385(atoB)

SAK: SAK_0568

LJO: LJ1609

LAC: LBA0626(thiL)

LSA: LSA1486

LDB: Ldb0879

LBU: LBUL_0804

LBR: LVIS_2218

LCA: LSEI_1787

LGA: LGAS_1374

LRE: Lreu_0052

EFA: EF1364

OOE: OEOE_0529

STH: STH2913 STH725 STH804

CAC: CAC2873 CA_P0078(thiL)

CPE: CPE2195(atoB)

CPF: CPF_2460

CPR: CPR_2170

CTC: CTC00312

CNO: NT01CX_0538 NT01CX_0603

CDF: CD1059(thlA1) CD2676(thlA2)

CBO: CBO3200(thl)

CBE: Cbei_0411 Cbei_3630

CKL: CKL_3696(thlA1) CKL_3697(thlA2) CKL_3698(thlA3)

AMT: Amet_4630

AOE: Clos_0084 Clos_0258

CHY: CHY_1288 CHY_1355(atoB) CHY_1604 CHY_1738

DSY: DSY0632 DSY0639 DSY1567 DSY1710 DSY2402 DSY3302

DRM: Dred_0400 Dred_1491 Dred_1784 Dred_1892

SWO: Swol_0308 Swol_0675 Swol_0789 Swol_1486 Swol_1934 Swol_2051

TTE: TTE0549(paaJ)

MTA: Moth_1260

MTU: Rv1135A Rv1323(fadA4) Rv3546(fadA5)

MTC: MT1365(phbA)

MBO: Mb1167 Mb1358(fadA4) Mb3576(fadA5) Mb3586c(fadA6)

MBB: BCG_1197 BCG_1385(fadA4) BCG_3610(fadA5) BCG_3620c(fadA6)

MLE: ML1158(fadA4)

MPA: MAP2407c(fadA3) MAP2436c(fadA4)

MAV: MAV_1544 MAV_1573 MAV_1863 MAV_5081

MSM: MSMEG_2224 MSMEG_4920

MUL: MUL_0357

MVA: Mvan_1976 Mvan_1988 Mvan_4305 Mvan_4677 Mvan_4891

MGI: Mflv_1347 Mflv_1484 Mflv_2040 Mflv_2340 Mflv_4356 Mflv_4368

MMC: Mmcs_1758 Mmcs_1769 Mmcs_3796 Mmcs_3864

MKM: Mkms_0251 Mkms_1540 Mkms_1805 Mkms_1816 Mkms_2836 Mkms_3159 Mkms_3286 Mkms_3869 Mkms_3938 Mkms_4227 Mkms_4411 Mkms_4580 Mkms_4724 Mkms_4764 Mkms_4776

MJL: Mjls_0231 Mjls_1739 Mjls_1750 Mjls_2819 Mjls_3119 Mjls_3235 Mjls_3800 Mjls_3850 Mjls_4110 Mjls_4383 Mjls_4705 Mjls_4876 Mjls_5018 Mjls_5063 Mjls_5075

CGL: NCgl2309(cgl2392)

CGB: cg2625(pcaF)

CEF: CE0731 CE2295

CJK: jk1543(fadA3)

NFA: nfa10750(fadA4)

RHA: RHA1_ro01455 RHA1_ro01623 RHA1_ro01876 RHA1_ro02517(catF) RHA1_ro03022 RHA1_ro03024 RHA1_ro03391 RHA1_ro03892 RHA1_ro04599 RHA1_ro05257 RHA1_ro08871

SCO: SCO5399(SC8F4.03)

SMA: SAV1384(fadA5) SAV2856(fadA1)

ART: Arth_1160 Arth_2986 Arth_3268 Arth_4073

NCA: Noca_1371 Noca_1797 Noca_1828 Noca_2764 Noca_4142

TFU: Tfu_1520 Tfu_2394

FRA: Francci3_3687

FRE: Franean1_1044 Franean1_2711 Franean1_2726 Franean1_3929 Franean1_4037 Franean1_4577

FAL: FRAAL2514 FRAAL2618 FRAAL5910(atoB)

ACE: Acel_0626 Acel_0672

SEN: SACE_1192(mmgA) SACE_2736(fadA6) SACE_4011(catF) SACE_6236(fadA4)

STP: Strop_3610

SAQ: Sare_1316 Sare_3991

RXY: Rxyl_1582 Rxyl_1842 Rxyl_2389 Rxyl_2530

FNU: FN0495

BGA: BG0110(fadA)

BAF: BAPKO_0110(fadA)

LIL: LA0457(thiL1) LA0828(thiL2) LA4139(fadA)

LIC: LIC10396(phbA)

LBJ: LBJ_2862(paaJ-4)

LBL: LBL_0209(paaJ-4)

SYN: slr1993(phaA)

SRU: SRU_1211(atoB) SRU_1547

CHU: CHU_1910(atoB)

GFO: GFO_1507(atoB)

FJO: Fjoh_4612

FPS: FP0770 FP1586 FP1725

RRS: RoseRS_3911 RoseRS_4348

RCA: Rcas_0702 Rcas_3206

HAU: Haur_0522

DRA: DR_1072 DR_1428 DR_1960 DR_2480 DR_A0053

DGE: Dgeo_0755 Dgeo_1305 Dgeo_1441 Dgeo_1883

TTH: TTC0191 TTC0330

TTJ: TTHA0559

TME: Tmel_1134

FNO: Fnod_0314

PMO: Pmob_0515

HMA: rrnAC0896(acaB3) rrnAC2815(aca2) rrnAC3497(yqeF)
rrnB0240(aca1) rrnB0242(acaB2) rrnB0309(acaB1)

TAC: Ta0582

TVO: TVN0649

PTO: PTO1505

APE: APE_2108

SSO: SSO2377(acaB-4)

STO: ST0514

SAI: Saci_0963 Saci_1361(acaB1)

MSE: Msed_0656

PAI: PAE1220

PIS: Pisl_0029 Pisl_1301

PCL: Pcal_0781

PAS: Pars_0309 Pars_1071

CMA: Cmaq_1941

Exemplary HMG-CoA synthase nucleic acids and polypeptides

HSA: 3157(HMGCS1) 3158(HMGCS2)

PTR: 457169(HMGCS2) 461892(HMGCS1)

MCC: 702553(HMGCS1) 713541(HMGCS2)

MMU: 15360(Hmgcs2) 208715(Hmgcs1)

RNO: 24450(Hmgcs2) 29637(Hmgcs1)

CFA: 479344(HMGCS1) 607923(HMGCS2)

BTA: 407767(HMGCS1)

SSC: 397673(CH242-38B5.1)

GGA: 396379(HMGCS1)

XLA: 380091(hmgcs1) 447204(MGC80816)

DRE: 394060(hmgcs1)

SPU: 578259(LOC578259)

DME: Dmel_CG4311(Hmgs)

CEL: F25B4.6

ATH: AT4G11820(BAP1)

OSA: 4331418 4347614

CME: CMM189C

SCE: YML126C(ERG13)

AGO: AGOS_ADL356C

PIC: PICST_83020

CAL: CaO19_7312(CaO19.7312)

CGR: CAGL0H04081g

SPO: SPAC4F8.14c(hcs)

MGR: MGG_01026

ANI: AN4923.2

AFM: AFUA_3G10660 AFUA_8G07210

AOR: AO090003000611 AO090010000487

CNE: CNC05080 CNG02670

UMA: UM05362.1

ECU: ECU10_0510

DDI: DDBDRAFT_0217522 DDB_0219924(hgsA)

TET: TTHERM_00691190

TBR: Tb927.8.6110

YPE: YPO1457

YPK: y2712(pksG)

YPM: YP_1349(pksG)

YPA: YPA_0750

YPN: YPN_2521

YPP: YPDSF_1517

YPS: YPTB1475

CBD: COXBU7E912_1931

TCX: Tcr_1719

DNO: DNO_0799

BMA: BMAA1212

BPS: BPSS1002

BPM: BURPS1710b_A2613

BPL: BURPS1106A_A1384

BPD: BURPS668_A1470

BTE: BTH_II1670

MXA: MXAN_3948(tac) MXAN_4267(mvaS)

BSU: BG10926(pksG)

OIH: OB2248

SAU: SA2334(mvaS)

SAV: SAV2546(mvaS)

SAM: MW2467(mvaS)

SAR: SAR2626(mvaS)

SAS: SAS2432

SAC: SACOL2561

SAB: SAB2420(mvaS)

SAA: SAUSA300_2484

SAO: SAOUHSC_02860

SAJ: SaurJH9_2569

SAH: SaurJH1_2622

SEP: SE2110

SER: SERP2122

SHA: SH0508(mvaS)

SSP: SSP0324

LMO: lmo1415

LMF: LMOf2365_1434(mvaS)

LIN: lin1454

LWE: lwe1432(mvaS)

LLA: L13187(hmcM)

LLC: LACR_1666

LLM: llmg_0929(hmcM)

SPY: SPy_0881(mvaS.2)

SPZ: M5005_Spy_0687(mvaS.1)

SPM: spyM18_0942(mvaS2)

SPG: SpyM3_0600(mvaS.2)

SPS: SPs1253

SPH: MGAS10270_Spy0745(mvaS1)

SPI: MGAS10750_Spy0779(mvaS1)

SPJ: MGAS2096_Spy0759(mvaS1)

SPK: MGAS9429_Spy0743(mvaS1)

SPF: SpyM51121(mvaS)

SPA: M6_Spy0704

SPB: M28_Spy0667(mvaS.1)

SPN: SP_1727

SPR: spr1571(mvaS)

SPD: SPD_1537(mvaS)

SAG: SAG1316

SAN: gbs1386

SAK: SAK_1347

SMU: SMU.943c

STC: str0577(mvaS)

STL: stu0577(mvaS)

STE: STER_0621

SSA: SSA_0338(mvaS)

SSU: SSU05_1641

SSV: SSU98_1652

SGO: SGO_0244

LPL: lp_2067(mvaS)

LJO: LJ1607

LAC: LBA0628(hmcS)

LSA: LSA1484(mvaS)

LSL: LSL_0526

LDB: Ldb0881(mvaS)

LBU: LBUL_0806

LBR: LVIS_1363

LCA: LSEI_1785

LGA: LGAS_1372

LRE: Lreu_0676

PPE: PEPE_0868

EFA: EF1363

OOE: OEOE_0968

LME: LEUM_1184

NFA: nfa22120

SEN: SACE_4570(pksG)

BBU: BB0683

BGA: BG0706

BAF: BAPKO_0727

FJO: Fjoh_0678

HAL: VNG1615G(mvaB)

HMA: rrnAC1740(mvaS)

HWA: HQ2868A(mvaB)

NPH: NP2608A(mvaB_1) NP4836A(mvaB_2)

Exemplary hydroxymethylglutaryl-CoA reductase nucleic acids and polypeptides

HSA: 3156(HMGCR)

PTR: 471516(HMGCR)

MCC: 705479(HMGCR)

MMU: 15357(Hmgcr)

RNO: 25675(Hmgcr)

CFA: 479182(HMGCR)

BTA: 407159(HMGCR)

GGA: 395145(RCJMB04_14m24)

SPU: 373355(LOC373355)

DME: Dmel_CG10367(Hmgcr)

CEL: F08F8.2

OSA: 4347443

SCE: YLR450W(HMG2) YML075C(HMG1)

AGO: AGOS_AER152W

CGR: CAGL0L11506g

SPO: SPCC162.09c(hmg1)

ANI: AN3817.2

AFM: AFUA_1G11230 AFUA_2G03700

AOR: AO090103000311 AO090120000217

CNE: CNF04830

UMA: UM03014.1

ECU: ECU10_1720

DDI: DDB_0191125(hmgA) DDB_0215357(hmgB)

TBR: Tb927.6.4540

TCR: 506831.40 509167.20

LMA: LmjF30.3190

VCH: VCA0723

VCO: VC0395_0662

VVU: VV2_0117

VVY: VVA0625

VPA: VPA0968

VFI: VFA0841

PAT: Patl_0427

CBU: CBU_0030 CBU_0610

CBD: COXBU7E912_0151 COXBU7E912_0622(hmgA)

TCX: Tcr_1717

DNO: DNO_0797

CVI: CV_1806

SUS: Acid_5728 Acid_6132

SAU: SA2333(mvaA)

SAV: SAV2545(mvaA)

SAM: MW2466(mvaA)

SAB: SAB2419c(mvaA)

SEP: SE2109

LWE: lwe0819(mvaA)

LLA: L10433(mvaA)

LLC: LACR_1664

LLM: llmg_0931(mvaA)

SPY: SPy_0880(mvaS.1)

SPM: spyM18_0941(mvaS1)

SPG: SpyM3_0599(mvaS.1)

SPS: SPs1254

SPH: MGAS10270_Spy0744

SPI: MGAS10750_Spy0778

SPJ: MGAS2096_Spy0758

SPK: MGAS9429_Spy0742

SPA: M6_Spy0703

SPN: SP_1726

SAG: SAG1317

SAN: gbs1387

STC: str0576(mvaA)

STL: stu0576(mvaA)

STE: STER_0620

SSA: SSA_0337(mvaA)

LPL: lp_0447(mvaA)

LJO: LJ1608

LSL: LSL_0224

LBR: LVIS_0450

LGA: LGAS_1373

EFA: EF1364

NFA: nfa22110

BGA: BG0708(mvaA)

SRU: SRU_2422

FPS: FP2341

MMP: MMP0087(hmgA)

MMQ: MmarC5_1589

MAC: MA3073(hmgA)

MBA: Mbar_A1972

MMA: MM_0335

MBU: Mbur_1098

MHU: Mhun_3004

MEM: Memar_2365

MBN: Mboo_0137

MTH: MTH562

MST: Msp_0584(hmgA)

MSI: Msm_0227

MKA: MK0355(HMG1)

AFU: AF1736(mvaA)

HAL: VNG1875G(mvaA)

HMA: rrnAC3412(mvaA)

HWA: HQ3215A(hmgR)

NPH: NP0368A(mvaA_2) NP2422A(mvaA_1)

TAC: Ta0406m

TVO: TVN1168

PTO: PTO1143

PAB: PAB2106(mvaA)

PFU: PF1848

TKO: TK0914

RCI: RCIX1027(hmgA) RCIX376(hmgA)

APE: APE_1869

IHO: Igni_0476

HBU: Hbut_1531

SSO: SSO0531

STO: ST1352

SAI: Saci_1359

PAI: PAE2182

PIS: Pisl_0814

PCL: Pcal_1085

PAS: Pars_0796

Exemplary mevalonate kinase nucleic acids and polypeptides

HSA: 4598(MVK)

MCC: 707645(MVK)

MMU: 17855(Mvk)

RNO: 81727(Mvk)

CFA: 486309(MVK)

BTA: 505792(MVK)

GGA: 768555(MVK)

DRE: 492477(zgc:103473)

SPU: 585785(LOC585785)

DME: Dmel_CG33671

OSA: 4348331

SCE: YMR208W(ERG12)

AGO: AGOS_AER335W

PIC: PICST_40742(ERG12)

CGR: CAGL0F03861g

SPO: SPAC13G6.11c

MGR: MGG_06946

ANI: AN3869.2

AFM: AFUA_4G07780

AOR: AO090023000793

CNE: CNK01740

ECU: ECU09_1780

DDI: DDBDRAFT_0168621

TET: TTHERM_00637680

TBR: Tb927.4.4070

TCR: 436521.9 509237.10

LMA: LmjF31.0560

CBU: CBU_0608 CBU_0609

CBD: COXBU7E912_0620(mvk)

LPN: lpg2039

LPF: lpl2017

LPP: lpp2022

BBA: Bd1027(lmbP) Bd1630(mvk)

MXA: MXAN_5019(mvk)

OIH: OB0225

SAU: SA0547(mvaK1)

SAV: SAV0590(mvaK1)

SAM: MW0545(mvaK1)

SAR: SAR0596(mvaK1)

SAS: SAS0549

SAC: SACOL0636(mvk)

SAB: SAB0540(mvaK1)

SAA: SAUSA300_0572(mvk)

SAO: SAOUHSC_00577

SEP: SE0361

SER: SERP0238(mvk)

SHA: SH2402(mvaK1)

SSP: SSP2122

LMO: lmo0010

LMF: LMOf2365_0011

LIN: lin0010

LWE: lwe0011(mvk)

LLA: L7866(yeaG)

LLC: LACR_0454

LLM: llmg_0425(mvk)

SPY: SPy_0876(mvaK1)

SPZ: M5005_Spy_0682(mvaK1)

SPM: spyM18_0937(mvaK1)

SPG: SpyM3_0595(mvaK1)

SPS: SPs1258

SPH: MGAS10270_Spy0740(mvaK1)

SPI: MGAS10750_Spy0774(mvaK1)

SPJ: MGAS2096_Spy0753(mvaK1)

SPK: MGAS9429_Spy0737(mvaK1)

SPF: SpyM51126(mvaK1)

SPA: M6_Spy0699

SPB: M28_Spy0662(mvaK1)

SPN: SP_0381

SPR: spr0338(mvk)

SPD: SPD_0346(mvk)

SAG: SAG1326

SAN: gbs1396

SAK: SAK_1357(mvk)

SMU: SMU.181

STC: str0559(mvaK1)

STL: stu0559(mvaK1)

STE: STER_0598

SSA: SSA_0333(mvaK1)

SSU: SSU05_0289

SSV: SSU98_0285

SGO: SGO_0239(mvk)

LPL: lp_1735(mvaK1)

LJO: LJ1205

LAC: LBA1167(mvaK)

LSA: LSA0908(mvaK1)

LSL: LSL_0685(eRG)

LDB: Ldb0999(mvk)

LBU: LBUL_0906

LBR: LVIS_0858

LCA: LSEI_1491

LGA: LGAS_1033

LRE: Lreu_0915

PPE: PEPE_0927

EFA: EF0904(mvk)

OOE: OEOE_1100

LME: LEUM_1385

NFA: nfa22070

BGA: BG0711

BAF: BAPKO_0732

FPS: FP0313

MMP: MMP1335

MAE: Maeo_0775

MAC: MA0602(mvk)

MBA: Mbar_A1421

MMA: MM_1762

MBU: Mbur_2395

MHU: Mhun_2890

MEM: Memar_1812

MBN: Mboo_2213

MST: Msp_0858(mvk)

MSI: Msm_1439

MKA: MK0993(ERG12)

HAL: VNG1145G(mvk)

HMA: rrnAC0077(mvk)

HWA: HQ2925A(mvk)

NPH: NP2850A(mvk)

PTO: PTO1352

PHO: PH1625

PAB: PAB0372(mvk)

PFU: PF1637(mvk)

TKO: TK1474

RCI: LRC399(mvk)

APE: APE_2439

HBU: Hbut_0877

SSO: SSO0383

STO: ST2185

SAI: Saci_2365(mvk)

MSE: Msed_1602

PAI: PAE3108

PIS: Pisl_0467

PCL: Pcal_1835

Exemplary mevalonate kinase nucleic acids and polypeptides homologus to Methanosarcina mazei mevalonate kinase

NP_633786.1 mevalonate kinase Methanosarcina mazei Go1

YP_304960.1 mevalonate kinase Methanosarcina barkeri str. Fusaro

NP_615566.1 mevalonate kinase Methanosarcina acetivorans C2A

YP_566996.1 mevalonate kinase Methanococcoides burtonii DSM 6242

YP_684687.1 mevalonate kinase uncultured methanogenic archaeon RC-I

YP_183887.1 mevalonate kinase Thermococcus kodakarensis KOD1

NP_126232.1 mevalonate kinase Pyrococcus abyssi GE5

NP_143478.1 mevalonate kinase Pyrococcus horikoshii OT3

NP_579366.1 mevalonate kinase Pyrococcus furiosus DSM 3638

YP_842907.1 mevalonate kinase Methanosaeta thermophila PT

YP_327075.1 mevalonate kinase Natronomonas pharaonis DSM 2160

YP_658630.1 mevalonate kinase Haloquadratum walsbyi DSM 16790

YP_134862.1 mevalonate kinase Haloarcula marismortui ATCC 43049

YP_001405370.1 mevalonate kinase Candidatus Methanoregula boonei 6A8

YP_001030120.1 mevalonate kinase Methanocorpusculum labreanum Z

YP_447890.1 putative mevalonate kinase Methanosphaera stadtmanae DSM 3091

YP_920295.1 mevalonate kinase Thermofilum pendens Hrk 5

ZP_02015315.1 mevalonate kinase Halorubrum lacusprofundi ATCC 49239

NP_280049.1 mevalonate kinase Halobacterium sp. NRC-1

YP_001274012.1 mevalonate kinase Methanobrevibacter smithii ATCC 35061

YP_001435347.1 mevalonate kinase Ignicoccus hospitalis KIN4/I

YP_001540788.1 mevalonate kinase Caldivirga maquilingensis IC-167

Q50559 KIME_METTH mevalonate kinase (MK)

NP_275189.1 mevalonate kinase Methanothermobacter thermautotrophicus str.

NP_071114.1 mevalonate kinase (mvk) Archaeoglobus fulgidus DSM 4304

YP_504301.1 mevalonate kinase Methanospirillum hungatei JF-1

YP_001040239.1 mevalonate kinase Staphylothermus marinus F1

YP_001047720.1 mevalonate kinase Methanoculleus marisnigri JR1

NP_614276.1 mevalonate kinase Methanopyrus kandleri AV19

YP_001737496.1 mevalonate kinase Candidatus Korarchaeum cryptofilum OPF8

YP_256937.1 mevalonate kinase Sulfolobus acidocaldarius DSM 639

NP_341921.1 mevalonate kinase Sulfolobus solfataricus P2

YP_001276466.1 mevalonate kinase Roseiflexus sp. RS-1

YP_001581649.1 mevalonate kinase Nitrosopumilus maritimus SCM1

NP_378182.1 hypothetical protein ST2185 Sulfolobus tokodaii str. 7

YP_001547075.1 mevalonate kinase Herpetosiphon aurantiacus ATCC 23779

YP_001056718.1 mevalonate kinase Pyrobaculum calidifontis JCM 11548

YP_001431846.1 mevalonate kinase Roseiflexus castenholzii DSM 13941

YP_001153805.1 mevalonate kinase Pyrobaculum arsenaticum DSM 13514

AAG02440.1AF290093_1 mevalonate kinase Enterococcus faecalis

NP_814642.1 mevalonate kinase Enterococcus faecalis V583

YP_001634502.1 mevalonate kinase Chloroflexus aurantiacus J-10-fl

XP_790690.1 similar to Mevalonate kinase (MK) Strongylocentrotus purpuratus

NP_560495.1 mevalonate kinase Pyrobaculum aerophilum str. IM2

YP_929988.1 mevalonate kinase Pyrobaculum islandicum DSM 4184

ZP_01465063.1 mevalonate kinase Stigmatella aurantiaca DW4/3-1

ZP_01906658.1 mevalonate kinase Plesiocystis pacifica SIR-1

NP_248080.1 mevalonate kinase Methanocaldococcus jannaschii DSM 2661

1KKHA chain A of the Methanococcus jannaschii mevalonate kinase

Exemplary mevalonate kinase nucleic acids and polypeptides homologus to Lactobacillus sakei mevalonate kinase

YP_395519.1 mevalonate kinase Lactobacillus sakei subsp. sakei 23K

YP_535578.1 mevalonate kinase Lactobacillus salivarius UCC118

YP_804427.1 mevalonate kinase Pediococcus pentosaceus ATCC 25745

YP_001271514.1 mevalonate kinase Lactobacillus reuteri F275

ZP_03073995.1 mevalonate kinase Lactobacillus reuteri 100-23

YP_795031.1 mevalonate kinase Lactobacillus brevis ATCC 367

ZP_02185318.1 mevalonate kinase Carnobacterium sp. AT7

YP_001844008.1 mevalonate kinase Lactobacillus fermentum IFO 3956

NP_266560.1 mevalonate kinase Lactococcus lactis subsp. lactis Il1403

YP_818851.1 mevalonate kinase Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293

NP_785308.1 mevalonate kinase Lactobacillus plantarum WCFS1

ZP_00604007.1 Mevalonate kinase Enterococcus faecium DO

YP_808480.1 mevalonate kinase Lactococcus lactis subsp. cremoris SK11

YP_001031775.1 mevalonate kinase Lactococcus lactis subsp. cremoris MG1363

NP_814642.1 mevalonate kinase Enterococcus faecalis V583

AAG02440.1 AF290093_1 mevalonate kinase Enterococcus faecalis

Exemplary mevalonate kinase nucleic acids and polypeptides homologus to Streptomyces sp. CL190 mevalonate kinase

BAB07790.1 mevalonate kinase Streptomyces sp. CL190

BAD86800.1 mevalonate kinase Streptomyces sp. KO-3988

BAB07817.1 mevalonate kinase Kitasatospora griseola

ABS50475.1 NapT6 Streptomyces sp. CNQ525

ABS50448.1 NapT6 Streptomyces aculeolatus

BAE78977.1 mevalonate kinase Streptomyces sp. KO-3988

CAL34097.1 putative mevalonate kinase Streptomyces cinnamonensis

BAD07375.1 mevalonate kinase Actinoplanes sp. A40644

YP_118418.1 putative mevalonate kinase Nocardia farcinica IFM 10152

YP_818851.1 mevalonate kinase Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293

YP_001620791.1 mevalonate kinase Acholeplasma laidlawii PG-8A

NP_720650.1 putative mevalonate kinase Streptococcus mutans UA159

YP_001031775.1 mevalonate kinase Lactococcus lactis subsp. cremoris MG1363

ZP_02689018.1 mevalonate kinase Listeria monocytogenes FSL J2-071

NP_266560.1 mevalonate kinase Lactococcus lactis subsp. lactis Il1403

YP_395519.1 mevalonate kinase Lactobacillus sakei subsp. sakei 23K

YP_808480.1 mevalonate kinase Lactococcus lactis subsp. cremoris SK11

ZP_01926008.1 mevalonate kinase Listeria monocytogenes FSL N1-017

ZP_01942559.1 mevalonate kinase Listeria monocytogenes HPB2262

YP_012624.1 mevalonate kinase Listeria monocytogenes str. 4b F2365

YP_001727922.1 mevalonate kinase Leuconostoc citreum KM20

NP_469357.1 hypothetical protein lin0010 Listeria innocua Clip11262

ZP_00875673.1 Mevalonate kinase Streptococcus suis 89/1591

ZP_00604007.1 Mevalonate kinase Enterococcus faecium DO

ZP_00230799.1 mevalonate kinase Listeria monocytogenes str. 4b H7858

YP_139080.1 mevalonate kinase Streptococcus thermophilus LMG 18311

YP_140970.1 mevalonate kinase Streptococcus thermophilus CNRZ1066

ZP_01544345.1 mevalonate kinase Oenococcus oeni ATCC BAA-1163

YP_001197657.1 mevalonate kinase Streptococcus suis 05ZYH33

YP_810664.1 mevalonate kinase Oenococcus oeni PSU-1

NP_463543.1 hypothetical protein lmo0010 Listeria monocytogenes EGD-e

YP_848214.1 mevalonate kinase Listeria welshimeri serovar 6b str. SLCC5334

ZP_01695505.1 mevalonate kinase Bacillus coagulans 36D1

YP_804427.1 mevalonate kinase Pediococcus pentosaceus ATCC 25745

YP_820062.1 mevalonate kinase Streptococcus thermophilus LMD-9

NP_814642.1 mevalonate kinase Enterococcus faecalis V583

AAG02440.1 AF290093_1 mevalonate kinase Enterococcus faecalis

YP_598349.1 mevalonate kinase Streptococcus pyogenes MGAS10270

YP_535578.1 mevalonate kinase Lactobacillus salivarius UCC118

YP_001851498.1 mevalonate kinase, Erg12 Mycobacterium marinum M

ZP_01817104.1 mevalonate kinase Streptococcus pneumoniae SP3-BS71

YP_002037061.1 mevalonate kinase Streptococcus pneumoniae G54

NP_357932.1 mevalonate kinase Streptococcus pneumoniae R6

ZP_02710031.1 mevalonate kinase Streptococcus pneumoniae CDC1087-00

NP_344908.1 mevalonate kinase Streptococcus pneumoniae TIGR4

YP_001547075.1 mevalonate kinase Herpetosiphon aurantiacus ATCC 23779

AAG02455.1 AF290099_1 mevalonate kinase Streptococcus pneumoniae

ZP_01819603.1 mevalonate kinase Streptococcus pneumoniae SP6-BS73

YP_001271514.1 mevalonate kinase Lactobacillus reuteri F275

NP_965060.1 mevalonate kinase Lactobacillus johnsonii NCC 533

ZP_02919501.1 hypothetical protein STRINF_00343 Streptococcus infantarius

YP_001034340.1 mevalonate kinase, putative Streptococcus sanguinis SK36

YP_001844008.1 mevalonate kinase Lactobacillus fermentum IFO 3956

ZP_03073995.1 mevalonate kinase Lactobacillus reuteri 100-23

NP_688324.1 mevalonate kinase, putative Streptococcus agalactiae 2603V/R

YP_907150.1 mevalonate kinase, Erg12 Mycobacterium ulcerans Agy99

NP_691146.1 mevalonate kinase Oceanobacillus iheyensis HTE831

YP_795031.1 mevalonate kinase Lactobacillus brevis ATCC 367

YP_002123449.1 mevalonate kinase Mvk Streptococcus equi subsp. zooepidemicus str. MGCS10565

YP_001449558.1 mevalonate kinase Streptococcus gordonii str. Challis substr. CH1

ZP_02185318.1 mevalonate kinase Carnobacterium sp. AT7

YP_001634502.1 mevalonate kinase Chloroflexus aurantiacus J-10-fl

YP_812921.1 mevalonate kinase Lactobacillus delbrueckii subsp. bulgaricus ATCC BAA-365

YP_814846.1 mevalonate kinase Lactobacillus gasseri ATCC 33323

YP_001987652.1 Mevalonate kinase Lactobacillus casei

YP_618979.1 mevalonate kinase Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842

NP_664399.1 mevalonate kinase Streptococcus pyogenes MGAS315

YP_806709.1 mevalonate kinase Lactobacillus casei ATCC 334

YP_060017.1 mevalonate kinase Streptococcus pyogenes MGAS10394

YP_280130.1 mevalonate kinase Streptococcus pyogenes MGAS6180

NP_269075.1 mevalonate kinase Streptococcus pyogenes M1 GAS

YP_001276466.1 mevalonate kinase Roseiflexus sp. RS-1

NP_607080.1 mevalonate kinase Streptococcus pyogenes MGAS8232

NP_785308.1 mevalonate kinase Lactobacillus plantarum WCFS1

ABH11598.1 GMP synthase, mevalonate kinase Lactobacillus helveticus CNRZ32

YP_001577580.1 mevalonate kinase Lactobacillus helveticus DPC 4571

YP_001431846.1 mevalonate kinase Roseiflexus castenholzii DSM 13941

YP_302212.1 mevalonate kinase Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305

YP_040044.1 mevalonate kinase Staphylococcus aureus subsp. aureus MRSA252

AAG02424.1 AF290087_1 mevalonate kinase Staphylococcus aureus

NP_645362.1 mevalonate kinase Staphylococcus aureus subsp. aureus MW2

ZP_01514039.1 mevalonate kinase Chloroflexus aggregans DSM 9485

YP_194037.1 mevalonate kinase Lactobacillus acidophilus NCFM

YP_254317.1 mevalonate kinase Staphylococcus haemolyticus JCSC1435

YP_187834.1 mevalonate kinase Staphylococcus epidermidis RP62A

AAG02435.1 AF290091_1 mevalonate kinase Staphylococcus epidermidis

YP_183887.1 mevalonate kinase Thermococcus kodakarensis KOD1

NP_143478.1 mevalonate kinase Pyrococcus horikoshii OT3

ZP_00780842.1 mevalonate kinase Streptococcus agalactiae 18RS21

NP_579366.1 mevalonate kinase Pyrococcus furiosus DSM 3638

NP_126232.1 mevalonate kinase Pyrococcus abyssi GE5

NP_371114.1 mevalonate kinase Staphylococcus aureus subsp. aureus Mu50

YP_001040239.1 mevalonate kinase Staphylothermus marinus F1

NP_763916.1 mevalonate kinase Staphylococcus epidermidis ATCC 12228

YP_633174.1 mevalonate kinase Myxococcus xanthus DK 1622

YP_920295.1 mevalonate kinase Thermofilum pendens Hrk 5

NP_148611.1 mevalonate kinase Aeropyrum pernix K1

NP_633786.1 mevalonate kinase Methanosarcina mazei Go1

Exemplary phosphomevalonate kinase nucleic acids and polypeptides

HSA: 10654(PMVK)

PTR: 457350(PMVK)

MCC: 717014(PMVK)

MMU: 68603(Pmvk)

CFA: 612251(PMVK)

BTA: 513533(PMVK)

DME: Dmel_CG10268

ATH: AT1G31910

OSA: 4332275

SCE: YMR220W(ERG8)

AGO: AGOS_AER354W

PIC: PICST_52257(ERG8)

CGR: CAGL0F03993g

SPO: SPAC343.01c

MGR: MGG_05812

ANI: AN2311.2

AFM: AFUA_5G10680

AOR: AO090010000471

CNE: CNM00100

UMA: UM00760.1

DDI: DDBDRAFT_0184512

TBR: Tb09.160.3690

TCR: 507913.20 508277.140

LMA: LmjF15.1460

MXA: MXAN_5017

OIH: OB0227

SAU: SA0549(mvaK2)

SAV: SAV0592(mvaK2)

SAM: MW0547(mvaK2)

SAR: SAR0598(mvaK2)

SAS: SAS0551

SAC: SACOL0638

SAB: SAB0542(mvaK2)

SAA: SAUSA300_0574

SAO: SAOUHSC_00579

SAJ: SaurJH9_0615

SEP: SE0363

SER: SERP0240

SHA: SH2400(mvaK2)

SSP: SSP2120

LMO: lmo0012

LMF: LMOf2365_0013

LIN: lin0012

LWE: lwe0013

LLA: L10014(yebA)

LLC: LACR_0456

LLM: llmg_0427

SPY: SPy_0878(mvaK2)

SPZ: M5005_Spy_0684(mvaK2)

SPM: spyM18_0939

SPG: SpyM3_0597(mvaK2)

SPS: SPs1256

SPH: MGAS10270_Spy0742(mvaK2)

SPI: MGAS10750_Spy0776(mvaK2)

SPJ: MGAS2096_Spy0755(mvaK2)

SPK: MGAS9429_Spy0739(mvaK2)

SPF: SpyM51124(mvaK2)

SPA: M6_Spy0701

SPB: M28_Spy0664(mvaK2)

SPN: SP_0383

SPR: spr0340(mvaK2)

SPD: SPD_0348(mvaK2)

SAG: SAG1324

SAN: gbs1394

SAK: SAK_1355

SMU: SMU.938

STC: str0561(mvaK2)

STL: stu0561(mvaK2)

STE: STER_0600

SSA: SSA_0335(mvaK2)

SSU: SSU05_0291

SSV: SSU98_0287

SGO: SGO_0241

LPL: lp_1733(mvaK2)

LJO: LJ1207

LAC: LBA1169

LSA: LSA0906(mvaK2)

LSL: LSL_0683

LDB: Ldb0997(mvaK)

LBU: LBUL_0904

LBR: LVIS_0860

LCA: LSEI_1092

LGA: LGAS_1035

LRE: Lreu_0913

PPE: PEPE_0925

EFA: EF0902

NFA: nfa22090

BGA: BG0710

BAF: BAPKO_0731

NPH: NP2852A

SSO: SSO2988

STO: ST0978

SAI: Saci_1244

Exemplary diphosphomevalonate decarboxylase nucleic acids and polypeptides

HSA: 4597(MVD)

PTR: 468069(MVD)

MCC: 696865(MVD)

MMU: 192156(Mvd)

RNO: 81726(Mvd)

CFA: 489663(MVD)

GGA: 425359(MVD)

DME: Dmel_CG8239

SCE: YNR043W(MVD1)

AGO: AGOS_AGL232C

PIC: PICST_90752

CGR: CAGL0C03630g

SPO: SPAC24C9.03

MGR: MGG_09750

ANI: AN4414.2

AFM: AFUA_4G07130

AOR: AO090023000862

CNE: CNL04950

UMA: UM05179.1

DDI: DDBDRAFT_0218058

TET: TTHERM_00849200

TBR: Tb10.05.0010 Tb10.61.2745

TCR: 507993.330 511281.40

LMA: LmjF18.0020

CBU: CBU_0607(mvaD)

CBD: COXBU7E912_0619(mvaD)

LPN: lpg2040

LPF: lpl2018

LPP: lpp2023

TCX: Tcr_1734

DNO: DNO_0504(mvaD)

BBA: Bd1629

MXA: MXAN_5018(mvaD)

OIH: OB0226

SAU: SA0548(mvaD)

SAV: SAV0591(mvaD)

SAM: MW0546(mvaD)

SAR: SAR0597(mvaD)

SAS: SAS0550

SAC: SACOL0637(mvaD)

SAB: SAB0541(mvaD)

SAA: SAUSA300_0573(mvaD)

SAO: SAOUHSC_00578

SAJ: SaurJH9_0614

SAH: SaurJH1_0629

SEP: SE0362

SER: SERP0239(mvaD)

SHA: SH2401(mvaD)

SSP: SSP2121

LMO: lmo0011

LMF: LMOf2365_0012(mvaD)

LIN: lin0011

LWE: lwe0012(mvaD)

LLA: L9089(yeaH)

LLC: LACR_0455

LLM: llmg_0426(mvaD)

SPY: SPy_0877(mvaD)

SPZ: M5005_Spy_0683(mvaD)

SPM: spyM18_0938(mvd)

SPG: SpyM3_0596(mvaD)

SPS: SPs1257

SPH: MGAS10270_Spy0741(mvaD)

SPI: MGAS10750_Spy0775(mvaD)

SPJ: MGAS2096_Spy0754(mvaD)

SPK: MGAS9429_Spy0738(mvaD)

SPF: SpyM51125(mvaD)

SPA: M6_Spy0700

SPB: M28_Spy0663(mvaD)

SPN: SP_0382

SPR: spr0339(mvd1)

SPD: SPD_0347(mvaD)

SAG: SAG1325(mvaD)

SAN: gbs1395

SAK: SAK_1356(mvaD)

SMU: SMU.937

STC: str0560(mvaD)

STL: stu0560(mvaD)

STE: STER_0599

SSA: SSA_0334(mvaD)

SSU: SSU05_0290

SSV: SSU98_0286

SGO: SGO_0240(mvaD)

LPL: lp_1734(mvaD)

LJO: LJ1206

LAC: LBA1168(mvaD)

LSA: LSA0907(mvaD)

LSL: LSL_0684

LDB: Ldb0998(mvaD)

LBU: LBUL_0905

LBR: LVIS_0859

LCA: LSEI_1492

LGA: LGAS_1034

LRE: Lreu_0914

PPE: PEPE_0926

EFA: EF0903(mvaD)

LME: LEUM_1386

NFA: nfa22080

BBU: BB0686

BGA: BG0709

BAF: BAPKO_0730

GFO: GFO_3632

FPS: FP0310(mvaD)

HAU: Haur_1612

HAL: VNG0593G(dmd)

HMA: rrnAC1489(dmd)

HWA: HQ1525A(mvaD)

NPH: NP1580A(mvaD)

PTO: PTO0478 PTO1356

SSO: SSO2989

STO: ST0977

SAI: Saci_1245(mvd)

MSE: Msed_1576

Exemplary isopentenyl phosphate kinases (IPK) nucleic acids and polypeptides

Methanobacterium thermoautotrophicum gi|2621082

Methanococcus jannaschii DSM 2661 gi|1590842 ;

Methanocaldococcus jannaschii gi|1590842

Methanothermobacter thermautotrophicus gi|2621082

Picrophilus torridus DSM9790 (IG-57) gi|48477569

Pyrococcus abyssi gi|14520758

Pyrococcus horikoshii OT3 gi|3258052

Archaeoglobus fulgidus DSM4304 gi|2648231

Exemplary isopentenyl-diphosphate Delta-isomerase (IDI) nucleic acids and polypeptides

HSA: 3422(IDI1) 91734(IDI2)

PTR: 450262(IDI2) 450263(IDI1)

MCC: 710052(LOC710052) 721730(LOC721730)

MMU: 319554(Idi1)

RNO: 89784(Idi1)

GGA: 420459(IDI1)

XLA: 494671(LOC494671)

XTR: 496783(idi2)

SPU: 586184(LOC586184)

CEL: K06H7.9(idi-1)

ATH: AT3G02780(IPP2)

OSA: 4338791 4343523

CME: CMB062C

SCE: YPL117C(IDI1)

AGO: AGOS_ADL268C

PIC: PICST_68990(IDI1)

CGR: CAGL0J06952g

SPO: SPBC106.15(idi1)

ANI: AN0579.2

AFM: AFUA_6G11160

AOR: AO090023000500

CNE: CNA02550

UMA: UM04838.1

ECU: ECU02_0230

DDI: DDB_0191342(ipi)

TET: TTHERM_00237280 TTHERM_00438860

TBR: Tb09.211.0700

TCR: 408799.19 510431.10

LMA: LmjF35.5330

EHI: 46.t00025

ECO: b2889(idi)

ECJ: JW2857(idi)

ECE: Z4227

ECS: ECs3761

ECC: c3467

ECI: UTI89_C3274

ECP: ECP_2882

ECV: APECO1_3638

ECW: EcE24377A_3215(idi)

ECX: EcHS_A3048

STY: STY3195

STT: t2957

SPT: SPA2907(idi)

SEC: SC2979(idi)

STM: STM3039(idi)

SFL: SF2875(idi)

SFX: S3074

SFV: SFV_2937

SSN: SSON_3042 SSON_3489(yhfK)

SBO: SBO_3103

SDY: SDY_3193

ECA: ECA2789

PLU: plu3987

ENT: Ent638_3307

SPE: Spro_2201

VPA: VPA0278

VFI: VF0403

PPR: PBPRA0469(mvaD)

PEN: PSEEN4850

CBU: CBU_0607(mvaD)

CBD: COXBU7E912_0619(mvaD)

LPN: lpg2051

LPF: lpl2029

LPP: lpp2034

TCX: Tcr_1718

HHA: Hhal_1623

DNO: DNO_0798

EBA: ebA5678 p2A143

DVU: DVU1679(idi)

DDE: Dde_1991

LIP: LI1134

BBA: Bd1626

AFW: Anae109_4082

MXA: MXAN_5021(fni)

RPR: RP452

RTY: RT0439(idi)

RCO: RC0744

RFE: RF_0785(fni)

RBE: RBE_0731(fni)

RAK: A1C_04190

RBO: A1I_04755

RCM: A1E_02555

RRI: A1G_04195

MLO: mlr6371

RET: RHE_PD00245(ypd00046)

XAU: Xaut_4134

SIL: SPO0131

SIT: TM1040_3442

RSP: RSP_0276

RSH: Rsph17029_1919

RSQ: Rsph17025_1019

JAN: Jann_0168

RDE: RD1_0147(idi)

DSH: Dshi_3527

BSU: BG11440(ypgA)

BAN: BA1520

BAR: GBAA1520

BAA: BA_2041

BAT: BAS1409

BCE: BC1499

BCA: BCE_1626

BCZ: BCZK1380(fni)

BCY: Bcer98_1222

BTK: BT9727_1381(fni)

BTL: BALH_1354

BLI: BL02217(fni)

BLD: BLi02426

BAY: RBAM_021020(fni)

BPU: BPUM_2020(fni)

OIH: OB0537

SAU: SA2136(fni)

SAV: SAV2346(fni)

SAM: MW2267(fni)

SAR: SAR2431(fni)

SAS: SAS2237

SAC: SACOL2341(fni)

SAB: SAB2225c(fni)

SAA: SAUSA300_2292(fni)

SAO: SAOUHSC_02623

SEP: SE1925

SER: SERP1937(fni-2)

SHA: SH0712(fni)

SSP: SSP0556

LMO: lmo1383

LMF: LMOf2365_1402(fni)

LIN: lin1420

LWE: lwe1399(fni)

LLA: L11083(yebB)

LLC: LACR_0457

LLM: llmg_0428(fni)

SPY: SPy_0879

SPZ: M5005_Spy_0685

SPM: spyM18_0940

SPG: SpyM3_0598

SPS: SPs1255

SPH: MGAS10270_Spy0743

SPI: MGAS10750_Spy0777

SPJ: MGAS2096_Spy0756

SPK: MGAS9429_Spy0740

SPF: SpyM51123(fni)

SPA: M6_Spy0702

SPB: M28_Spy0665

SPN: SP_0384

SPR: spr0341(fni)

SPD: SPD_0349(fni)

SAG: SAG1323

SAN: gbs1393

SAK: SAK_1354(fni)

SMU: SMU.939

STC: str0562(idi)

STL: stu0562(idi)

STE: STER_0601

SSA: SSA_0336

SGO: SGO_0242

LPL: lp_1732(idi1)

LJO: LJ1208

LAC: LBA1171

LSA: LSA0905(idi)

LSL: LSL_0682

LDB: Ldb0996(fni)

LBU: LBUL_0903

LBR: LVIS_0861

LCA: LSEI_1493

LGA: LGAS_1036

LRE: Lreu_0912

EFA: EF0901

OOE: OEOE_1103

STH: STH1674

CBE: Cbei_3081

DRM: Dred_0474

SWO: Swol_1341

MTA: Moth_1328

MTU: Rv1745c(idi)

MTC: MT1787(idi)

MBO: Mb1774c(idi)

MBB: BCG_1784c(idi)

MPA: MAP3079c

MAV: MAV_3894(fni)

MSM: MSMEG_1057(fni) MSMEG_2337(fni)

MUL: MUL_0380(idi2)

MVA: Mvan_1582 Mvan_2176

MGI: Mflv_1842 Mflv_4187

MMC: Mmcs_1954

MKM: Mkms_2000

MJL: Mjls_1934

CGL: NCgl2223(cgl2305)

CGB: cg2531(idi)

CEF: CE2207

CDI: DIP1730(idi)

NFA: nfa19790 nfa22100

RHA: RHA1_ro00239

SCO: SCO6750(SC5F2A.33c)

SMA: SAV1663(idi)

LXX: Lxx23810(idi)

CMI: CMM_2889(idiA)

AAU: AAur_0321(idi)

PAC: PPA2115

FRA: Francci3_4188

FRE: Franean1_5570

FAL: FRAAL6504(idi)

KRA: Krad_3991

SEN: SACE_2627(idiB_2) SACE_5210(idi)

STP: Strop_4438

SAQ: Sare_4564 Sare_4928

RXY: Rxyl_0400

BBU: BB0684

BGA: BG0707

SYN: sll1556

SYC: syc2161_c

SYF: Synpcc7942_1933

CYA: CYA_2395(fni)

CYB: CYB_2691(fni)

TEL: tll1403

ANA: all4591

AVA: Ava_2461 Ava_B0346

TER: Tery_1589

SRU: SRU_1900(idi)

CHU: CHU_0674(idi)

GFO: GFO_2363(idi)

FJO: Fjoh_0269

FPS: FP1792(idi)

CTE: CT0257

CCH: Cag_1445

CPH: Cpha266_0385

PVI: Cvib_1545

PLT: Plut_1764

RRS: RoseRS_2437

RCA: Rcas_2215

HAU: Haur_4687

DRA: DR_1087

DGE: Dgeo_1381

TTH: TT_P0067

TTJ: TTHB110

MJA: MJ0862

MMP: MMP0043

MMQ: MmarC5_1637

MMX: MmarC6_0906

MMZ: MmarC7_1040

MAE: Maeo_1184

MVN: Mevan_1058

MAC: MA0604(idi)

MBA: Mbar_A1419

MMA: MM_1764

MBU: Mbur_2397

MTP: Mthe_0474

MHU: Mhun_2888

MLA: Mlab_1665

MEM: Memar_1814

MBN: Mboo_2211

MTH: MTH48

MST: Msp_0856(fni)

MSI: Msm_1441

MKA: MK0776(lldD)

AFU: AF2287

HAL: VNG1818G(idi) VNG6081G(crt_1) VNG6445G(crt_2) VNG7060 VNG7149

HMA: rrnAC3484(idi)

HWA: HQ2772A(idiA) HQ2847A(idiB)

NPH: NP0360A(idiB_1) NP4826A(idiA) NP5124A(idiB_2)

TAC: Ta0102

TVO: TVN0179

PTO: PTO0496

PHO: PH1202

PAB: PAB1662

PFU: PF0856

TKO: TK1470

RCI: LRC397(fni)

APE: APE_1765.1

SMR: Smar_0822

IHO: Igni_0804

HBU: Hbut_0539

SSO: SSO0063

STO: ST2059

SAI: Saci_0091

MSE: Msed_2136

PAI: PAE0801

PIS: Pisl_1093

PCL: Pcal_0017

PAS: Pars_0051

TPE: Tpen_0272

Exemplary isoprene synthase nucleic acids and polypeptides

Genbank Accession Nos.

AY341431

AY316691

AY279379

AJ457070

AY182241

CLAIMS

What is claimed is:

1. Cells in culture comprising a nucleic acid encoding a heterologous isoprene synthase polypeptide and one or more nucleic acids encoding MVA pathway polypeptides, wherein the cells further comprise i) one or more copies of a nucleic acid encoding a mevalonate kinase polypeptide, or ii) a nucleic acid encoding a mevalonate kinase polypeptide under the control of a strong promoter, and wherein the cells express the mevalonate kinase polypeptide at a level that is at least about 2-fold higher than the level of expression in cells that do not comprise one or more copies of a nucleic acid encoding a mevalonate kinase polypeptide or a nucleic acid encoding a mevalonate kinase polypeptide under the control of a strong promoter.

2. The cells of claim 1, wherein the cells produce greater than about 400 nmole/g_{wcm}/hr of isoprene.

3. The cells of claim 1, wherein the mevalonate kinase polypeptide is *M. mazei* mevalonate kinase.

4. The cells of claim 1, wherein the MVA pathway polypeptide is selected from the group consisting of Lactobacillus mevalonate kinase polypeptide, *Lactobacillus sakei* mevalonate kinase polypeptide, yeast mevalonate kinase polypeptide, *Saccharomyces cerevisiae* mevalonate kinase polypeptide, Streptococcus mevalonate kinase polypeptide, *Streptococcus pneumoniae* mevalonate kinase polypeptide, and Streptomyces mevalonate kinase polypeptide, Streptomyces CL190 mevalonate kinase polypeptide.

5. The cells of claim 1, wherein the MVA pathway polypeptide is a polypeptide from *Saccharomyces cerevicia* or *Enterococcus faecalis*.

6. The cells of claim 1, wherein the isoprene synthase polypeptide is a polypeptide from *Pueraria* or *Populus* or a hybrid, *Populus alba x Populus tremula.*

7. The cells of claim 6, wherein the isoprene synthase polypeptide is selected from the group consisting of *Pueraria montana or Pueraria lobata, Populus tremuloides*, *Populus alba*, *Populus nigra*, and *Populus trichocarpa*.

8. The cells of claim 1, wherein the cells are gram-positive bacterial cells, *Streptomyces* cells, gram-negative bacterial cells, *Escherichia* cells, *Pantoea* cells, fungal cells, filamentous fungal cells, *Trichoderma* cells, *Aspergillus* cells, or yeast cells.

9. The cells of claim 8, wherein the cells are selected from the group consisting of *Bacillus subtilis, Streptomyces lividans*, *Streptomyces coelicolor*, *Streptomyces griseus, Escherichia coli*, *Pantoea citrea, Trichoderma reesei, Aspergillus oryzae* and *Aspergillus niger, Saccharomyces cerevisiae* and *Yarrowia lipolytica*.

10. The cells of claim 1, wherein the concentration of MVA is between about 0 to about 120 g/L.

11. A composition for producing isoprene comprising cells of claim 1.

12. A method of producing isoprene, the method comprising (a) culturing cells of claim 1 under suitable culture conditions for the production of isoprene, and (b) producing isoprene.

13. The method of claim 12, wherein the cells in culture produce greater than about 400 nmole/g_{wcm}/hr of isoprene.

14. The method of claim 12, wherein the mevalonate kinase polypeptide is *M. mazei* mevalonate kinase.

15. The method of claim 12, further comprising recovering the isoprene.

16. A method of manufacturing a tire, wherein the improvement comprises using the cells of claim 1 to produce isoprene for the manufacture of the tire.

17. Use of isoprene prepared by the method of claim 12 in the manufacture of a tire.

Figure 1

1-
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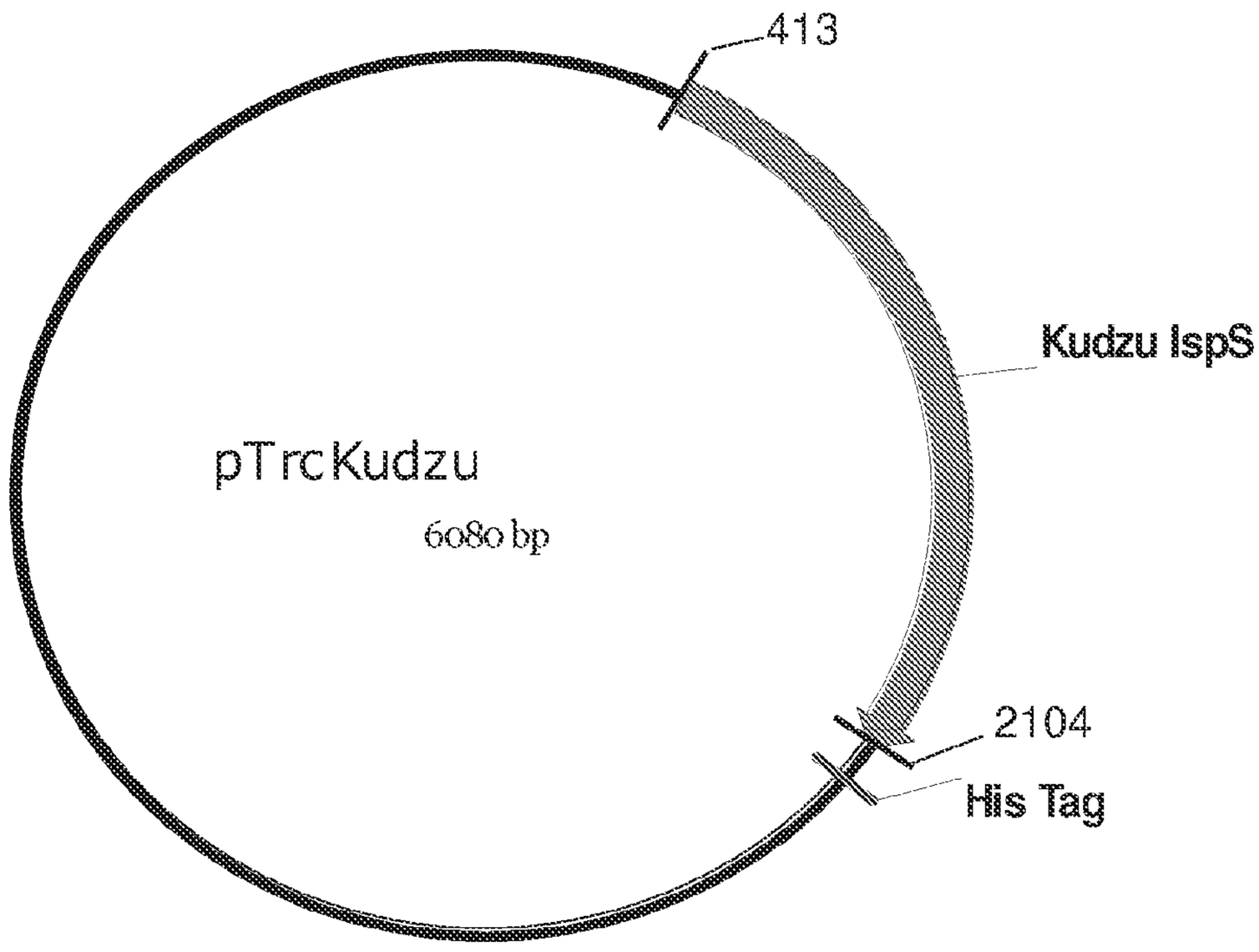

Figure 3A

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Figure 3B

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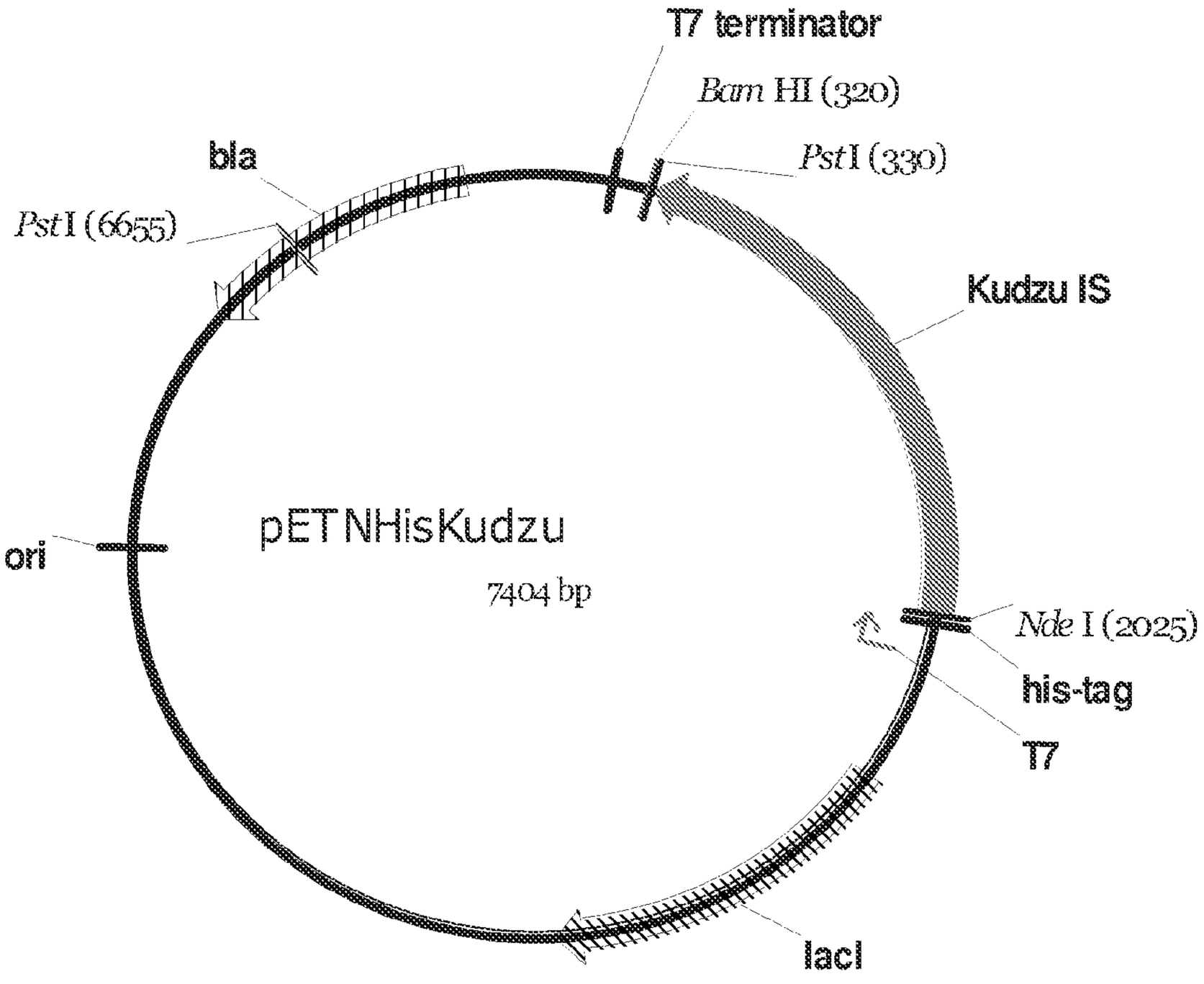
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Figure 5A

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Figure 5B

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Figure 5C

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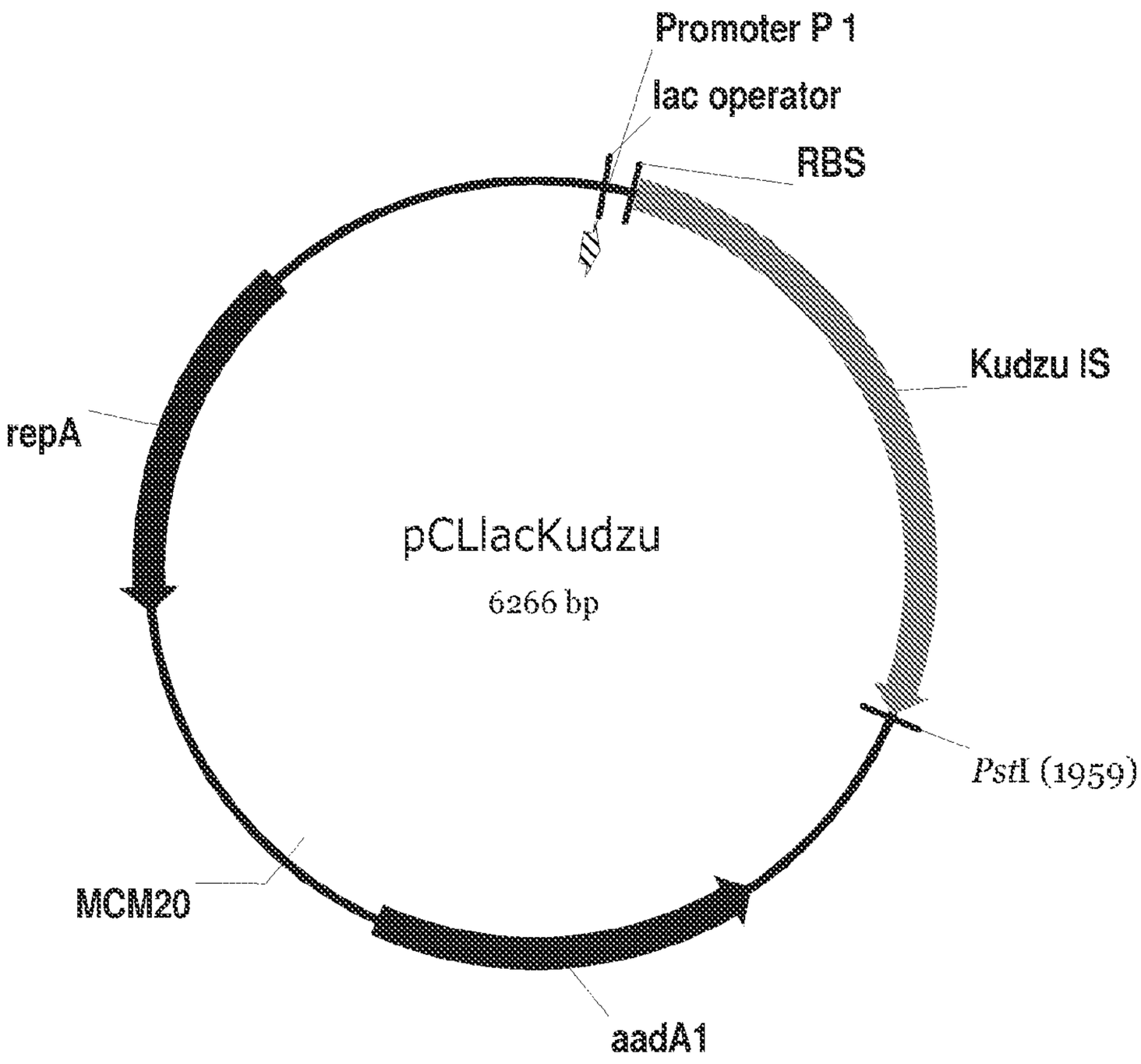
(SEQ ID NO:5)

Figure 6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lac operator
RBS
Kudzu IS
repA
pCLlacKudzu
6266 bp
PstI (1959)
MCM20
aadA1

Figure 7A

1-
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Figure 7B

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Figure 7C

Aattctgctagacctttgctggaaaacttgtaaattctgctagaccctctgtaaattccgctag
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Figure 8A

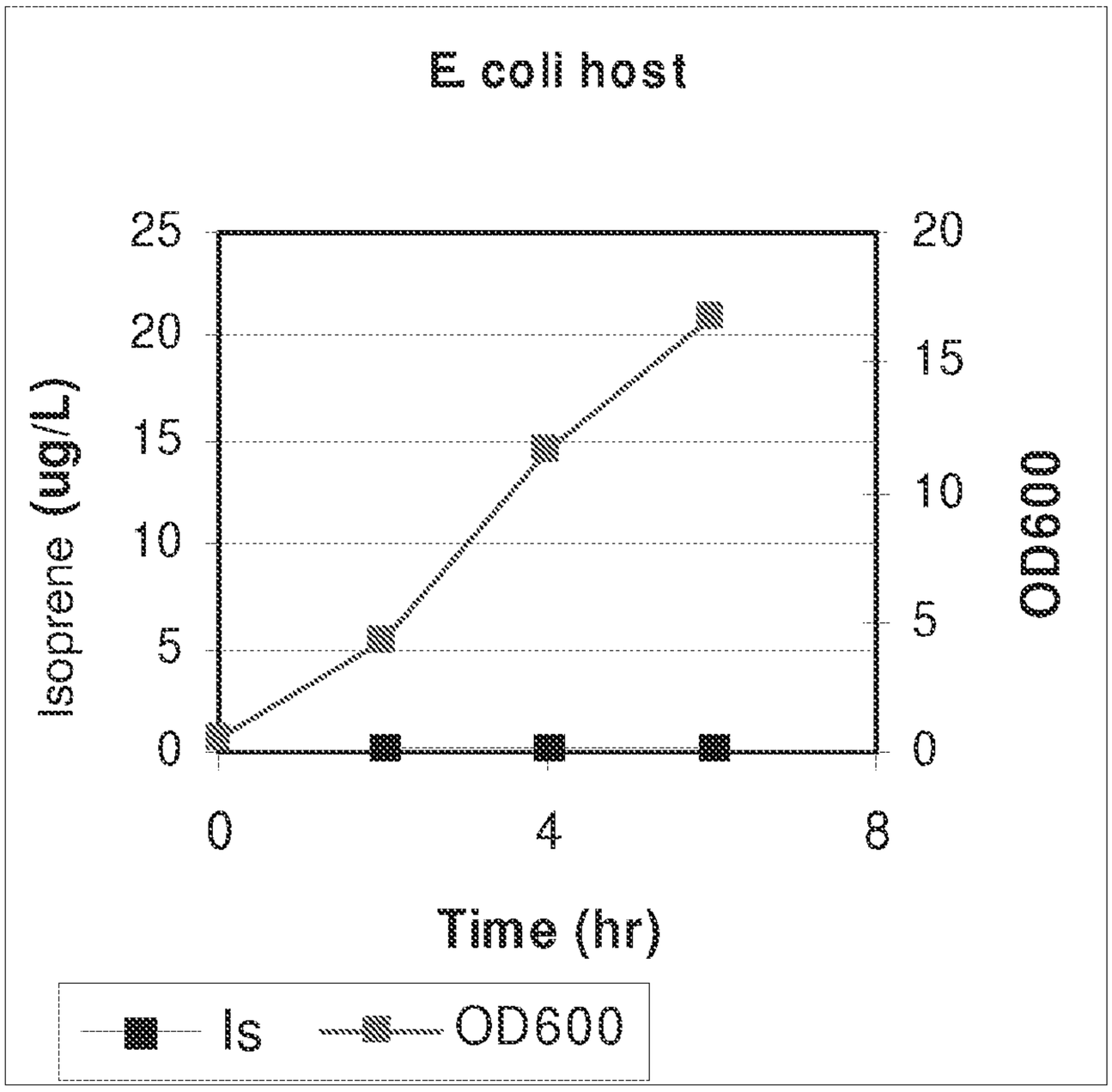

E coli host
Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 8B

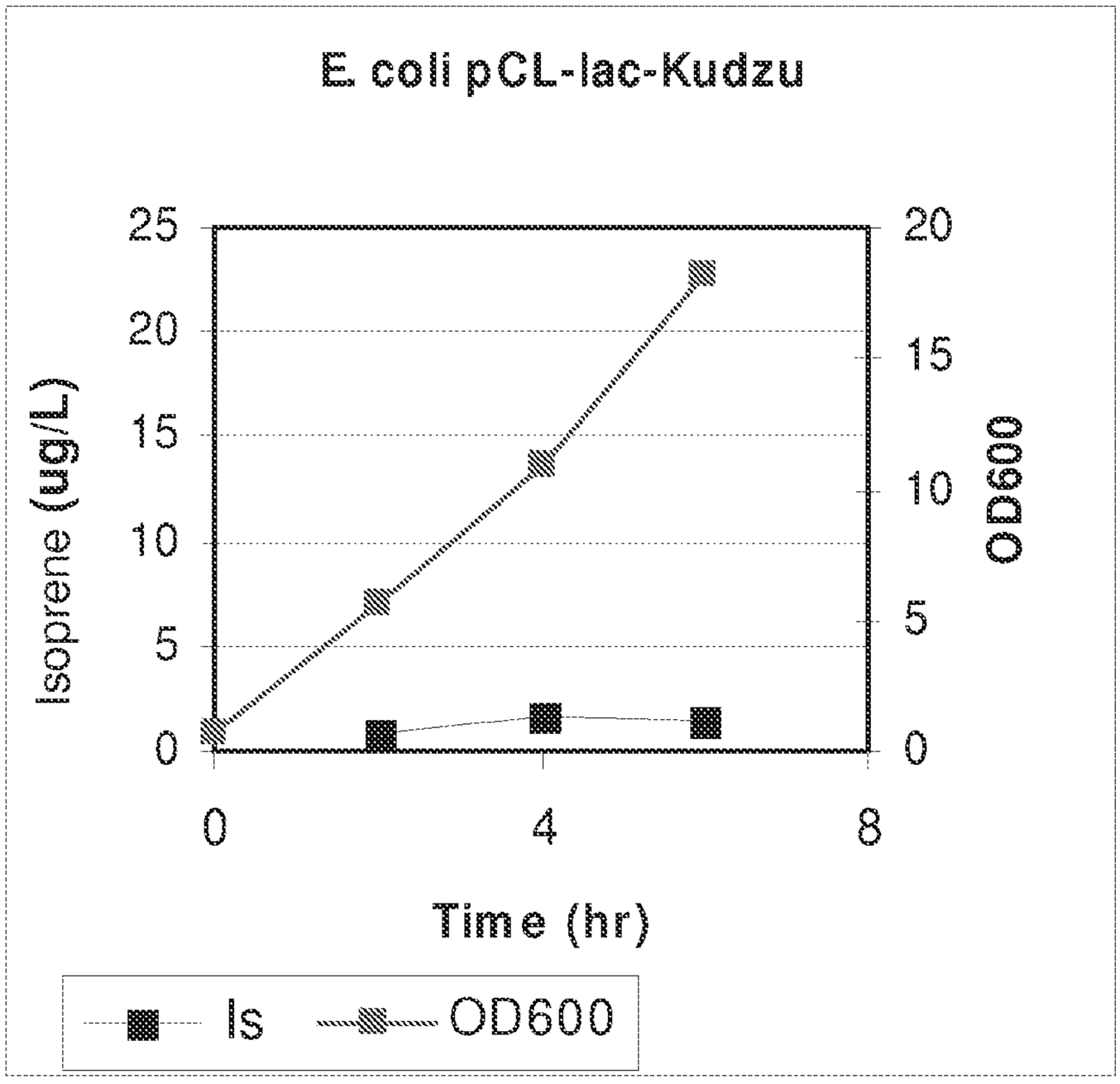

E. coli pCL-lac-Kudzu
Isoprene (ug/L)
OD600
0
5
10
15
20
25
0
5
10
15
20
0
4
8
Time (hr)
Is
OD600

Figure 8C

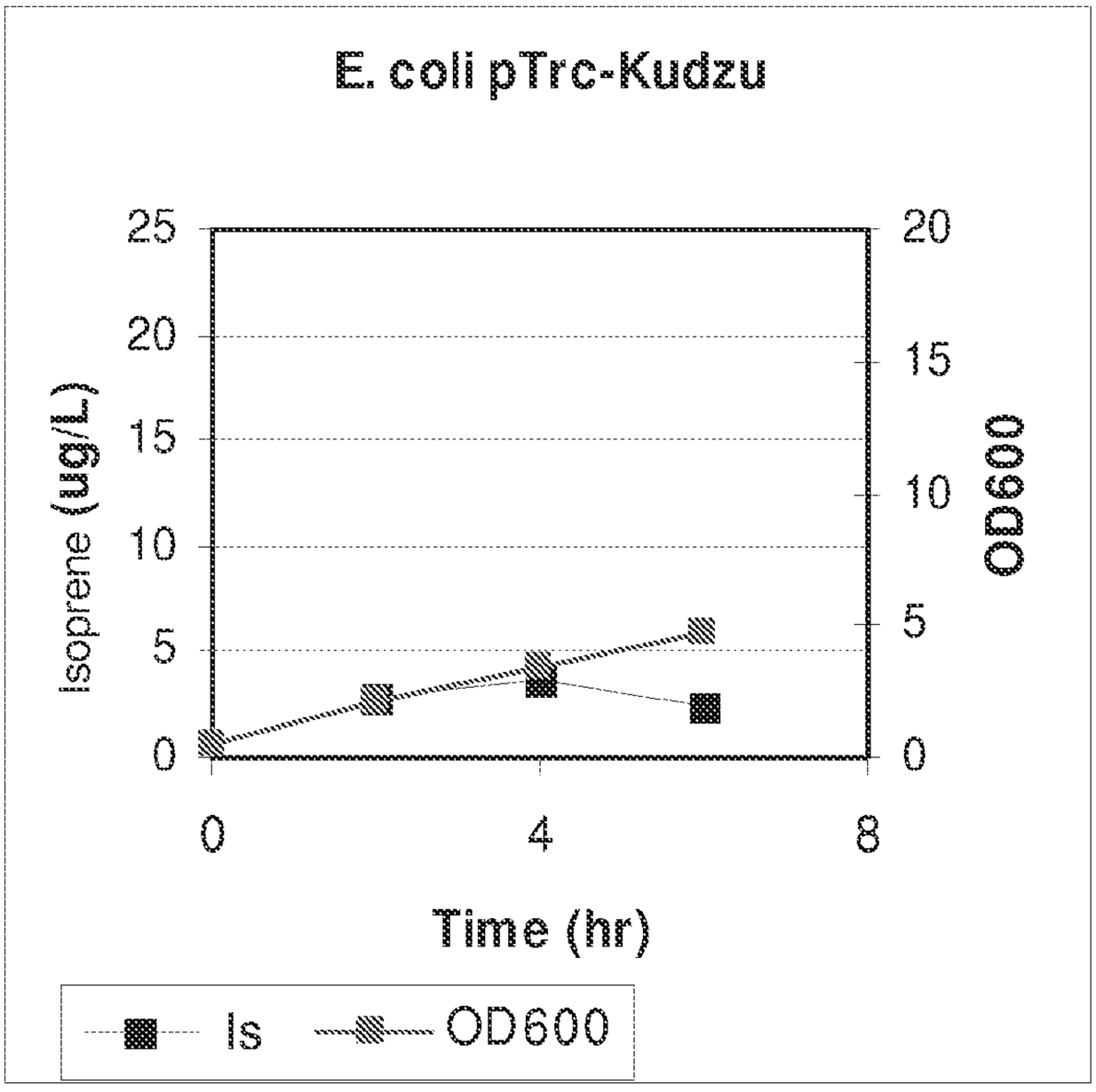

E. coli pTrc-Kudzu
Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 8D

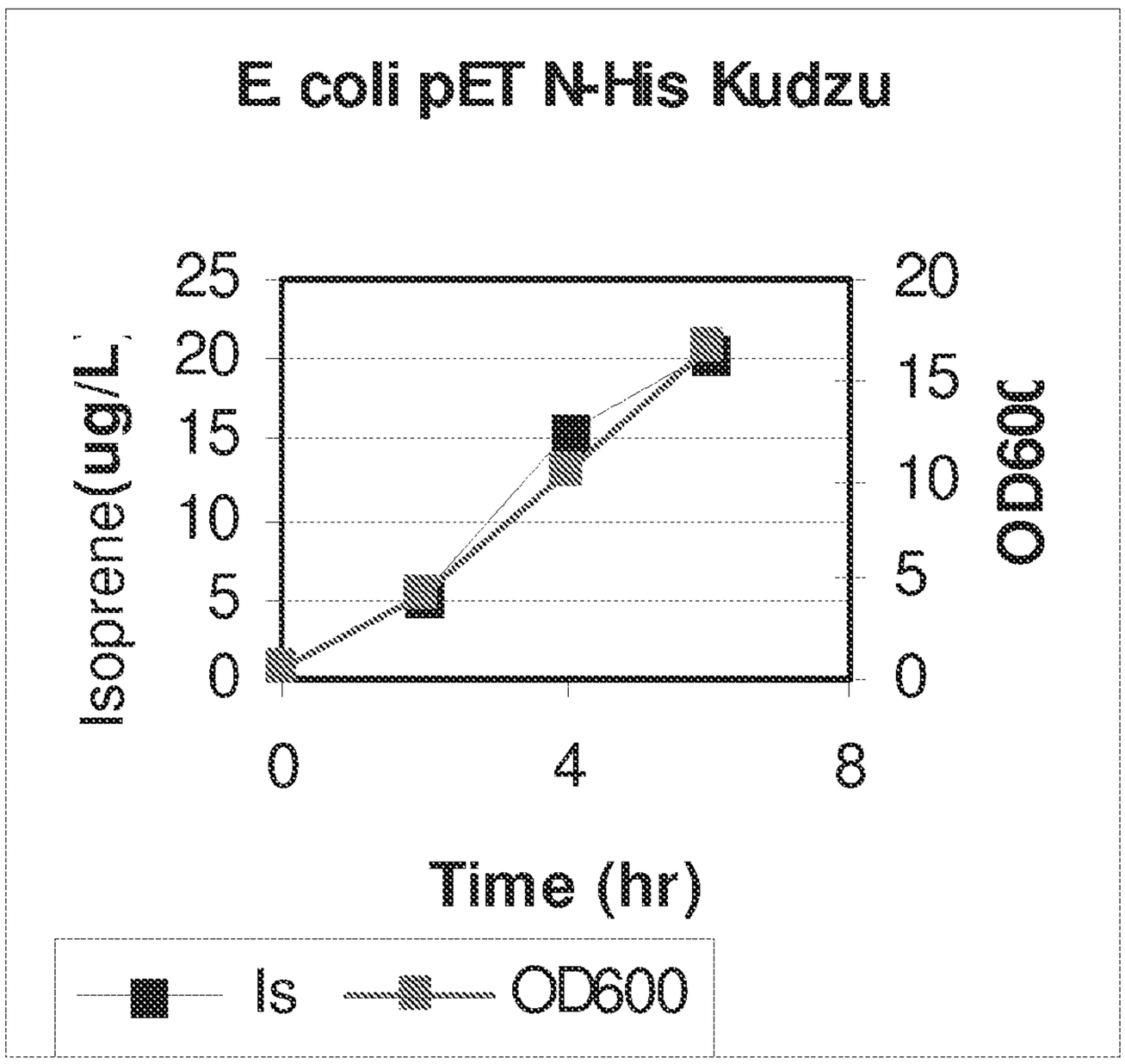
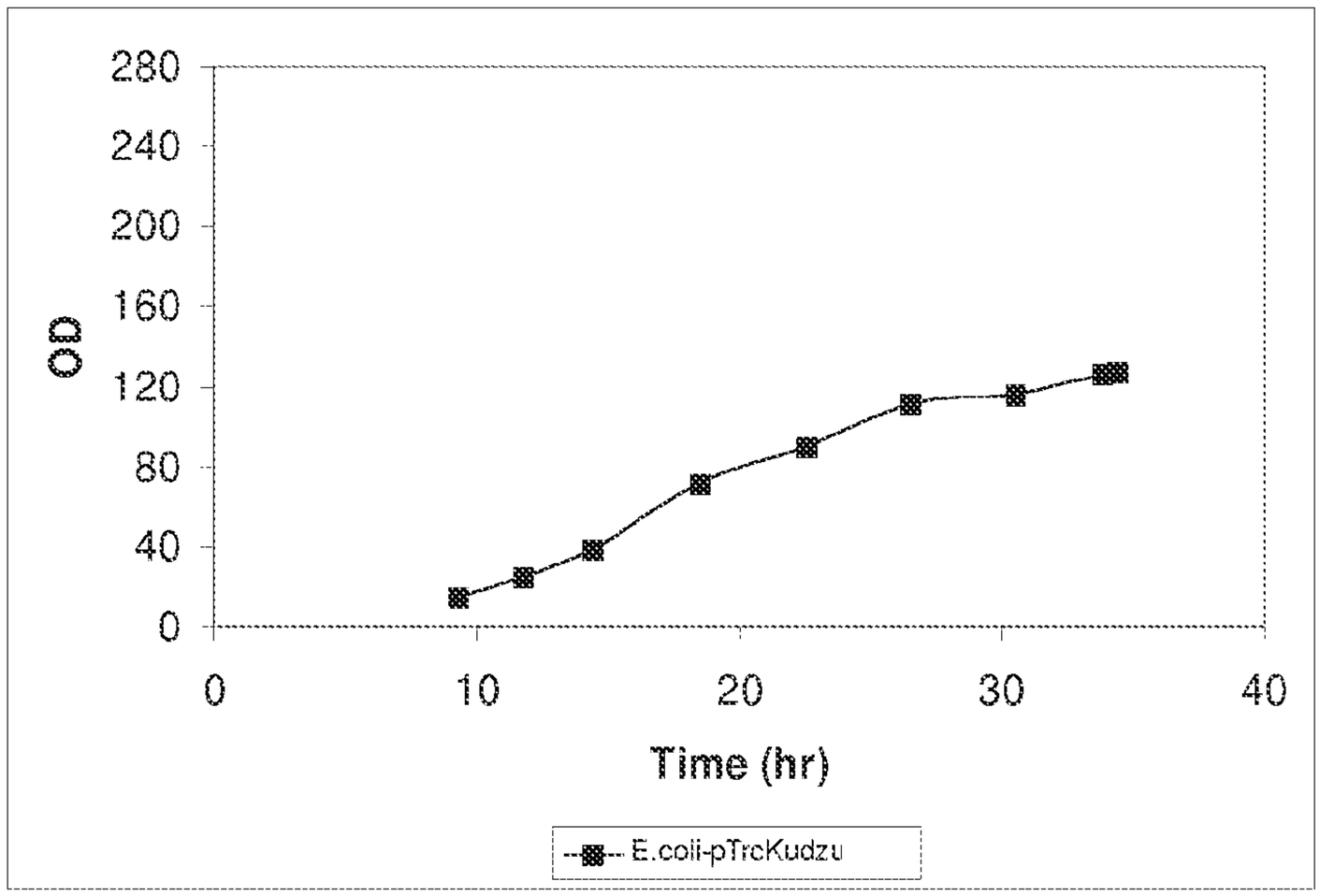

E coli pET N-His Kudzu
Isoprene(ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 9

A.


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

B.

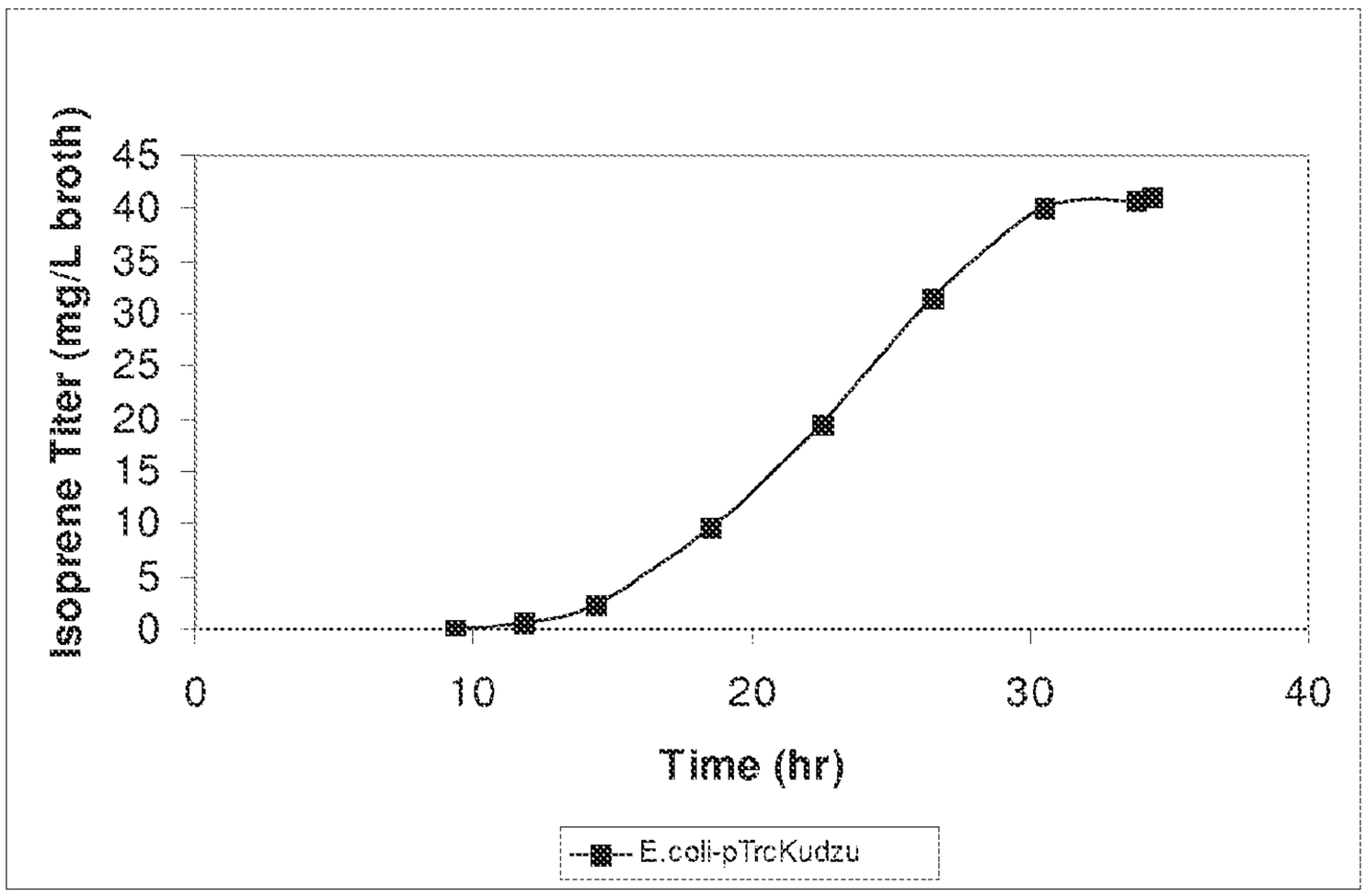

Isoprene Titer (mg/L broth)
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
Time (hr)
E.coli-pTrcKudzu

Figure 10A

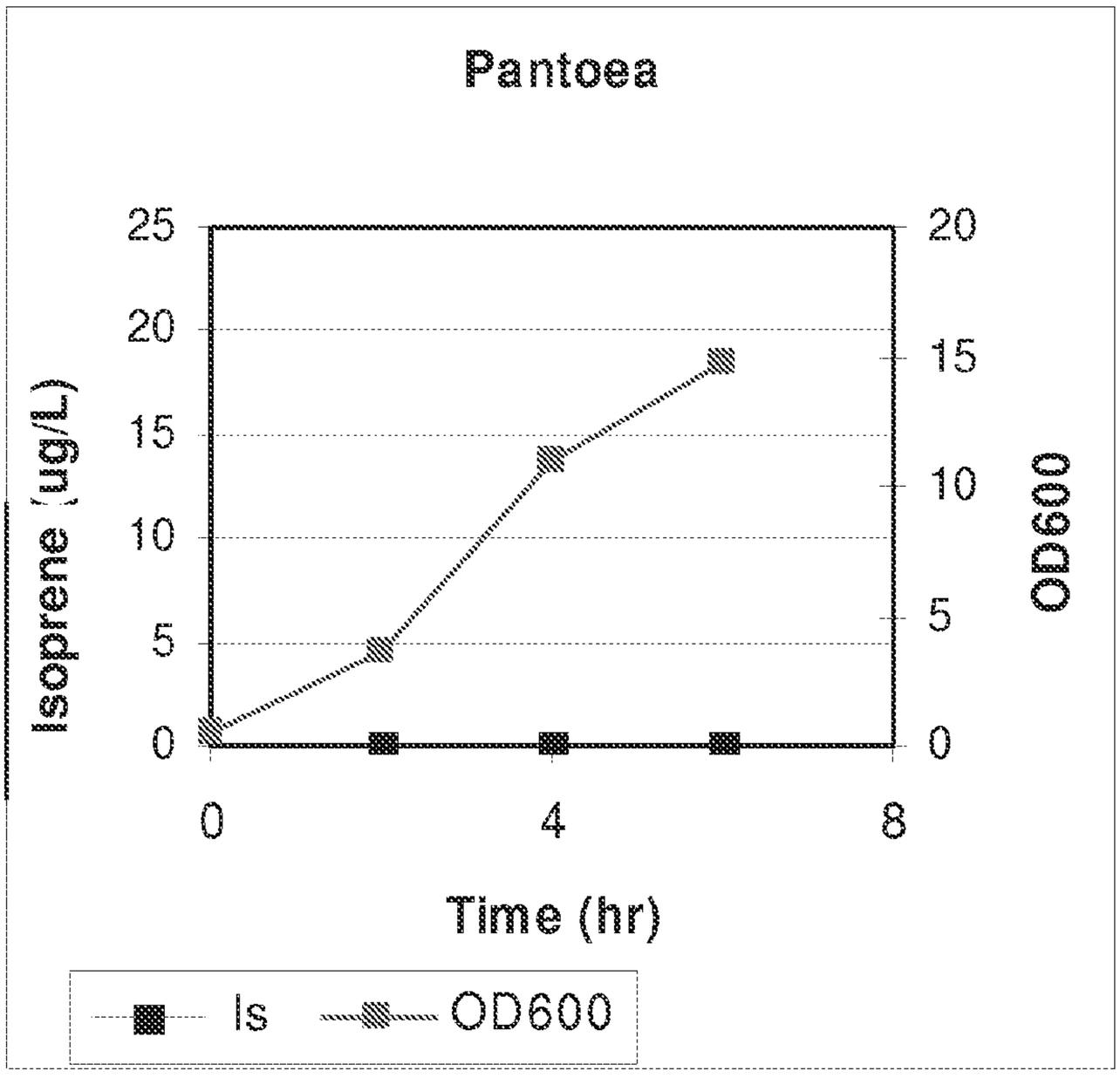

Pantoea
Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 10B

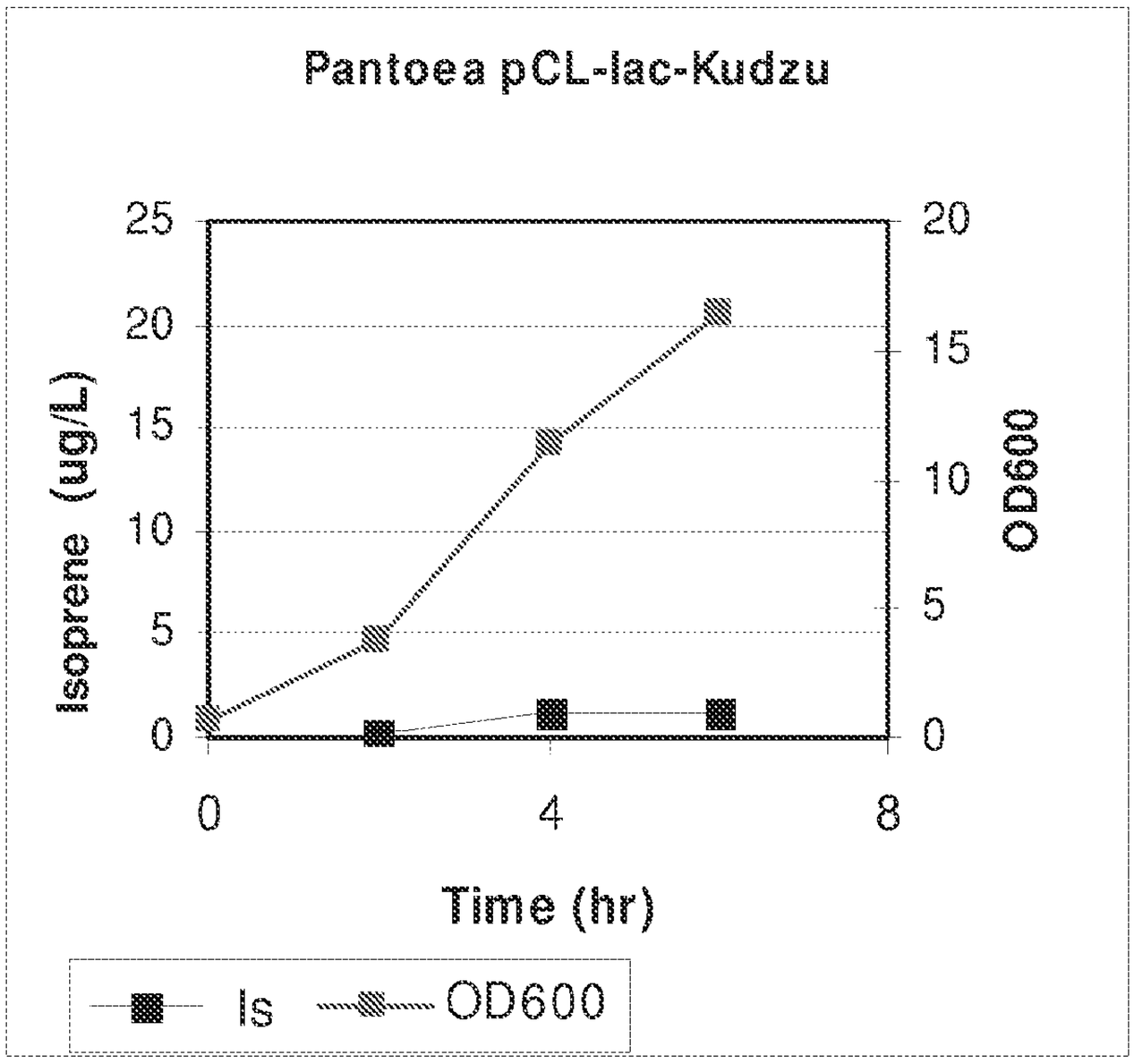

Pantoea pCL-lac-Kudzu
Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 10C

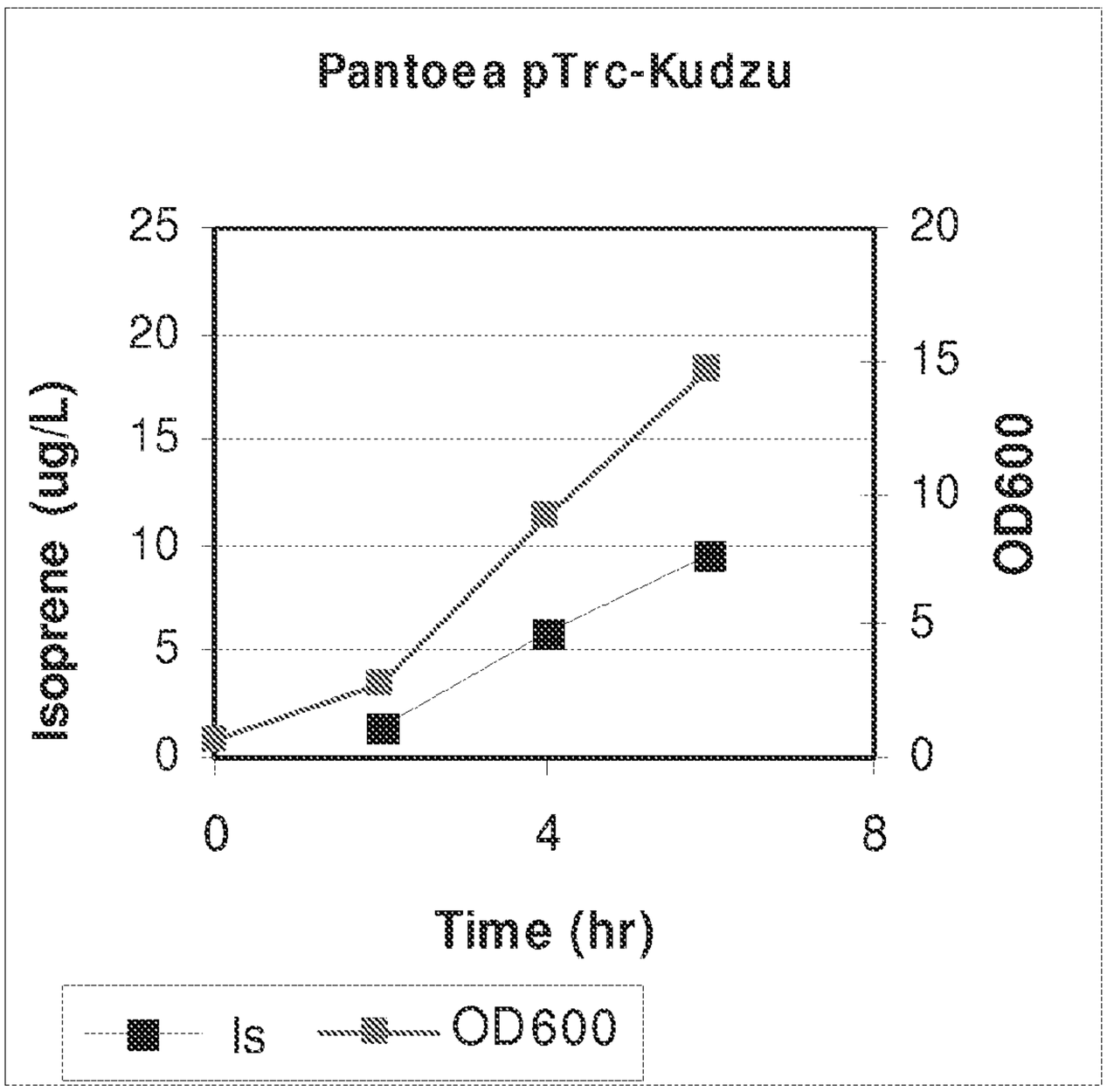

Pantoea pTrc-Kudzu
Isoprene (ug/L)
OD600
25
20
15
10
5
0
20
15
10
5
0
0
4
8
Time (hr)
Is
OD600

Figure 11

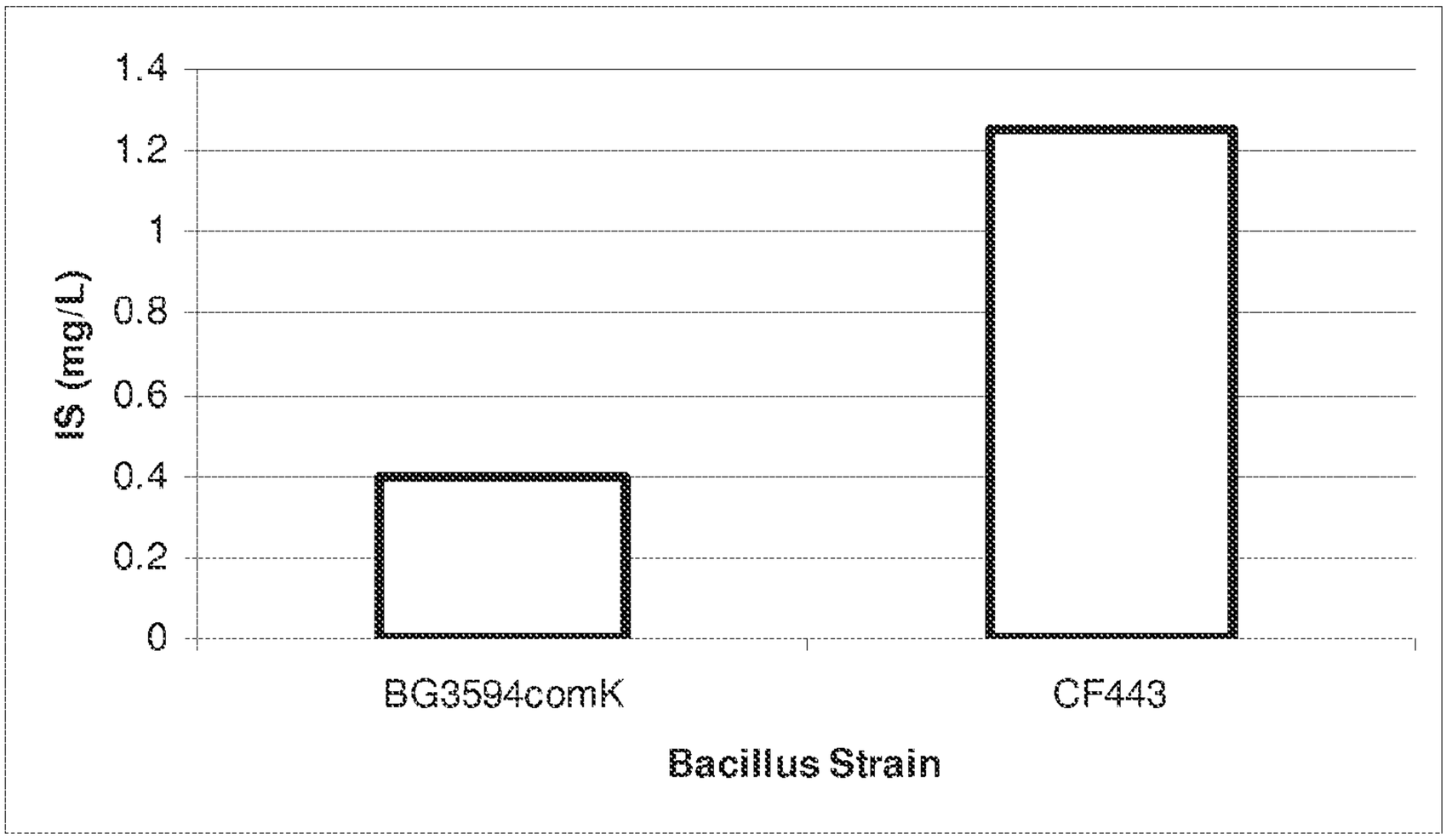


Figure 12A

1-
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Figure 12B

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Figure 12C

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(SEQ ID NO:57)

Figure 13

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(SEQ ID NO:8)

Figure 14

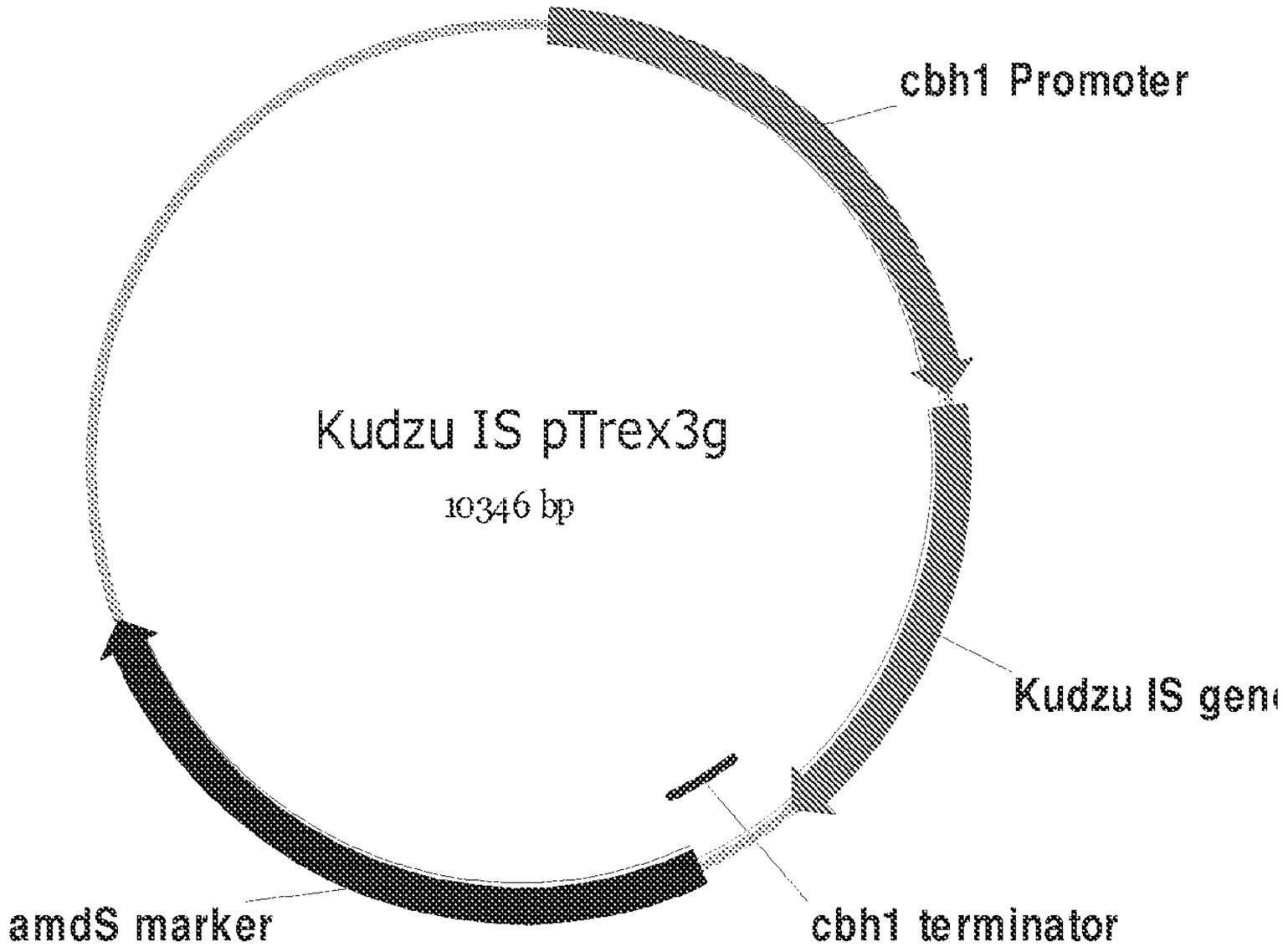
cbh1 Promoter
Kudzu IS pTrex3g
10346 bp
Kudzu IS gen
amdS marker
cbh1 terminator

Figure 15A

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  61 AAAGAAAGCA ATTGAAAACA AAACAAAACA ATTTTCATTC CTTCTCTTAT CATTCCTTTT
 121 CTTTTCTTTT CTCTCATTCA ACGCACTCCA TCGTATCCGT ATTCCTCTTA TTTTTTCTCT
 181 TTCTCTATAT CCATTTCTTT CTCTCTAGGT GTGTCCTCTC TCTCTCTTCA ATTTCTCTAC
 241 TCCGCATTCC AACGCATCCT TCCCCCAACC TCCCATTTCC TCCTTACGGC CCGATAGCGA
 301 TCGTCTTTCC CTCGCTATCA CTCGCTACCG GCCCCTCCTC TGCACCGTAA CCTCCTACGT
 361 ATTTACCATA TCATAAAGTT TTTTCCGACG CTTATCGCTG ACCCCCTGTC GCCCTCCTAT
 421 TGGCTTCCGG ATTATCTTCT TGTCCATAAG GTGATCCATG CTTCCTGAAG ATTCCCGAAA
 481 TGTGTCCACT TTGGCGGGGA ATCATTCCAT CCACTTCTTT CTCTCTCGCT TTCCTCATTC
 541 GGCGCTCCCC TTCCGCGTCT CATTGGTCTT CCGCTCCGTT TTTGCTTTGC CGATGTTACT
 601 TGGGGAGAGG TGCGATAATC CTTTCGCAAA AACTCGGTTT GACGCCTCCC ATGGTATAAA
 661 TAGTGGGTGG TGGACAGGTG CCTTCGCTTT TCTTTAAGCA AGAGAATCCC ATTGTCTTGA
 721 CTATCACGAA TTCACATACA TTATGAAGAT CACCGCTGTC ATTGCCCTTT TATTCTCACT
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 901 CCTTAAGAAG AGAAATGATA CACCTGCAAG TGGATATCAA GTTGAAAAAG TCGTAATTTT
 961 GTCACGTCAC GGTGTTAGGG CCCCTACAAA AATGACTCAA ACCATGCGTG ATGTCACTCC
1021 TAATACATGG CCAGAATGGC CCGTTAAATT AGGATATATT ACACCAAGAG GTGAACACTT
1081 GATATCACTT ATGGGCGGTT TTTACCGTCA AAAATTCCAG CAACAAGGAA TCCTTTCTCA
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1201 AAAAACTGGT GAAGCATTCC TTGCTGGTTT GGCACCACAA TGTGGCTTGA CAATTCATCA
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1981 TCAAACACTA GAACAACTTC GATCACAGAC TCCCCTTTCT CTAAATCAGC CTGCCGGATC
2041 TGTTCAACTT AAAATTCCAG GTTGCAATGA TCAAACAGCC GAGGGTTACT GTCCTCTTTC
2101 CACTTTTACA AGAGTTGTTT CCCAATCTGT TGAACCTGGA TGCCAACTTC AATAATGAGG
2161 ATCCAAGTAA GGGAATGAGA ATGTGATCCA CTTTTAATTC CTAATGAATA CATGCCTATA
2221 GTTCTTTTCT TTTGTTCTTT ATGTCGTTTT TCGATGGTAC GGCCGTTGTC AATCTCAGTT
2281 TGTGTGCTTG GTTGCAGCTT GGTTTCAAAT CTGTTCATCT CATGAATCTT TTACCATTTC
2341 ACCACACGTT TATACCATTC TCTCATAGAA TCTTCATCAA ACCATCTCGG GGTTAGAGTG
2401 GAAAGAAAGT CTTGTTCTTT TATTTCCTTT TTTCCATCTT CAAGGCTTTT CTTTTCTTCC
2461 TCCTCCTCGT TCATCTTGAG GTTTGACGTG TCTGTTTAGA ATTTTGAGCT GTTGCAGCAT
2521 CTTATTTTTT GTTTTGCGAA AACGAAGCGC TTTACTCTCT TCATCAGTTG GACGATTGTA
2581 CCTTTGAAAA CCAACTACTT TTGCATGTTT TGTATAGAAA TCAATGATAT TAGAATCCCA
2641 TCCTTTAATT TCTTTCAAAG TAGTTGAGCT ATAGTTAAGT GTAAGGGCCC TACTGCGAAA
2701 GCATTTGCCA AGGATGTTTT CATTAATCAA GAACGAAAGT TAGGGGATCG AAGACGATCA
2761 GATACCGTCG TAGTCTTAAC CATAAACTAT GCCGACTAGG GATCGGGCAA TGTTTCATTT
2821 ATCGACTTGC TCGGCACCTT ACGAGAAATC AAAGTCTTTG GGTTCCGGGG GGAGTATGGT
2881 CGCAAGGCTG AAACTTAAAG GAATTGACGG AAGGGCACCA CAATGGAGTG GAGCCTGCGG
2941 CTTAATTTGA CTCAACACGG GGAAACTCAC CAGGTCCAGA CATAGTAAGG ATTGACAGAT
3001 TGAGAGCTCT TTCTTGATTC TATGGGTGGT GGTGCATGGC CGTTCTTAGT TGGTGGAGTG
3061 ATTTGTCTGC TTAATTGCGA TAACGAACGA GACCTTAACC TGCTAAATAG CTGGATCAGC
3121 CATTTTGGCT GATCATTAGC TTCTTAGAGG GACTATTGGC ATAAAGCCAA TGGAAGTTTG
3181 AGGCAATAAC AGGTCTGTGA TGCCCTTAGA TGTTCTGGGC CGCACGCGCG CTACACTGAC
3241 GGAGCCAACG AGTTGAAAAA AATCTTTTGA TTTTTTATCC TTGGCCGGAA GGTCTGGGTA
3301 ATCTTGTTAA ACTCCGTCGT GCTGGGGATA GAGCATTGCA ATTATTGCGG CCGCTCCTCA
3361 ATTCGATGTT GCAGATTTTA CAAGTTTTTA AAATGTATTT CATTATTACT TTTTATATGC
3421 CTAATAAAAA AGCCATAGTT TAATCTATAG ATAACTTTTT TTCCAGTGCA CTAACGGACG
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Figure 15B

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3661 GTTGACTTGC TTTATCGAAT AATTACCGTA CCTGTAATTG GCTTGCTGGA TATAGCGGTA
3721 GTCTAATATC TAGCAAAAAT CTTTTGGGTG AAAAGGCTTG CAATTTCACG ACACCGAACT
3781 ATTTGTCATT TTTTAATAAG GAAGTTTTCC ATAAATTCCT GTAATTCTCG GTTGATCTAA
3841 TTGAAAAGAG TAGTTTTGCA TCACGATGAG GAGGGCTTTT GTAGAAAGAA ATACGAACGA
3901 AACGAAAATC AGCGTTGCCA TCGCTTTGGA CAAAGCTCCC TTACCTGAAG AGTCGAATTT
3961 TATTGATGAA CTTATAACTT CCAAGCATGC AAACCAAAAG GGAGAACAAG TAATCCAAGT
4021 AGACACGGGA ATTGGATTCT TGGATCACAT GTATCATGCA CTGGCTAAAC ATGCAGGCTG
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4321 GTCCTGTGAA ATGATCCCTC ACTTACTATA TTCCTTTTCG GTAGCAGCTG GAATTACTTT
4381 GCATGTTACC TGCTTATATG GTAGTAATGA CCATCATCGT GCTGAAAGCG CTTTTAAATC
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4621 CGTTAATTAG AAGCGTTATC TCGTGAAGGA ATATAGTACG TAGCCGTATA AATTGAATTG
4681 AATGTTCAGC TTATAGAATA GAGACACTTT GCTGTTCAAT GCGTCGTCAC TTACCATACT
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4861 CCATAGCTTC AAAATGTTTC TACTCCTTTT TTACTCTTCC AGATTTTCTC GGACTCCGCG
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4981 TCTAGGGTGT CGTTAATTAC CCGTACTAAA GGTTTGGAAA AGAAAAAAGA GACCGCCTCG
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5161 GTCTTCAATT TCTCAAGTTT CAGTTTCATT TTTCTTGTTC TATTACAACT TTTTTTACTT
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5281 TCGGCATAGT ATATCGGCAT AGTATAATAC GACAAGGTGA GGAACTAAAC CATGGCCAAG
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5761 CTTTGTCGAT ATCATGTAAT TAGTTATGTC ACGCTTACAT TCACGCCCTC CCCCCACATC
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5941 CAGACGCGAG CTTCCCAGTA AATGTGCCAT CTCGTAGGCA GAAAACGGTT CCCCCGTAGG
6001 GTCTCTCTCT TGGCCTCCTT TCTAGGTCGG GCTGATTGCT CTTGAAGCTC TCTAGGGGGG
6061 CTCACACCAT AGGCAGATAA CGTTCCCCAC CGGCTCGCCT CGTAAGCGCA CAAGGACTGC
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6241 CAAGGGAAAA CGCAAGCGCA AAGAGAAAGC AGGTAGCTTG CAGTGGGCTT ACATGGCGAT
6301 AGCTAGACTG GGCGGTTTTA TGGACAGCAA GCGAACCGGA ATTGCCAGCT GGGGCGCCCT
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6601 GGCGCCCGGT TCTTTTTGTC AAGACCGACC TGTCCGGTGC CCTGAATGAA CTGCAGGACG
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Figure 15C

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7201 TTTACGGTAT CGCCGCTCCC GATTCGCAGC GCATCGCCTT CTATCGCCTT CTTGACGAGT
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7801 GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCATTGA GAAAGCGCCA CGCTTCCCGA
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7921 GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC GCCACCTCTG
7981 ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA AAAACGCCAG
8041 CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT TTTGCTCACA TGTTCTTTCC
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8161 TCGCCGCAGC CGAACGACCG AGCGCAGCGA G
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(SEQ ID NO:11)

Figure 16

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   1 GAATTCAAAA CAAAATGTGT GCAACCTCCT CCCAGTTTAC TCAGATTACC GAGCATAATT
  61 CTCGACGATC TGCTAACTAC CAGCCGAACC TTTGGAACTT TGAGTTTCTC CAGTCTCTCG
 121 AAAATGACCT GAAGGTGGAA AAGCTCGAGG AGAAGGCGAC CAAACTCGAG GAGGAGGTGC
 181 GATGTATGAT CAACAGAGTT GACACCCAAC CCCTGTCTTT GCTGGAGCTG ATCGACGATG
 241 TGCAGCGGTT GGGTTTGACT TATAAATTCG AGAAGGACAT TATCAAGGCA CTGGAGAACA
 301 TTGTGCTCCT CGACGAGAAC AAGAAGAACA AGTCTGATCT TCACGCTACC GCTCTCTCTT
 361 TCCGACTTCT TCGACAACAC GGCTTCGAGG TGTCGCAGGA CGTCTTCGAG AGATTTAAGG
 421 ACAAGGAGGG AGGATTTAGC GGCGAGCTGA AGGGAGACGT TCAGGGTCTT CTCTCCTTGT
 481 ACGAGGCGTC CTACCTGGGA TTCGAGGGAG AGAACCTCCT GGAGGAAGCT CGTACATTTT
 541 CCATCACTCA CCTTAAGAAT AACCTTAAGG AGGGAATTAA CACCAAGGTG GCCGAGCAGG
 601 TTTCTCACGC CCTGGAGCTC CCCTACCACC AACGGCTCCA TAGACTGGAG GCTCGTTGGT
 661 TCCTGGACAA ATATGAGCCA AAGGAGCCTC ATCATCAGTT GCTGTTGGAG TTGGCCAAGC
 721 TGGACTTCAA TATGGTTCAG ACGCTGCACC AAAAGGAGTT GCAGGACCTG TCTCGATGGT
 781 GGACCGAGAT GGGATTGGCC TCGAAGCTGG ATTTTGTCCG TGACCGACTT ATGGAGGTCT
 841 ATTTTTGGGC CCTTGGAATG GCGCCTGACC CCCAGTTCGG AGAGTGCCGG AAGGCGGTGA
 901 CGAAGATGTT CGGTCTTGTG ACTATCATCG ACGACGTCTA CGATGTCTAC GGCACACTCG
 961 ACGAGTTGCA GCTGTTCACT GACGCCGTCG AGCGATGGGA TGTGAACGCC ATTAATACTC
1021 TCCCTGACTA TATGAAGCTG TGCTTCCTGG CTCTGTACAA CACTGTCAAC GATACCTCGT
1081 ACTCTATCCT CAAGGAGAAG GGACACAACA ATCTCTCCTA CTTGACCAAA TCCTGGCGAG
1141 AACTGTGCAA GGCTTTTCTG CAGGAGGCTA AATGGTCCAA TAACAAGATC ATTCCTGCTT
1201 TTTCTAAATA CCTGGAAAAT GCCTCGGTGT CGAGCTCTGG CGTCGCCCTT CTGGCCCCTT
1261 CCTACTTCTC CGTCTGCCAG CAGCAGGAGG ATATTTCCGA TCATGCTCTT AGATCGCTGA
1321 CCGATTTTCA CGGCCTCGTG CGATCTTCCT GCGTGATTTT TCGGTTGTGT AATGACCTTG
1381 CGACCTCTGC TGCTGAGCTG GAACGAGGCG AGACTACAAA TTCCATTATT TCTTACATGC
1441 ACGAAAACGA TGGAACATCT GAAGAACAGG CTAGAGAGGA ACTGCGAAAG TTGATCGACG
1501 CCGAGTGGAA GAAGATGAAC AGAGAGCGGG TGTCCGACTC TACCCTGCTT CCCAAGGCCT
1561 TCATGGAGAT CGCCGTGAAC ATGGCTCGAG TTTCCCATTG TACTTACCAG TACGGTGACG
1621 GCCTGGGTCG TCCGGACTAC GCTACAGAGA ACCGAATCAA GCTGCTGCTC ATCGACCCCT
1681 TCCCTATCAA CCAATTGATG TACGTGTAAT AGTCTAGAGG ATCC
```

(SEQ ID NO:12)

Figure 17

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   1 GAATTCAACA AAAATGTGCT CTGTTTCCAC TGAGAACGTG TCCTTTACTG AGACTGAGAC
  61 TGAAGCACGT AGAAGCGCCA ACTACGAACC CAACTCCTGG GATTATGACT TTCTGCTGTC
 121 TTCTGACACC GACGAGTCGA TCGAGGTTTA TAAGGATAAG GCCAAGAAAC TTGAGGCCGA
 181 GGTCAGACGA GAGATTAACA ACGAGAAGGC CGAGTTCCTG ACCCTTCTTG AGCTGATCGA
 241 CAACGTTCAA CGACTTGGTC TTGGTTACCG TTTCGAATCC GATATCCGAC GTGCATTGGA
 301 TCGATTTGTC TCGTCCGGAG GTTTCGATGG TGTGACTAAG ACGTCGCTGC ACGCCACAGC
 361 TCTTTCCTTC AGACTGTTGC GGCAGCATGG ATTTGAGGTT TCCCAGGAAG CCTTTTCTGG
 421 TTTCAAGGAT CAGAACGGAA ACTTTTTGGA GAATCTCAAG GAGGACACCA AGGCCATCCT
 481 GTCGTTGTAT GAGGCCTCGT TCCTGGCTCT TGAGGGCGAG AATATTCTGG ATGAGGCTCG
 541 GGTTTTCGCT ATTTCGCACC TGAAGGAGTT GTCGGAGGAA AAGATCGGAA AGGAACTGGC
 601 CGAGCAGGTC AACCATGCAC TTGAACTTCC CCTGCATCGA CGTACCCAGC GACTGGAGGC
 661 CGTGTGGAGC ATCGAGGCGT ACAGAAAAAA GGAGGATGCT AATCAGGTTC TGCTCGAACT
 721 CGCTATCCTC GACTATAACA TGATTCAGAG CGTGTACCAG CGTGACTTGC GAGAGACAAG
 781 CCGGTGGTGG CGACGGGTGG GACTGGCCAC GAAGCTCCAC TTTGCTAAAG ATCGATTGAT
 841 TGAGTCGTTC TACTGGGCAG TGGGTGTGGC CTTTGAGCCT CAGTACTCCG ACTGCCGAAA
 901 CTCCGTTGCA AAGATGTTTT CTTTTGTCAC TATCATCGAC GACATCTACG ATGTTTACGG
 961 CACTCTCGAT GAACTCGAAC TCTTCACGGA CGCTGTCGAG CGATGGGATG TGAATGCCAT
1021 TAATGATCTG CCAGATTATA TGAAGTTGTG TTTCTTGGCG CTCTACAACA CAATTAATGA
1081 AATTGCCTAC GACAACCTCA AGGACAAGGG AGAGAACATT CTGCCCTACC TTACTAAAGC
1141 CTGGGCCGAC CTGTGTAACG CCTTTTTGCA GGAAGCCAAG TGGCTCTATA ACAAATCTAC
1201 TCCTACATTT GATGACTACT TCGGCAACGC TTGGAAGTCT TCCAGCGGCC CTCTCCAGTT
1261 GATCTTCGCT TACTTTGCAG TGGTCCAGAA CATCAAGAAA GAGGAGATTG AGAACCTCCA
1321 GAAGTATCAC GACATCATCT CCCGACCTTC GCACATCTTT CGACTGTGCA ATGACCTTGC
1381 CTCCGCATCC GCTGAGATTG CCCGAGGAGA AACAGCCAAT TCTGTGTCGT GTTACATGCG
1441 TACAAAGGGC ATCTCCGAGG AGCTGGCTAC CGAGTCTGTG ATGAACCTGA TCGATGAAAC
1501 CTGTAAGAAG ATGAACAAAG AGAAACTGGG CGGTTCTCTG TTCGCCAAAC CATTTGTTGA
1561 AACCGCGATC AATCTGGCTC GTCAGTCTCA TTGTACTTAC CATAACGGTG ACGCGCACAC
1621 TTCGCCGGAC GAATTGACCC GTAAGCGTGT GCTTTCGGTG ATTACCGAGC CGATCCTGCC
1681 GTTCGAAAGA TAATAGGATC C
```

(SEQ ID NO:13)

Figure 18A1

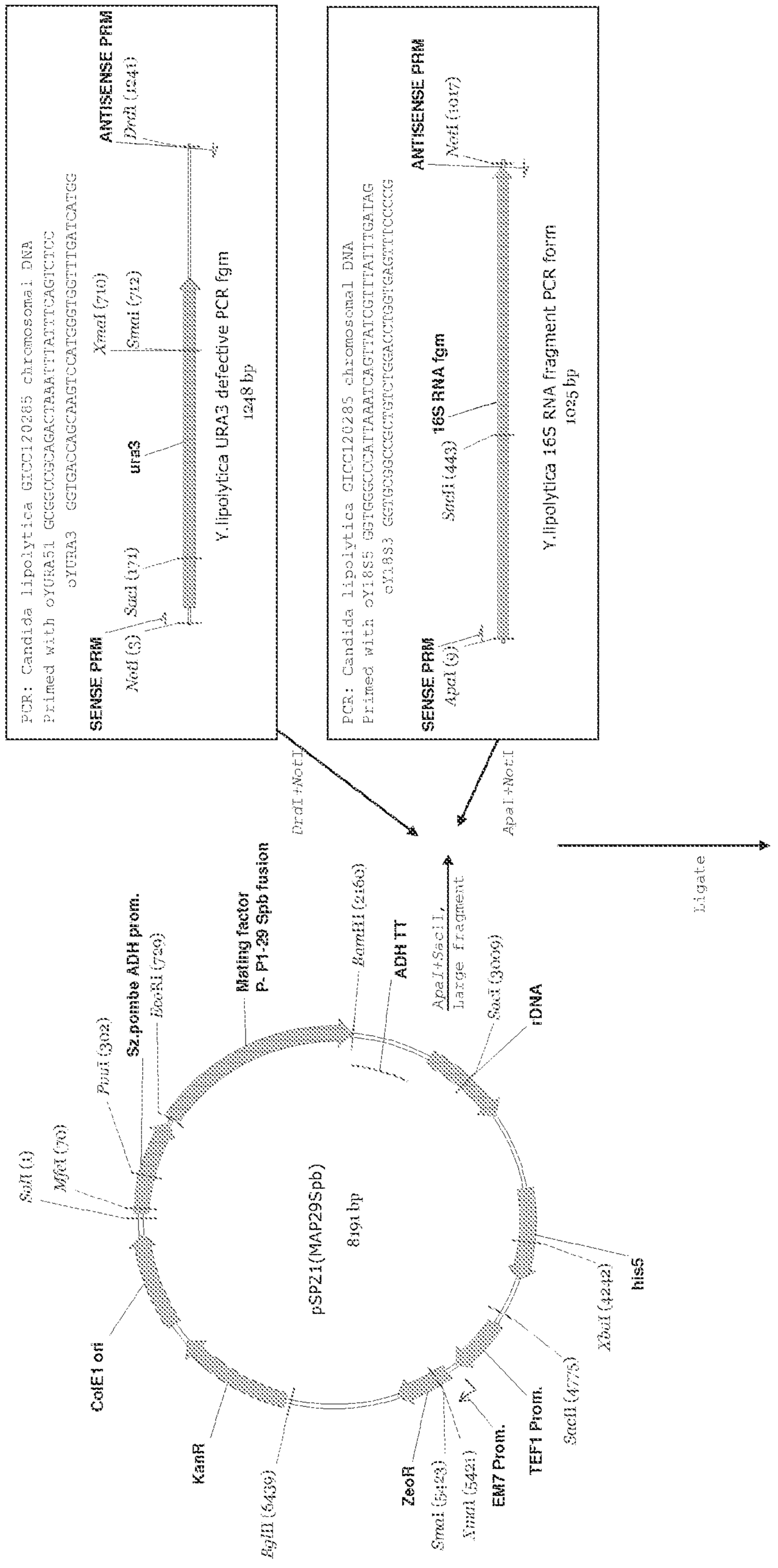

PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oYURA51 GCGGCCGCAGACTAAATTTATTCAGTCTCC
oYURA3 GGTGACCAGCAAGTCCATGGGTGGTTTGATCATGG
SENSE PRM
NotI (8)
SalI (171)
ura3
XmaI (710)
SmaI (712)
Y.lipolytica URA3 defective PCR fgm
1248 bp
DraI+NotI
PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oY18S5 GGTGGGCCCATTAAATCAGTTATTTGATAG
oY18S3 GGTGCGGCCGCTGTCTGGACCTGGTGAGTTTCCCCG
SENSE PRM
ApaI (9)
SacII (443)
16S RNA fgm
Y.lipolytica 16S RNA fragment PCR form
1025 bp
ApaI+NotI
Ligate
pSPZ1(MAP29Spb)
8191 bp
SalI (1)
MfeI (70)
PmaI (302)
Sz.pombe ADH prom.
BsoBI (729)
Mating factor
P- P1-29 Spb fusion
BamHI (2168)
ADH TT
ApaI+SacII
Large fragment
SacI (3009)
rDNA
his5
XbaI (4242)
SacII (4775)
TEF1 Prom.
EM7 Prom.
SmaI (5421)
SmaI (5423)
ZeoR
BglII (5439)
KanR
ColE1 ori

Figure 18A2

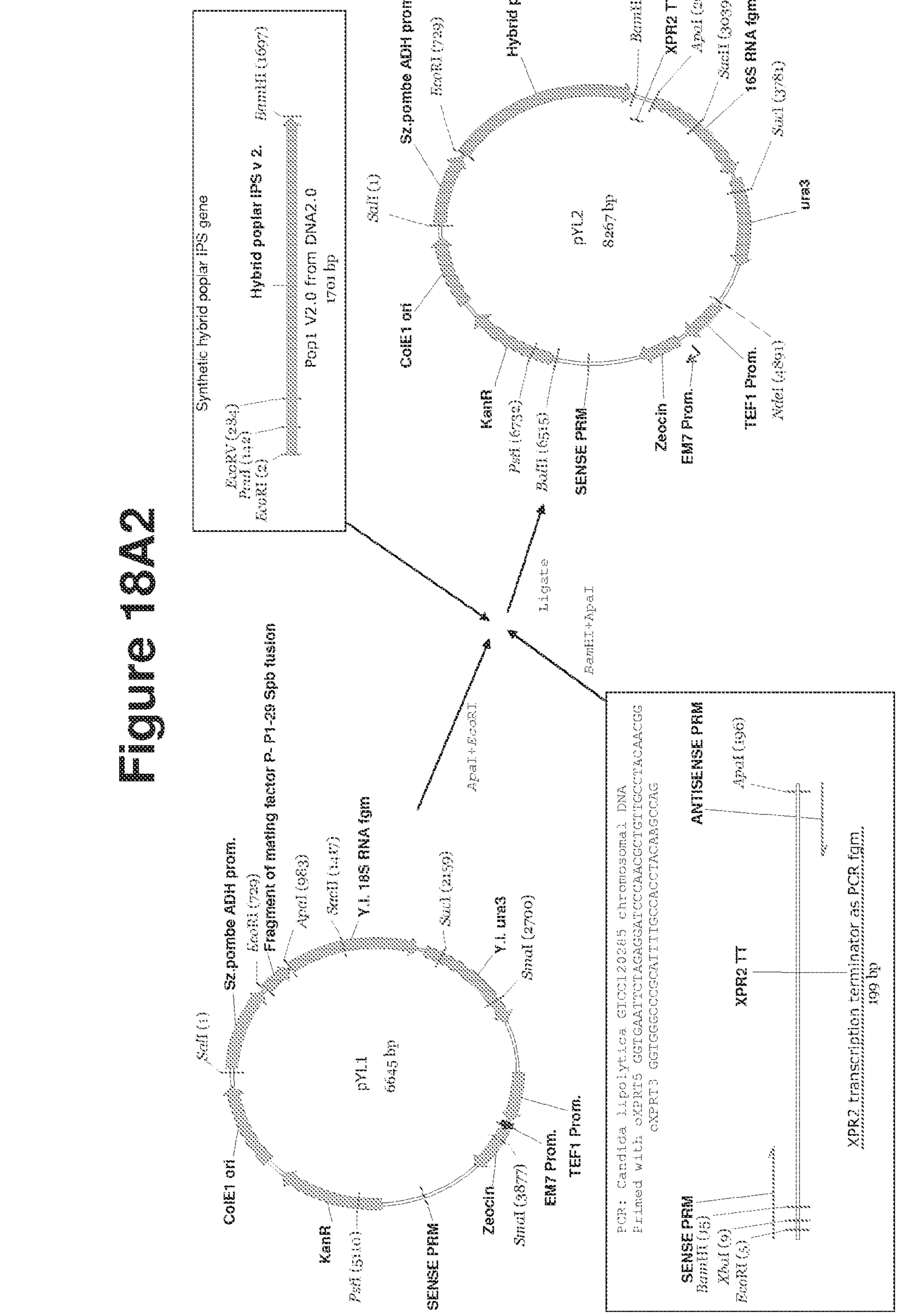

Synthetic hybrid poplar iPS gene
EcoRV (284)
PmlI (142)
EcoRI (2)
Hybrid poplar iPS v 2.
BamHI (1697)
Hybrid poplar iPS v 2
Pop1 V2.0 from DNA2.0
1701 bp
Ligate
ApaI
Sz.pombe ADH prom.
EcoRI (729)
Hybrid poplar iPS v2
BamHI (2424)
XPR2 TT
ApaI (2605)
SacII (3039)
16S RNA fgm
SalI (3781)
SalI (1)
CoIE1 ori
KanR
PstI (6732)
BclI (6515)
SENSE PRM
Zeocin
EM7 Prom.
TEF1 Prom.
NdeI (4891)
ura3
pYL2
8267 bp
ApaI+EcoRI
BamHI+
SalI (1)
Sz.pombe ADH prom.
EcoRI (729)
Fragment of mating factor P- P1-29 Spb fusion
ApaI (983)
SacII (1447)
Y.l. 18S RNA fgm
SacI (2159)
Y.l. ura3
SmaI (2700)
CoIE1 ori
KanR
PstI (5810)
SENSE PRM
Zeocin
SmaI (3877)
EM7 Prom.
TEF1 Prom.
pYL1.1
6645 bp
Candida lipolytica GICC120285 chromosomal DNA
Primed with oXPRT5 GGTGAATTCTAGAGGATCCCAACGCTGTTGCCTACAACGG
oXPRT3 GGTGGGCCCGCATTTGCCACCTACAAGCCAG
SENSE PRM
BamHI (15)
XbaI (9)
EcoRI (6)
XPR2 TT
ApaI (196)
ANTISENSE PRM
XPR2 transcription terminator as PCR fgm
199 bp

Figure 18B

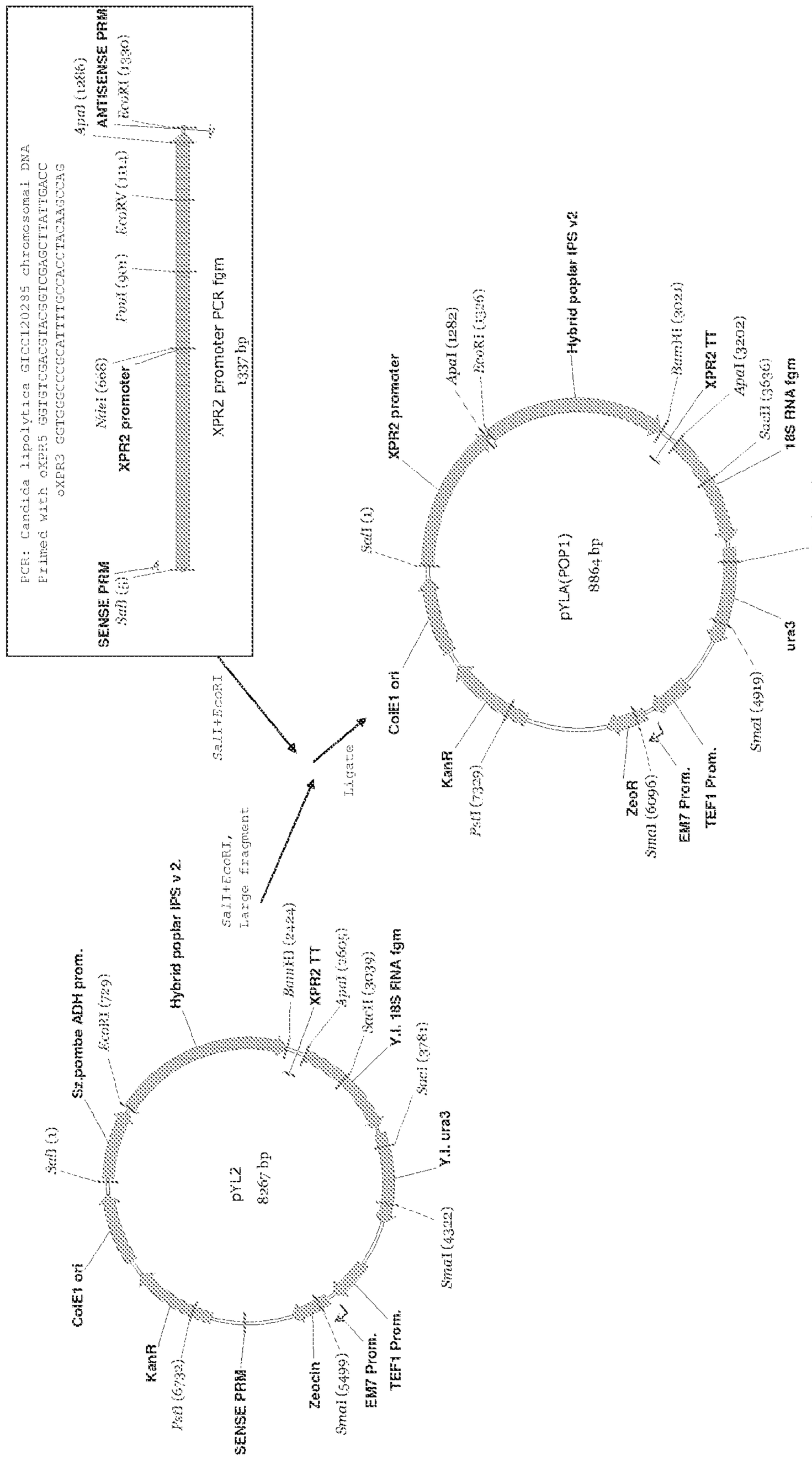

PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oXPR5 GGTGTCGACGIACGGTCGAGCTTATTGACC
oXPR3 GGTGGGCCCCGCATTTGCCACCTACAAGCCAG
SENSE PRM
SalI (6)
NdeI (668)
XPR2 promoter
PmlI (901)
EcoRV (1114)
ApaI (1286)
ANTISENSE PRM
EcoRI (1330)
XPR2 promoter PCR fgm
1337 bp
SalI+EcoRI
SalI+EcoRI,
Large fragment
Ligate
pYLA(POP1)
8864 bp
SalI (1)
XPR2 promoter
ApaI (1282)
EcoRI (1326)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
SacI (4478)
ura3
SmaI (4919)
ColE1 ori
KanR
PstI (7329)
ZeoR
SmaI (6096)
EM7 Prom.
TEF1 Prom.
pYL2
8267 bp
SalI (1)
Sz.pombe ADH prom.
EcoRI (729)
Hybrid poplar IPS v 2.
BamHI (2424)
XPR2 TT
ApaI (3605)
SacII (3039)
Y.l. 18S RNA fgm
SacI (3781)
Y.l. ura3
SmaI (4322)
ColE1 ori
KanR
PstI (6739)
SENSE PRM
Zeocin
SmaI (5499)
EM7 Prom.
TEF1 Prom.

Figure 18C

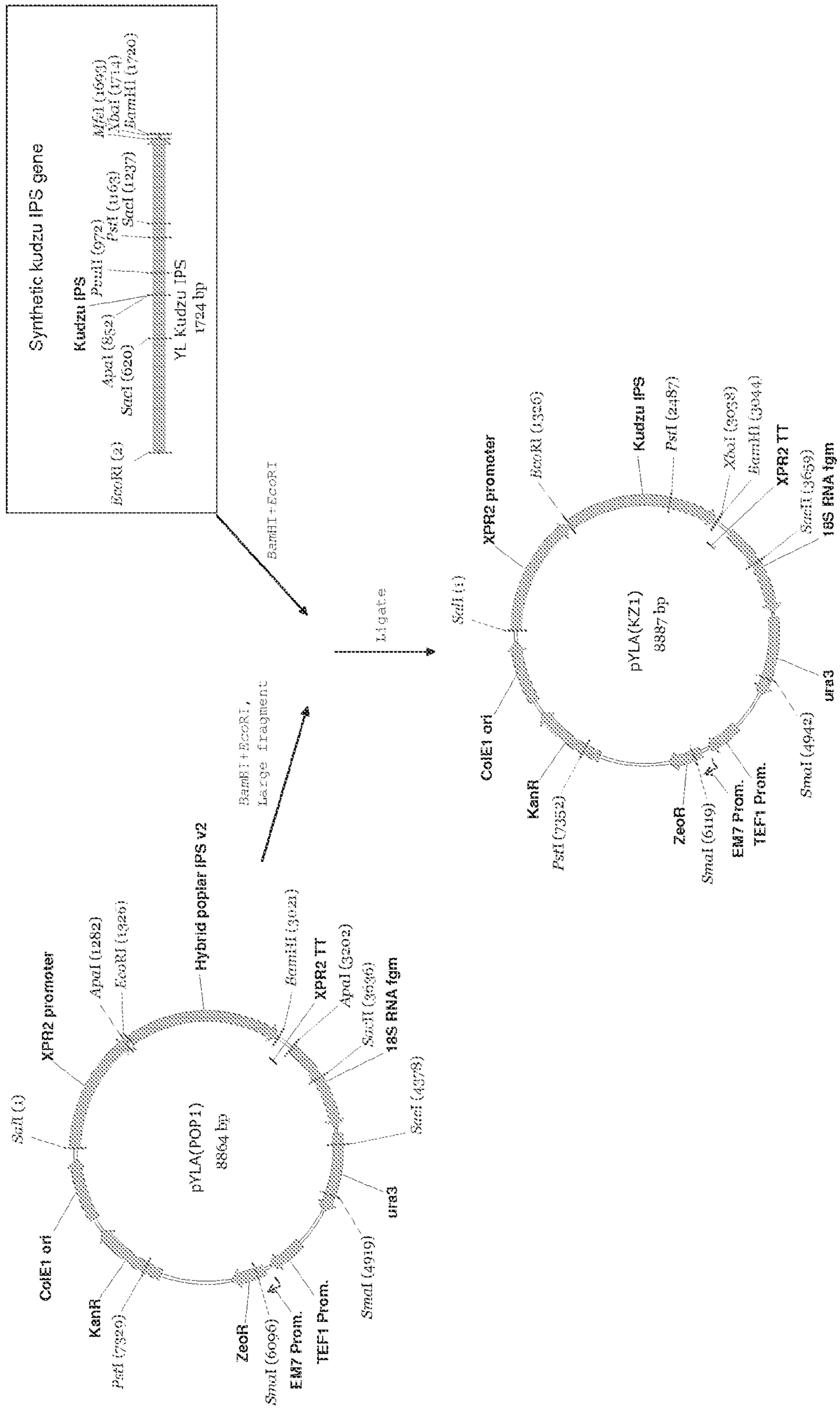

Synthetic kudzu IPS gene
Kudzu IPS
EcoRI (2)
ApaI (852)
SacI (620)
PmlI (972)
PstI (1163)
SacI (1237)
MfeI (1693)
XbaI (1714)
BamHI (1720)
YL Kudzu IPS
1724 bp
BamHI+EcoRI
Ligate
BamHI+EcoRI,
Large fragment
pYLA(KZ1)
8887 bp
SalI (1)
XPR2 promoter
EcoRI (1326)
Kudzu IPS
PstI (2487)
XbaI (3038)
BamHI (3044)
XPR2 TT
SacII (3659)
18S RNA tgm
ura3
SmaI (4942)
ColE1 ori
KanR
PstI (7352)
ZeoR
SmaI (6119)
EM7 Prom.
TEF1 Prom.
pYLA(POP1)
8864 bp
SalI (1)
XPR2 promoter
ApaI (1282)
EcoRI (1326)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA tgm
SacI (4378)
ura3
SmaI (4919)
ColE1 ori
KanR
PstI (7329)
ZeoR
SmaI (6996)
EM7 Prom.
TEF1 Prom.

Figure 18D

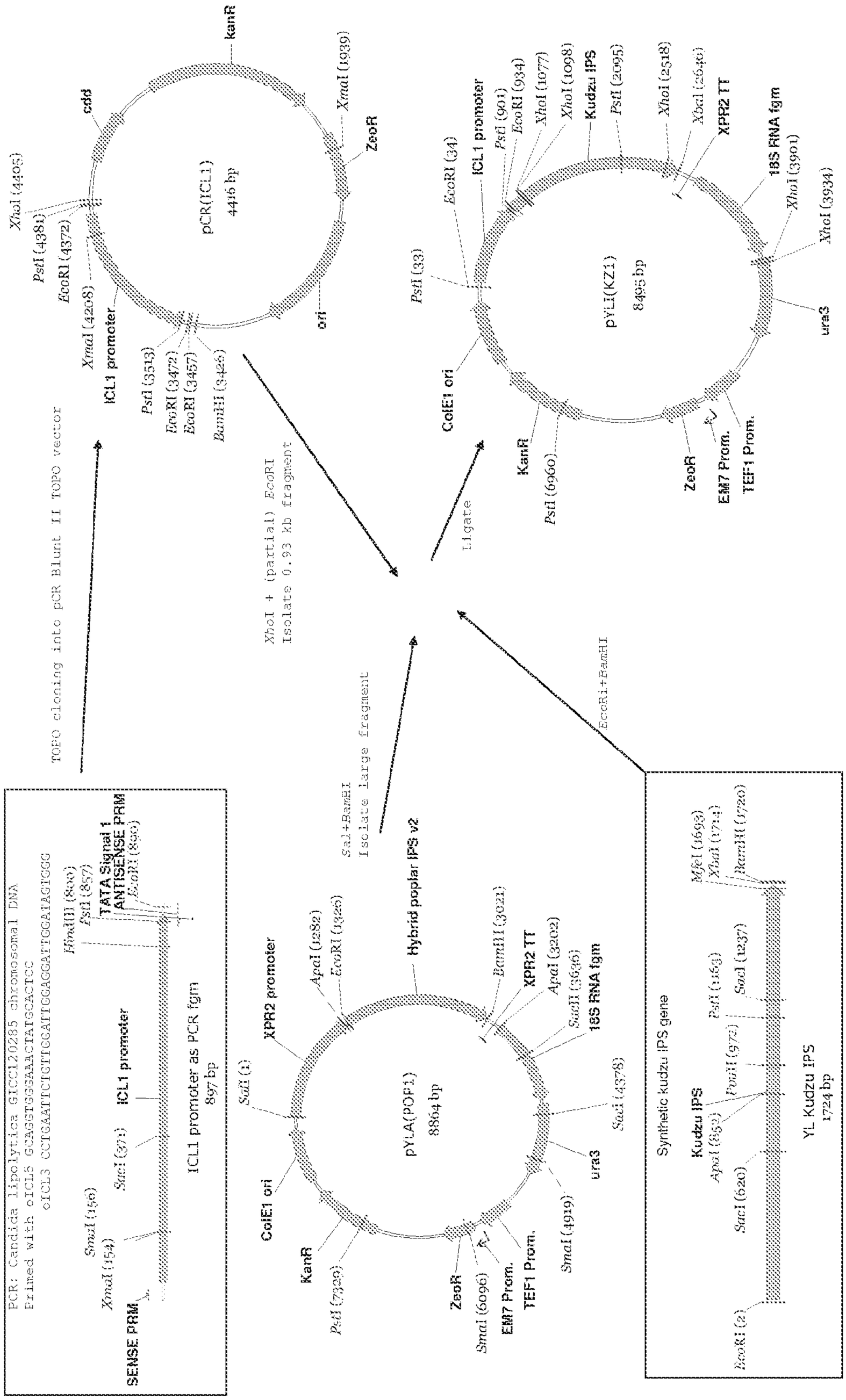

PCR: Candida lipolytica GICC120285 chromosomal DNA
Primed with oICL5 GCAGGTGGGAAACTATGCACTCC
oICL3 CCTGAATTCTGTTGGATTGGAGGATTGGATAGTGGG
ICL1 promoter as PCR fgm
897 bp
TOPO cloning into pCR Blunt II TOPO vector
pCR(ICL1)
4416 bp
kanR
cdd
ZeoR
ori
XhoI (4405)
PstI (4381)
EcoRI (4372)
SmaI (156)
XmaI (154)
SacI (371)
ICL1 promoter
TATA Signal 1
ANTISENSE PRM
EcoRI (890)
BmgBI (800)
PstI (857)
XmaI (1939)
XhoI + (partial) EcoRI
Isolate 0.93 kb fragment
Ligate
pYLA(POP1)
8864 bp
SalI (1)
XPR2 promoter
ApaI (1282)
EcoRI (1325)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
SacI (4378)
ura3
SmaI (4919)
ColE1 ori
KanR
PstI (7329)
ZeoR
SmaI (6096)
EM7 Prom.
TEF1 Prom.
Sal+BamHI
Isolate large fragment
EccRi+BamHI
pYLI(KZ1)
8495 bp
EcoRI (34)
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)
PstI (33)
ColE1 ori
KanR
PstI (6960)
ZeoR
EM7 Prom.
TEF1 Prom.
ura3
Synthetic kudzu IPS gene
Kudzu IPS
ApaI (852)
SacI (620)
PmlI (972)
PstI (1168)
SacI (1237)
MfeI (1693)
XbaI (1714)
BamHI (1720)
YL Kudzu IPS
1724 bp
EcoRI (2)

Figure 18E

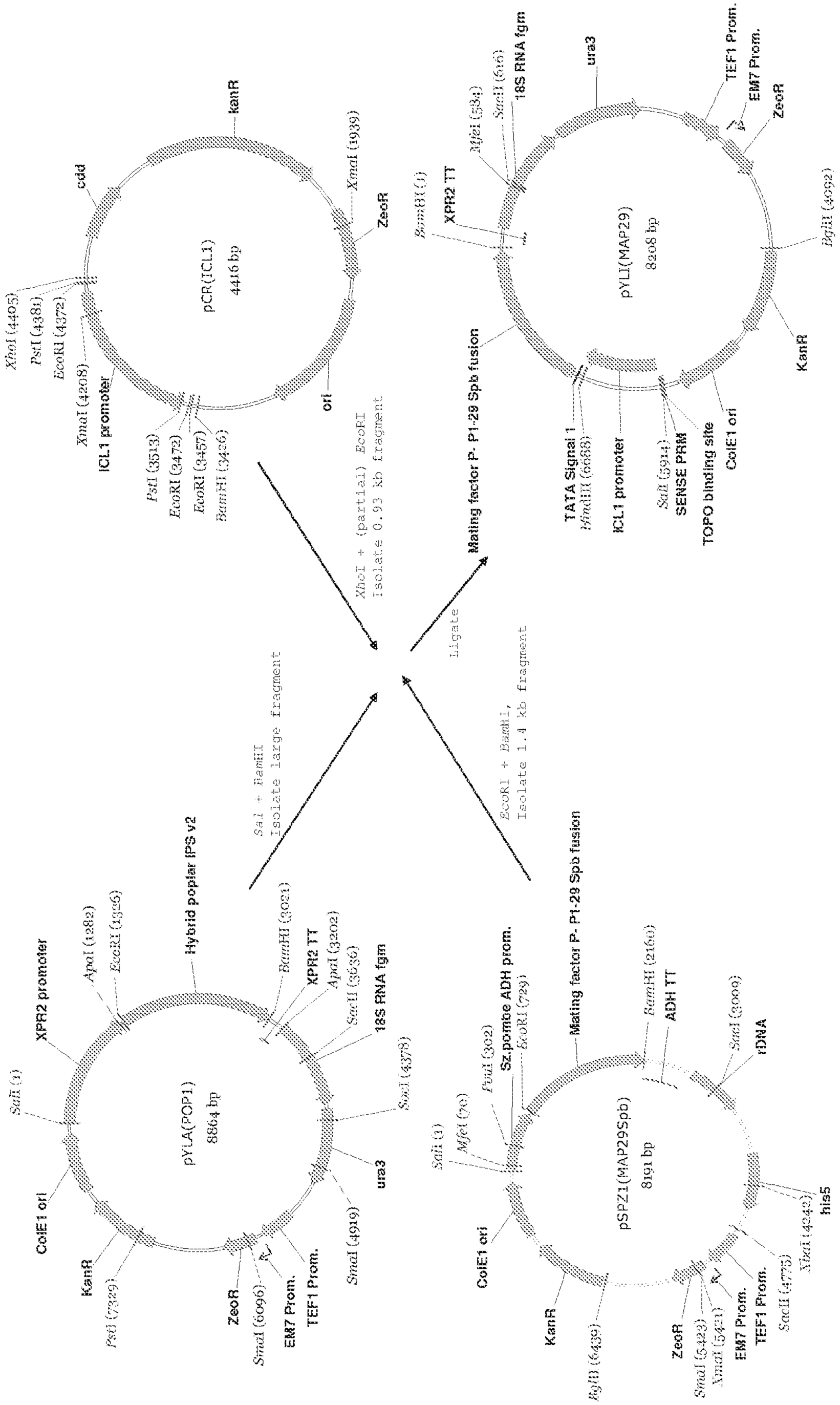

cdd
kanR
XmaI (1939)
ZeoR
XhoI (4405)
PstI (4381)
EcoRI (4372)
XmaI (4208)
ICL1 promoter
pCR(ICL1)
4416 bp
PstI (3513)
EcoRI (3472)
EcoRI (3457)
BamHI (3426)
ori
XhoI + (partial) EcoRI
Isolate 0.93 kb fragment
Ligate
BamHI (1)
XPR2 TT
MfeI (584)
SacII (616)
18S RNA fgm
ura3
TEF1 Prom.
EM7 Prom.
ZeoR
BglII (4092)
pYLI(MAP29)
8208 bp
Mating factor P- P1-29 Spb fusion
TATA Signal 1
HindIII (6683)
ICL1 promoter
SalI (3914)
SENSE PRM
TOPO binding site
ColE1 ori
KanR
SalI (1)
XPR2 promoter
ApaI (1282)
EcoRI (1326)
Hybrid poplar IPS v2
BamHI (3021)
XPR2 TT
ApaI (3202)
SacII (3636)
18S RNA fgm
SmaI (4378)
ura3
SmaI (4919)
ori
pYLA(POP1)
8864 bp
PstI (7329)
KanR
ColE1 ori
ZeoR
SmaI (6096)
EM7 Prom.
TEF1 Prom.
Sal + BamHI
Isolate large fragment
EcoRI + BamHI,
Isolate 1.4 kb fragment
SalI (1)
MfeI (70)
FseI (302)
Sz.pombe ADH prom.
EcoRI (729)
Mating factor P- P1-29 Spb fusion
BamHI (2160)
ADH TT
SmaI (3009)
rDNA
pSPZ1(MAP29Spb)
8191 bp
ColE1 ori
KanR
BglII (6439)
ZeoR
SmaI (5423)
XmaI (5421)
EM7 Prom.
TEF1 Prom.
SacII (4775)
XbaI (4242)
his5

Figure 18F

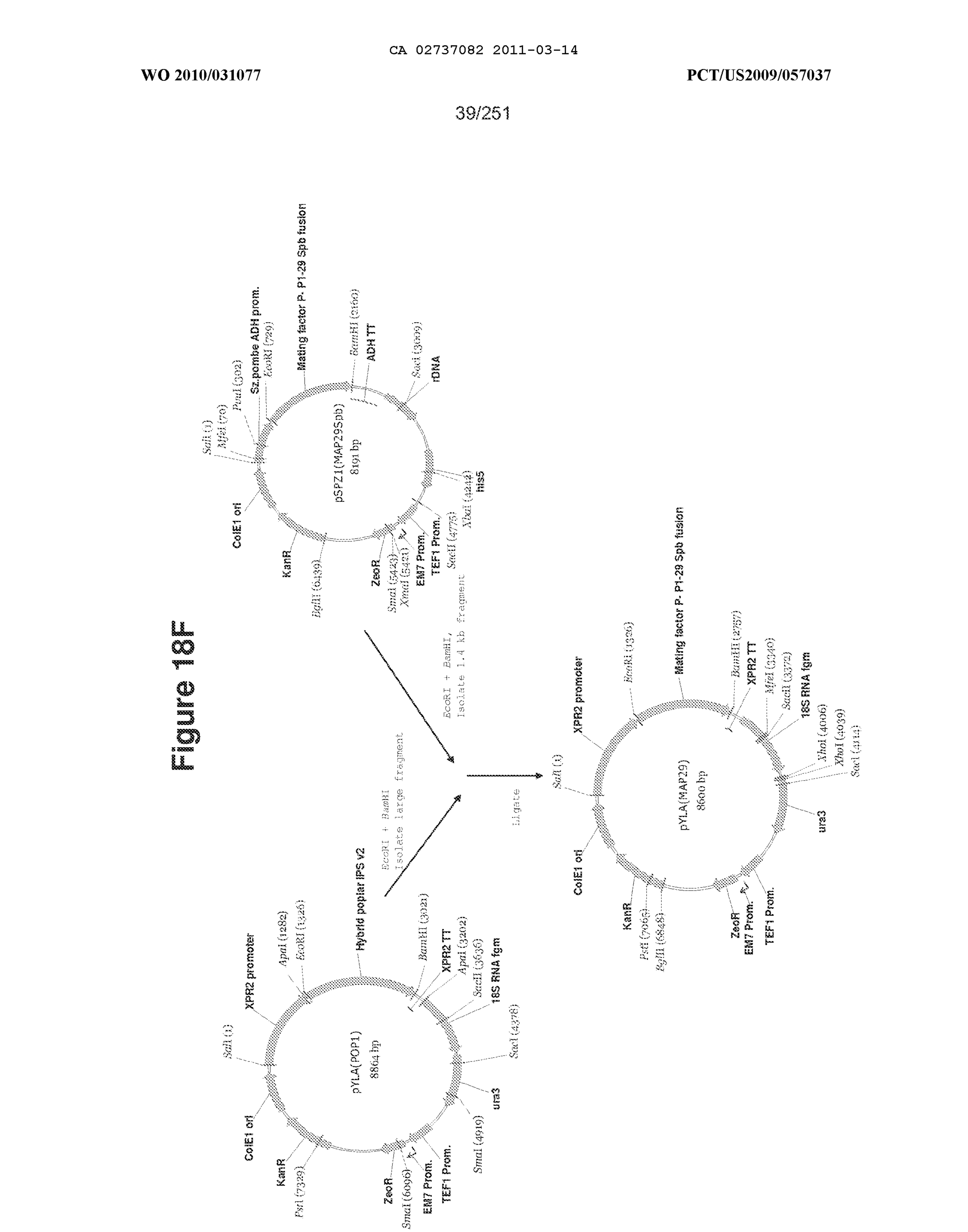

pSPZ1(MAP29Spb)
8191 bp
ColE1 ori
KanR
ZeoR
EM7 Prom.
TEF1 Prom.
Mating factor P-
Sz.pombe ADH prom
ADH TT
rDNA
his5
SalI (1)
MfeI (70)
PmaI (302)
EcoRI (729)
BamHI (2160)
SacI (3609)
BglII (6439)
SmaI (5443)
XmaI (5421)
SacII (4775)
XbaI (4244)
EcoRI + BamHI
Isolate large fragment
Ligate
EcoRI + BamHI,
Isolate 1.4 kb fragment
pYLA(MAP29)
8600 bp
XPR2 promoter
Hybrid poplar IPS v2
XPR2 TT
18S RNA fgm
ura3
ApaI (1282)
EcoRI (1526)
BamHI (3021)
ApaI (3202)
SacII (3636)
SacI (4378)
pYLA(POP1)
8864 bp
PstI (7329)
SmaI (6096)
SmaI (4919)
BamHI (2757)
MfeI (3340)
SacII (3372)
XhoI (4006)
XhoI (4039)
SacI (4144)
PstI (7068)
BglII (6848)

Figure 19A

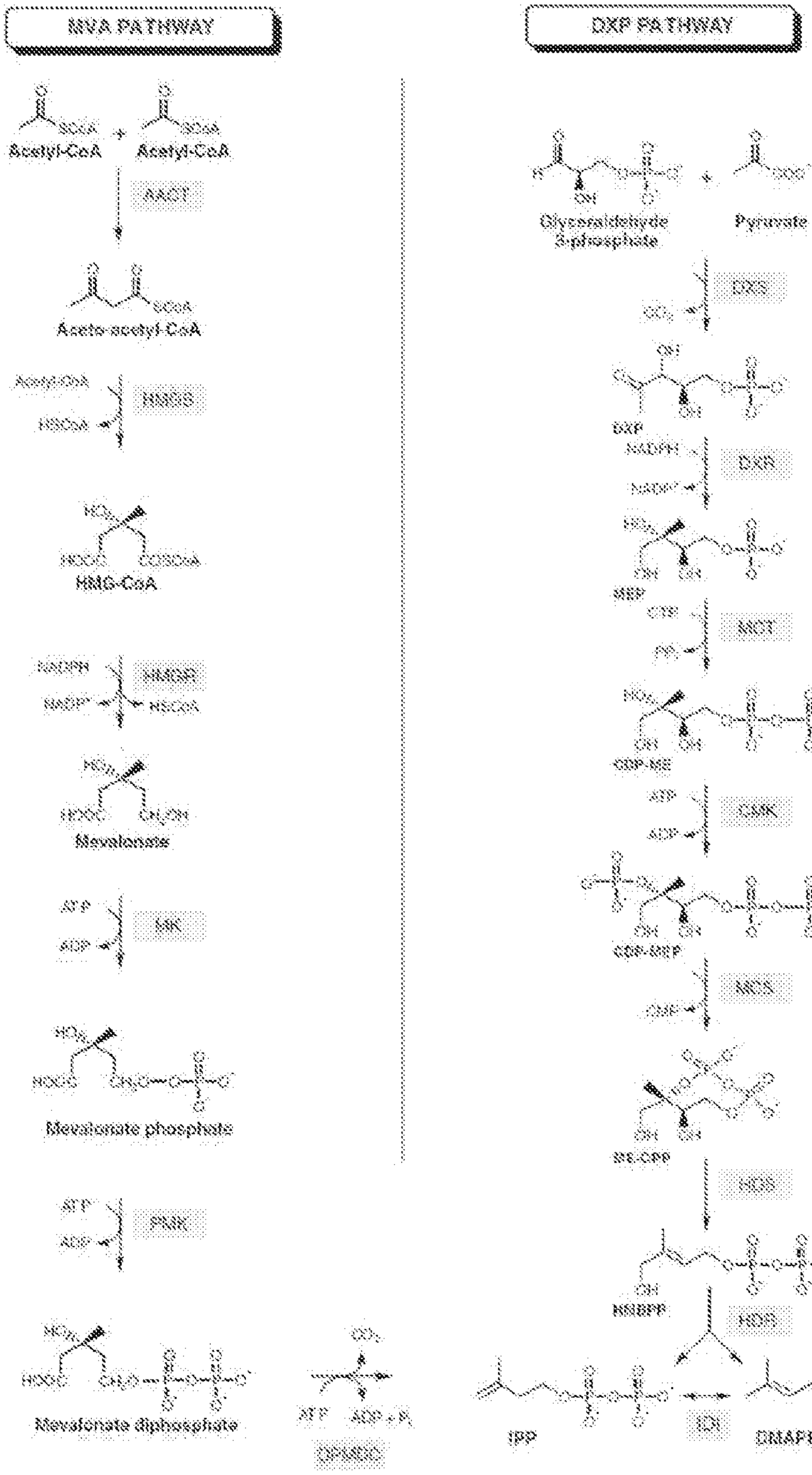
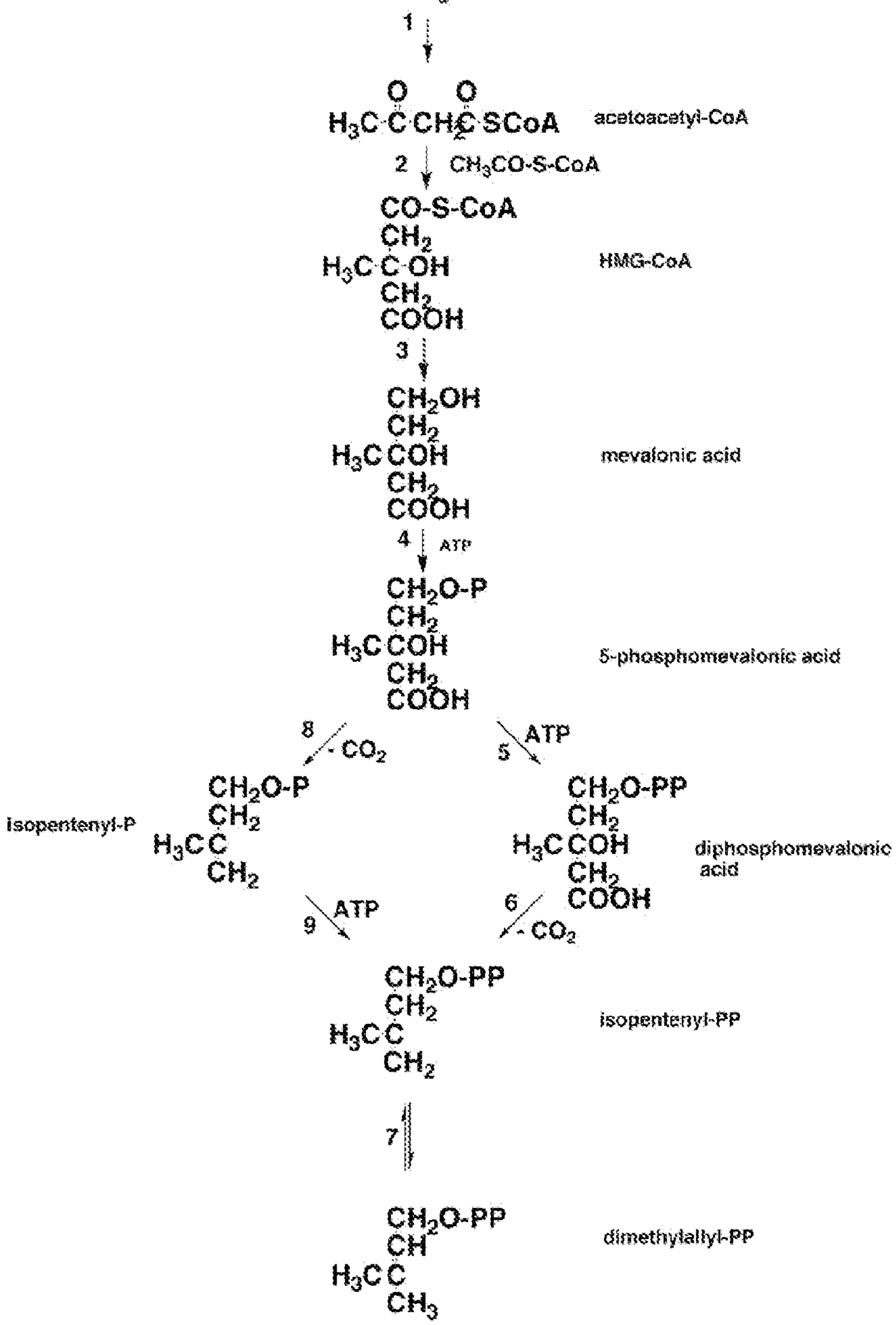

MVA PATHWAY
DXP PATHWAY
Acetyl-CoA
Acetyl-CoA
AACT
Aceto-acetyl-CoA
HMGS
HMG-CoA
HMGR
Mevalonate
MK
Mevalonate phosphate
PMK
Mevalonate diphosphate
MDD
IPP
IDI
DMAPP
Glyceraldehyde 3-phosphate
Pyruvate
DXS
DXP
DXR
MEP
MCT
CDP-ME
CMK
CDP-MEP
MCS
ME-cPP
HDS
HMBPP
HDR

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

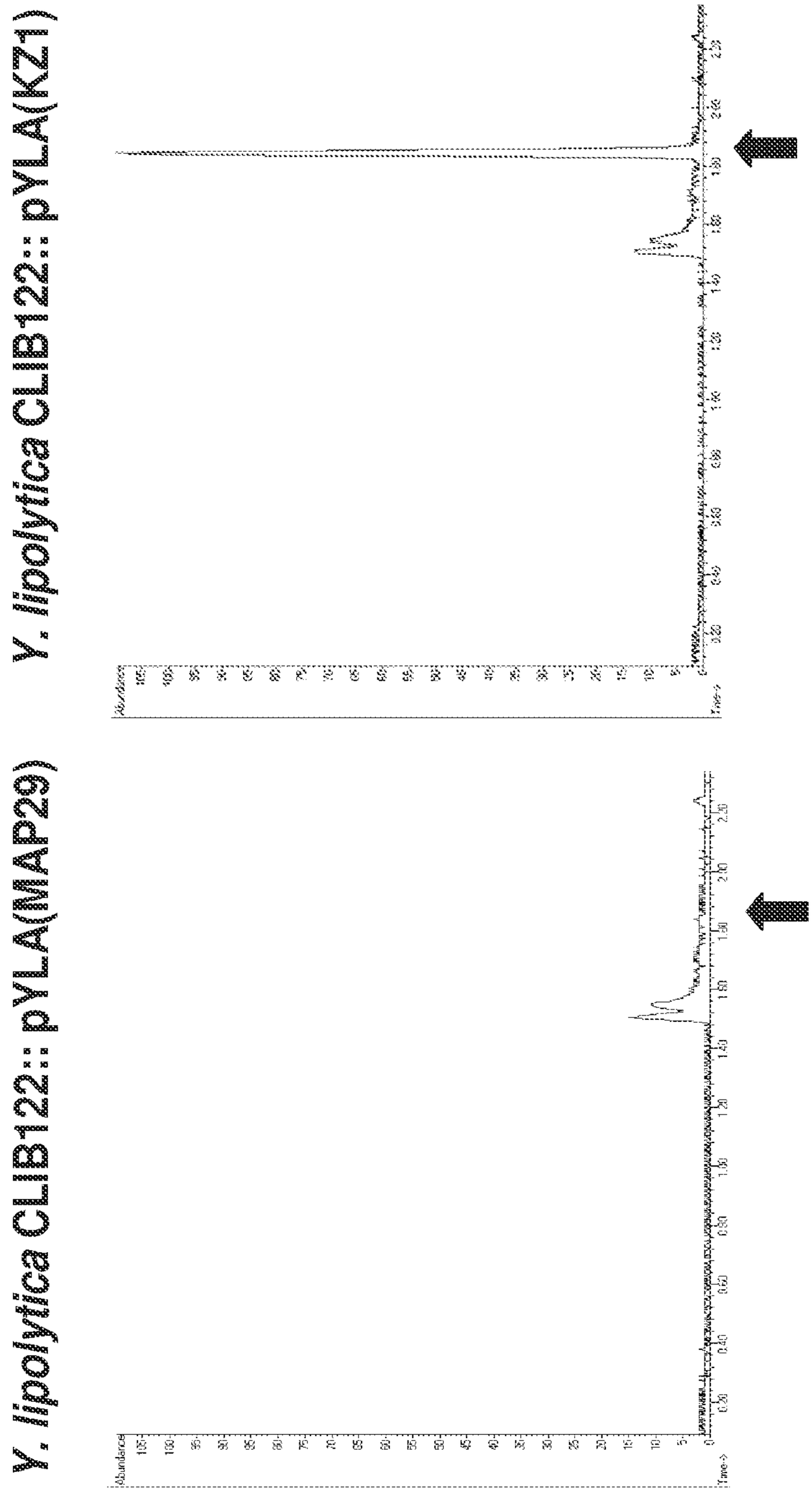

Y. lipolytica CLIB122::pYLA(KZ1)
Y. lipolytica CLIB122::pYLA(MAP29)
Abundance
Time->

Figure 20

Figure 21

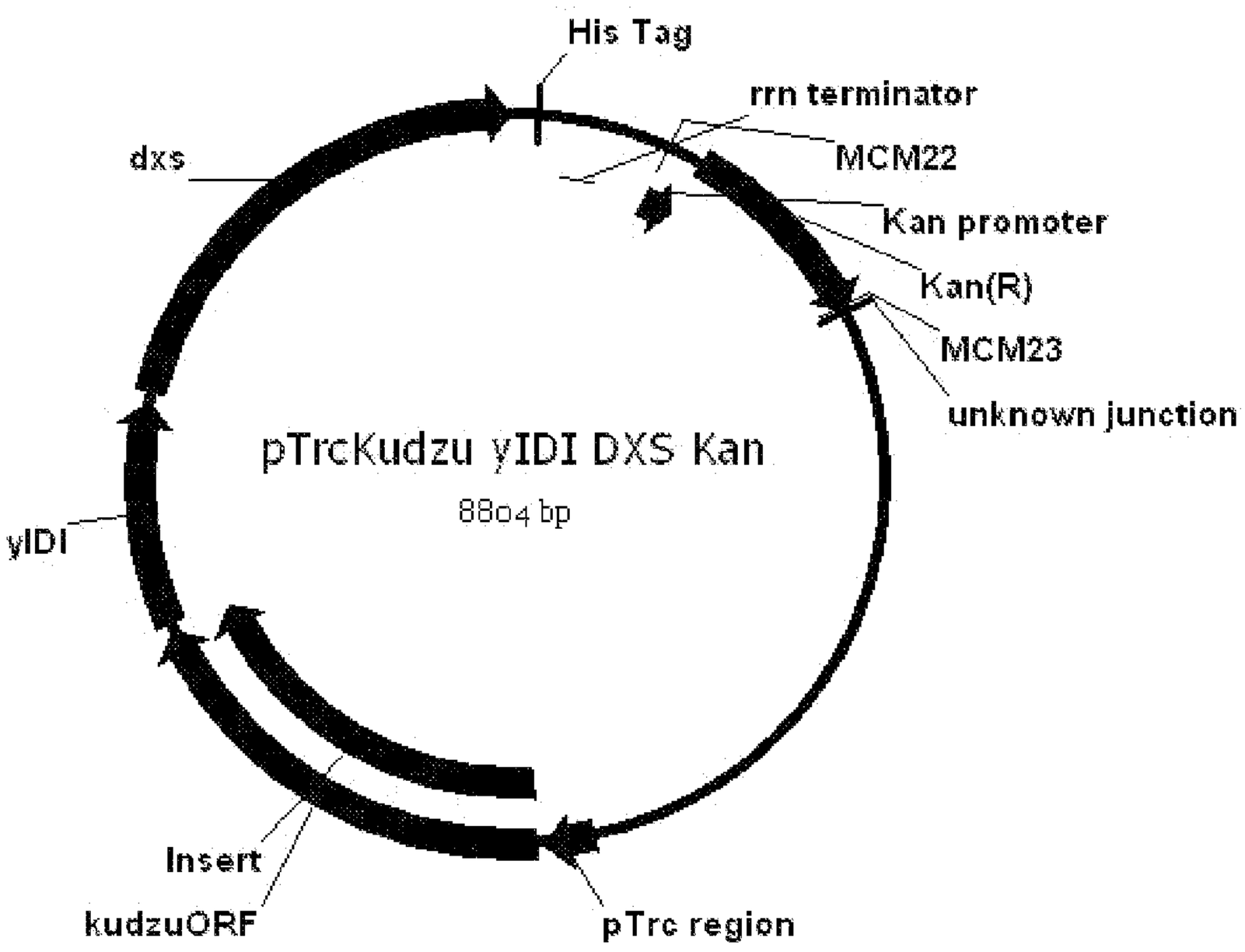

His Tag
rrn terminator
MCM22
dxs
Kan promoter
Kan(R)
MCM23
unknown junction
pTrcKudzu yIDI DXS Kan
8804 bp
yIDI
Insert
kudzuORF
pTrc region

Figure 22A

1-
gctggtaccatatgggaattcgaagctttctagaacaaaaaactcatctcagaagaggatctgaa
tagcgccgtcgaccatcatcatcatcattgagtttaaacggtctccagcttggctgttttg
gcggatgagagaagattttcagcctgatacagattaaatcagaacgcagaagcggtctgataaa
acagaatttgcctggcggcagtagcgcggtggtcccacctgaccccatgccgaactcagaagtg
aaacgccgtagcgccgatggtagtgtggggtctccccatgcgagagtagggaactgccaggcat
caaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtga
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Figure 22B

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Figure 22C

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Figure 22D

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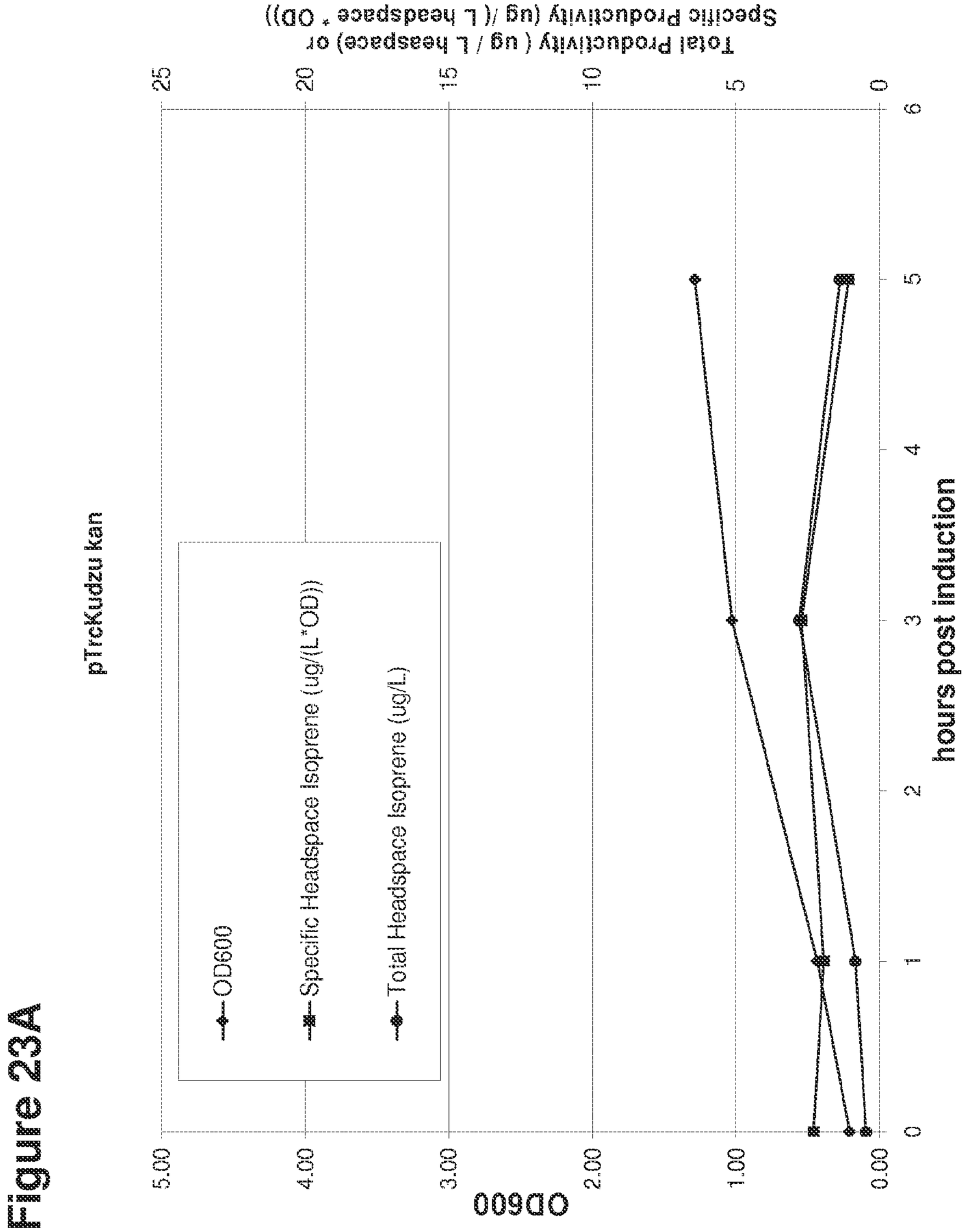
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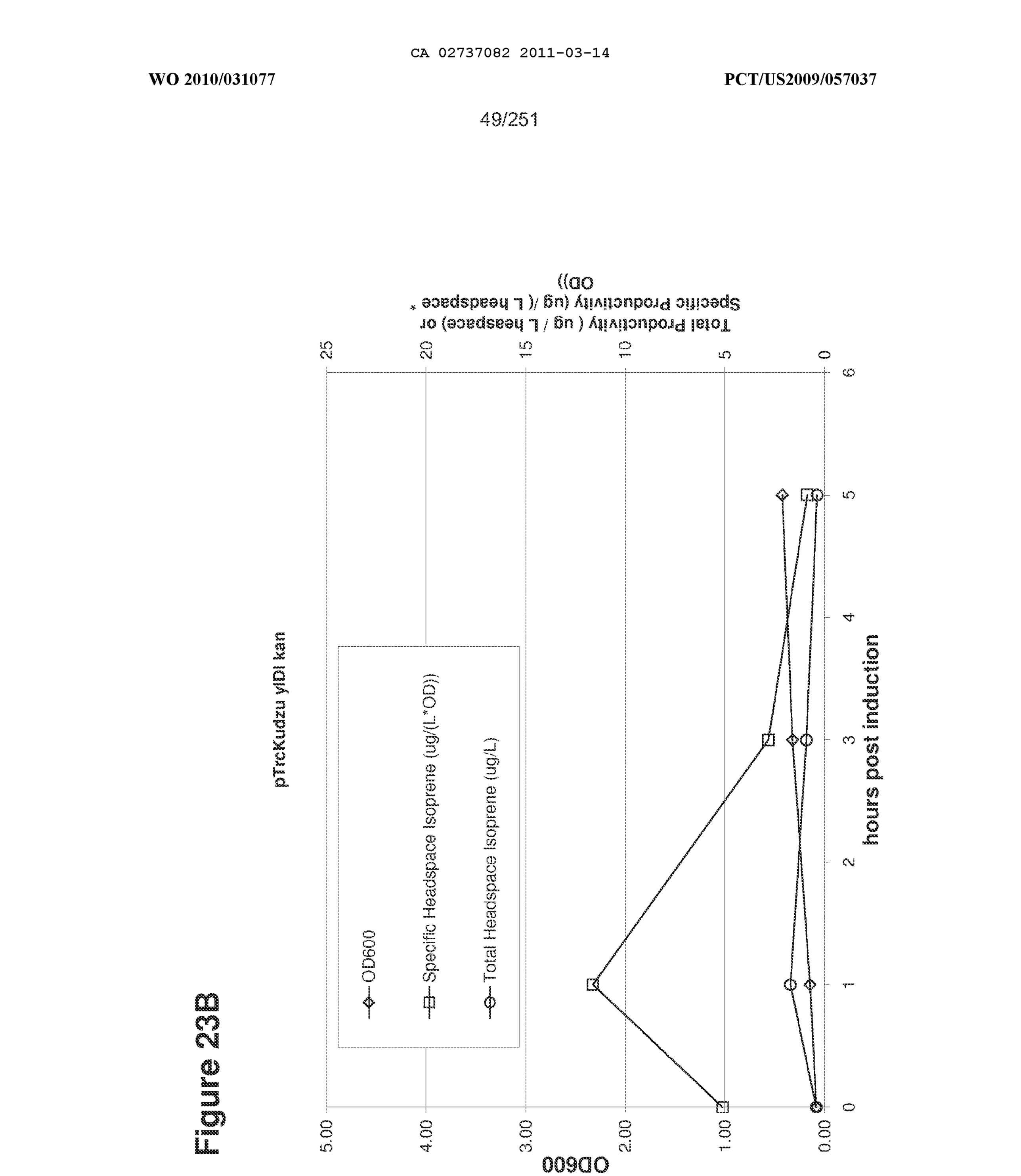
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
hours post induction
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
OD600
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
1
2
3
4
5
6

Fig. 23A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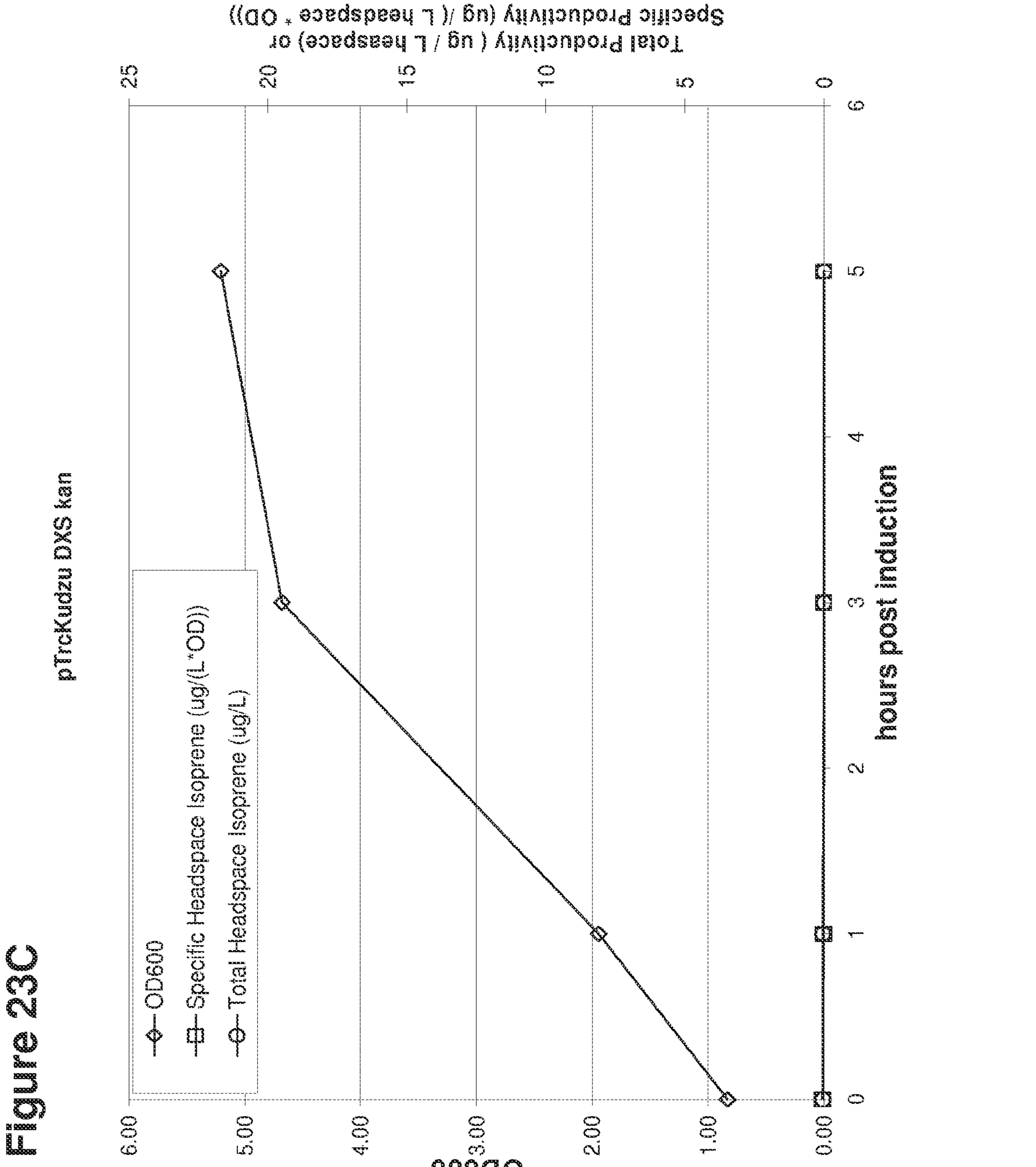
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
hours post induction
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
OD600

Figure 23B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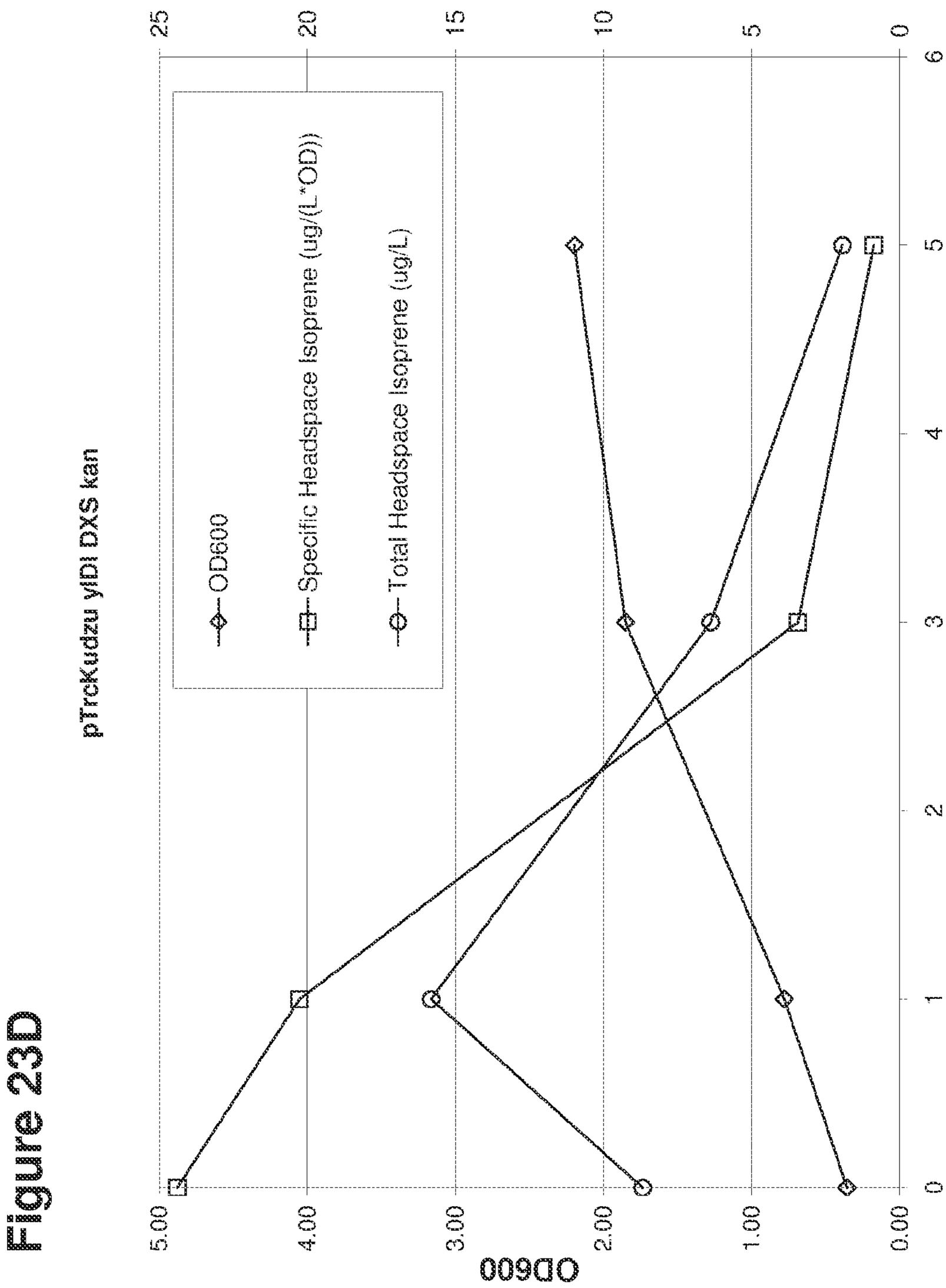
Total Headspace Isoprene (ug/L)
hours post induction
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
OD600
0.00
1.00
2.00
3.00
4.00
5.00
6.00
0
1
2
3
4
5
6
10
15
20
25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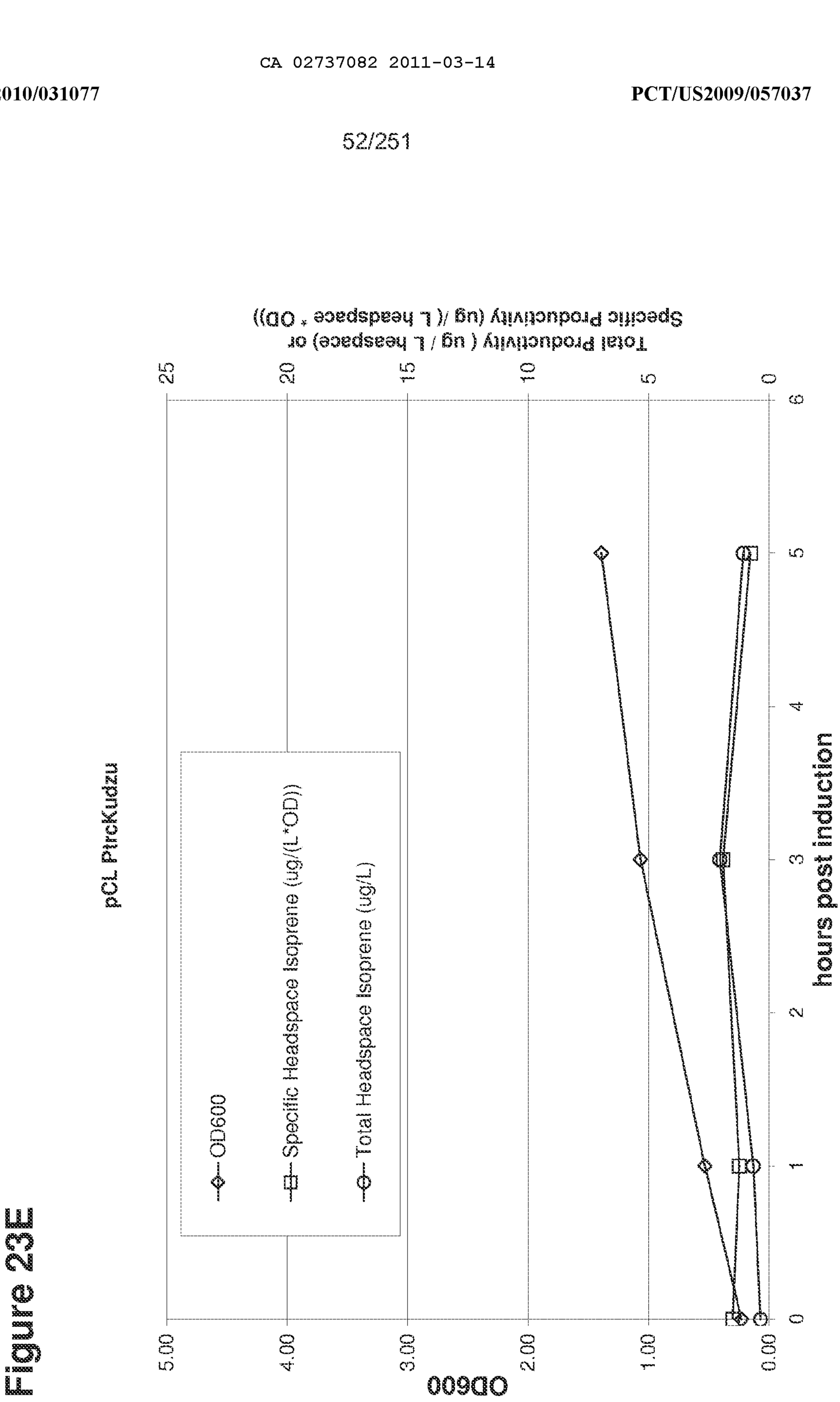
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
hours post induction
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
OD600

Figure 23D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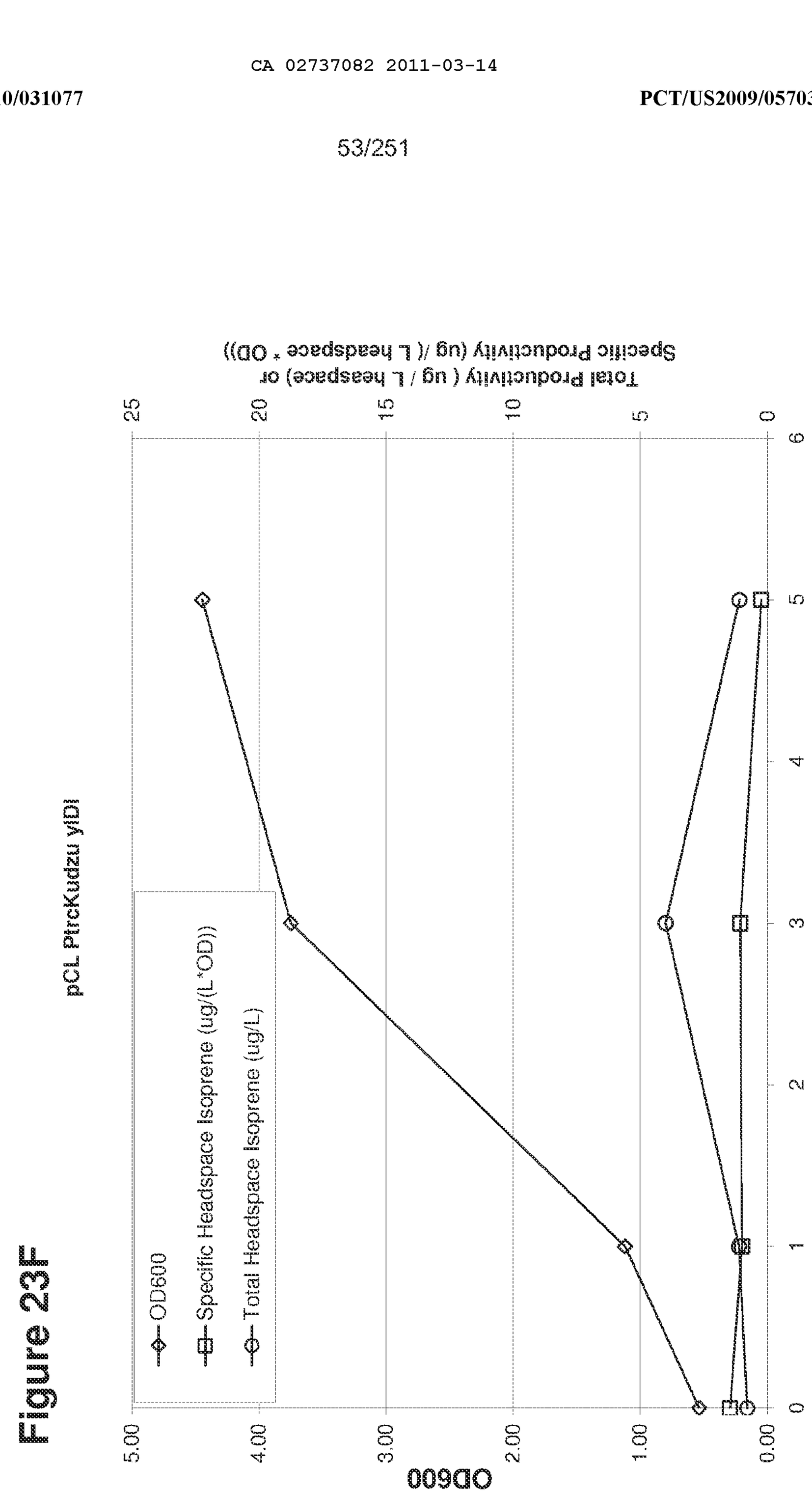
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
hours post induction
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))

Figure 23E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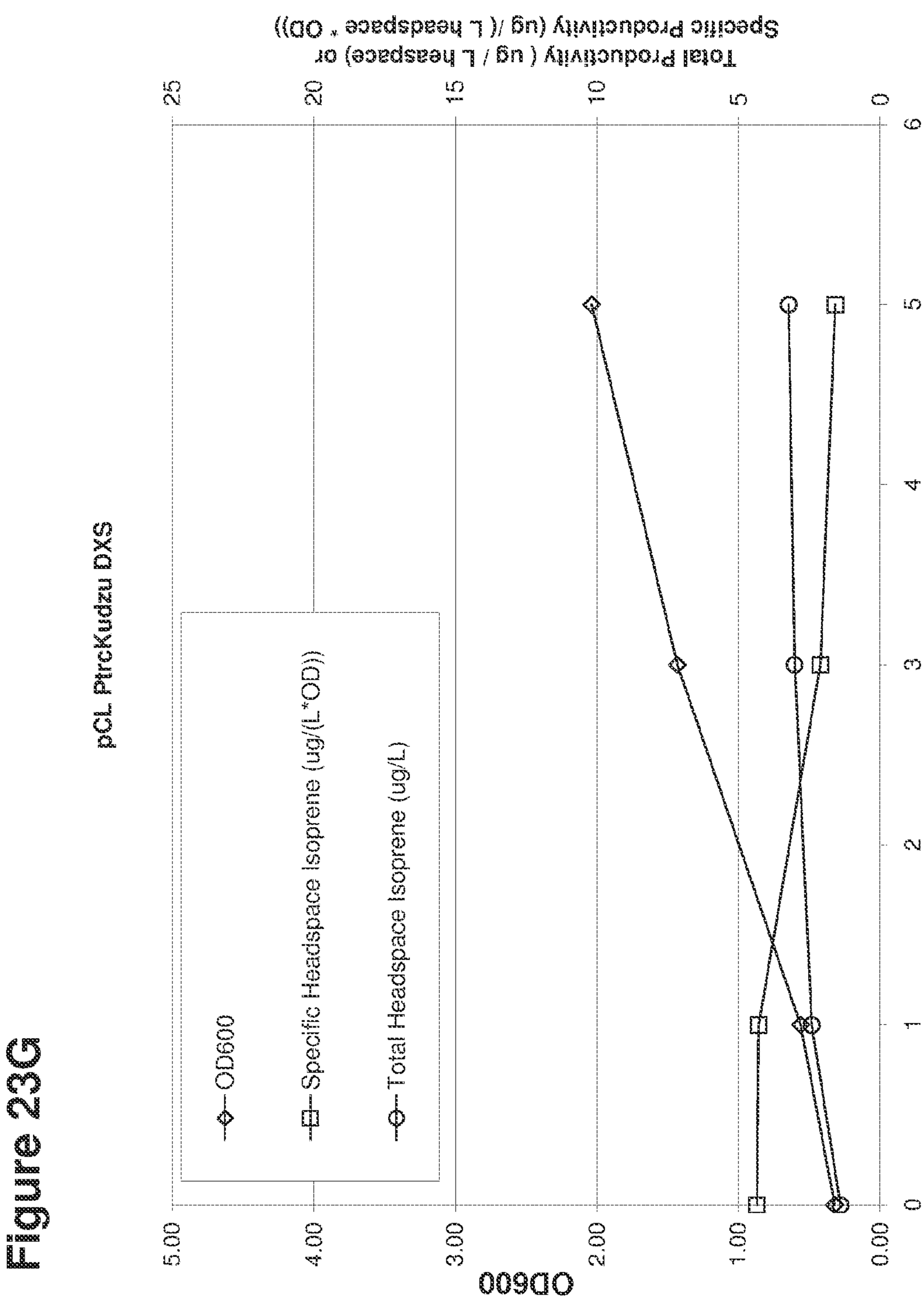
Total Headspace Isoprene (ug/L)
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
hours post induction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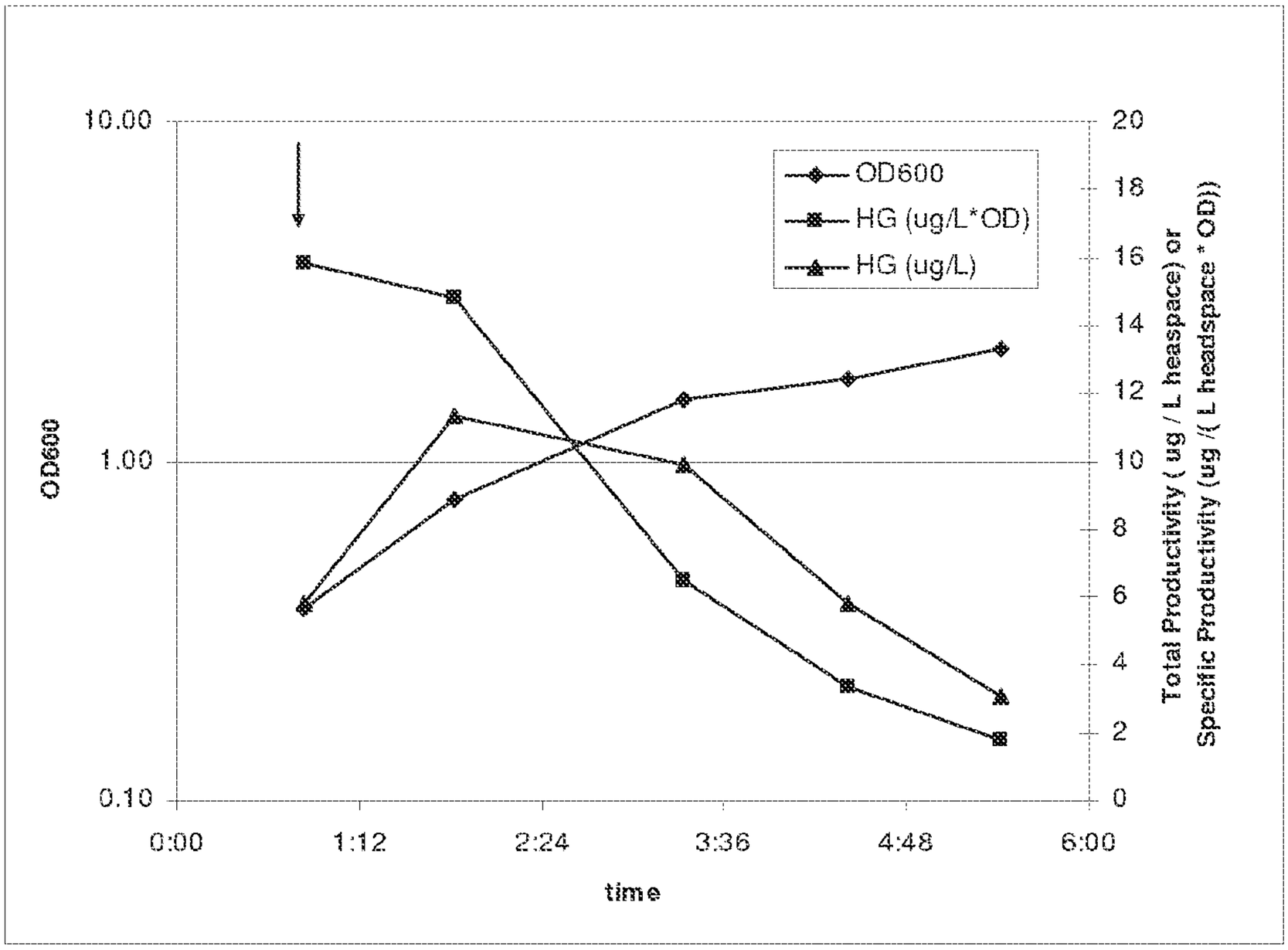
OD600
Specific Headspace Isoprene (ug/(L*OD))
Total Headspace Isoprene (ug/L)
hours post induction
OD600
Total Productivity (ug / L headspace) or
Specific Productivity (ug /(L headspace * OD))
0.00
1.00
2.00
3.00
4.00
5.00
0
5
10
15
20
25
1
2
3
4
5
6

Figure 23G

Figure 23H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1.00
0.10
OD600
20
18
16
14
12
10
8
6
4
2
0
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Specific Productivity (ug /(L headspace * OD))
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HG (ug/L*OD)
HG (ug/L)
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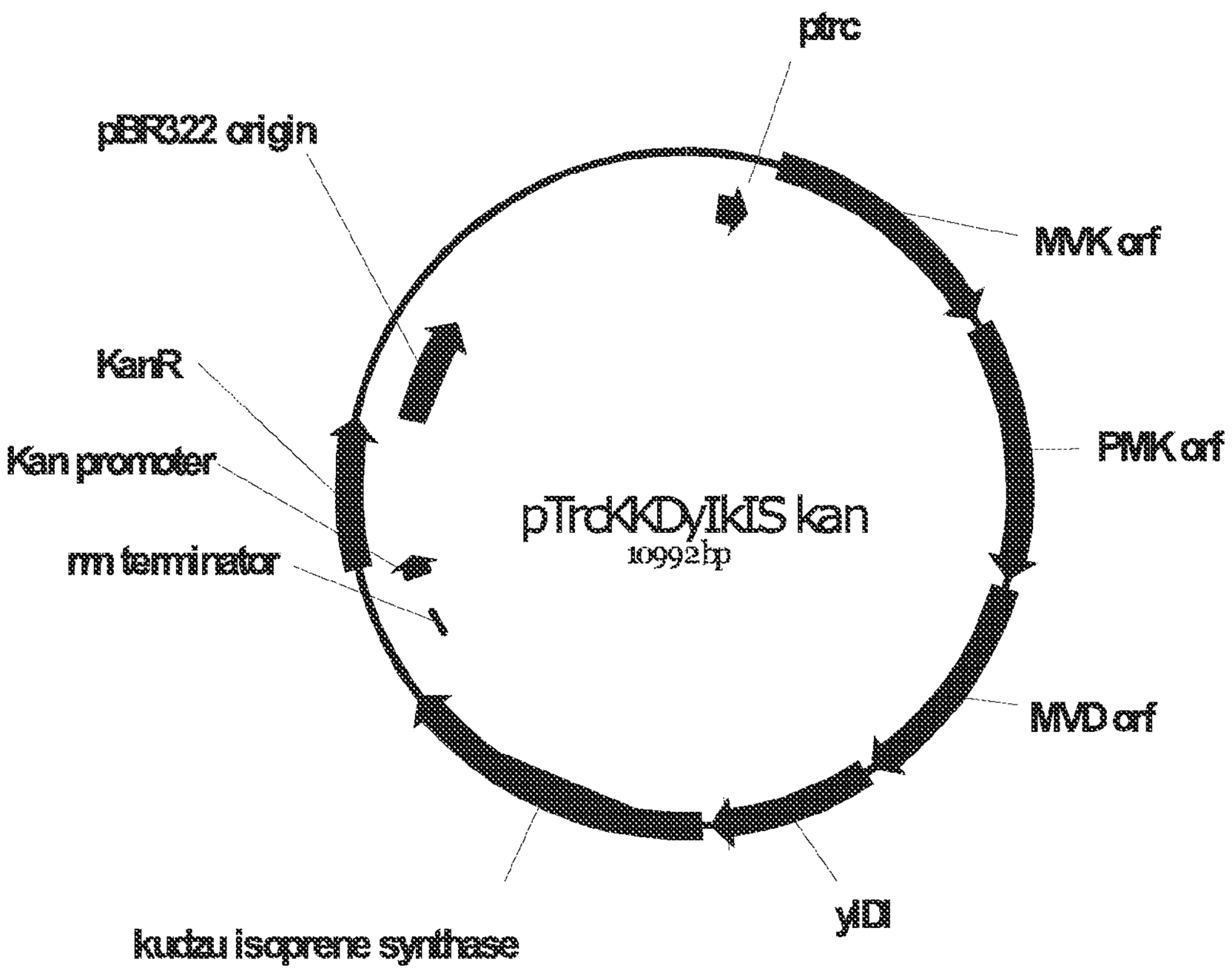
1:12
2:24
3:36
4:48
6:00
time

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

Figure 25A

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Figure 25B

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Figure 25C

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Figure 25D

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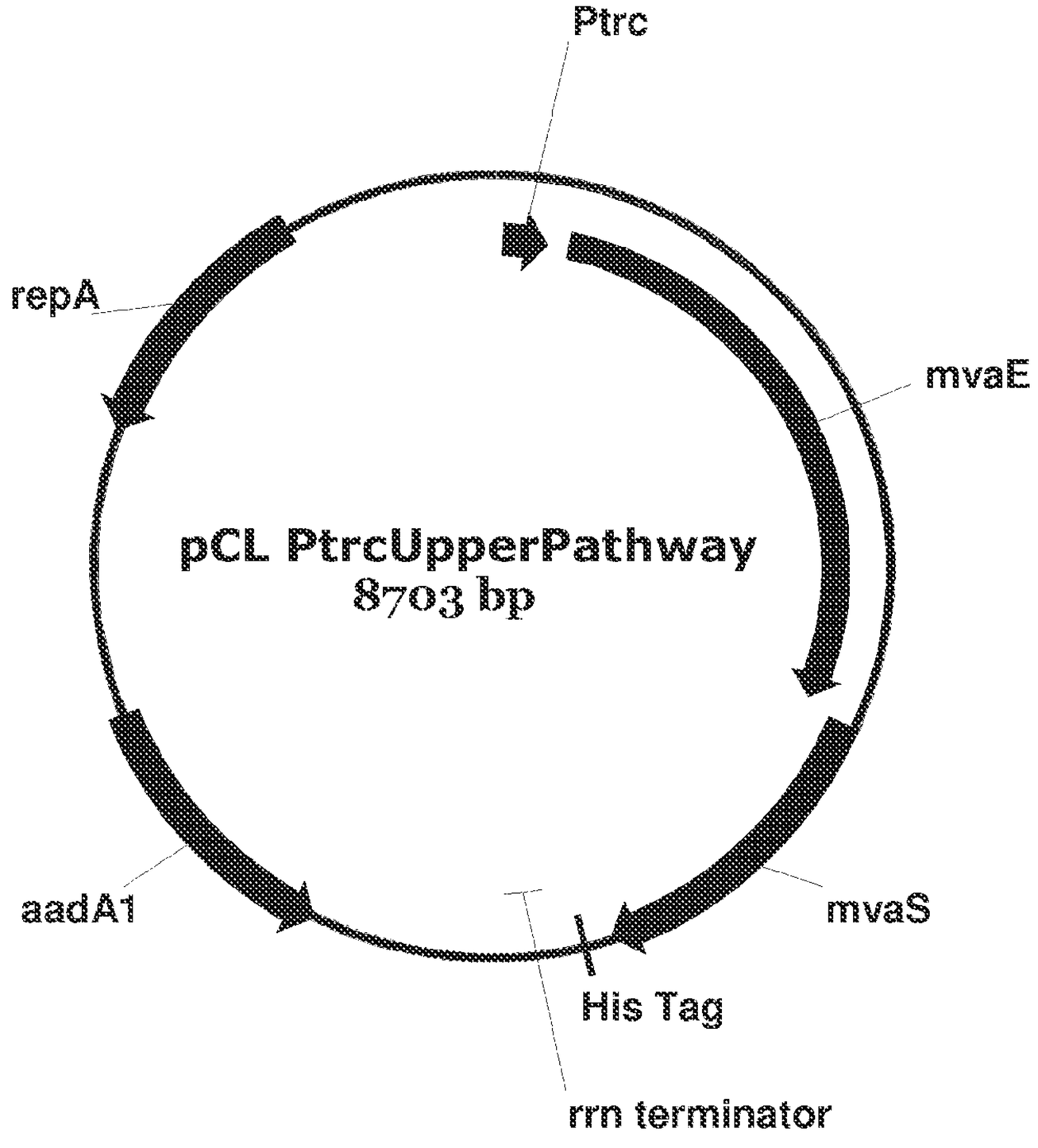
Figure 26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mvaE
pCL PtrcUpperPathway
8703 bp
aadA1
mvaS
His Tag
rrn terminator

Figure 27A

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Figure 27B

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Figure 27C

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Figure 27D

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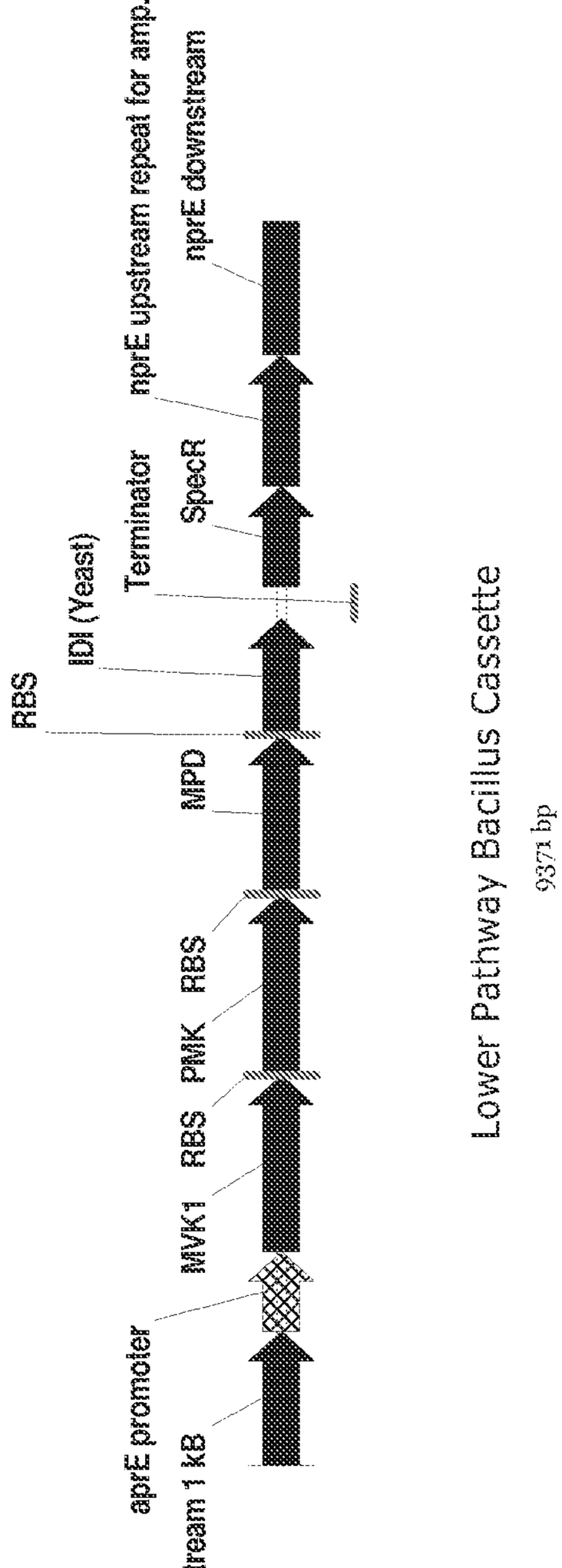
aprE upstream repeat for amplification
aprE promoter
MVK1
RBS
PMK
RBS
MPD
RBS
IDI (Yeast)
Terminator
SpecR
nprE upstream repeat for amplification
nprE downstream
Lower Pathway Bacillus Cassette
9371 bp

Fig 28

Figure 29A

5′ –
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Figure 29B

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Figure 29C

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Figure 29D

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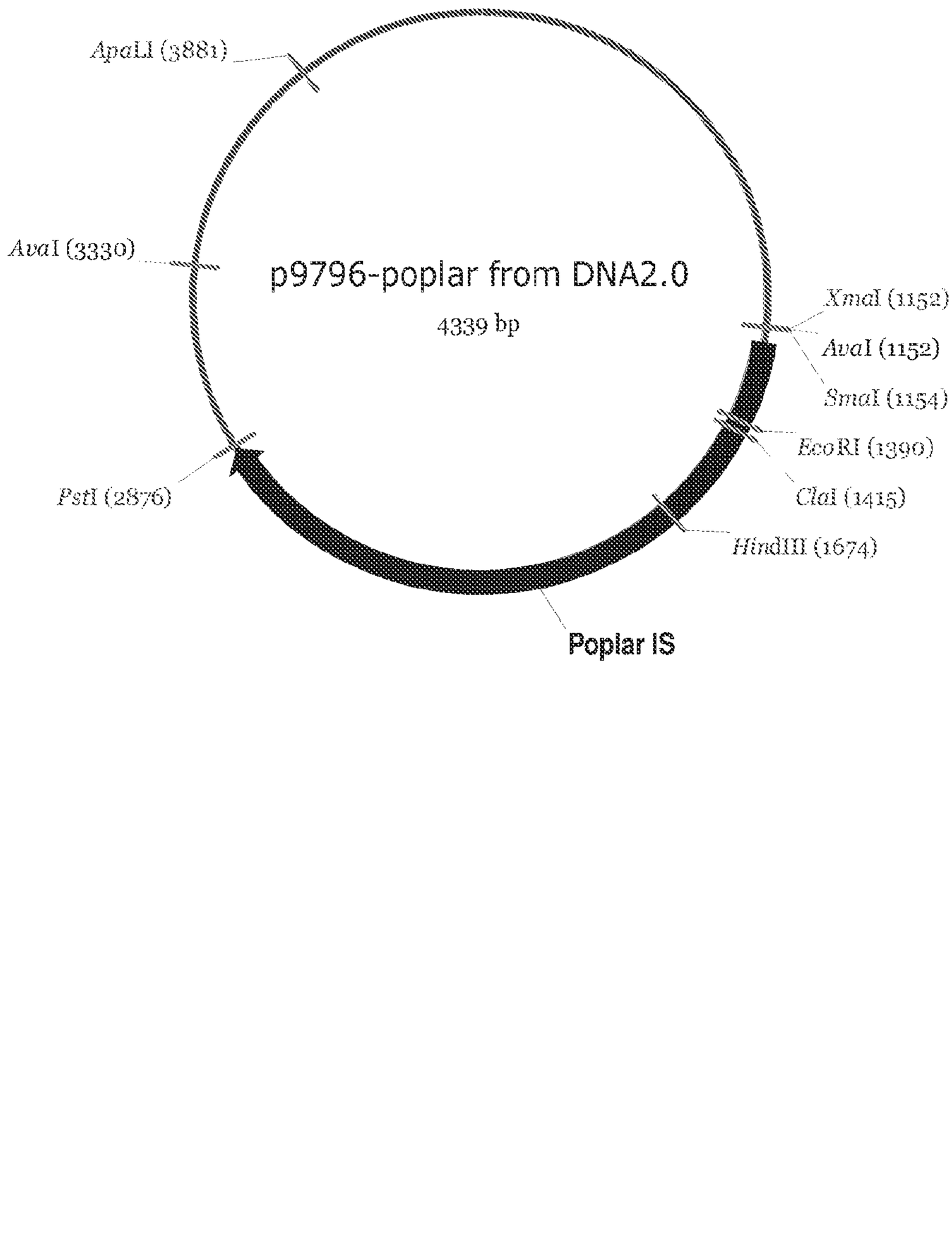
Figure 30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AvaI (3330)
PstI (2876)
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SmaI (1154)
EcoRI (1390)
ClaI (1415)
HindIII (1674)
Poplar IS

Figure 31A

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Figure 31B

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Figure 32

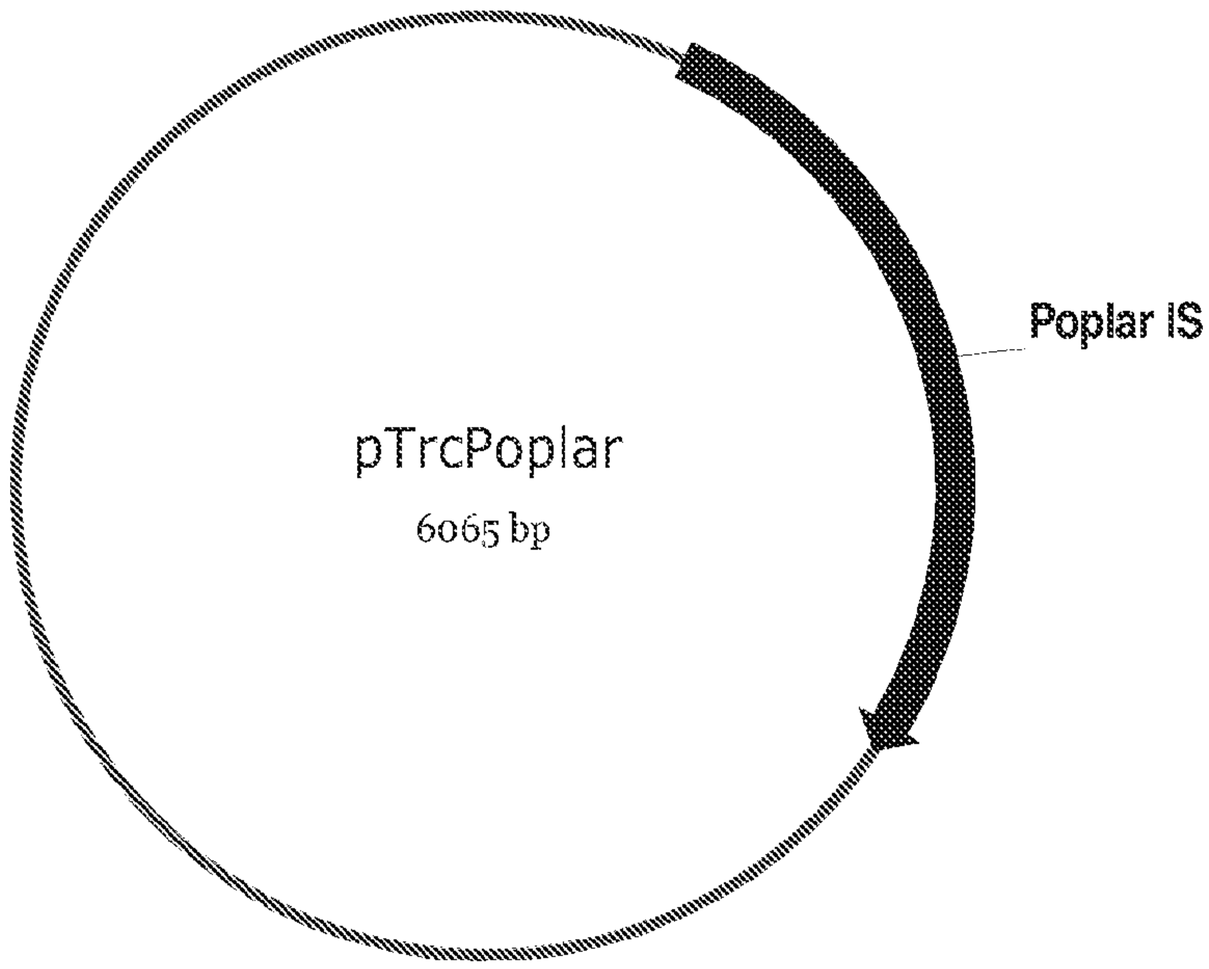

Figure 33A

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Figure 33B

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Figure 33C

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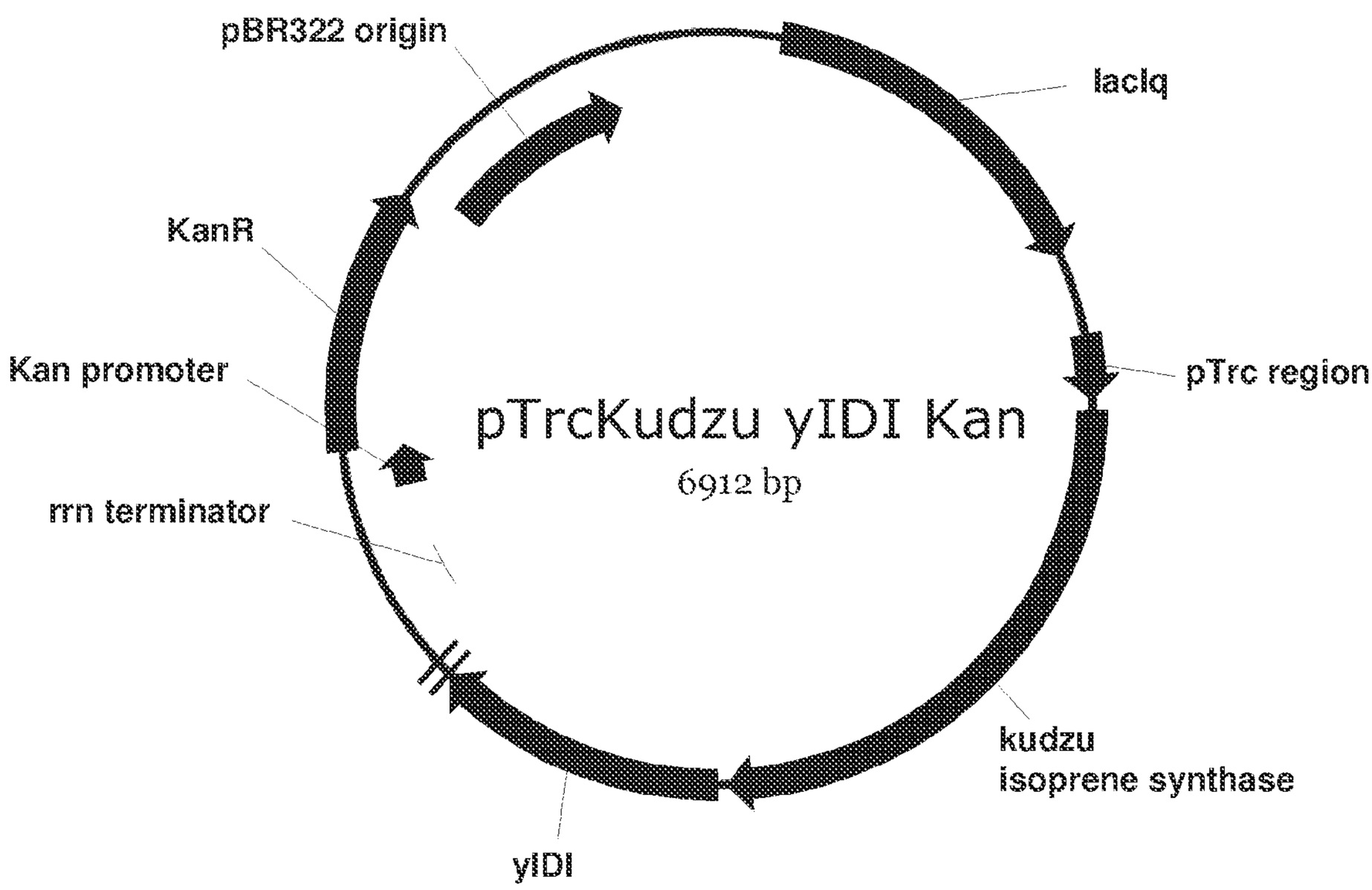
Figure 34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rrn terminator
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Figure 35A

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Figure 35B

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Figure 35C

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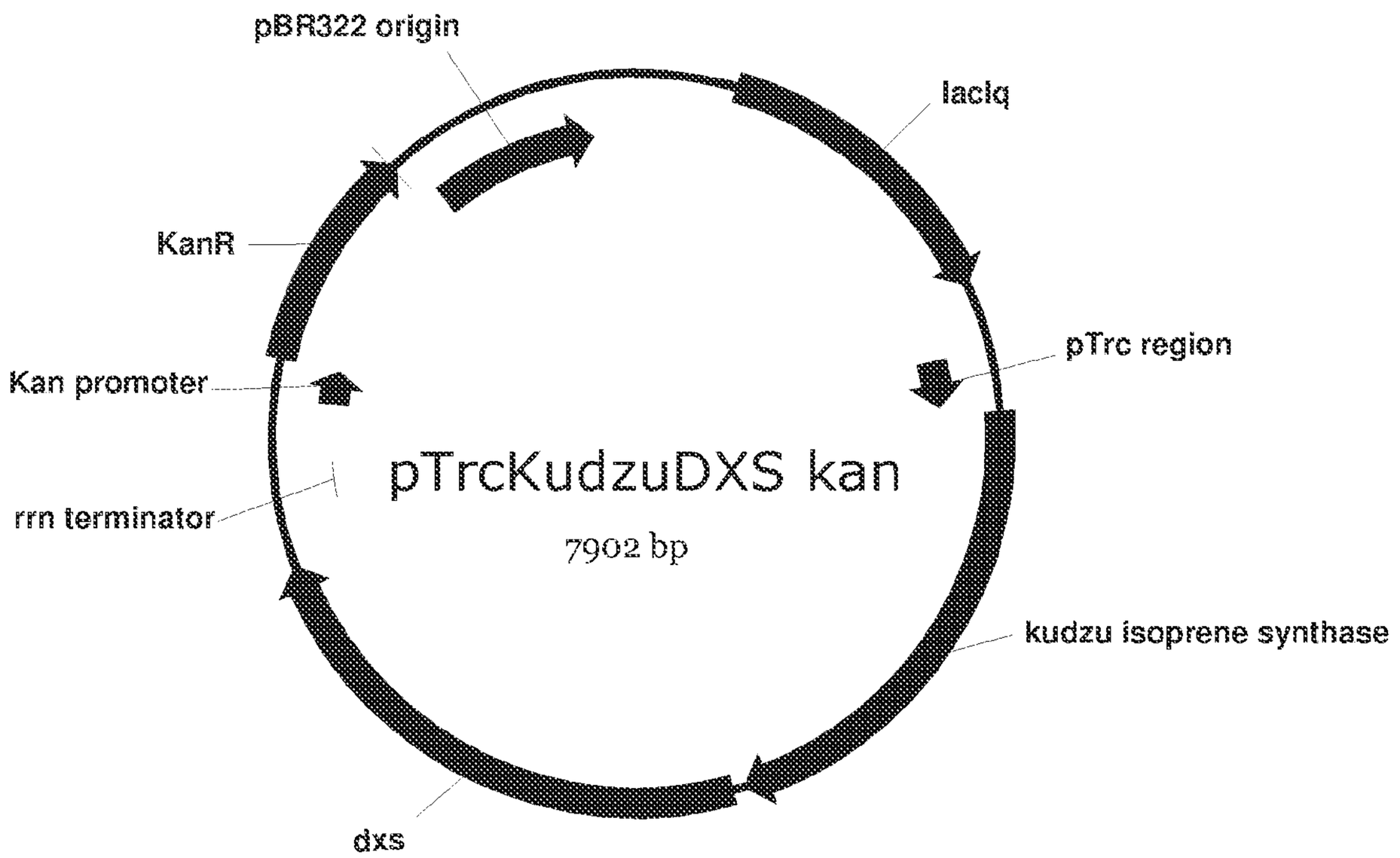
Figure 36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rrn terminator
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Figure 37A

5'-

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Figure 37B

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Figure 37C

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Figure 38

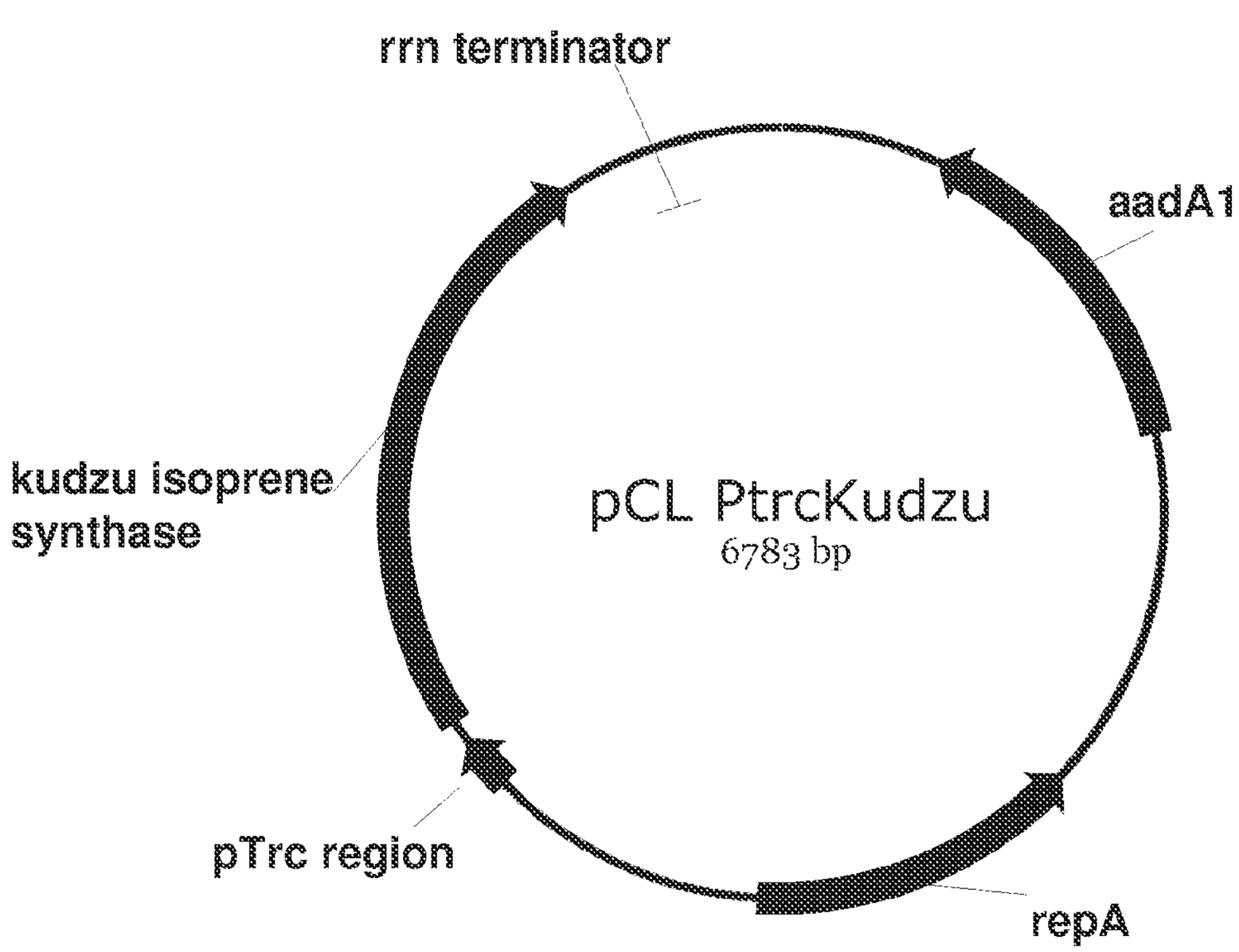
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Figure 39A

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Figure 39B

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Figure 39C

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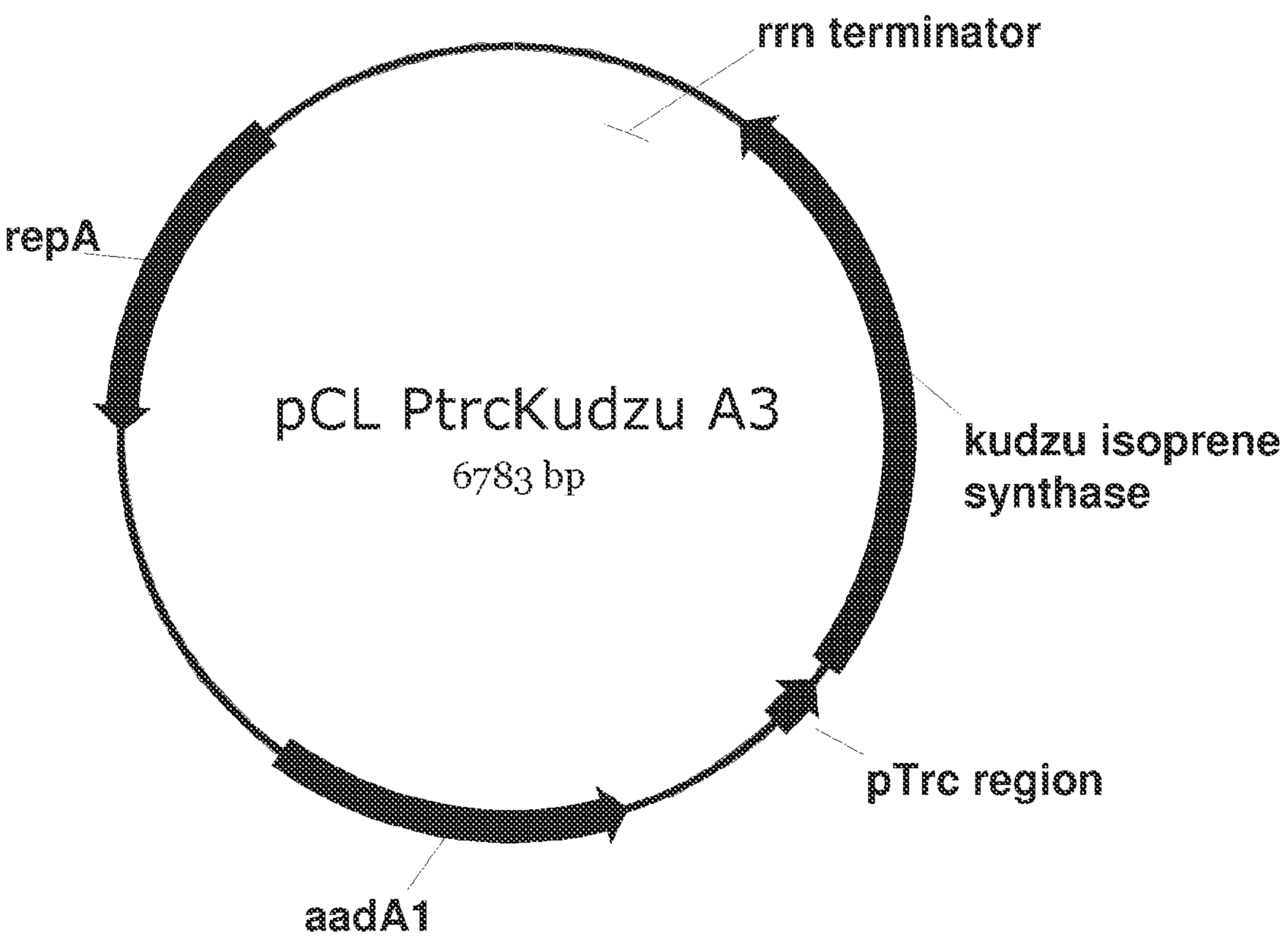
Figure 40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kudzu isoprene synthase
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Figure 41A

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Figure 41B

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Figure 41C

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Figure 42

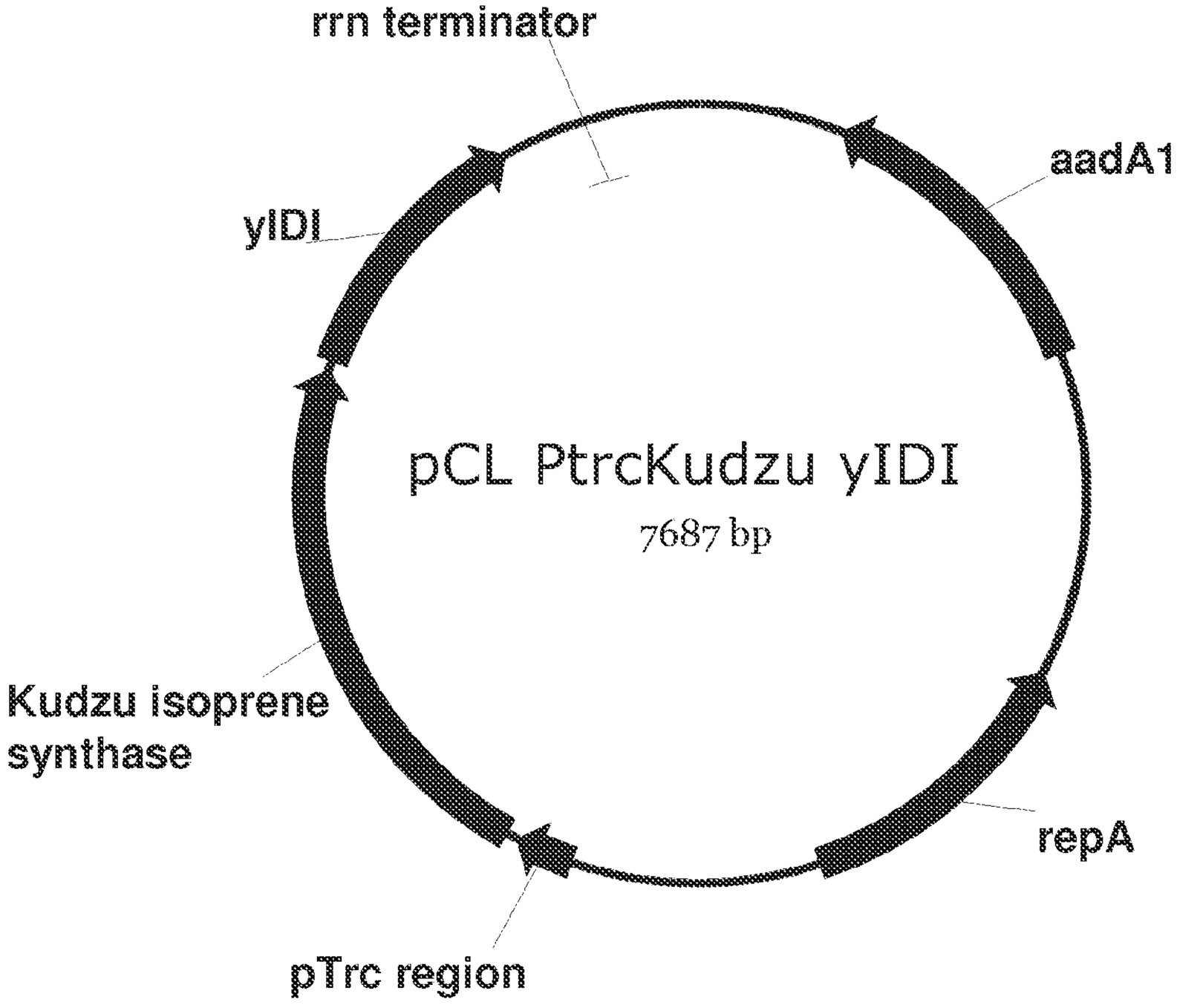
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Figure 43A

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Figure 43B

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Figure 43C

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Figure 44

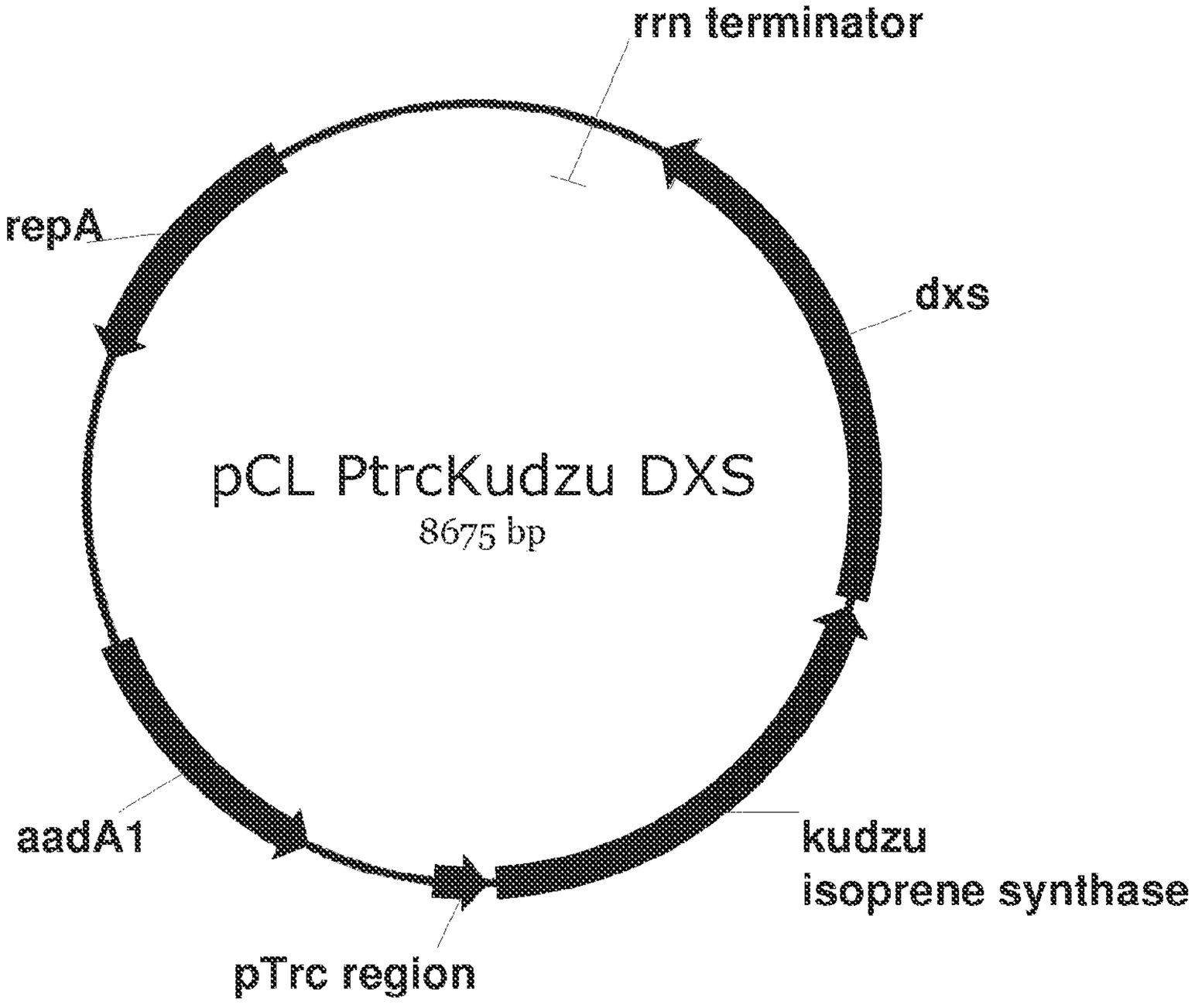
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Figure 45A

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Figure 45B

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Figure 45C

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Figure 45D

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Figure 46A

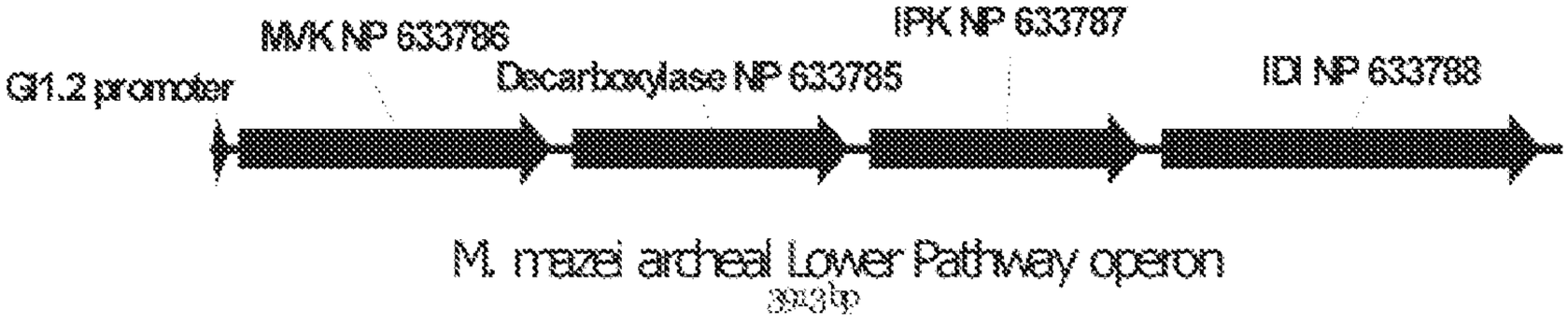
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Figure 46B

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Figure 46C

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Figure 47A

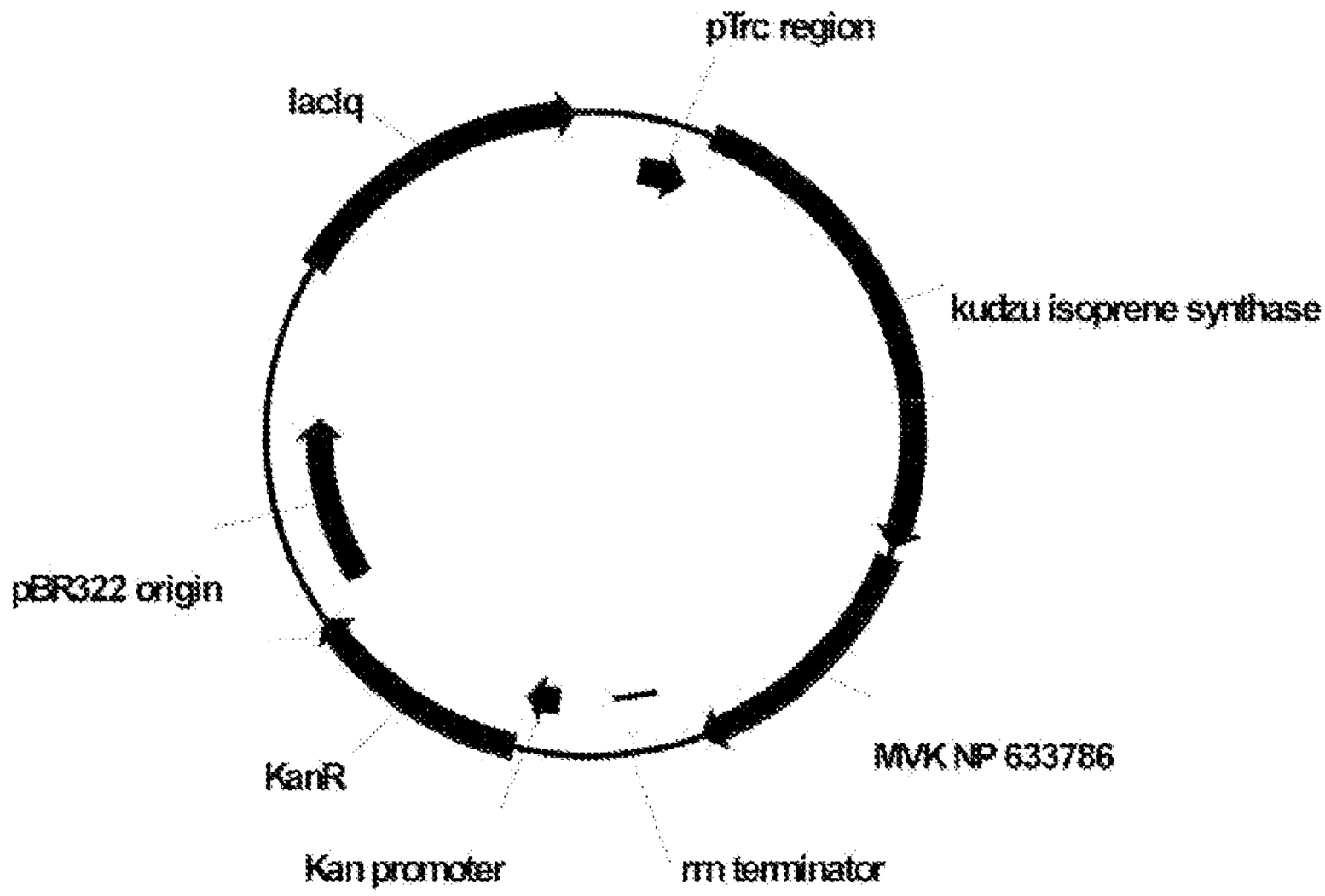

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Figure 47B

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Figure 47C

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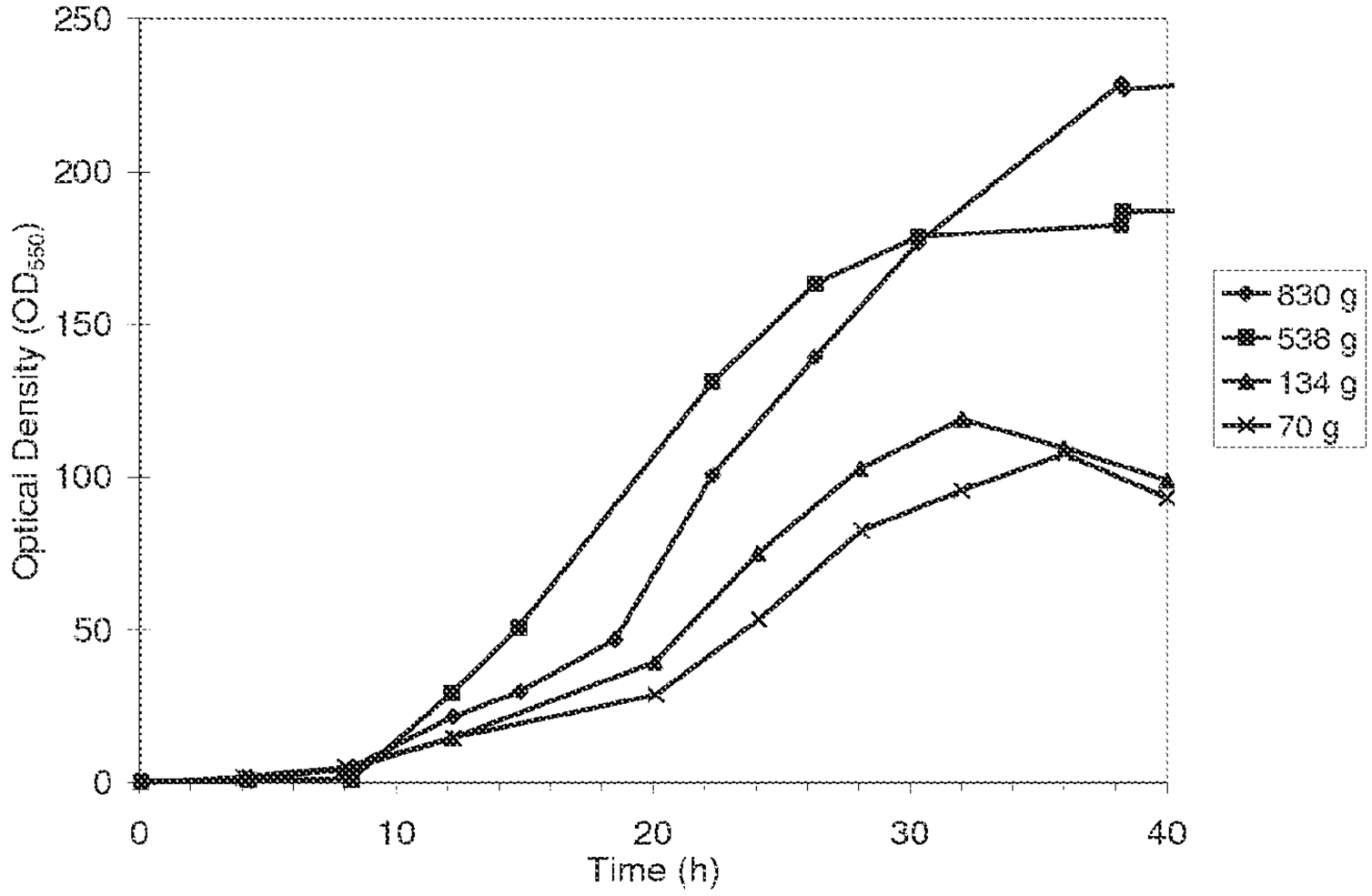


Figure 48B

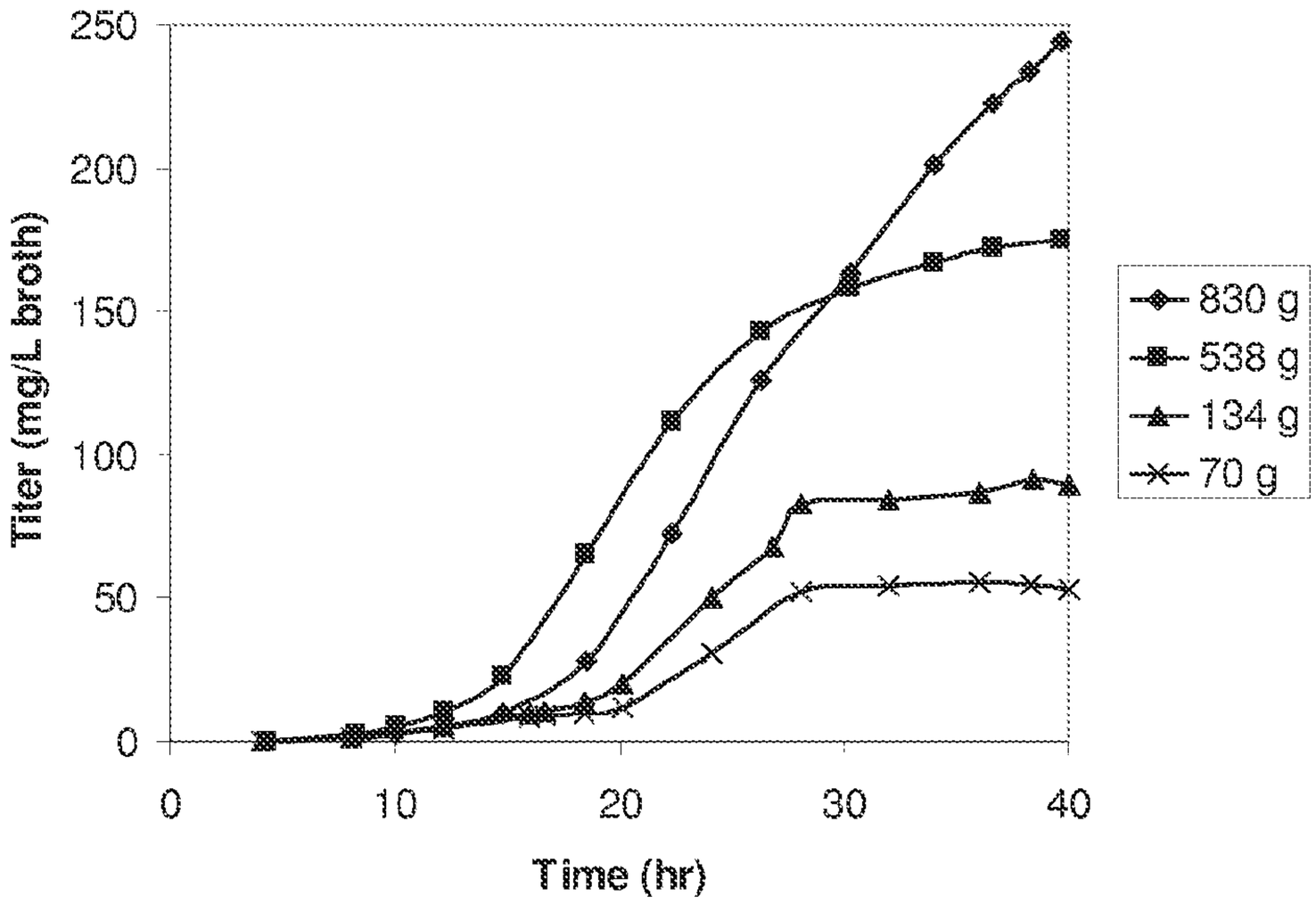
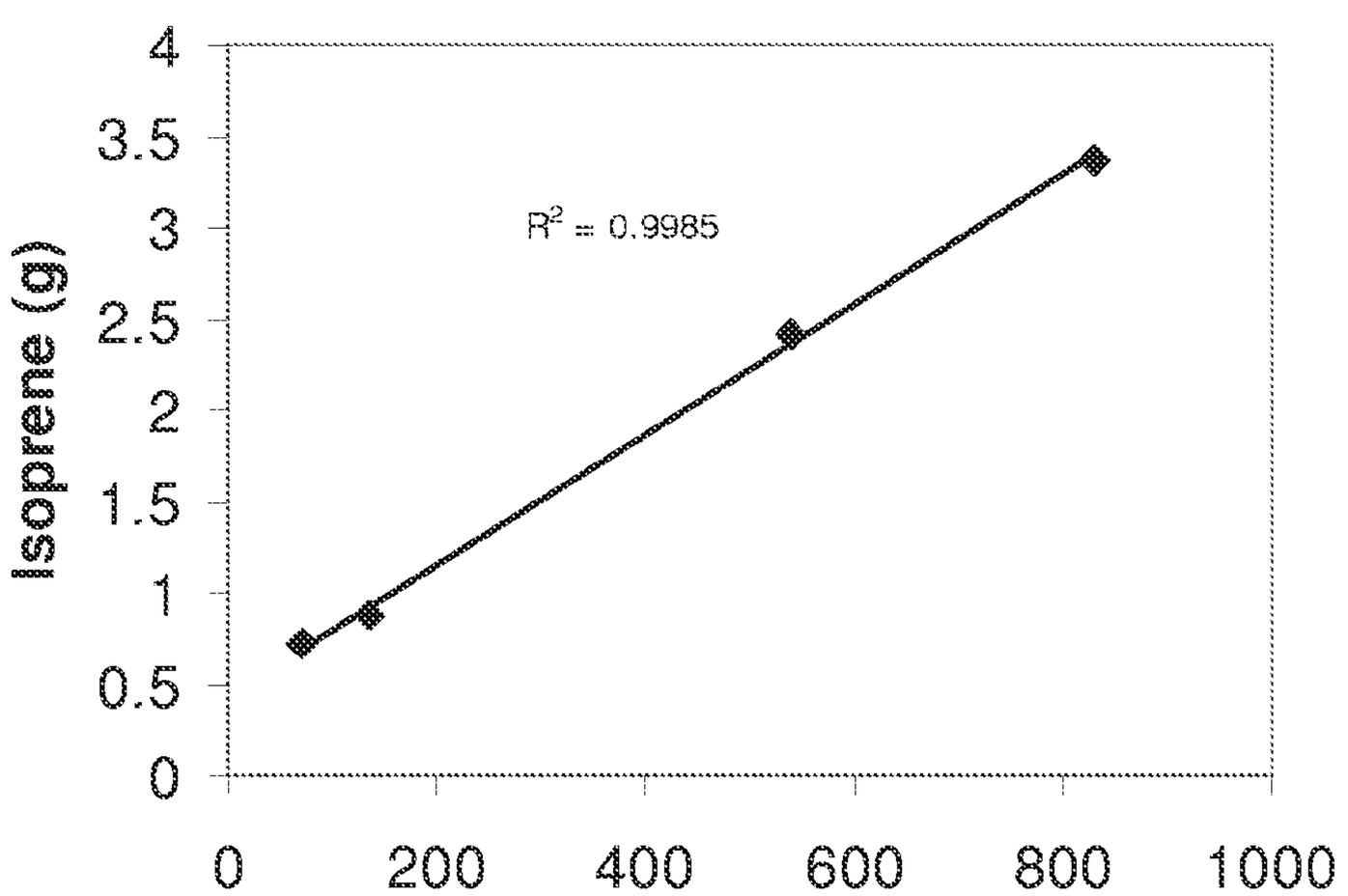


Figure 48C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3
2.5
2
1.5
1
0.5
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400
600
800
1000
Yeast extract (g)

Figure 49A

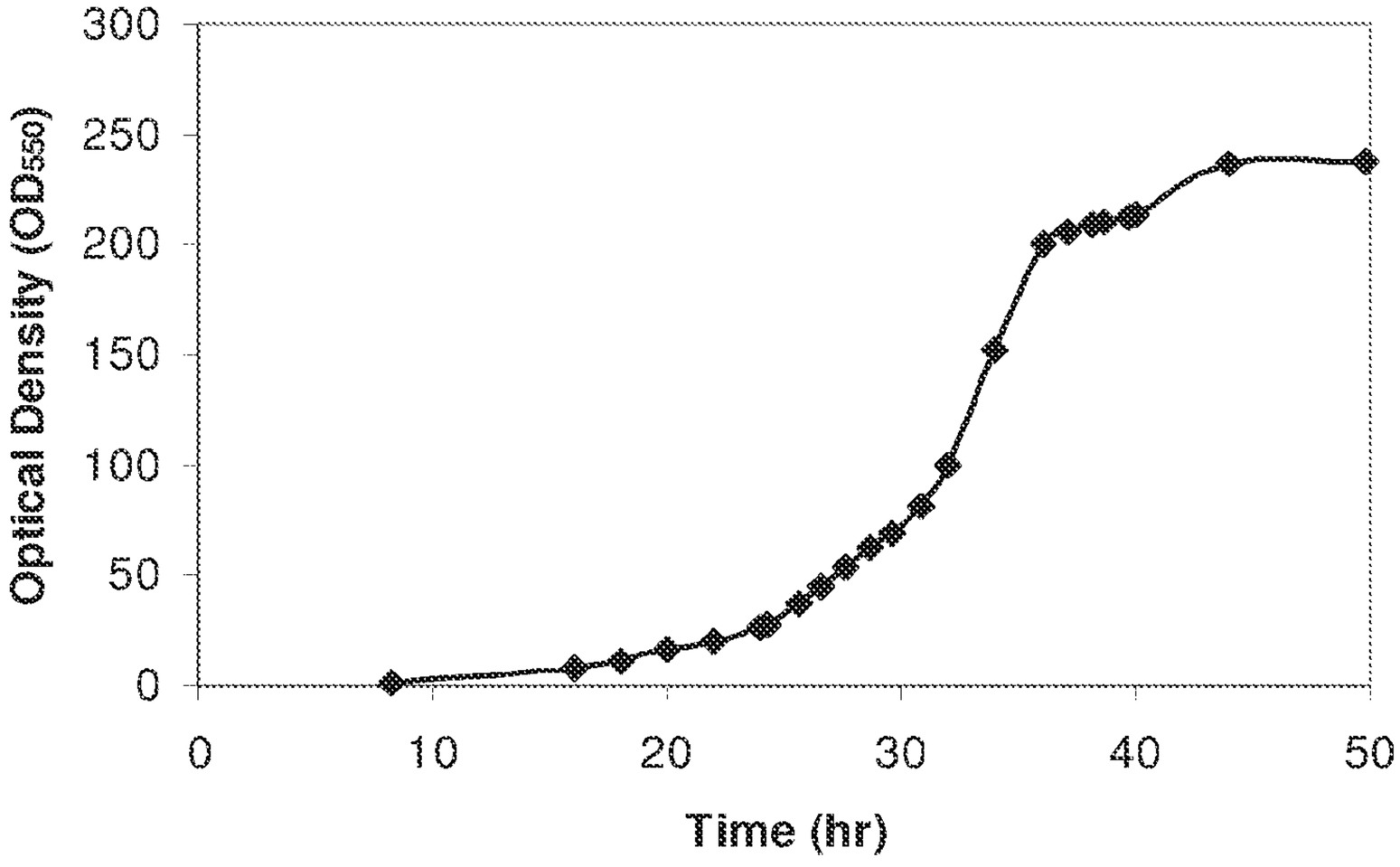
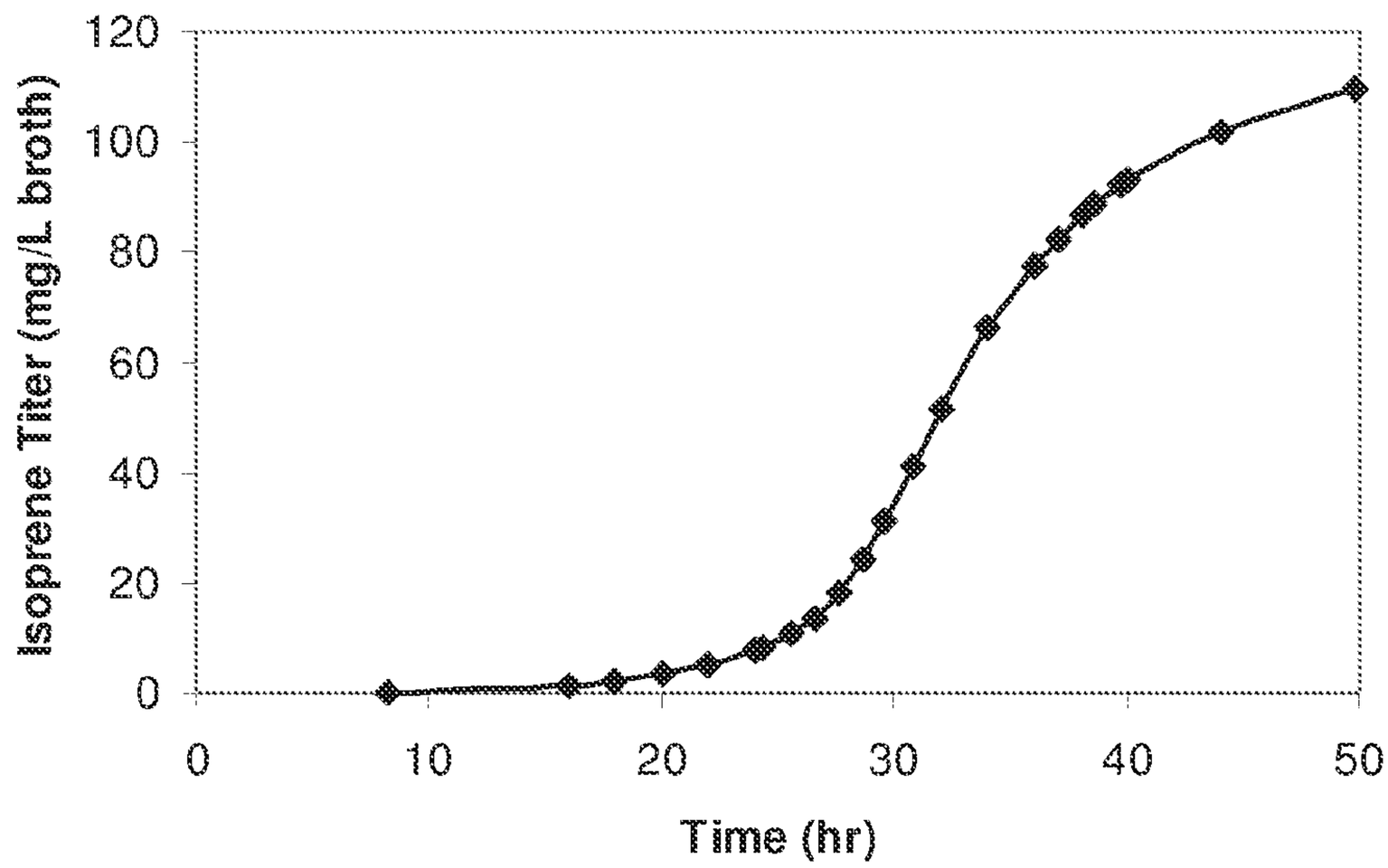


Figure 49B



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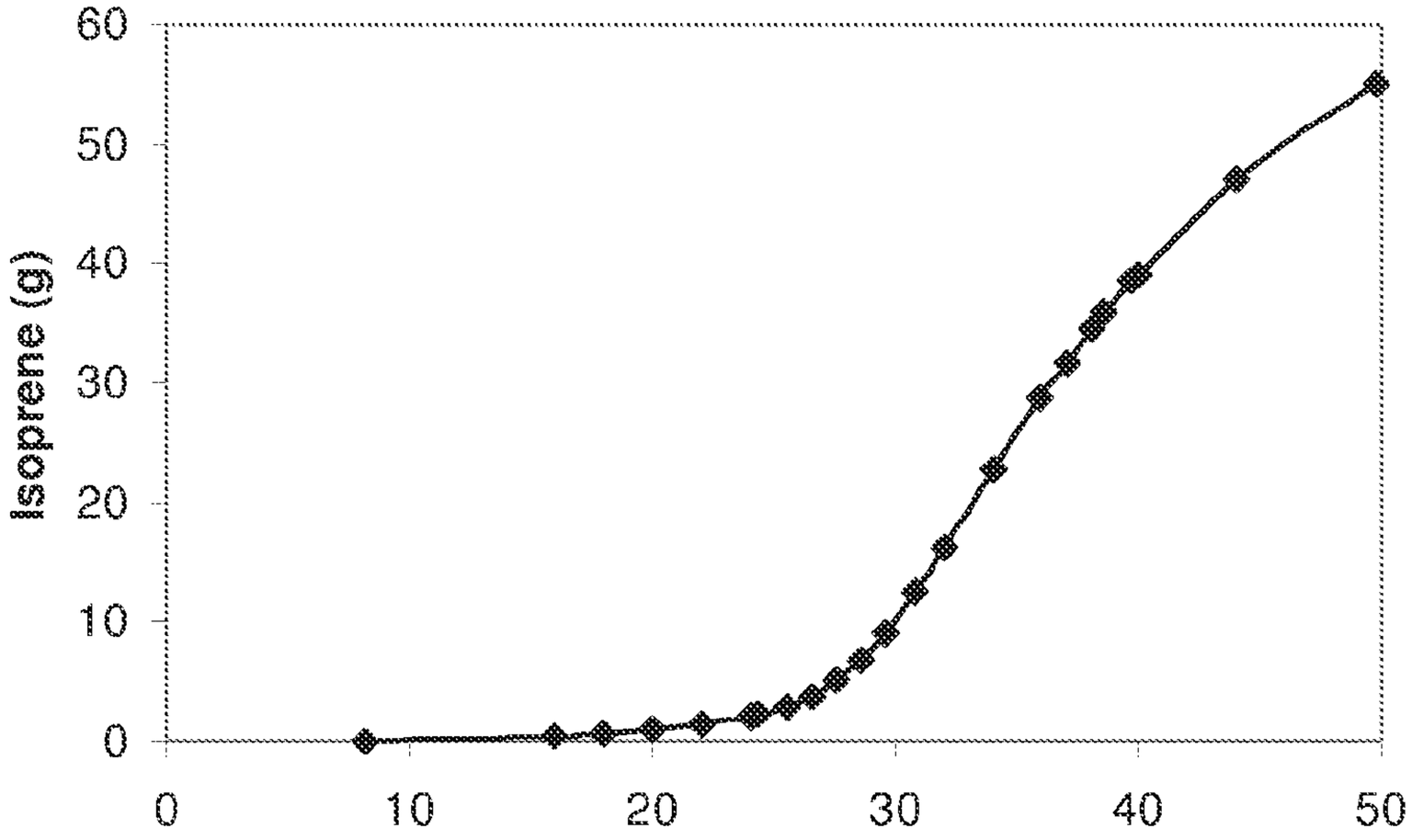

Figure 50

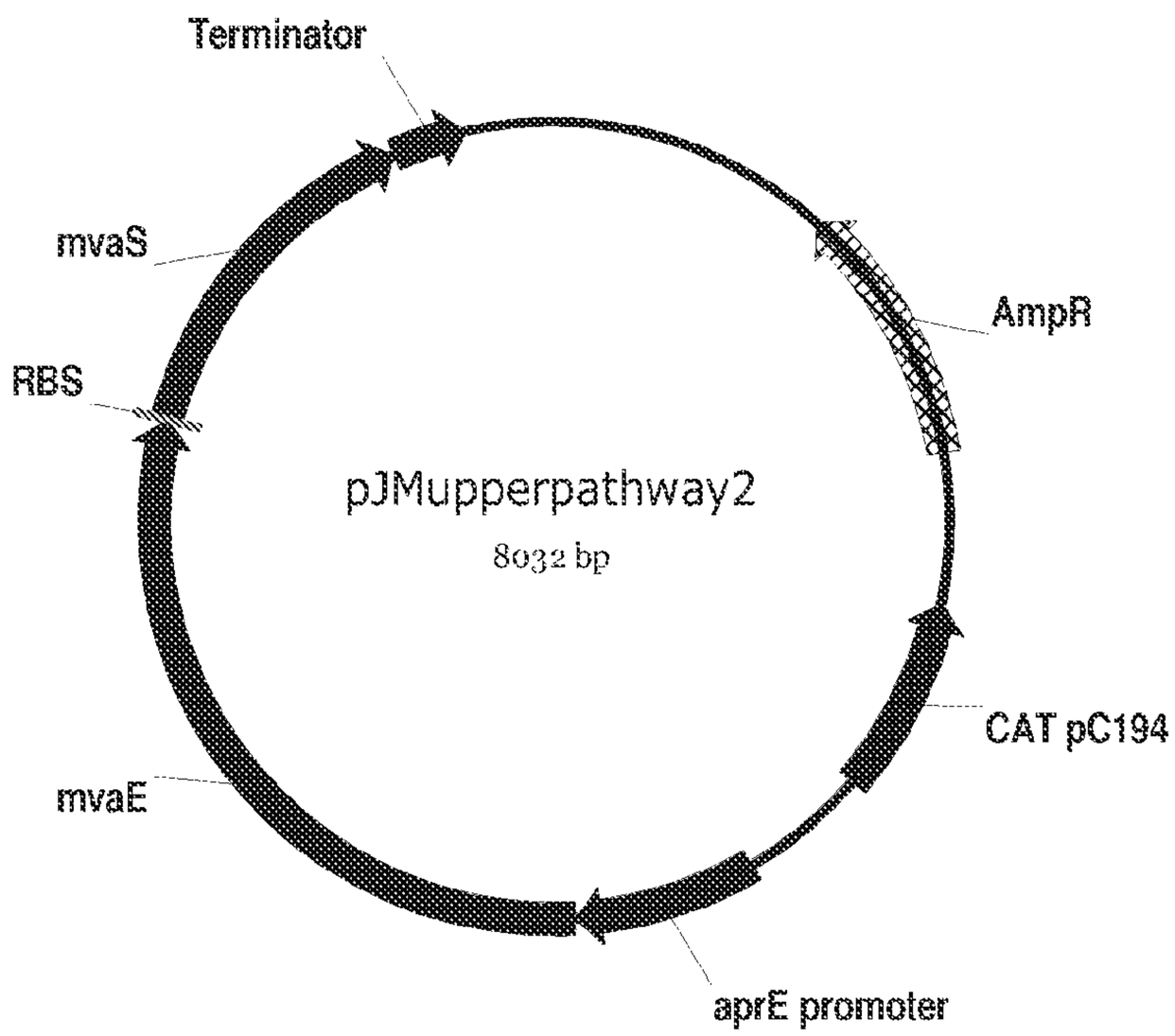
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Figure 51A

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Figure 51B

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Figure 51C

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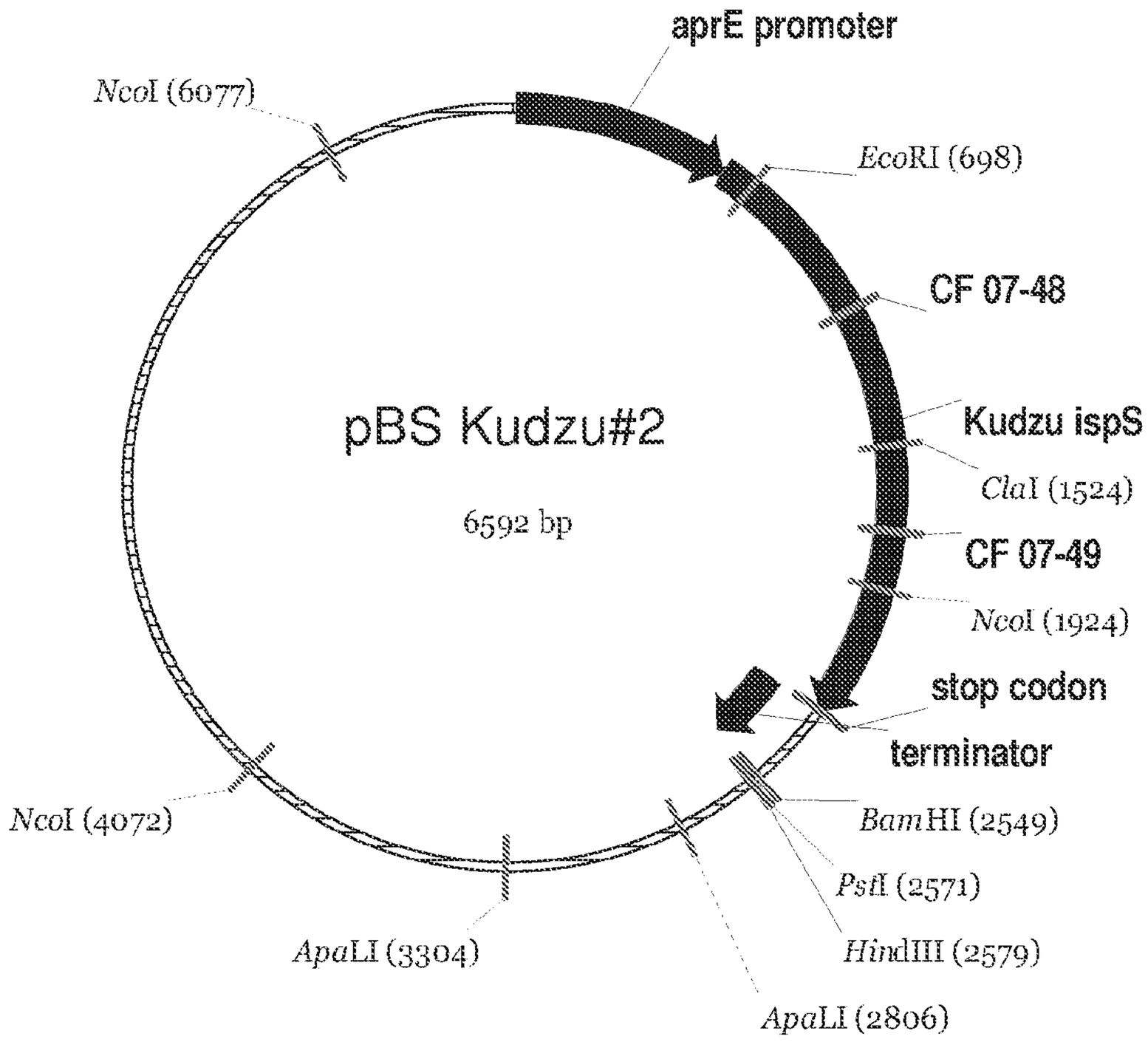
Figure 52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stop codon
terminator
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Figure 53A

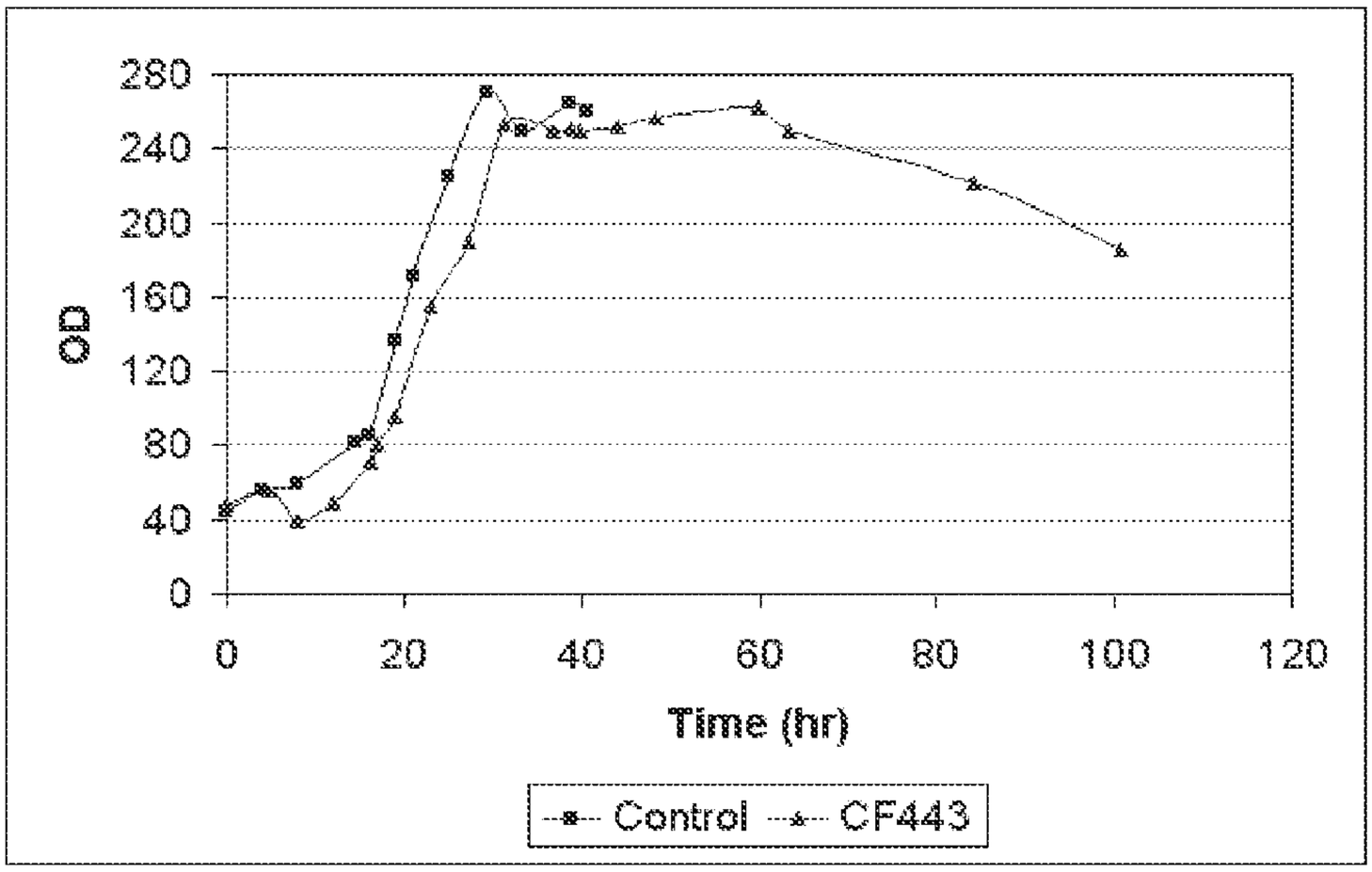
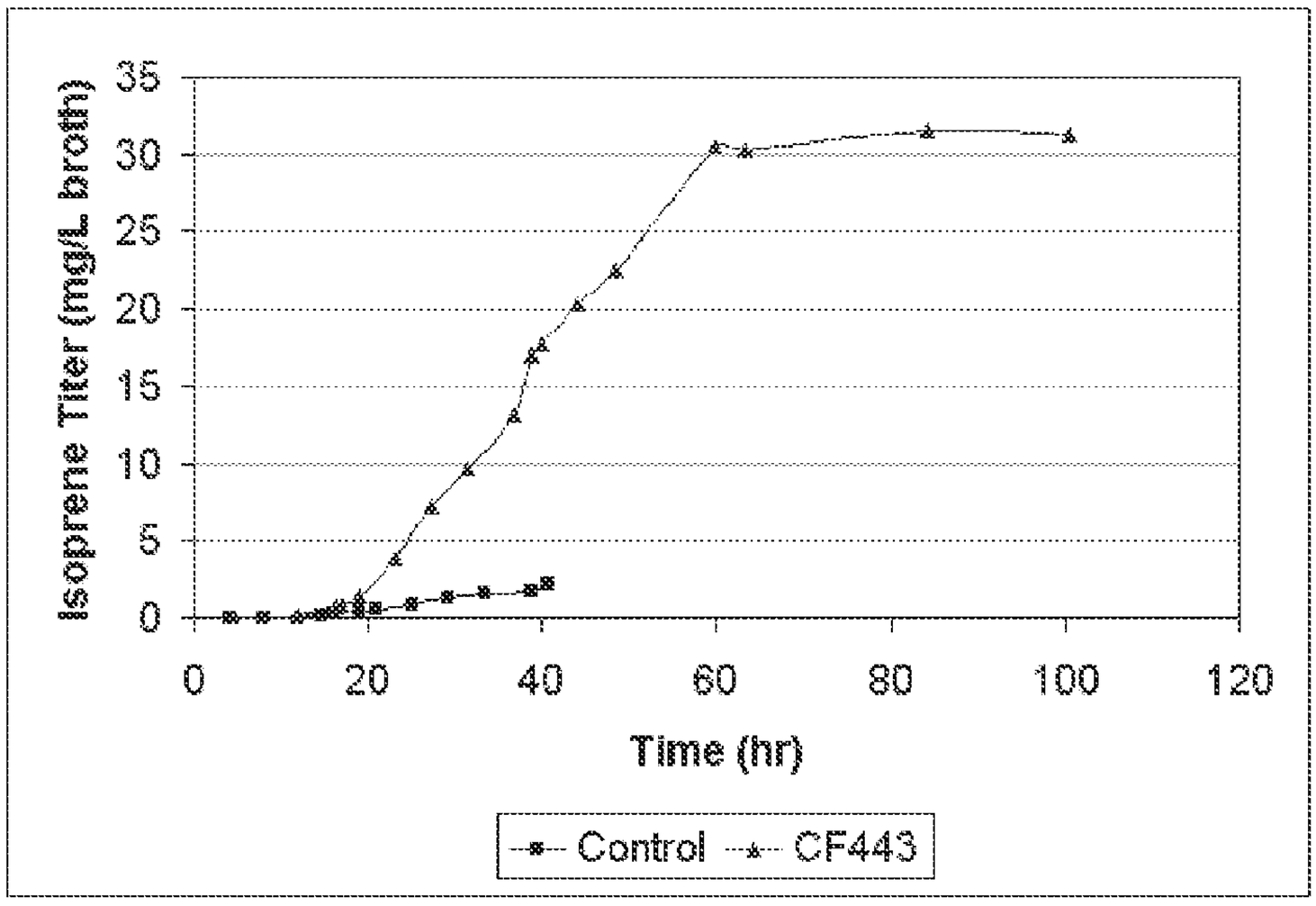


Figure 53B



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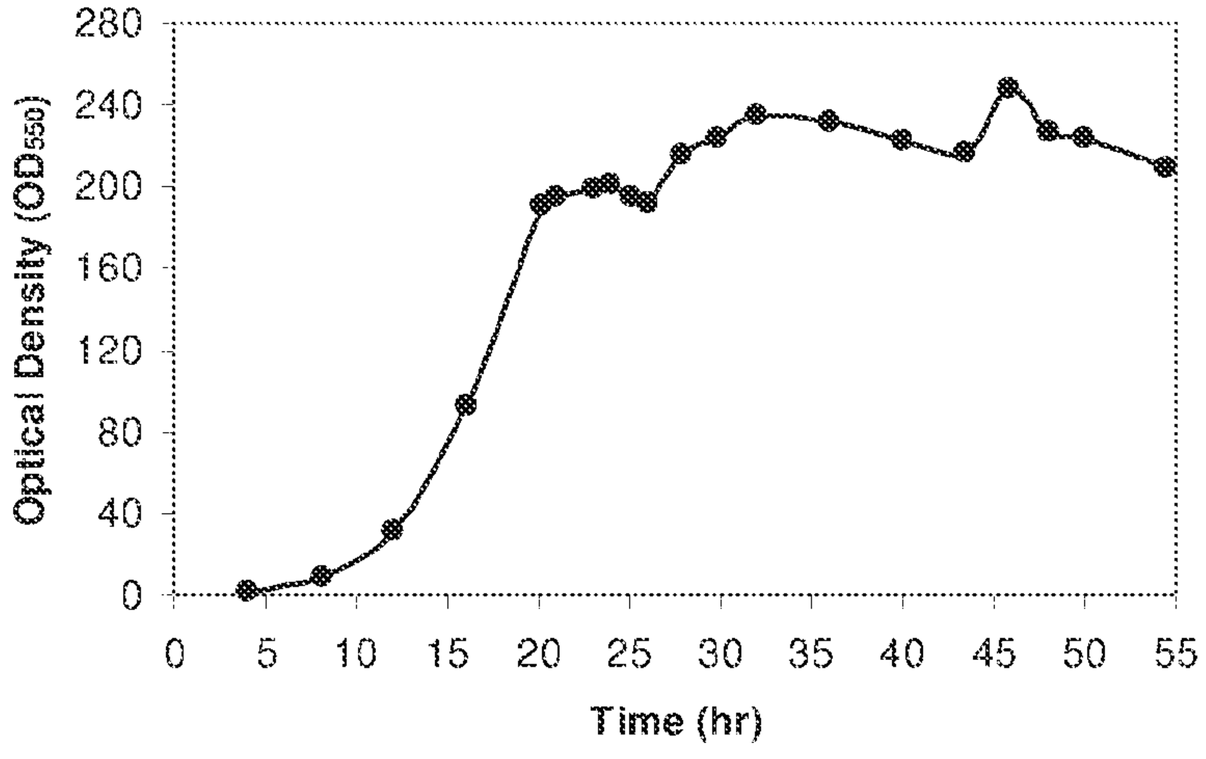

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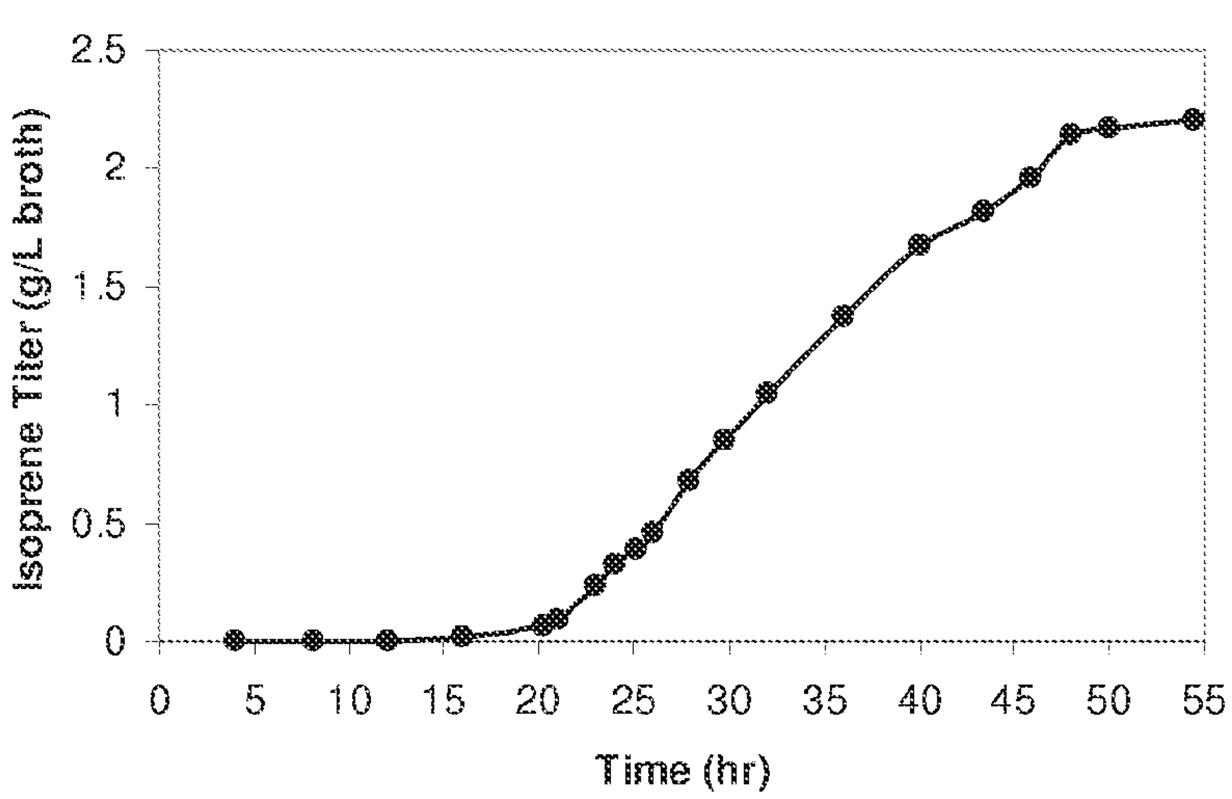

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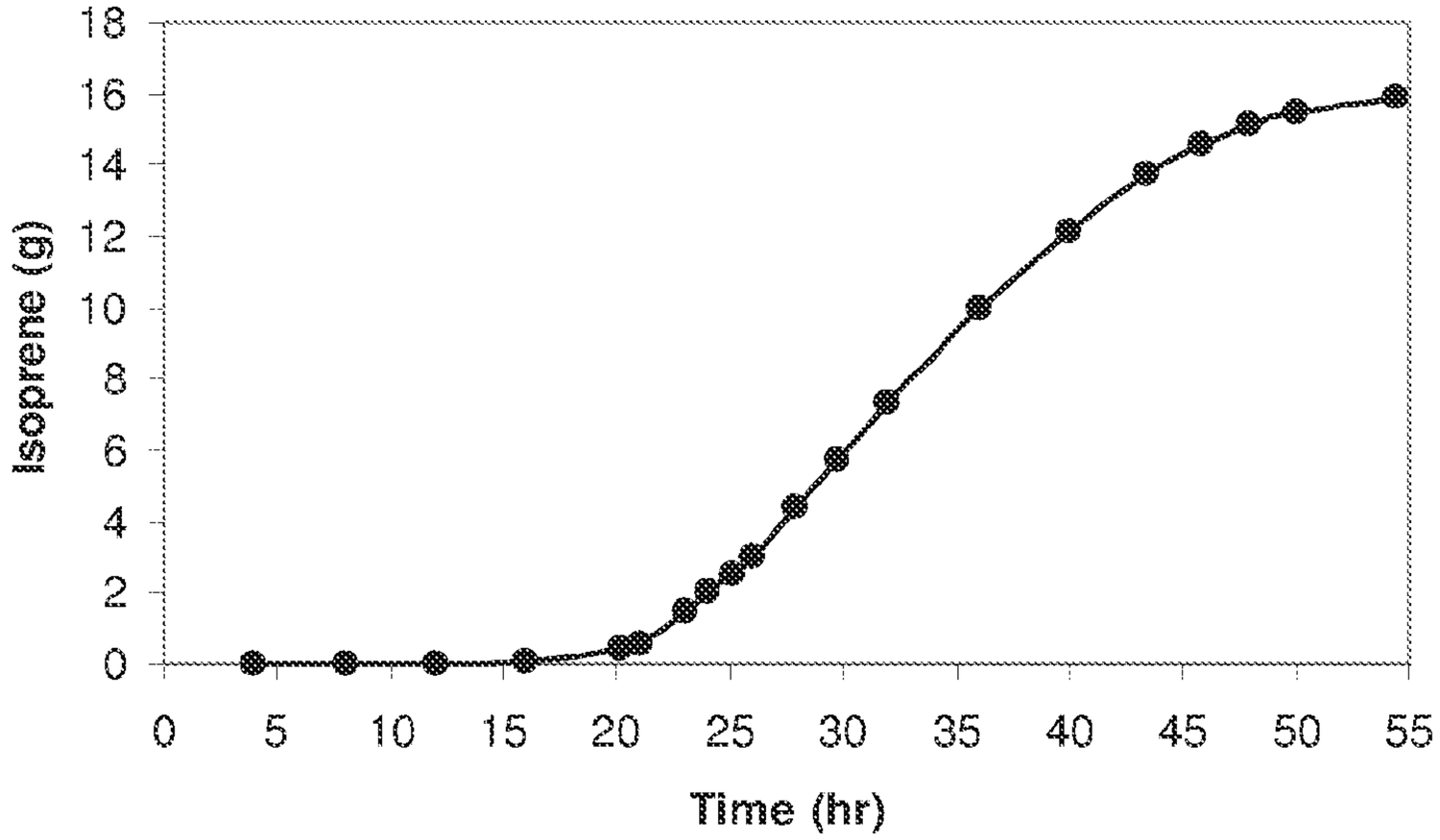
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55
Time (hr)

Figure 57A

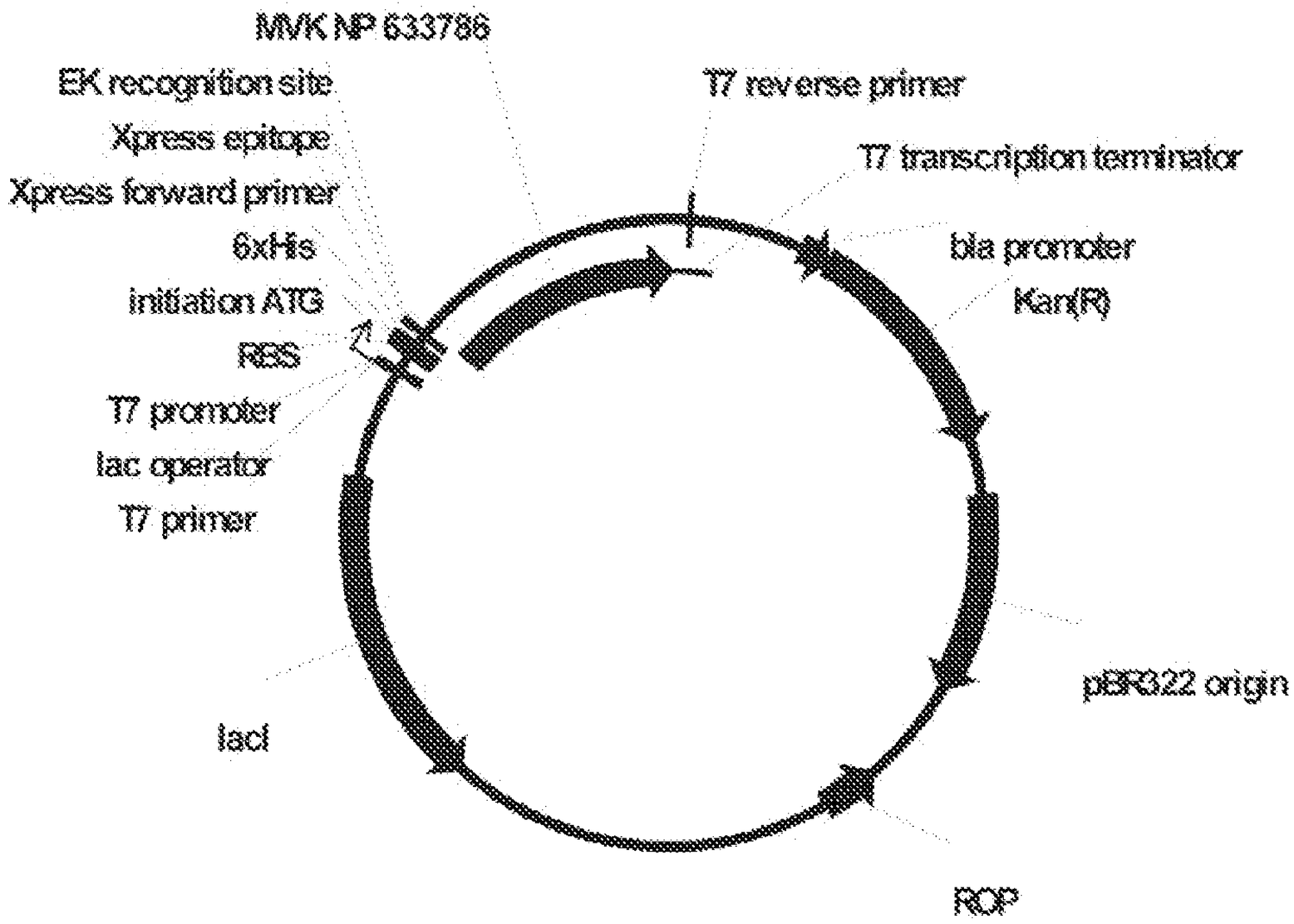

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Figure 57B

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Figure 57C

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Figure 58A

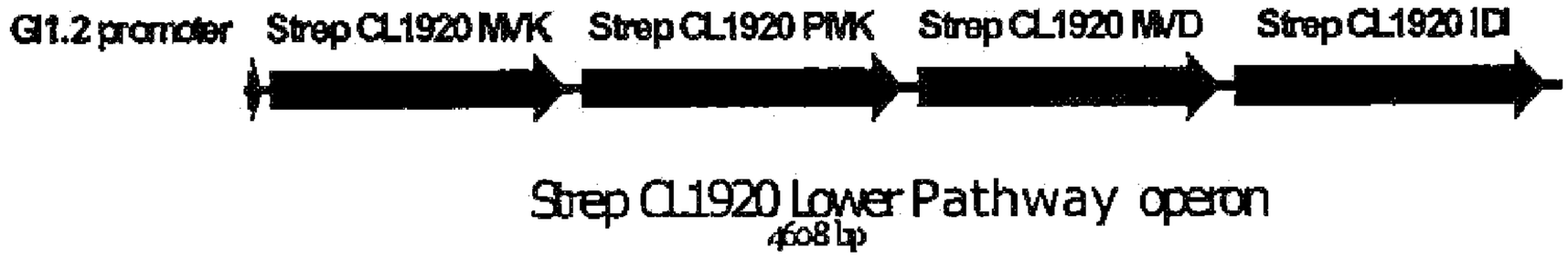
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Figure 58B

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Figure 58C

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Figure 59A

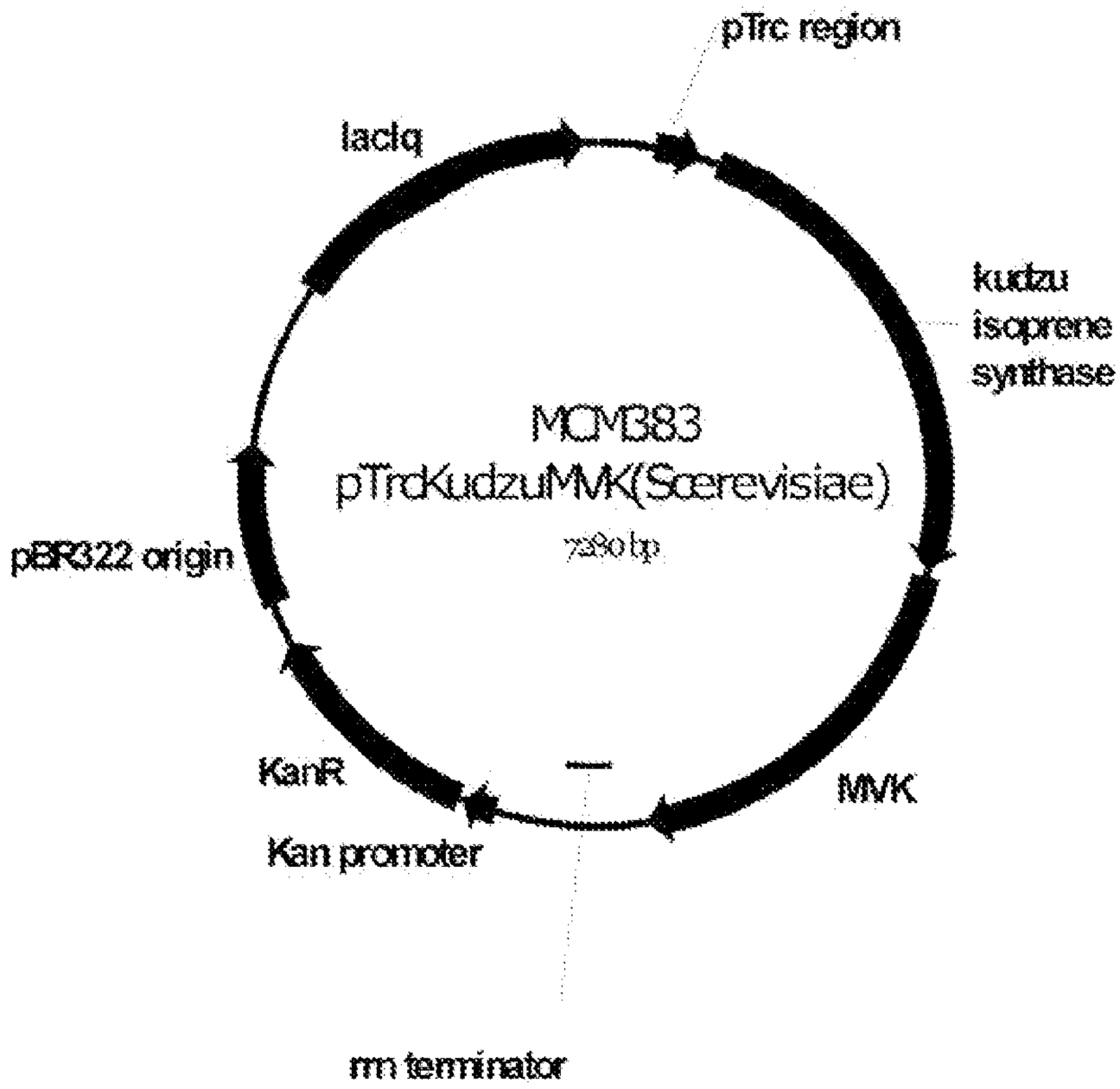
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Figure 59B

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Figure 59C

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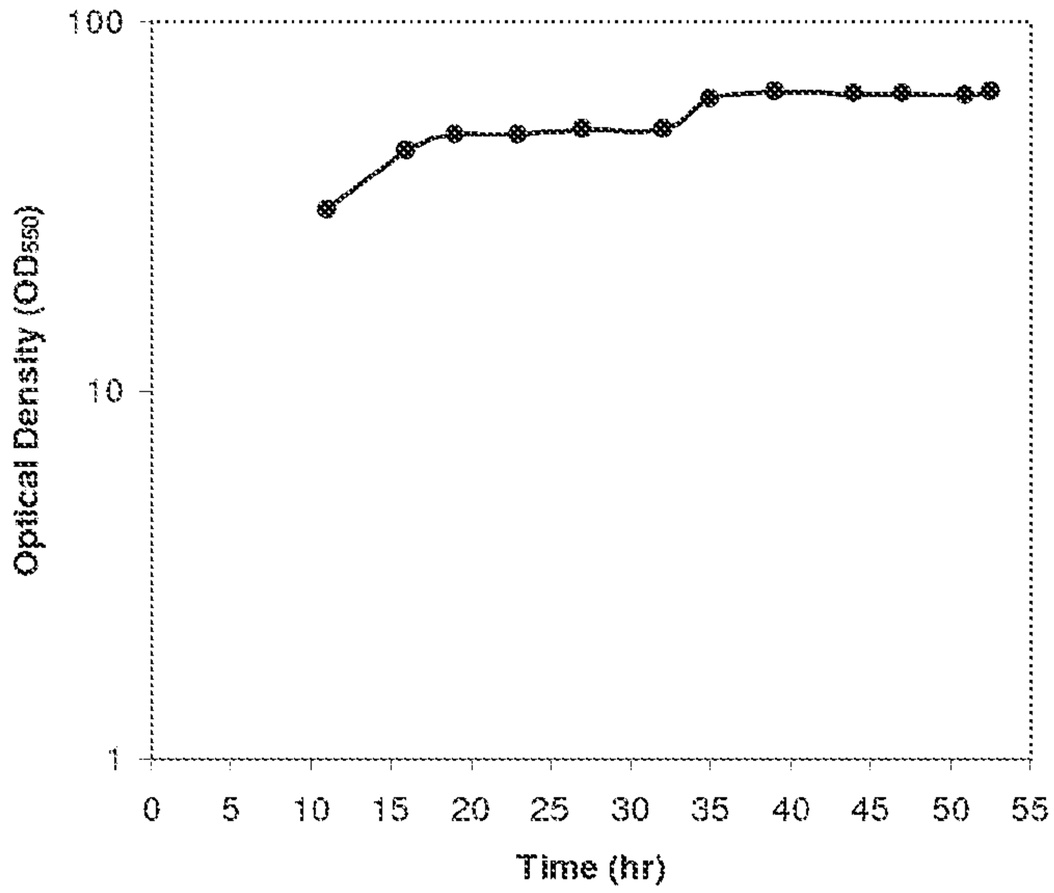

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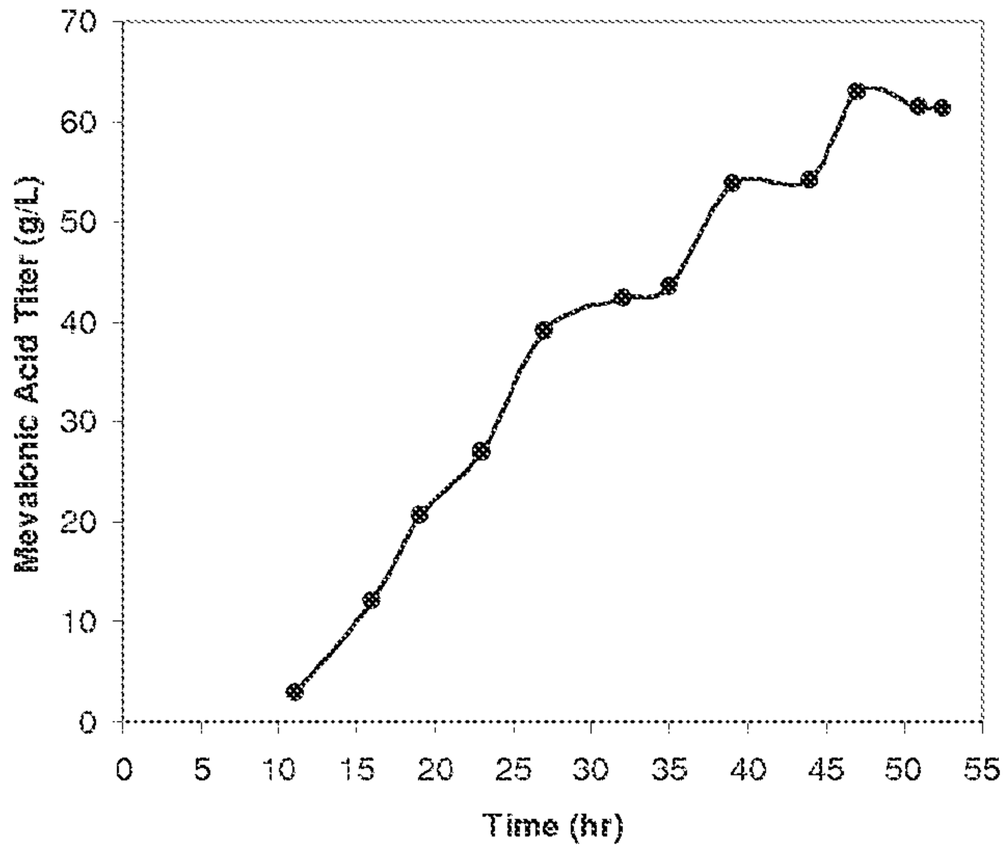

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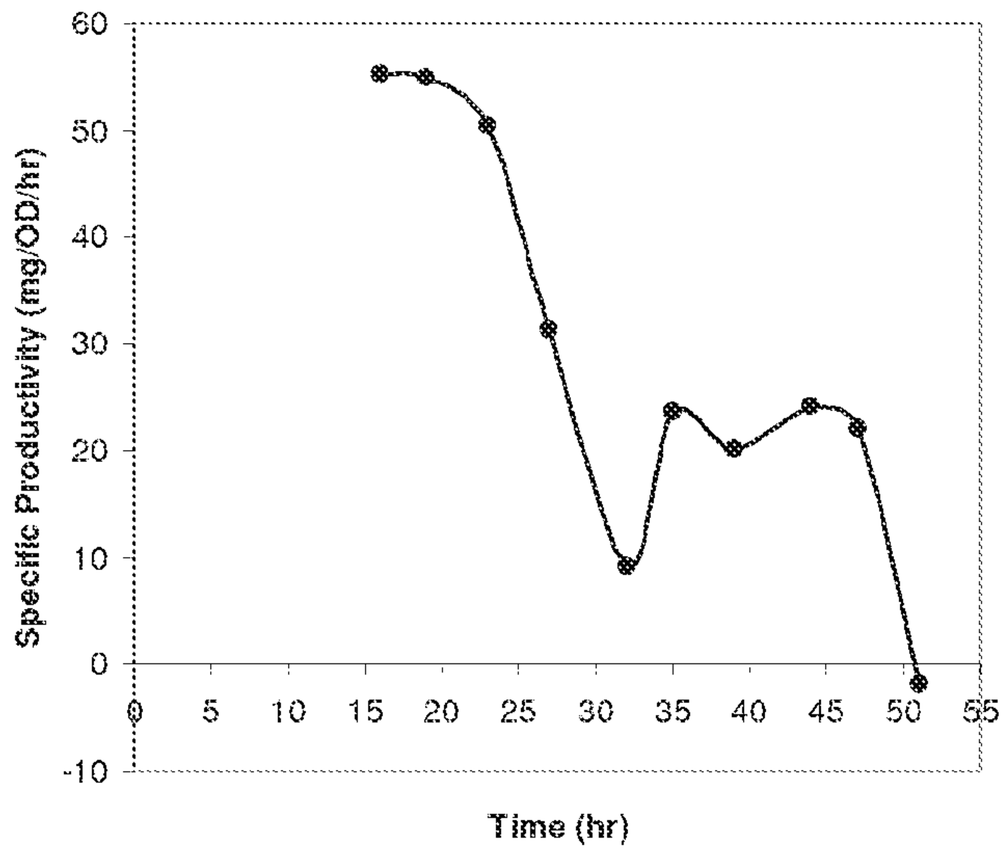

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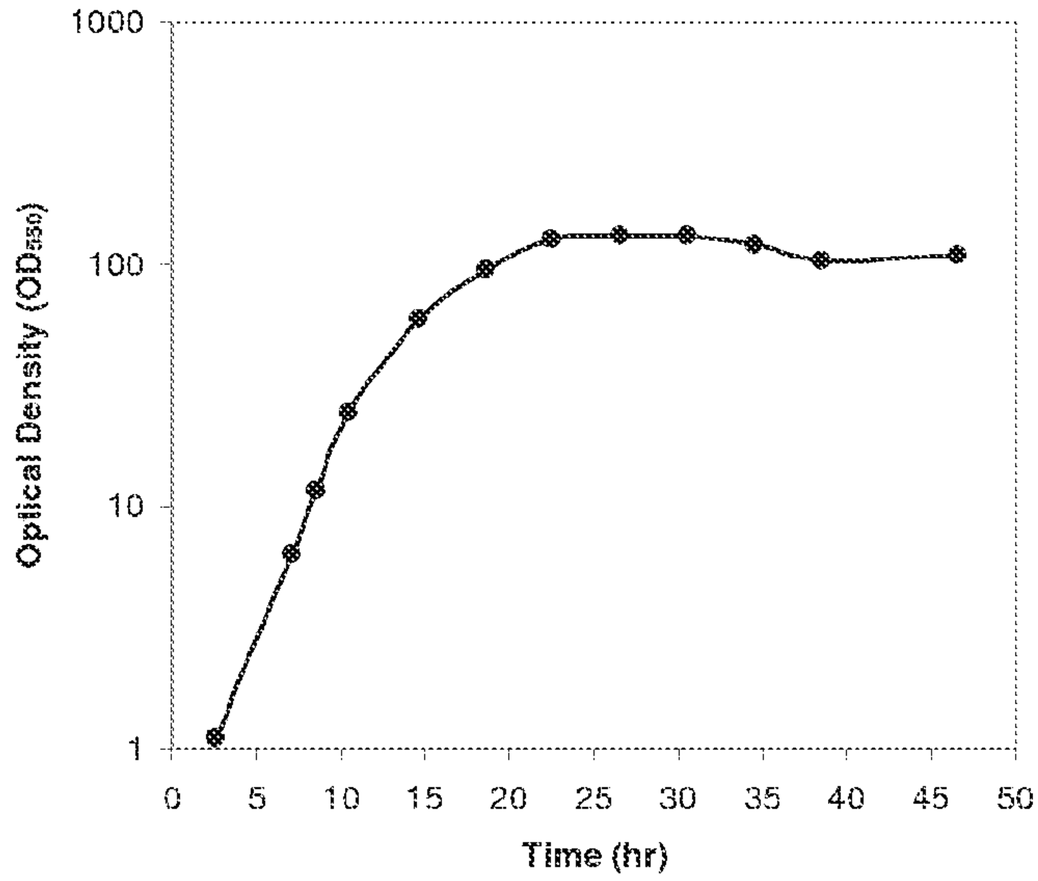

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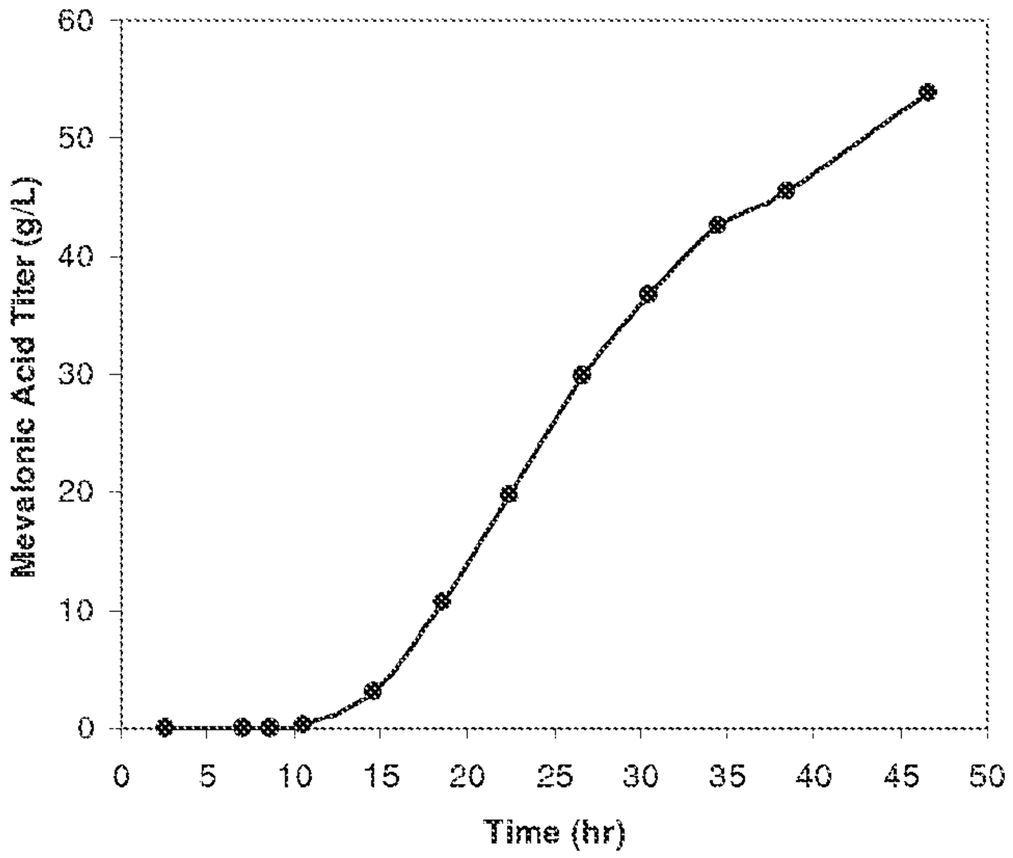

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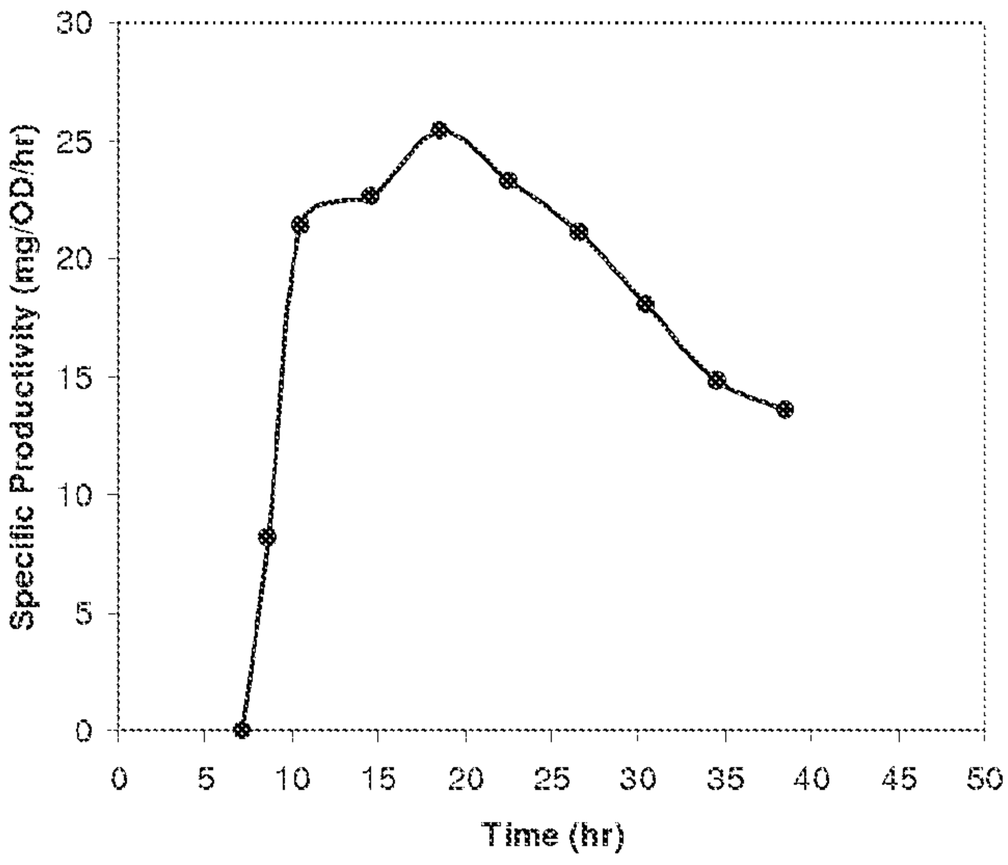

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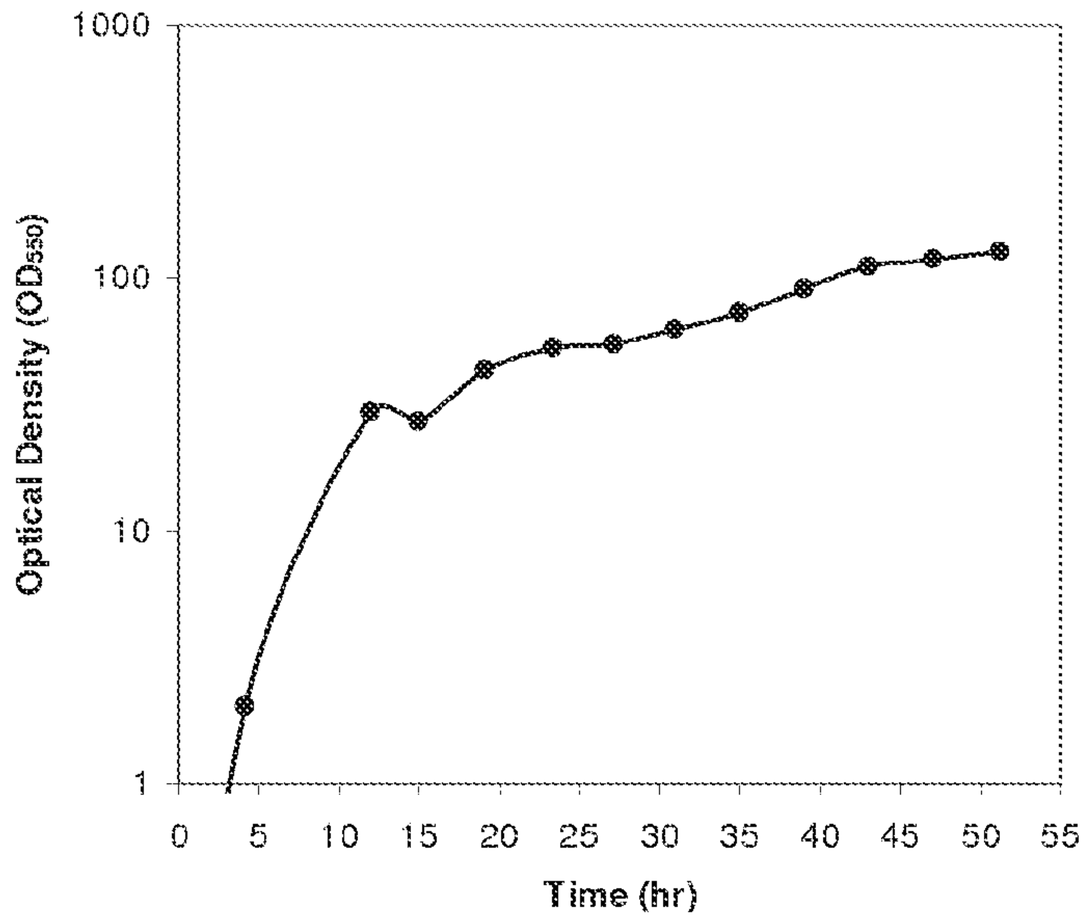

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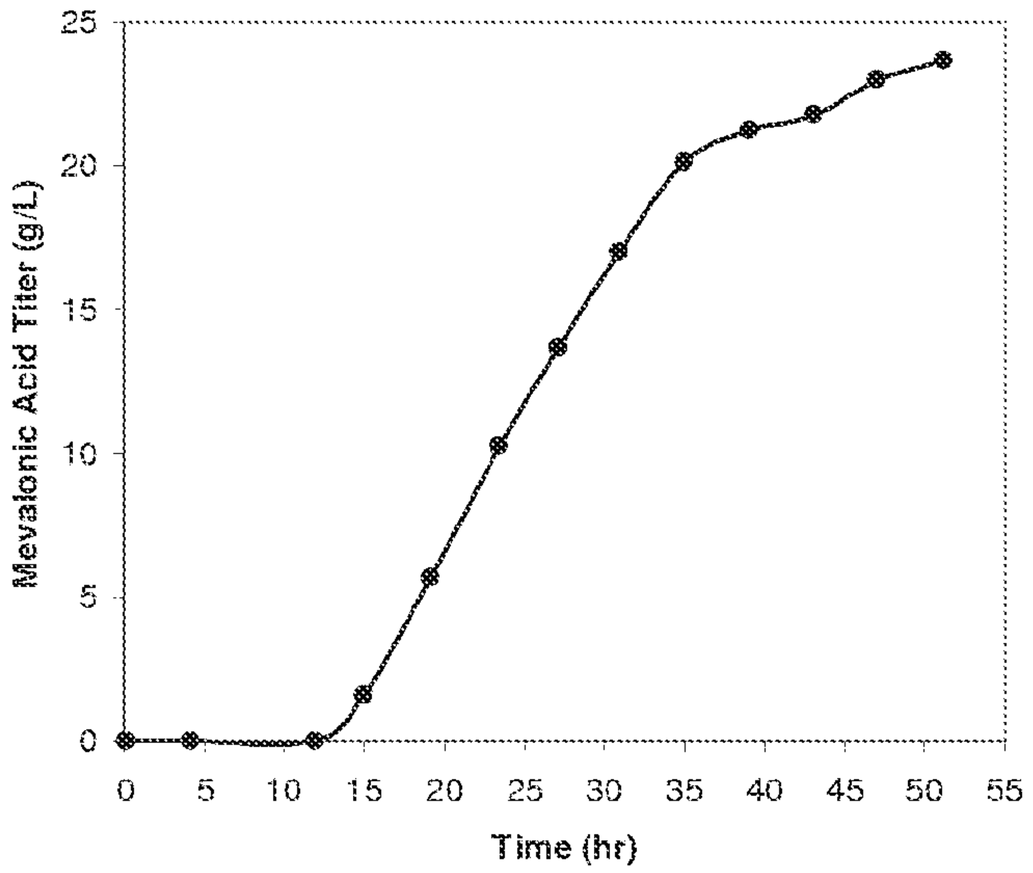

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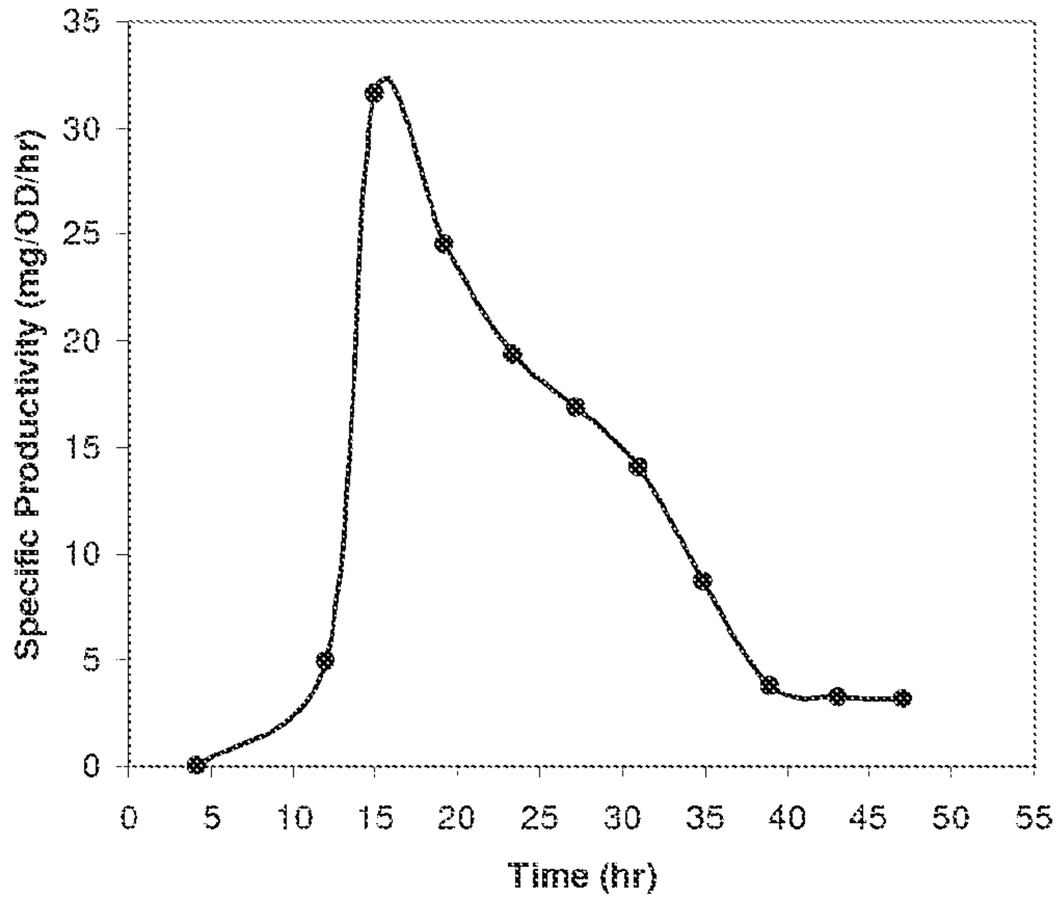

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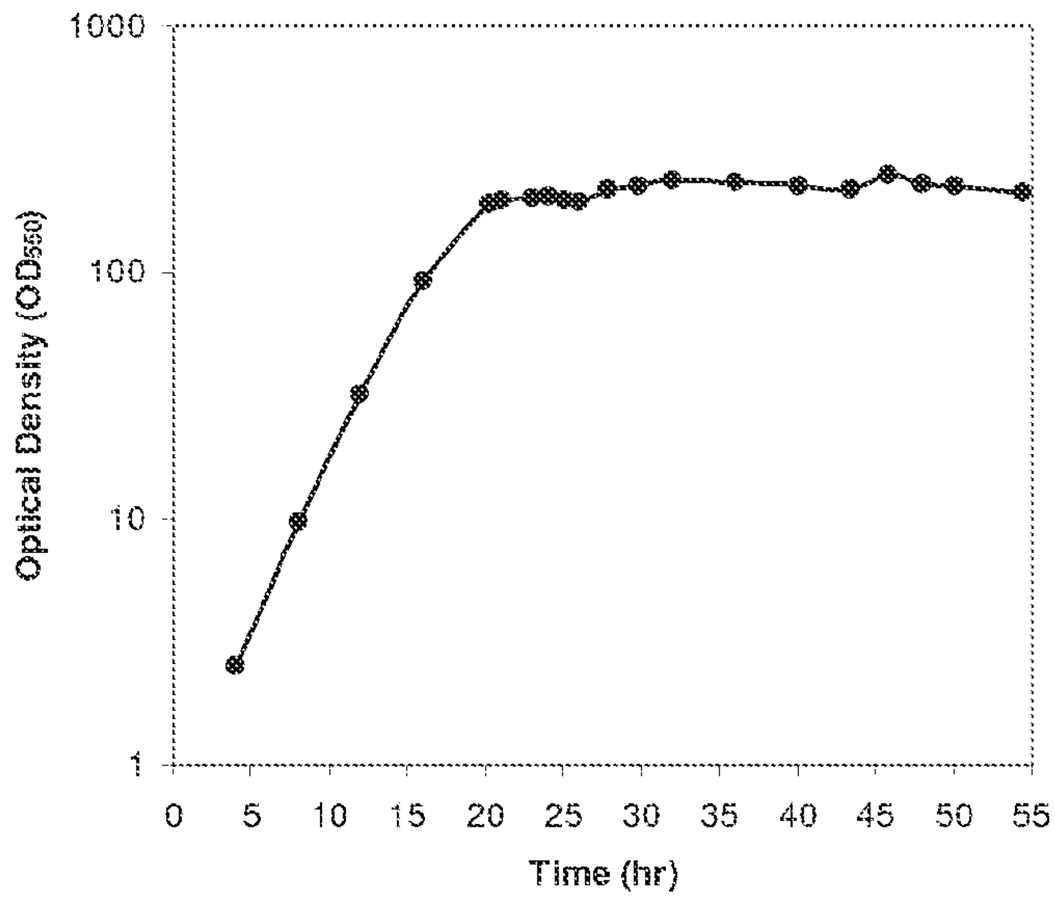

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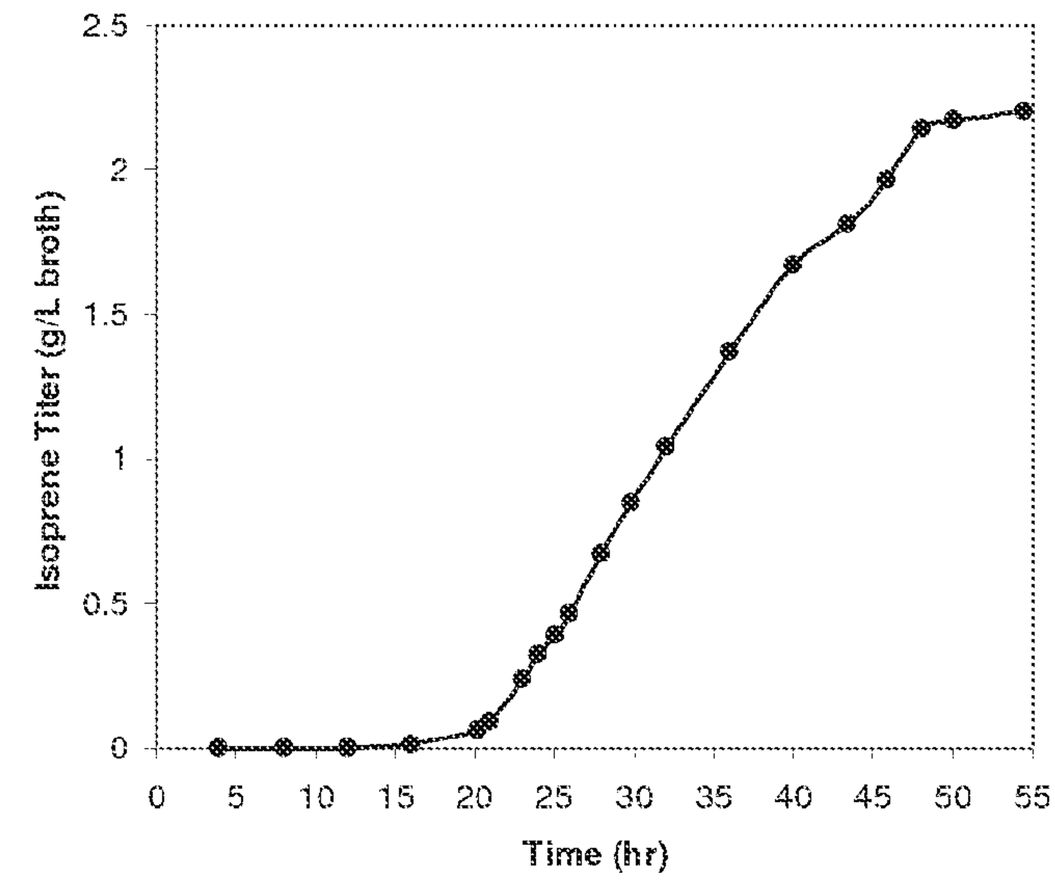

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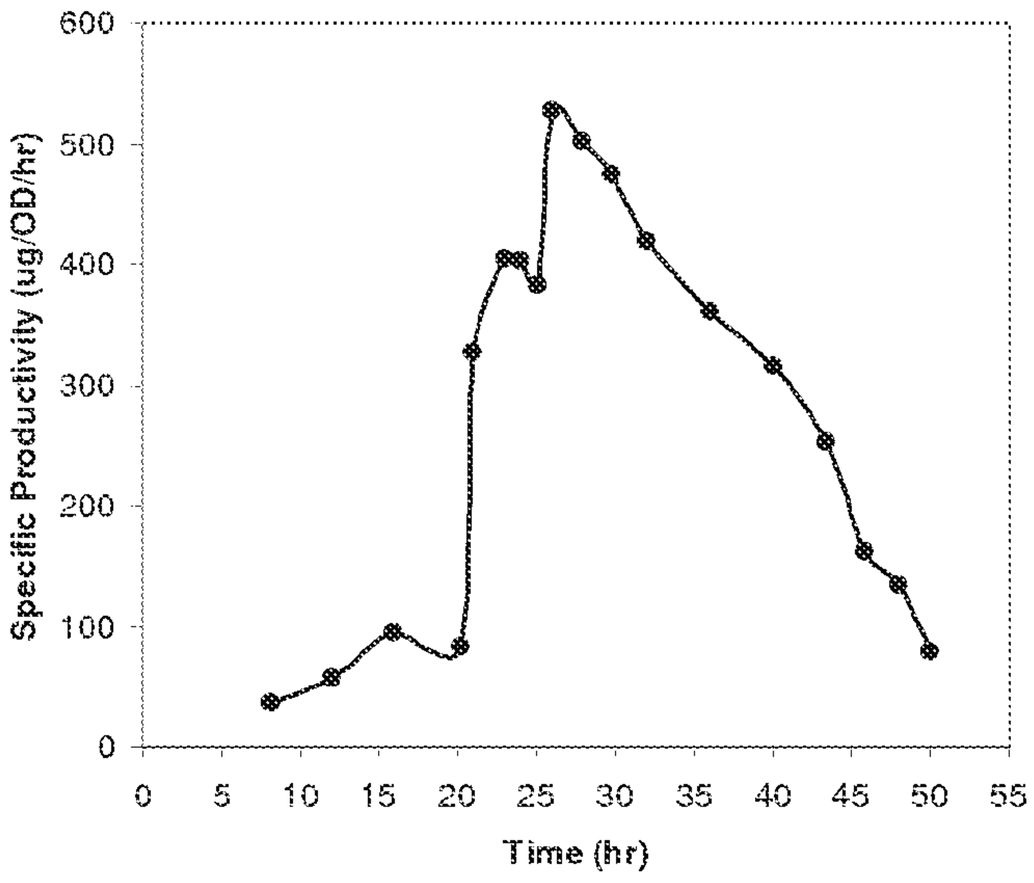

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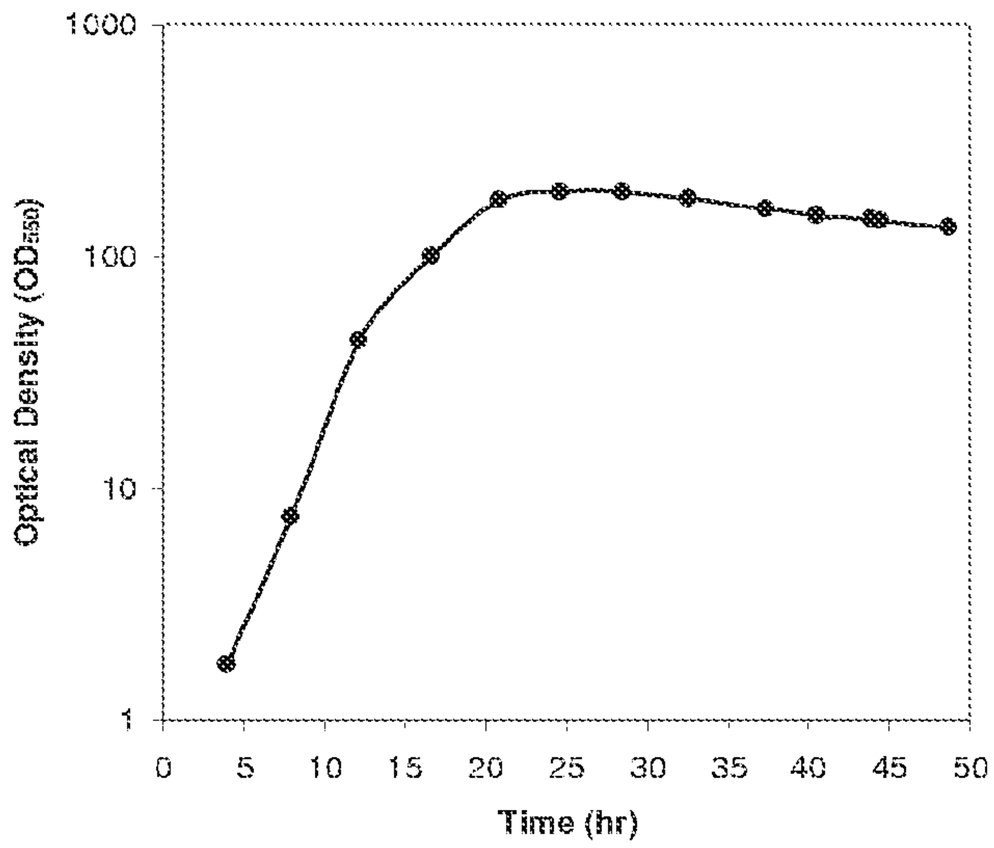

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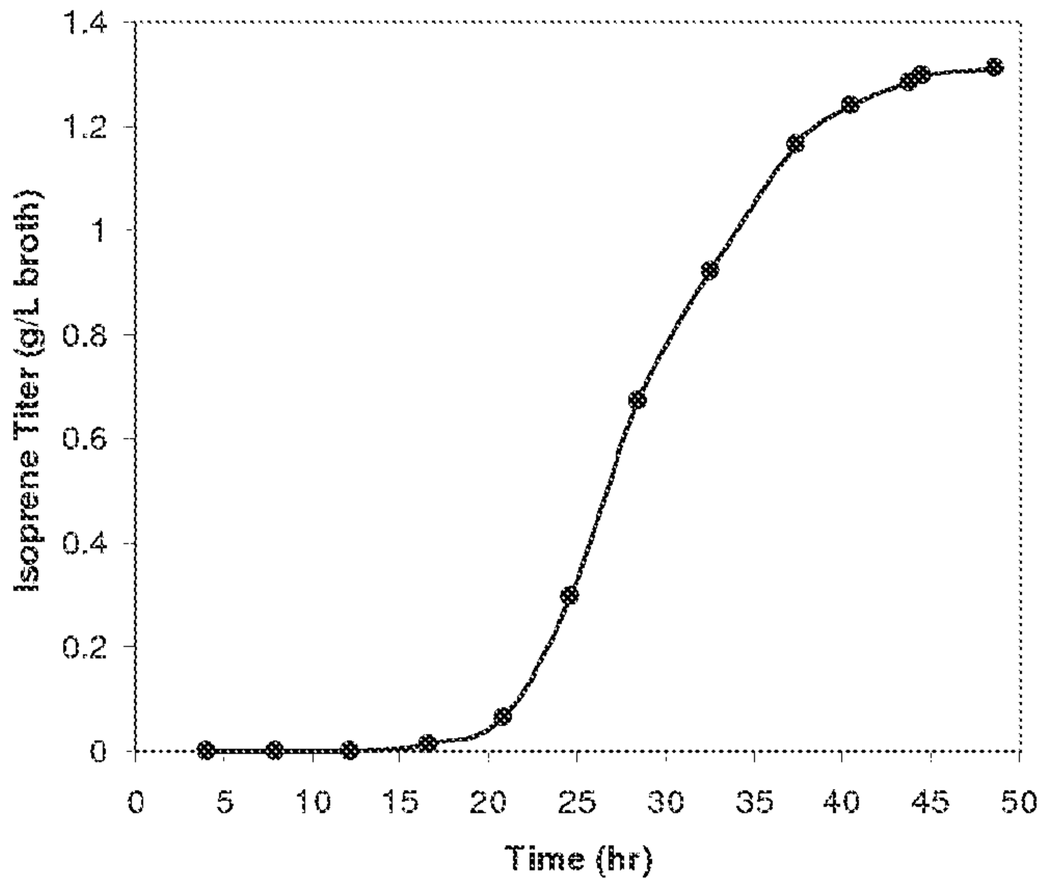

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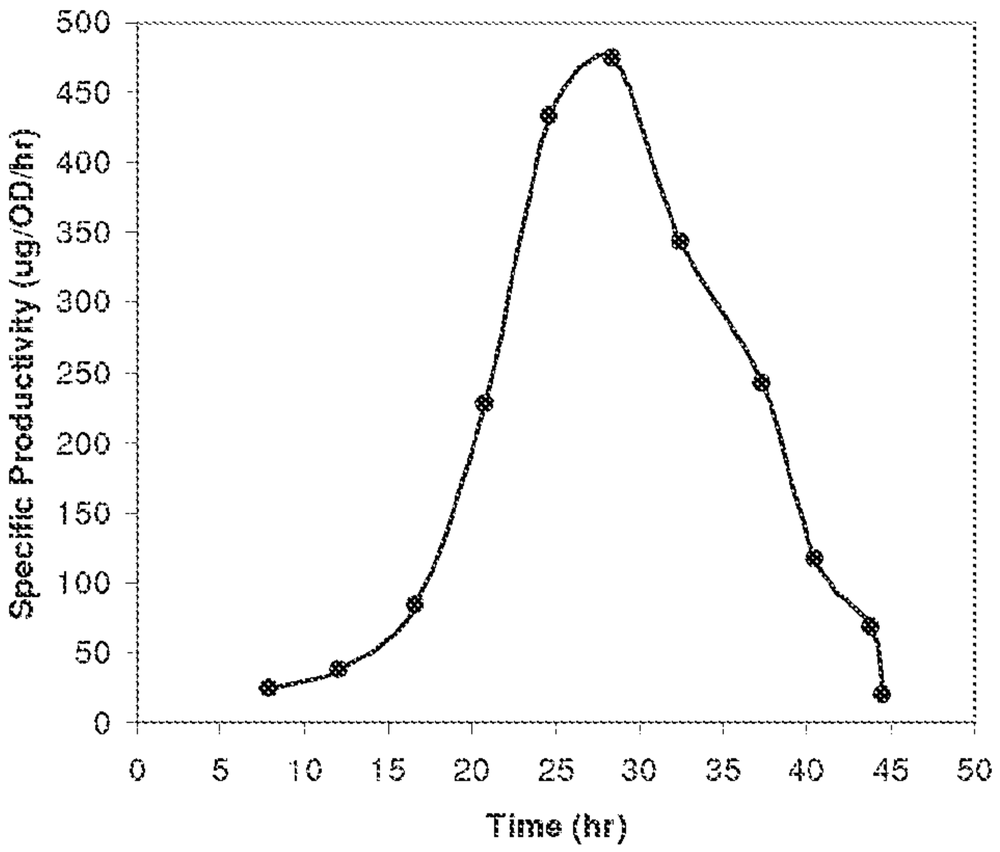

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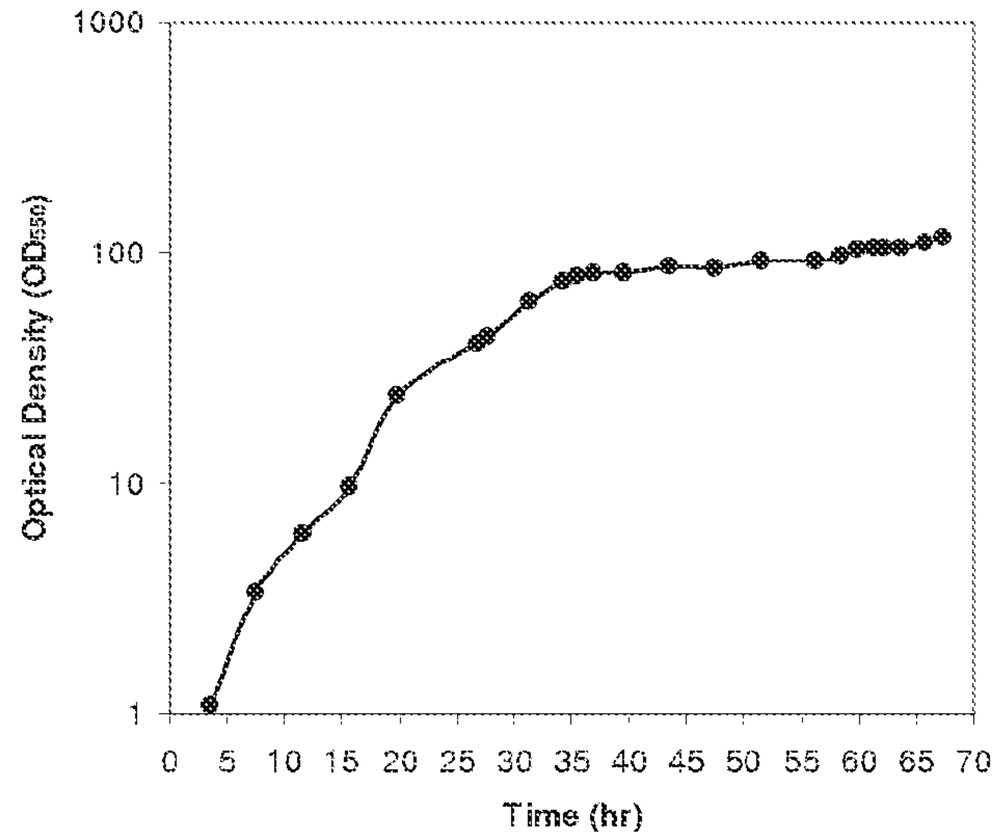

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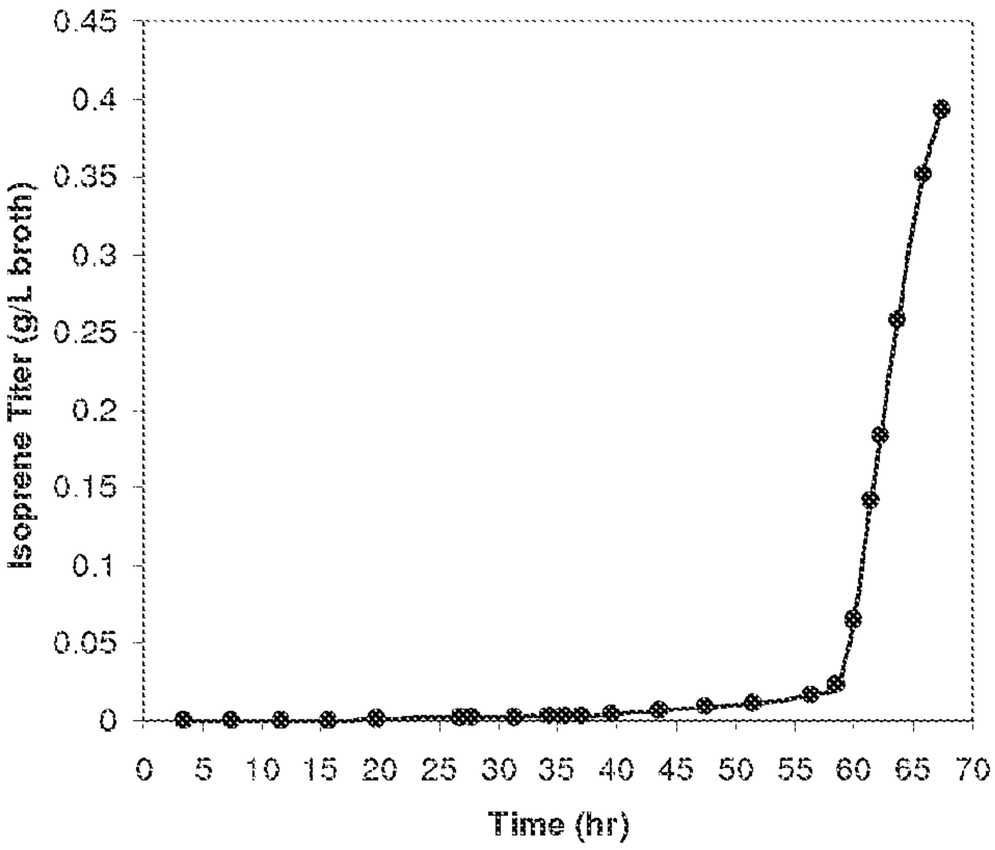


C

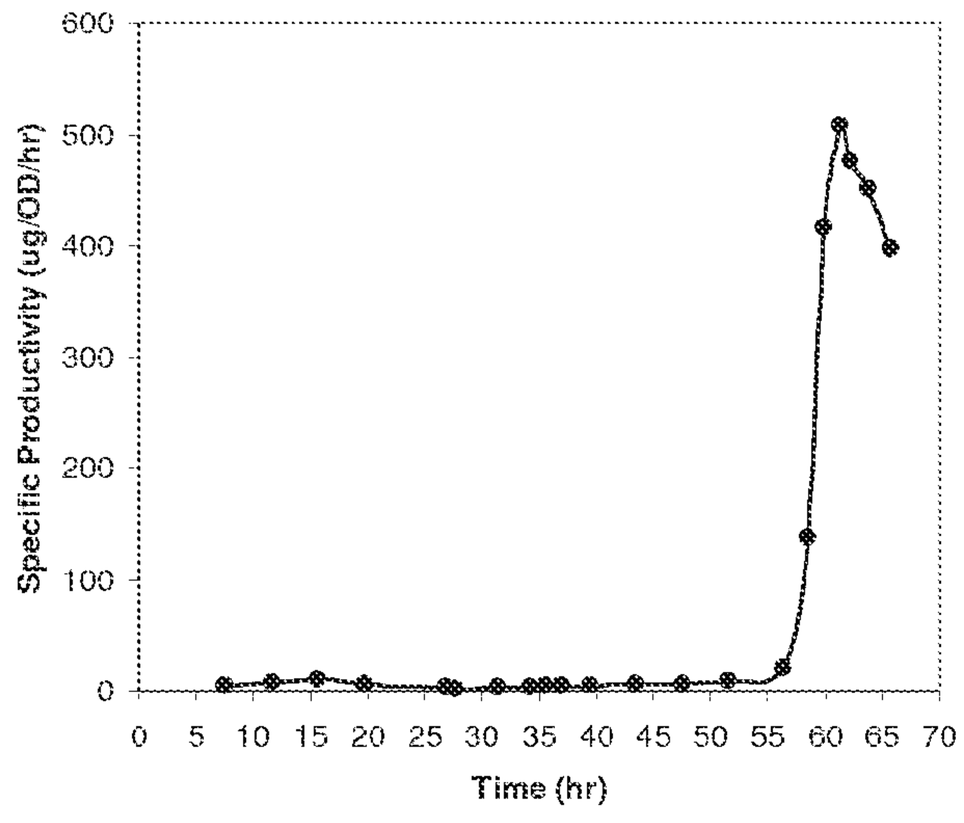


Figure 66

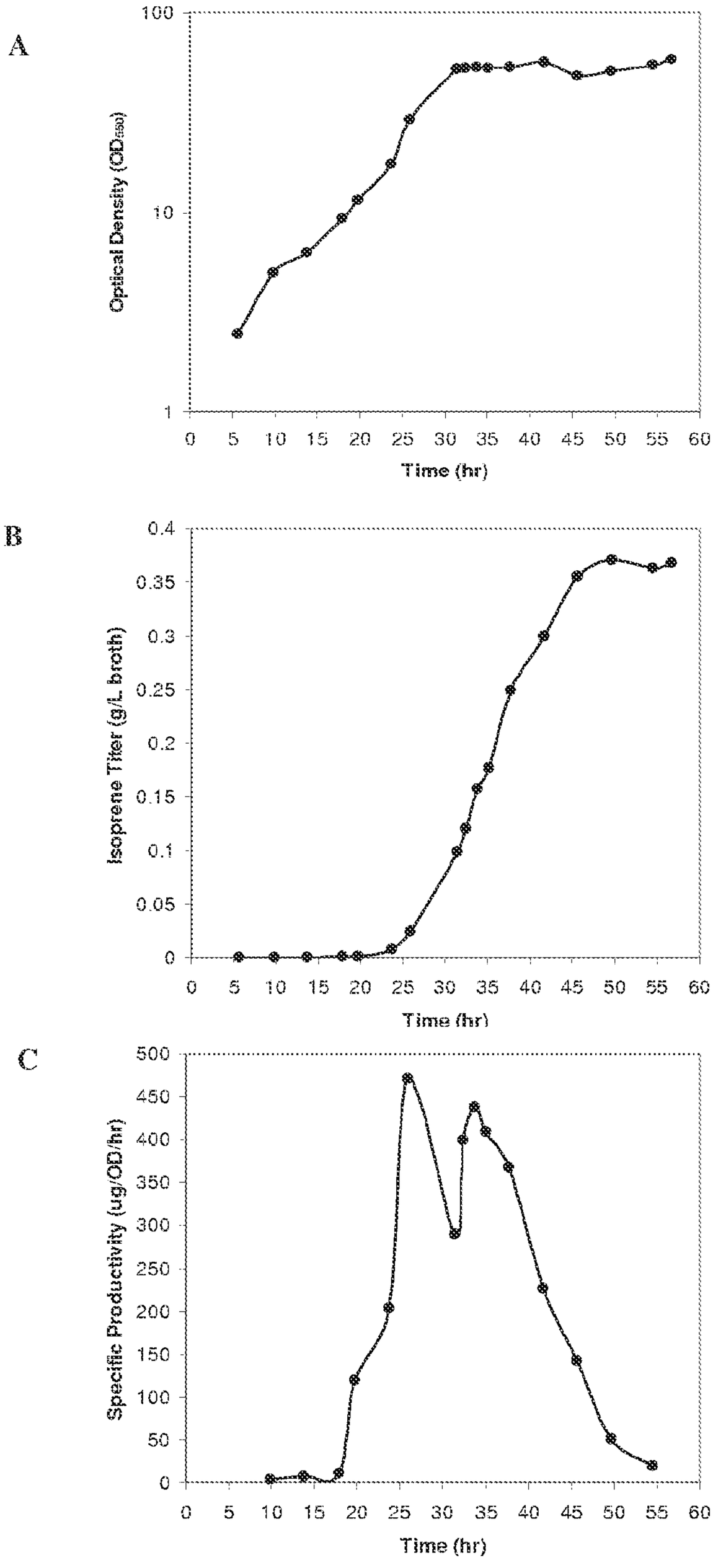
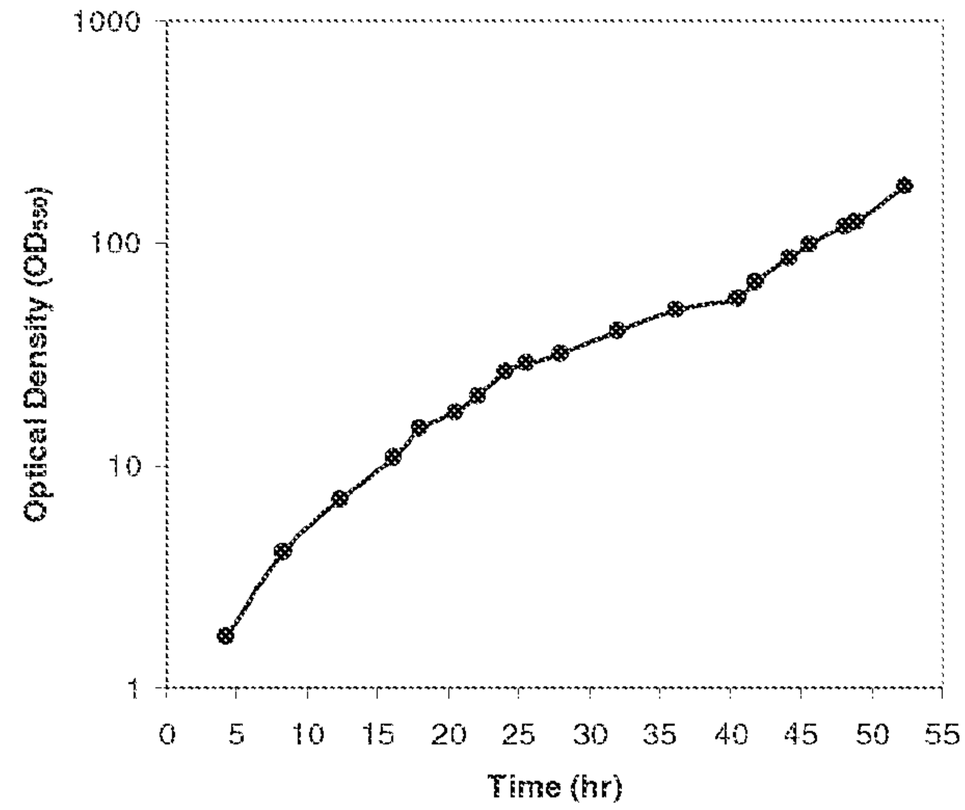
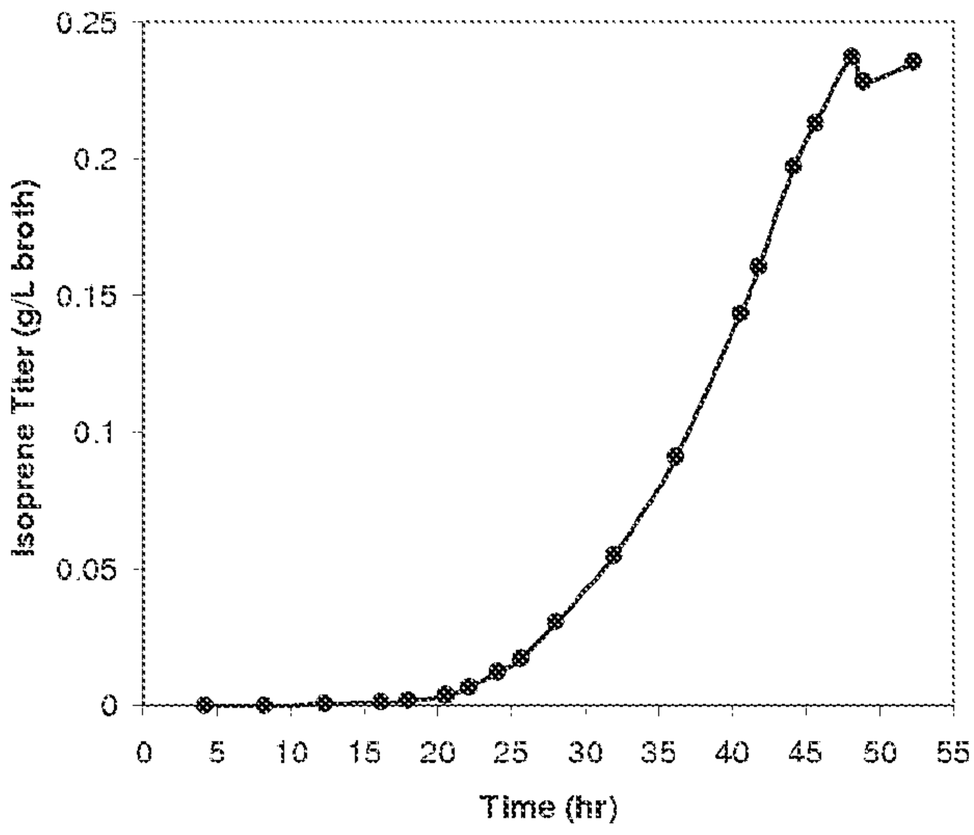

A
100
10
1
Optical Density (OD550)
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (hr)
B
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Isoprene Titer (g/L broth)
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (hr)
C
500
450
400
350
300
250
200
150
100
50
0
Specific Productivity (ug/OD/hr)
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (hr)

Figure 67

A



B



C

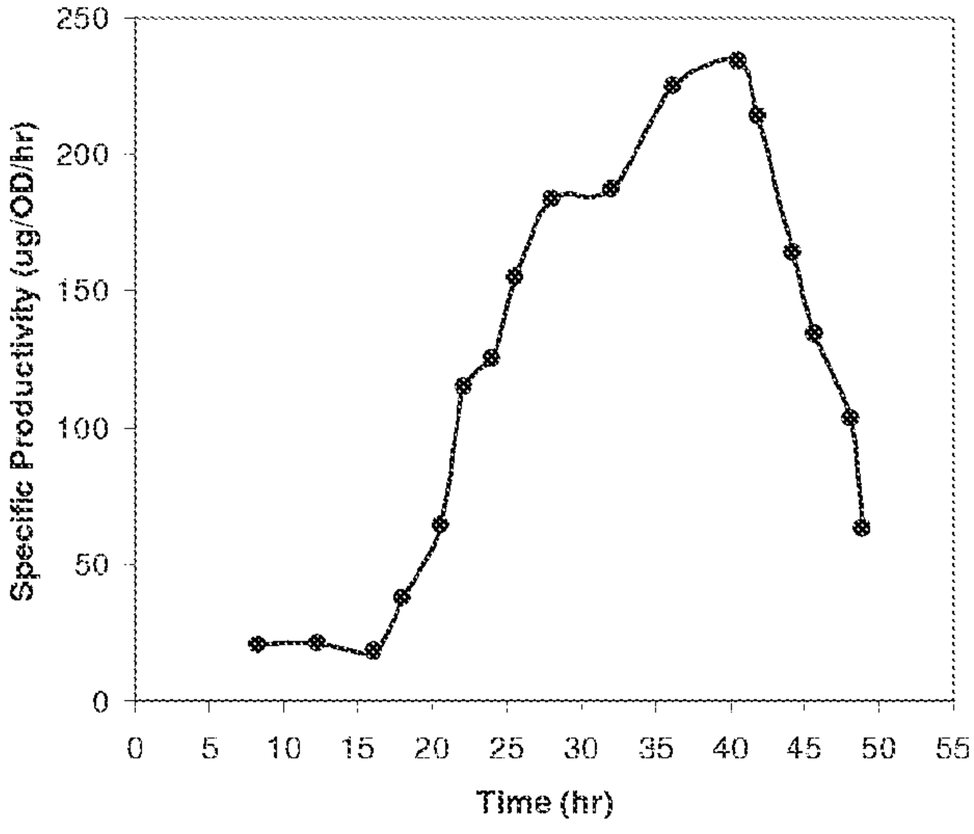


Figure 68

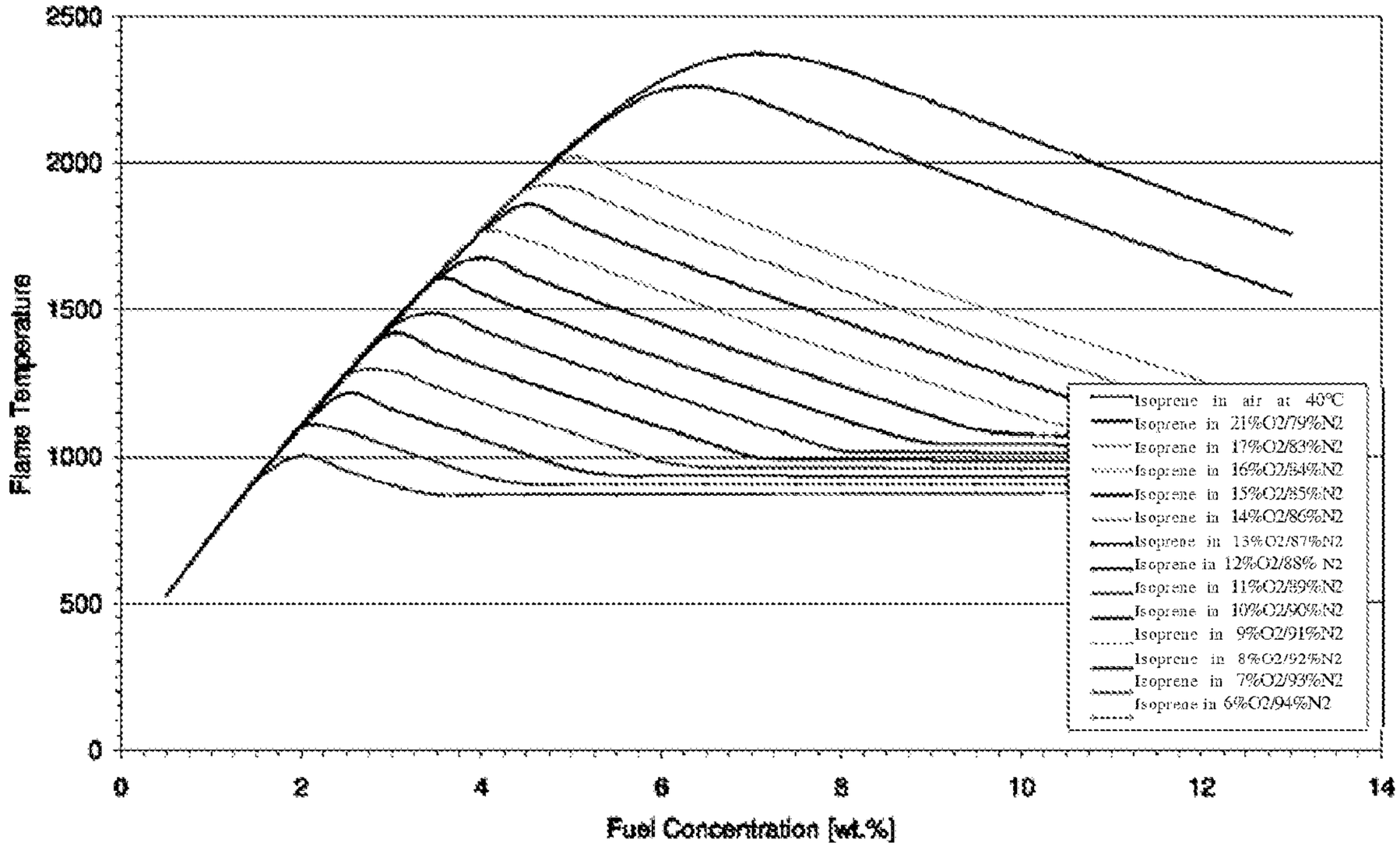

Isoprene in Oxygen/Nitrogen Environments
Flame Temperature
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Fuel Concentration [wt.%]
Isoprene in air at 40°C
Isoprene in 21%O2/79%N2
Isoprene in 17%O2/83%N2
Isoprene in 16%O2/84%N2
Isoprene in 15%O2/85%N2
Isoprene in 14%O2/86%N2
Isoprene in 13%O2/87%N2
Isoprene 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

Figure 69

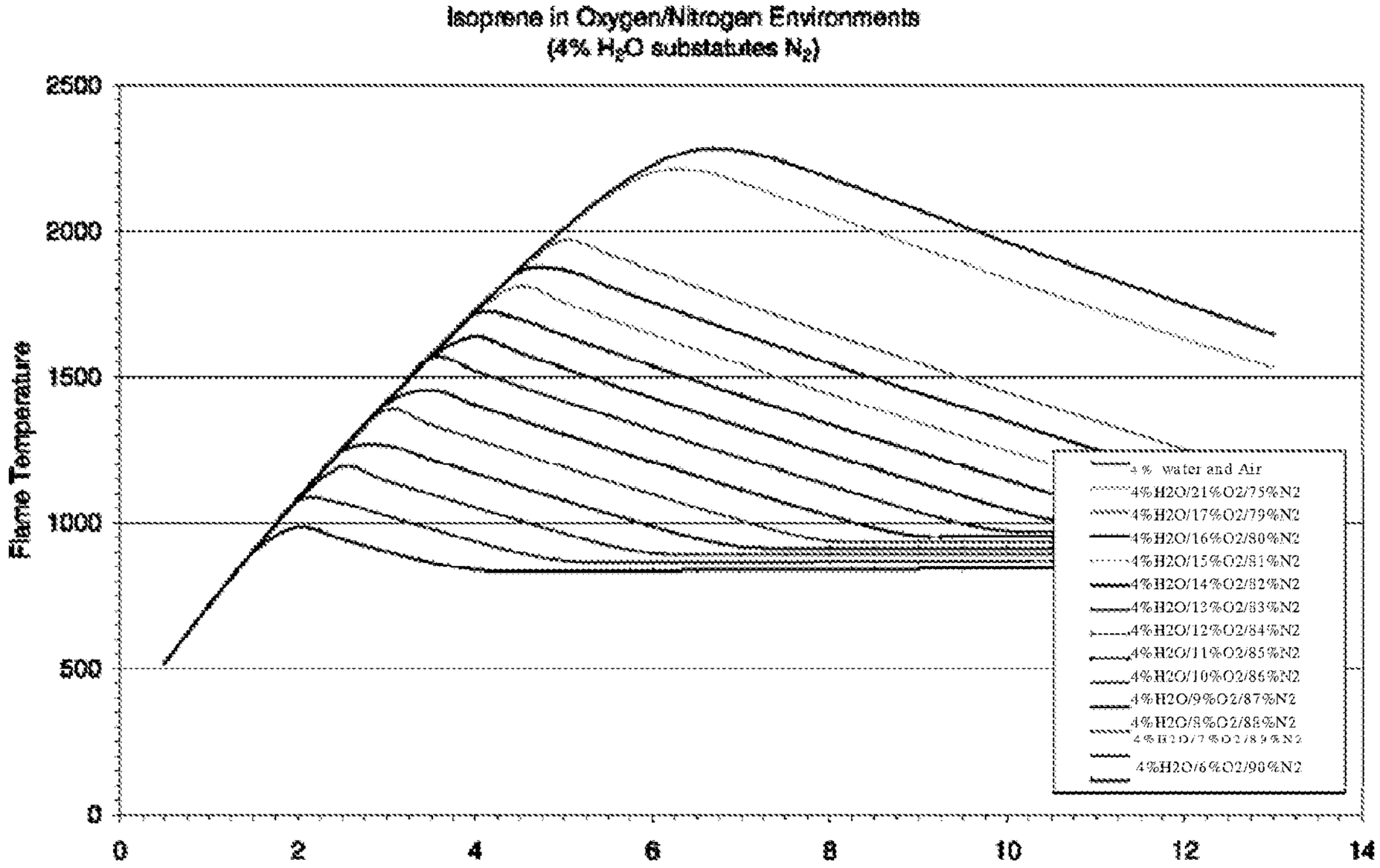

Isoprene in Oxygen/Nitrogen Environments
(4% H_2O substatutes N_2)
Flame Temperature
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Fuel Concentration [wt.%]
4% water and Air
4%H2O/21%O2/75%N2
4%H2O/17%O2/79%N2
4%H2O/16%O2/80%N2
4%H2O/15%O2/81%N2
4%H2O/14%O2/82%N2
4%H2O/13%O2/83%N2
4%H2O/12%O2/84%N2
4%H2O/11%O2/85%N2
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

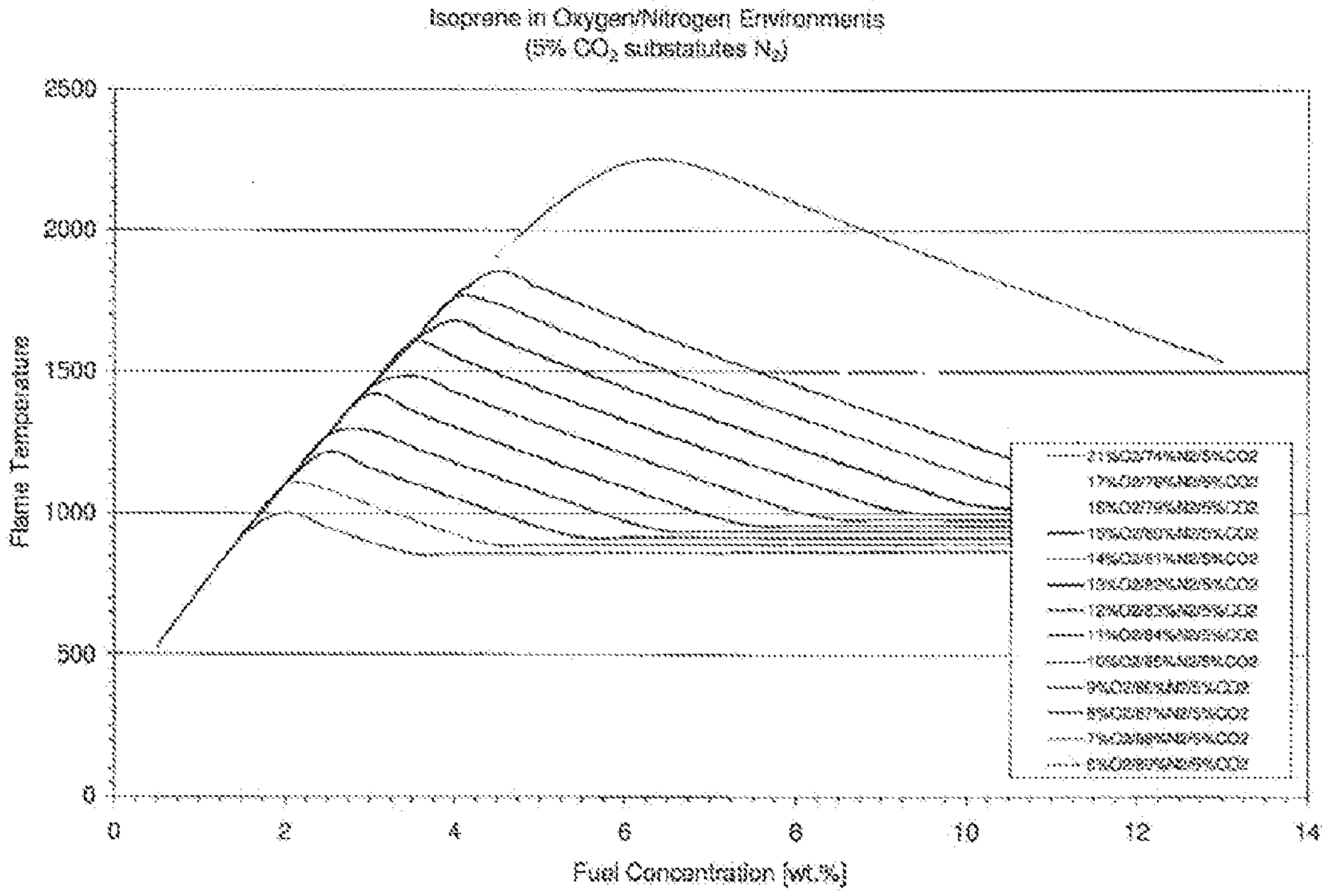

Isoprene in Oxygen/Nitrogen Environments
(5% CO_2 substitutes N_2)
Flame Temperature
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Fuel Concentration (wt.%)

Figure 71

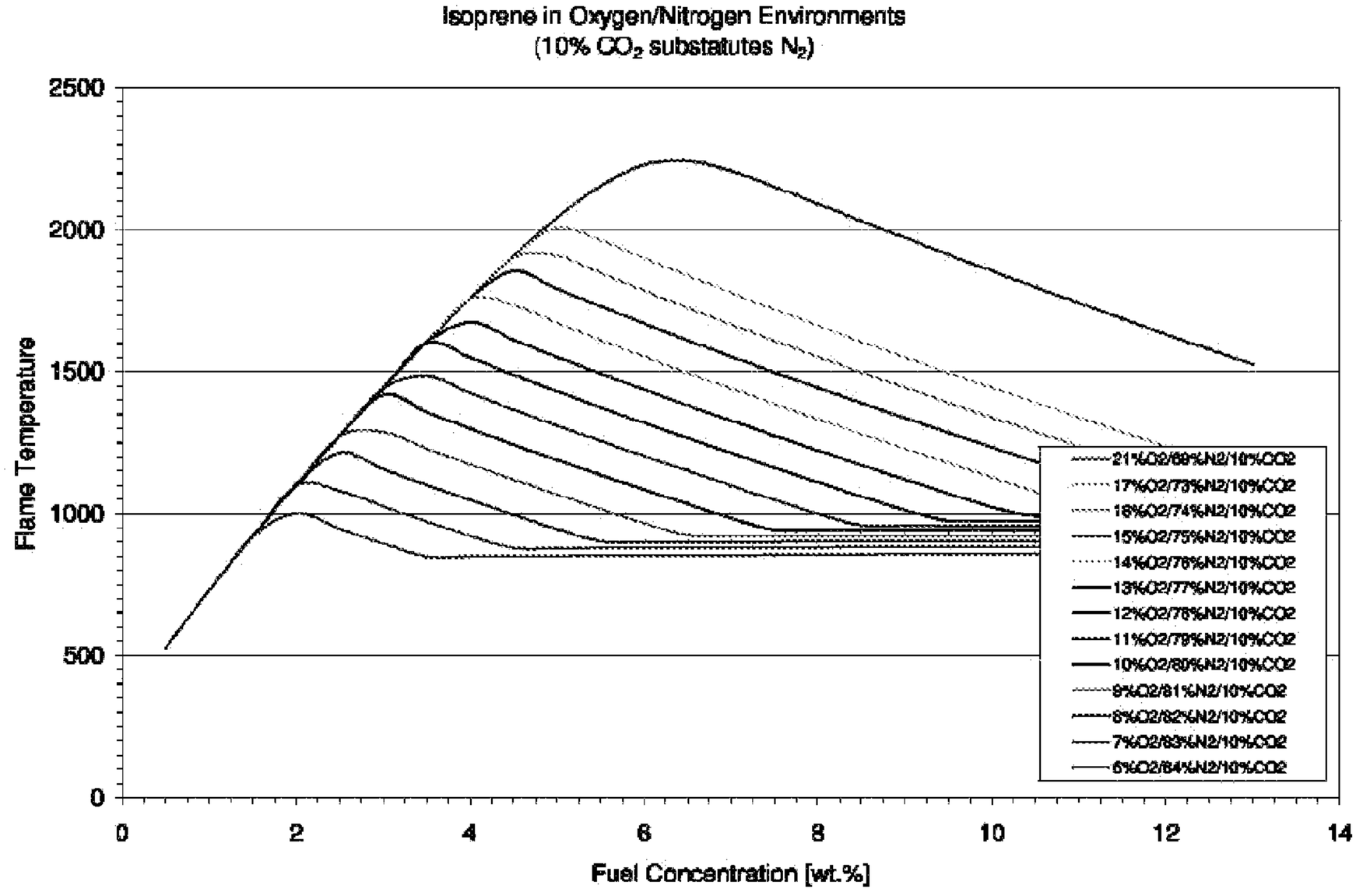

Isoprene in Oxygen/Nitrogen Environments
(10% CO_2 substatutes N_2)
Flame Temperature
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Fuel Concentration [wt.%]
21%O2/69%N2/10%CO2
17%O2/73%N2/10%CO2
16%O2/74%N2/10%CO2
15%O2/75%N2/10%CO2
14%O2/76%N2/10%CO2
13%O2/77%N2/10%CO2
12%O2/78%N2/10%CO2
11%O2/79%N2/10%CO2
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

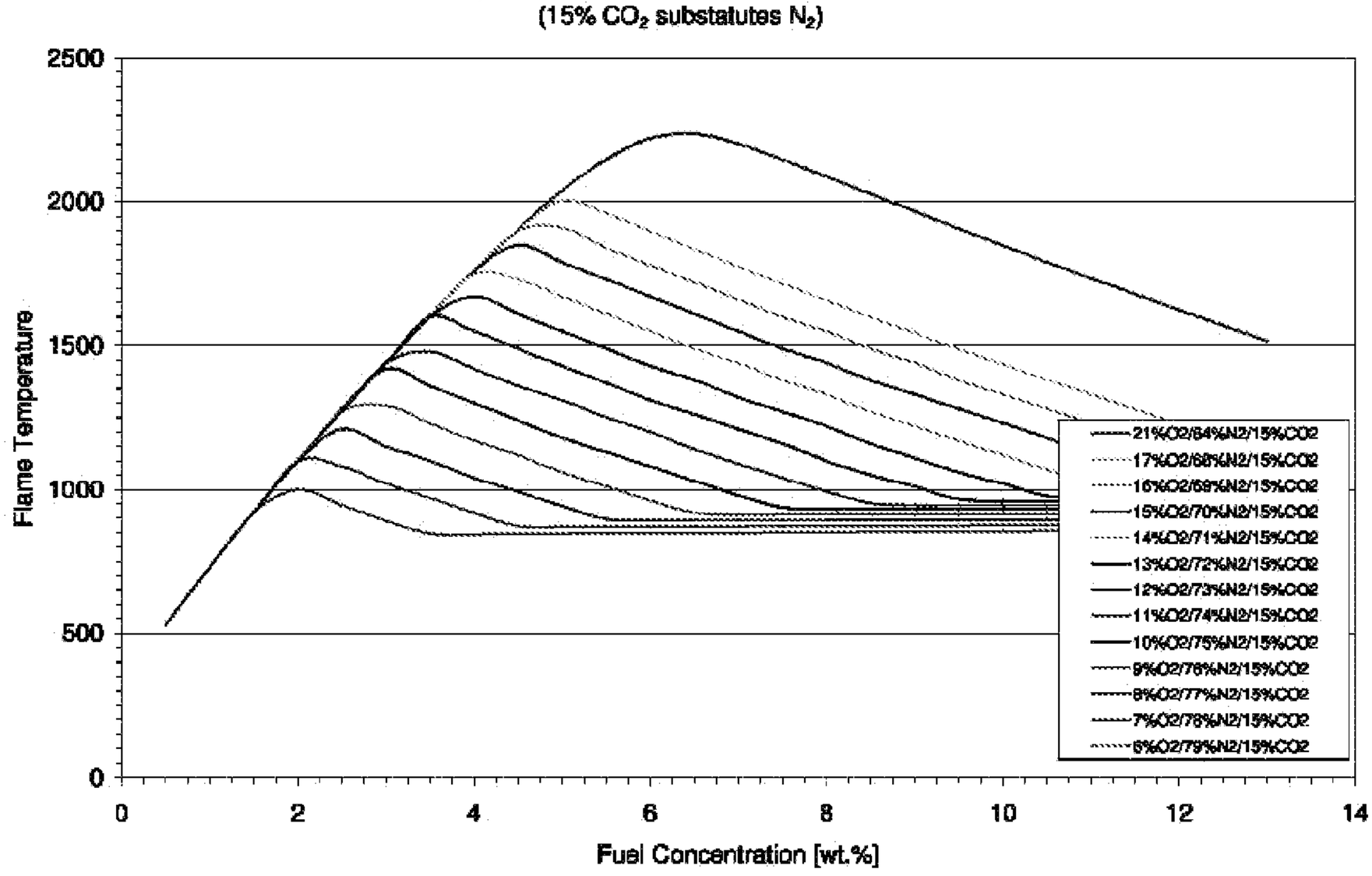

Isoprene in Oxygen/Nitrogen Environments
(15% CO_2 substatutes N_2)
Flame Temperature
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Fuel Concentration [wt.%]
21%O2/64%N2/15%CO2
17%O2/68%N2/15%CO2
16%O2/69%N2/15%CO2
15%O2/70%N2/15%CO2
14%O2/71%N2/15%CO2
13%O2/72%N2/15%CO2
12%O2/73%N2/15%CO2
11%O2/74%N2/15%CO2
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

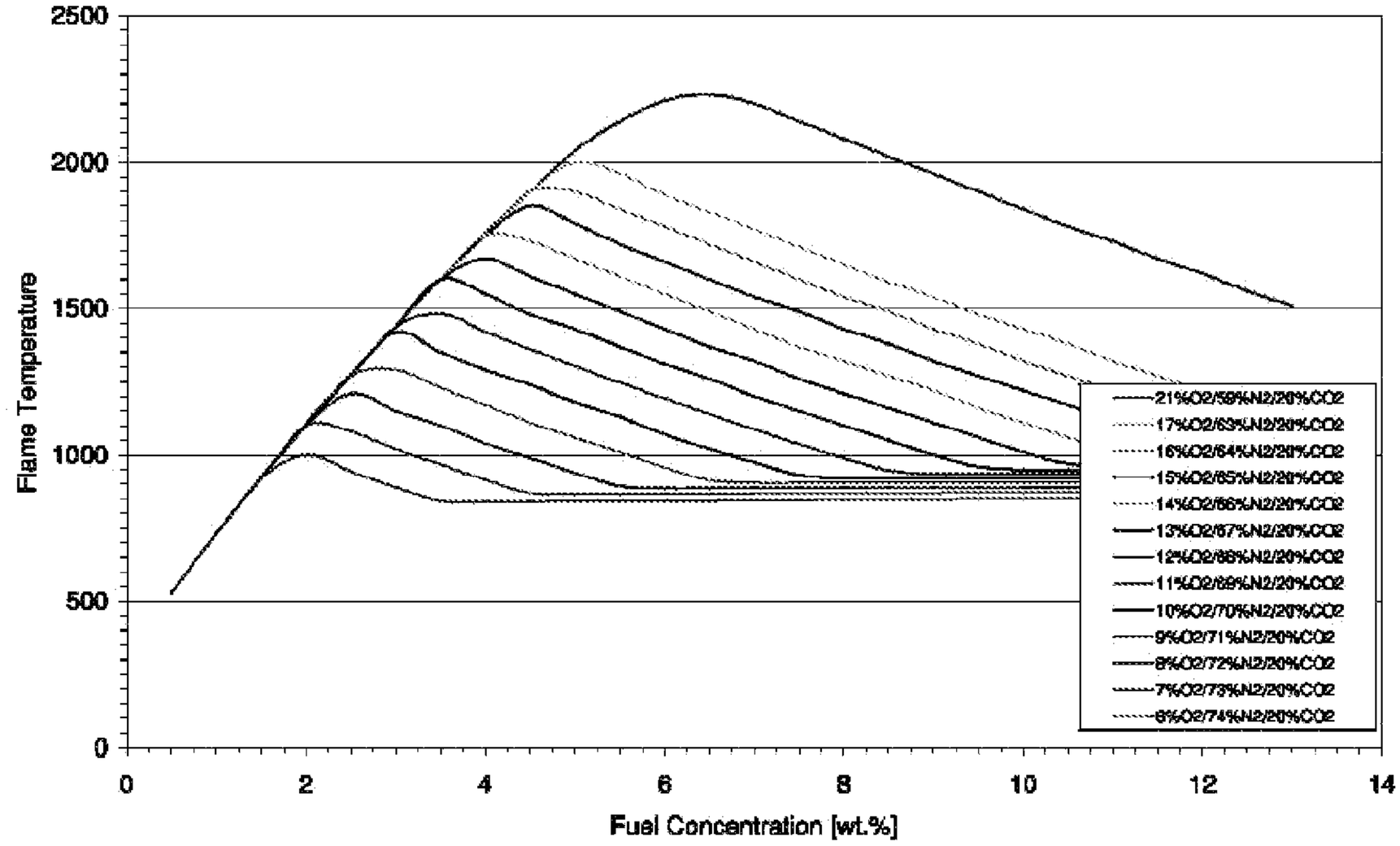

Isoprene in Oxygen/Nitrogen Environments
(20% CO_2 substatutes N_2)
Flame Temperature
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Fuel Concentration [wt.%]
21%O2/59%N2/20%CO2
17%O2/63%N2/20%CO2
16%O2/64%N2/20%CO2
15%O2/65%N2/20%CO2
14%O2/66%N2/20%CO2
13%O2/67%N2/20%CO2
12%O2/68%N2/20%CO2
11%O2/69%N2/20%CO2
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

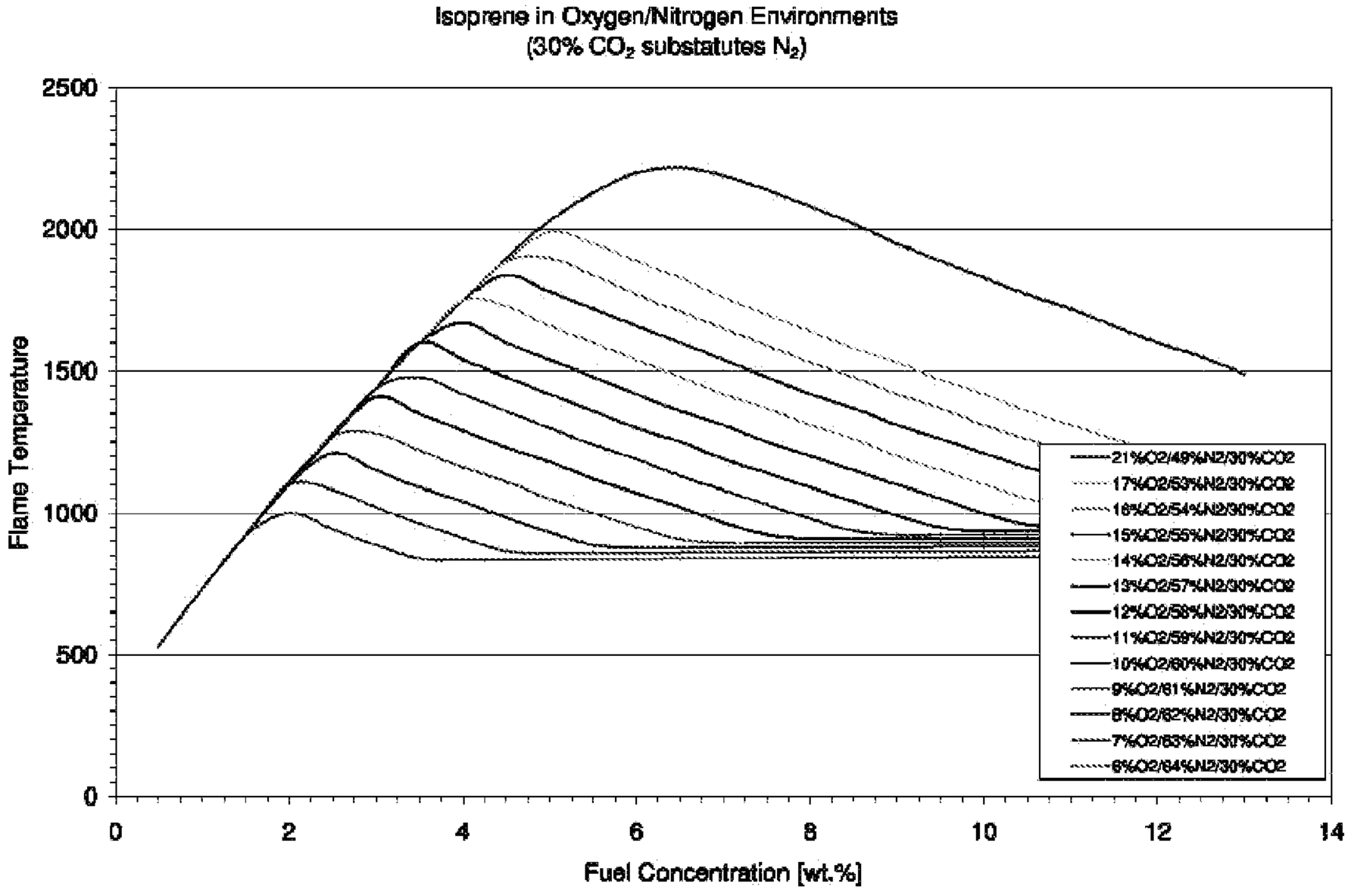

Isoprene in Oxygen/Nitrogen Environments
(30% CO_2 substatutes N_2)
Flame Temperature
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Fuel Concentration [wt.%]
21%O2/49%N2/30%CO2
17%O2/53%N2/30%CO2
16%O2/54%N2/30%CO2
15%O2/55%N2/30%CO2
14%O2/56%N2/30%CO2
13%O2/57%N2/30%CO2
12%O2/58%N2/30%CO2
11%O2/59%N2/30%CO2
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. (wt.%)	Oxidizer Conc. (wt.%)	Isoprene (wt. %)	H_2O (wt. %)	O_2 (wt. %)	N_2 (wt. %)	Isoprene (mole)	H_20 (mole)	O_2 (mole)	N_2 (mole)	Total (mole)	Isoprene (vol. %)	O_2 (vol. %)	N_2 (vol. %)	H_2O (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
13.50	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

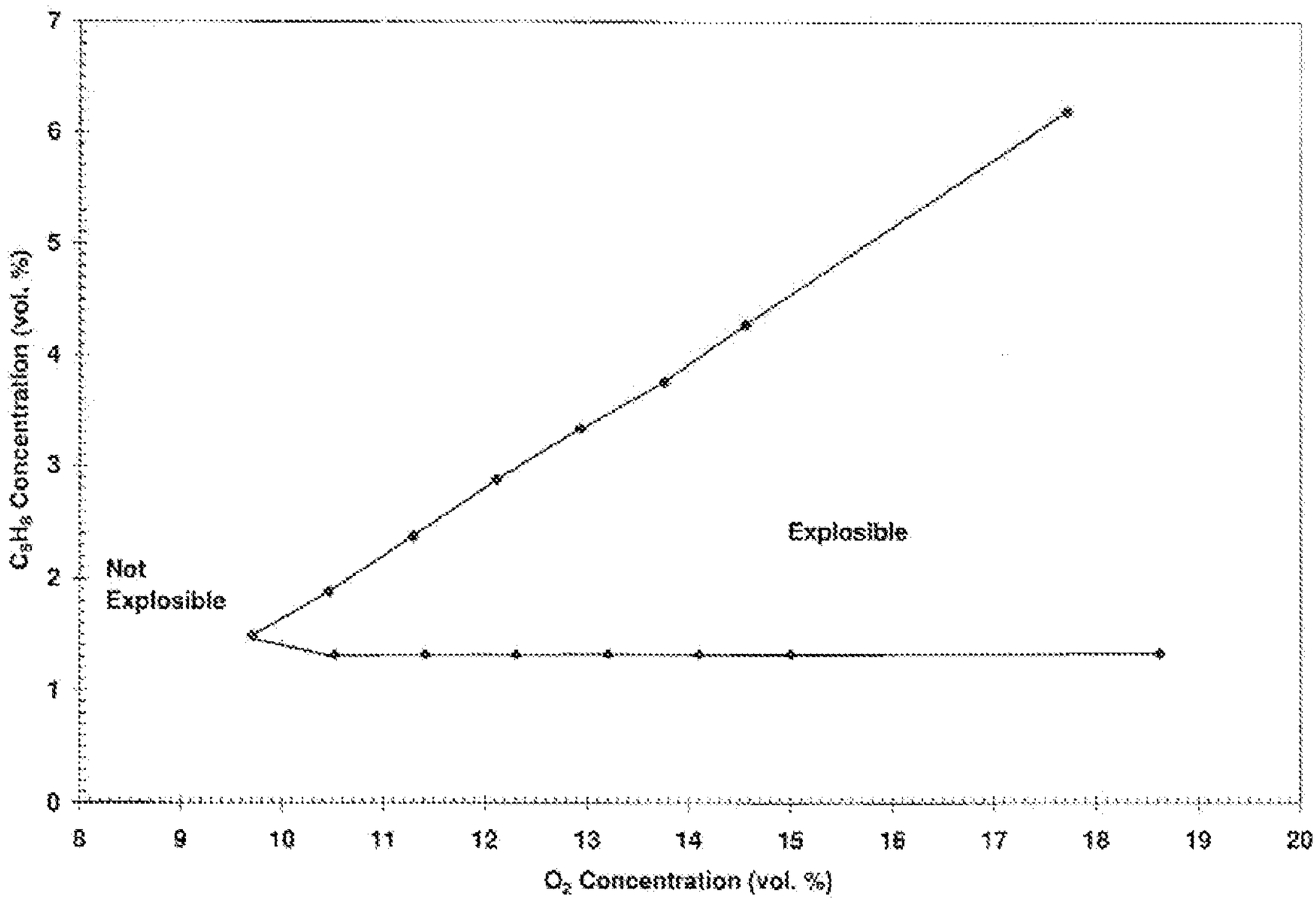

C_3H_8 Concentration (vol. %)
0
1
2
3
4
5
6
Not Explosible
Explosible
8
9
10
11
12
13
14
15
16
17
18
19
20
O_2 Concentration (vol. %)

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. (wt.%)	Oxidizer Conc. (wt.%)	Isoprene (wt. %)	H_2O (wt. %)	O_2 (wt. %)	N_2 (wt. %)	Isoprene (mole)	H_20 (mole)	O_2 (mole)	N_2 (mole)	Total (mole)	Isoprene (vol. %)	O_2 (vol. %)	N_2 (vol. %)	H_2O (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	34.68	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	3.68	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

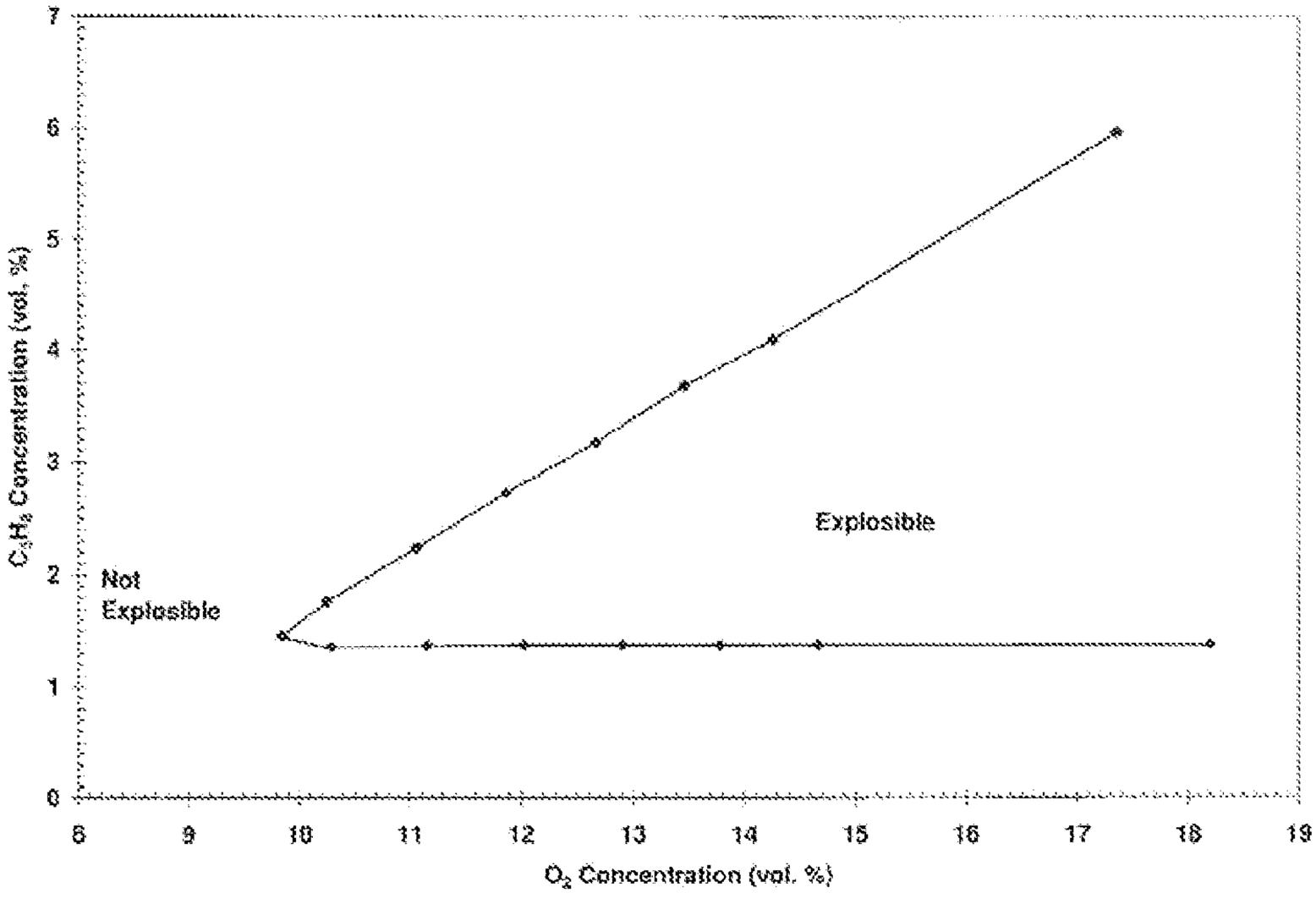
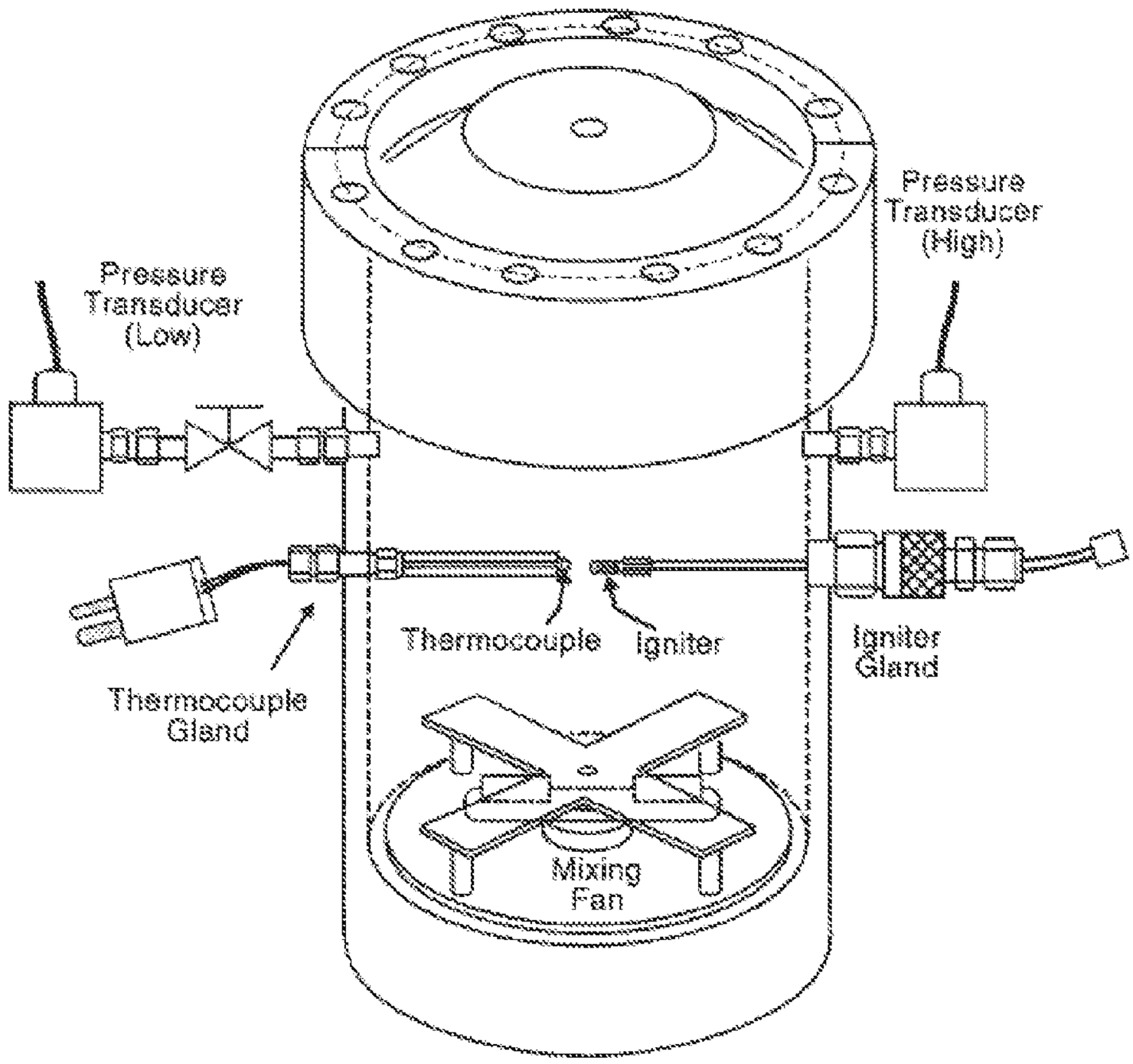

C_3H_8 Concentration (vol. %)
0
1
2
3
4
5
6
Not Explosible
Explosible
8
9
10
11
12
13
14
15
16
17
18
19
O_2 Concentration (vol. %)

Figure 77


Pressure Transducer (High)
Pressure Transducer (Low)
Thermocouple
Igniter
Igniter Gland
Thermocouple Gland
Mixing Fan

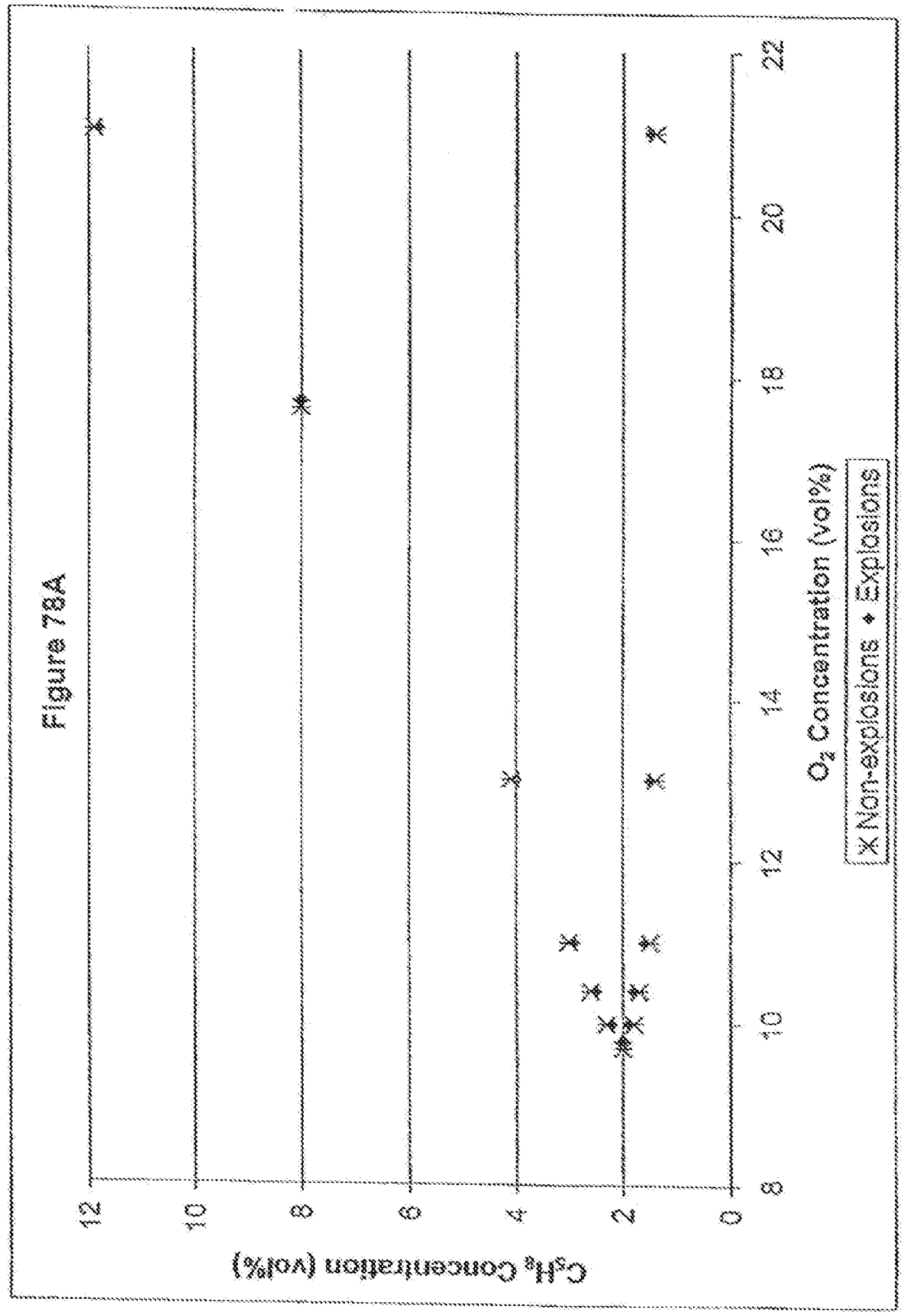


Figure 78A
C2H4 Concentration (vol%)
O2 Concentration (vol%)
X Non-explosions • Explosions

Figure 78B

Explosions		Non-explosions	
O_2 Concentration (vol. %)	C_5H_8 Concentration (vol. %)	O_2 Concentration (vol. %)	C_5H_8 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

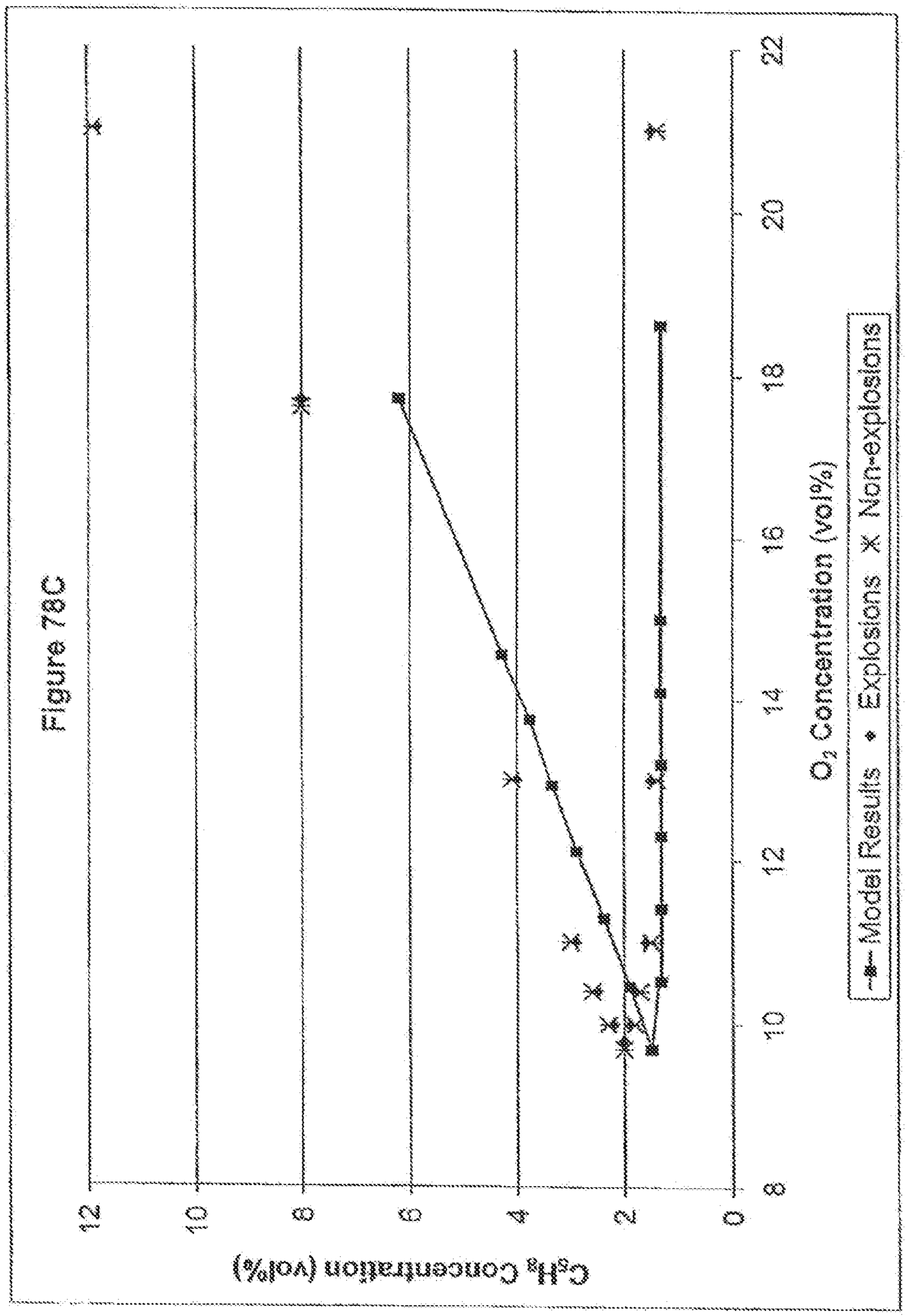


Figure 78C
C_3H_8 Concentration (vol%)
O_2 Concentration (vol%)
Explosions
Non-explosions
Model Results
0
2
4
6
8
10
12
14
16
18
20
22

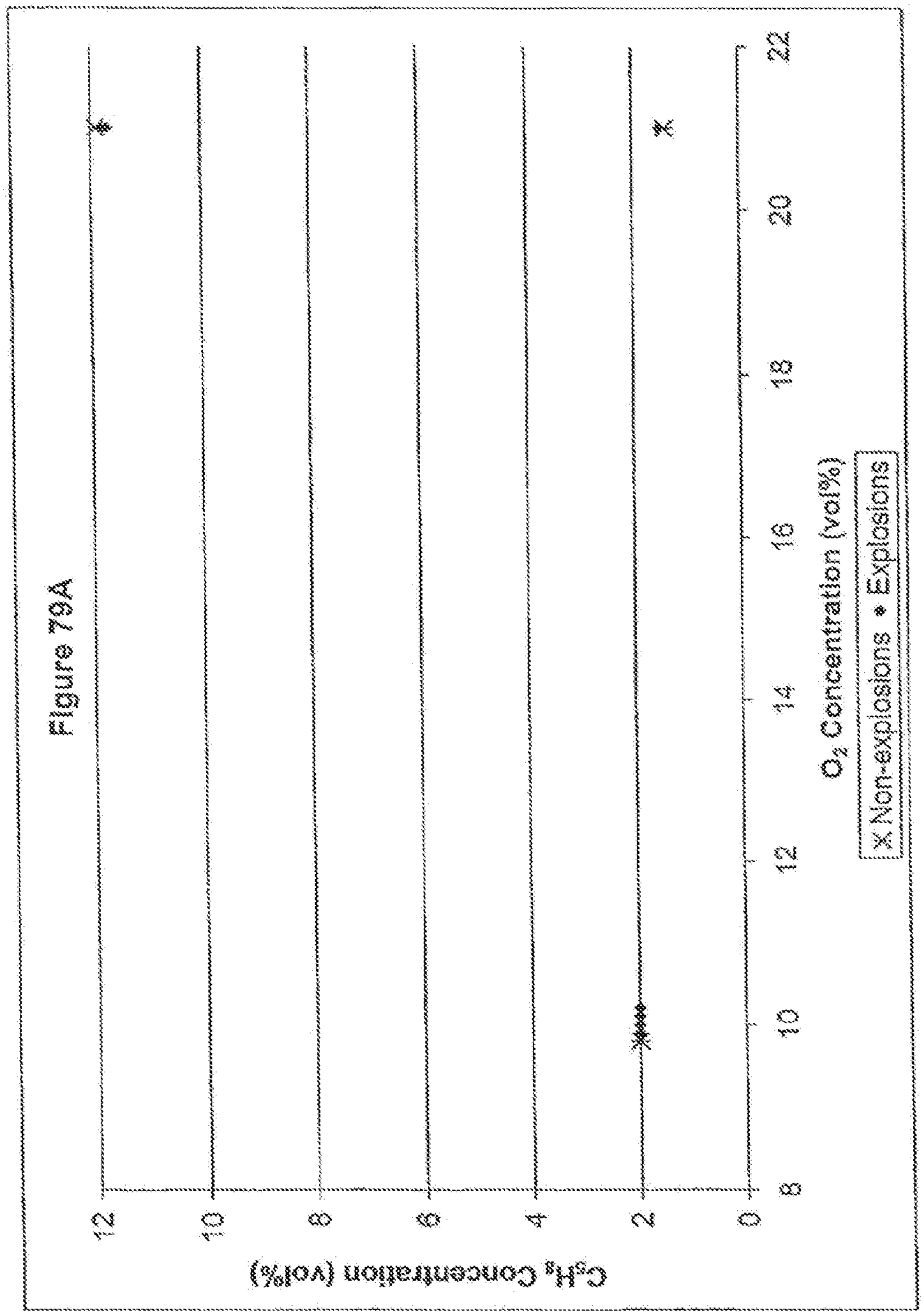


Figure 79A
C2H4 Concentration (vol%)
O2 Concentration (vol%)
X Non-explosions • Explosions

Figure 79B

Explosions		Non-explosions	
O_2 Concentration (vol. %)	C_5H_8 Concentration (vol. %)	O_2 Concentration (vol. %)	C_5H_8 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

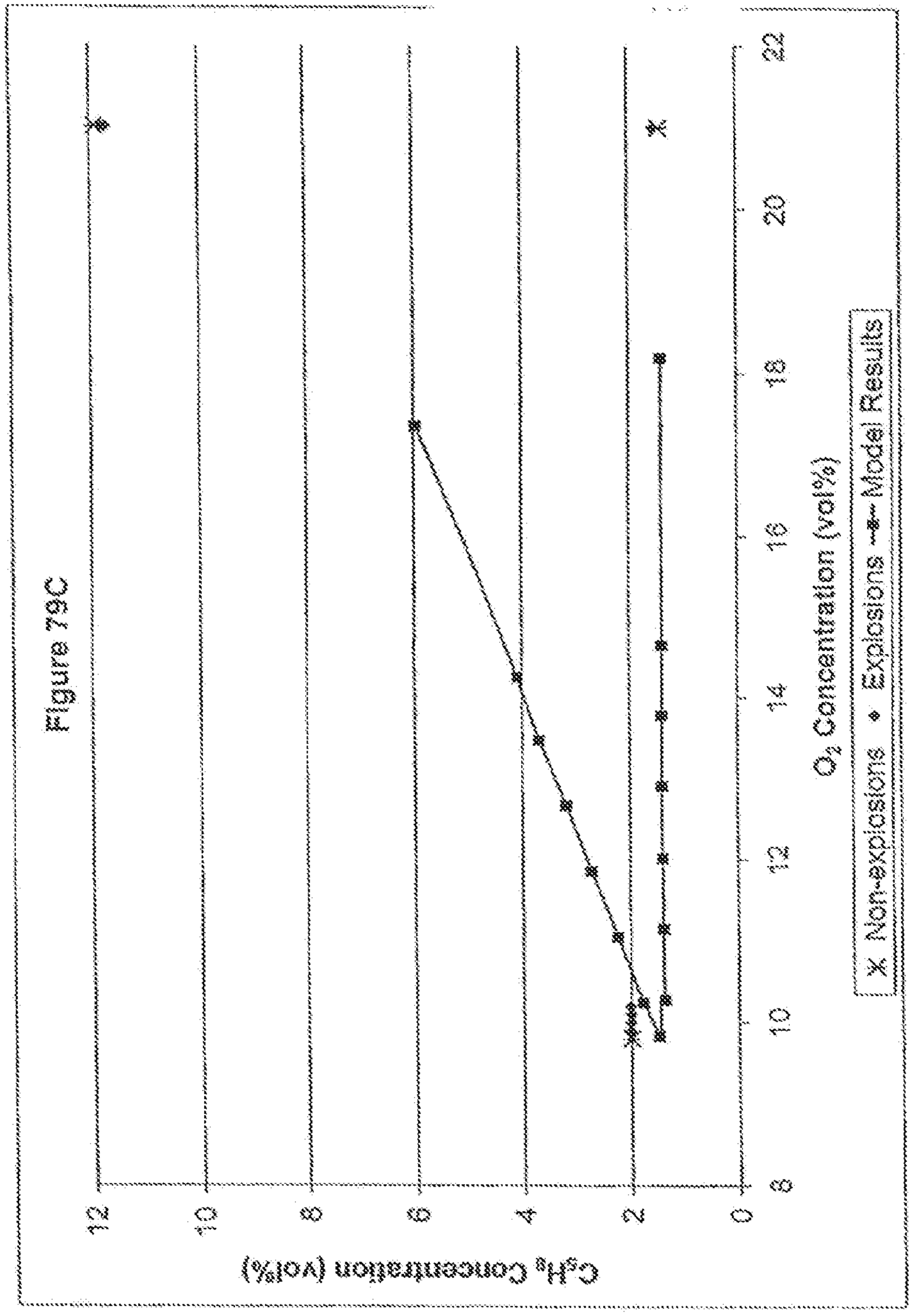


Figure 79C
C_3H_8 Concentration (vol%)
0
2
4
6
8
10
12
14
16
18
20
22
O_2 Concentration (vol%)
X Non-explosions • Explosions —•— Model Results

Figure 80A

TEST SERIES 1

Test	Data File Name	Temp °C	Initial Pressure bara	Partial Pressures C_3H_8 mbar	Partial Pressures N_2 mbar	Partial Pressures O_2 mbar	Concentrations C_3H_8 vol. %	Concentrations N_2 vol. %	Concentrations O_2 vol. %	Result	Pex 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	1.08
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	86.5	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	1.08
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	99	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	781	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 °C	Initial Pressure bara	Partial Pressures H_2O mbar	C_3H_8 mbar	N_2 mbar	O_2 mbar	Concentrations H_2O vol. %	C_3H_8 vol. %	N_2 vol. %	O_2 vol. %	Result	Pex 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

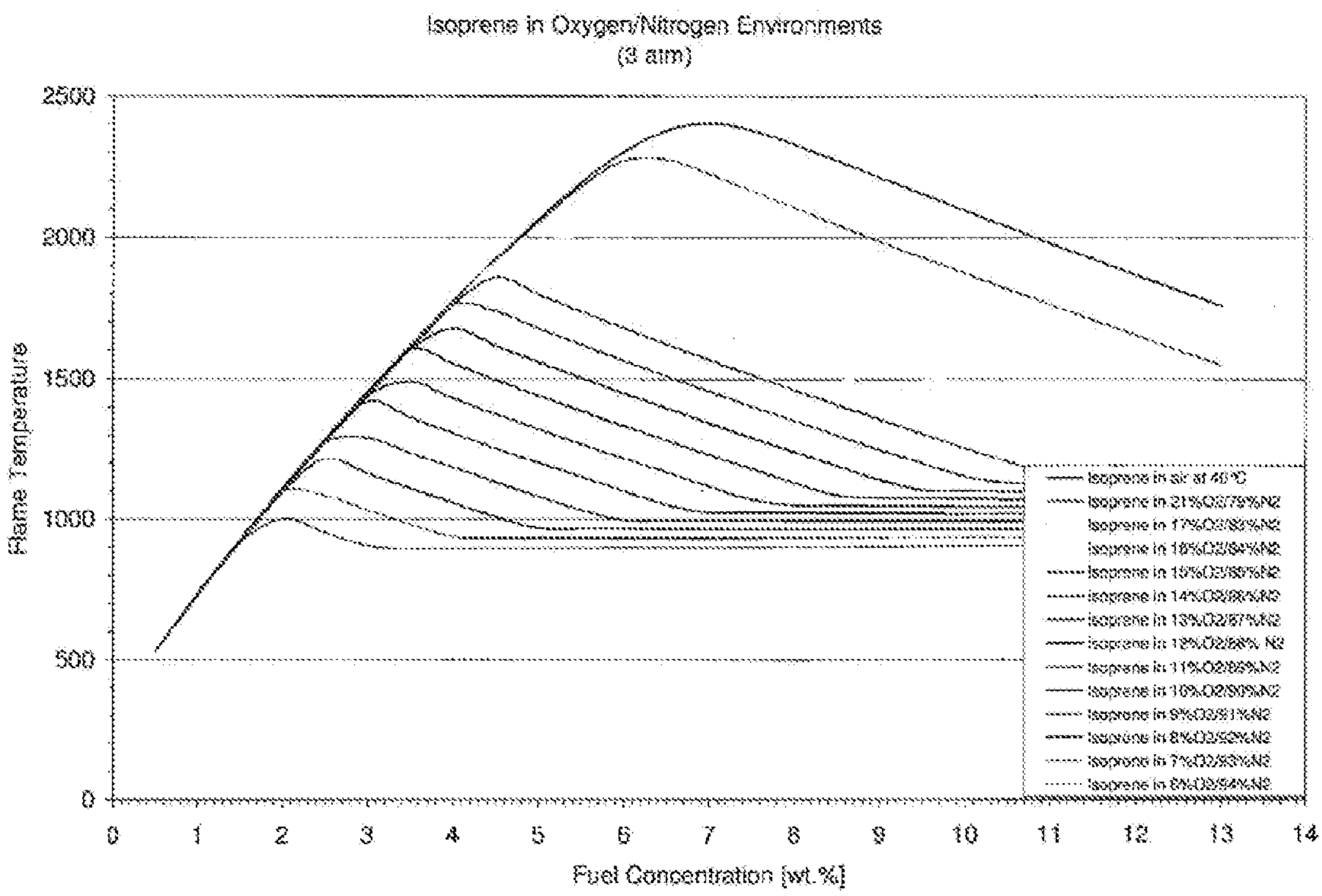

Isoprene in Oxygen/Nitrogen Environments
(3 atm)
Flame Temperature
2500
2000
1500
1000
500
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Fuel Concentration [wt.%]
Isoprene in air at 40°C
Isoprene in 21%O2/79%N2
Isoprene in 17%O2/83%N2
Isoprene in 16%O2/84%N2
Isoprene in 15%O2/85%N2
Isoprene in 14%O2/86%N2
Isoprene 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

Figure 83

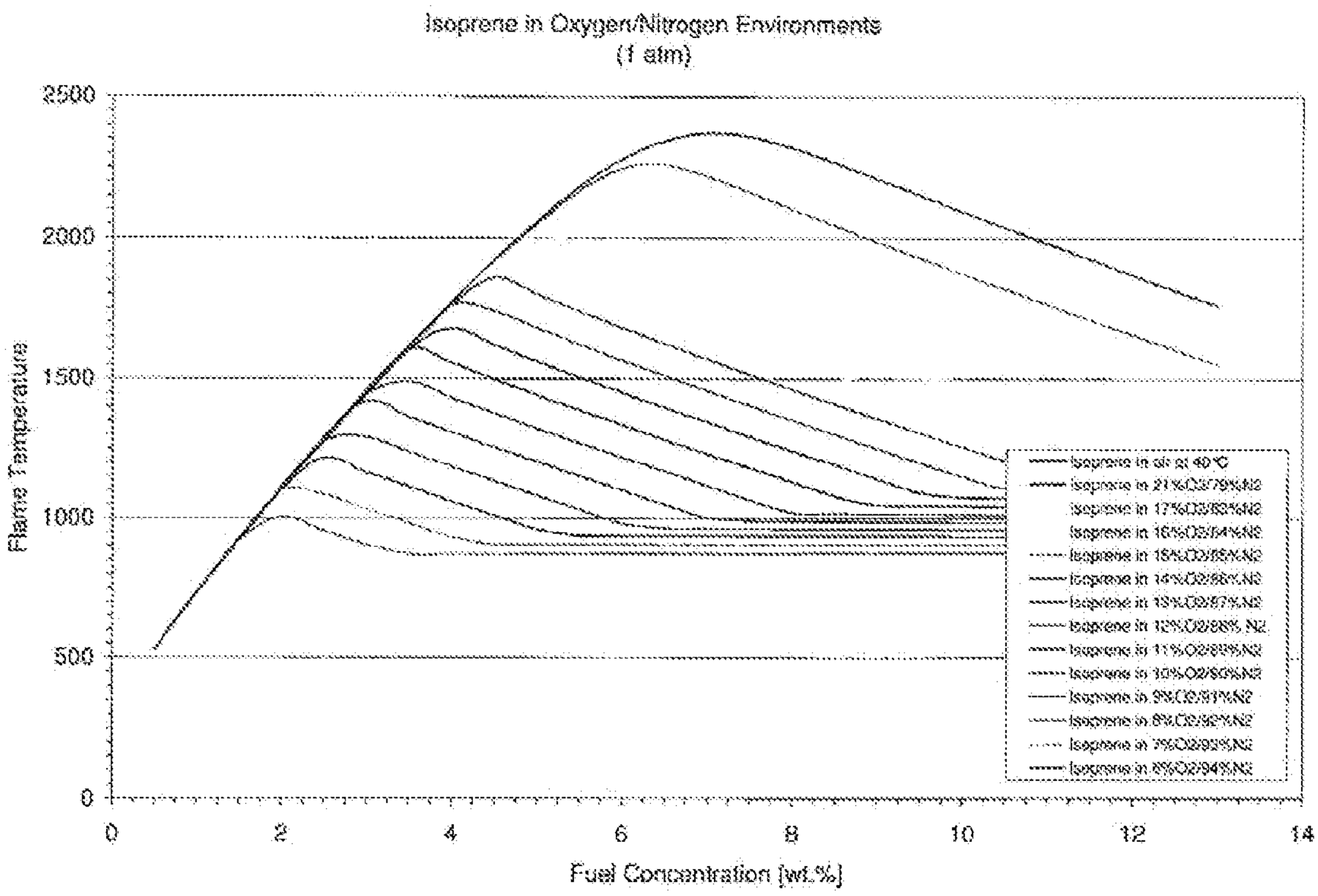

Isoprene in Oxygen/Nitrogen Environments
(1 atm)
Flame Temperature
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Fuel Concentration [wt.%]
Isoprene in air at 40°C
Isoprene in 21%O2/79%N2
Isoprene in 17%O2/83%N2
Isoprene in 16%O2/84%N2
Isoprene in 15%O2/85%N2
Isoprene in 14%O2/86%N2
Isoprene in 13%O2/87%N2
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

Figure 84

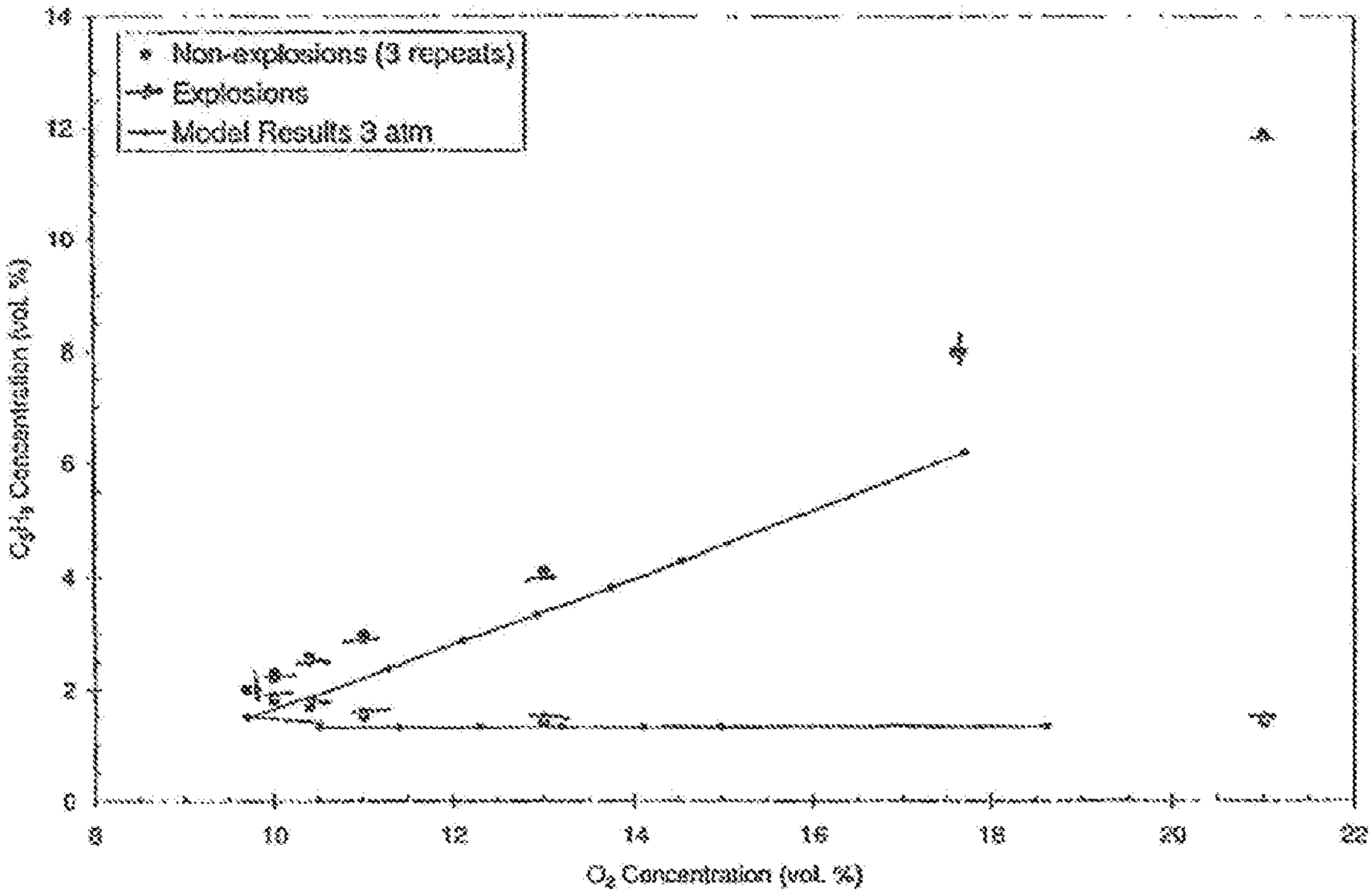

Non-explosions (3 repeats)
Explosions
Model Results 3 atm
C_3H_8 Concentration (vol. %)
0
2
4
6
8
10
12
14
8
10
12
14
16
18
20
22
O_2 Concentration (vol. %)

Figure 85

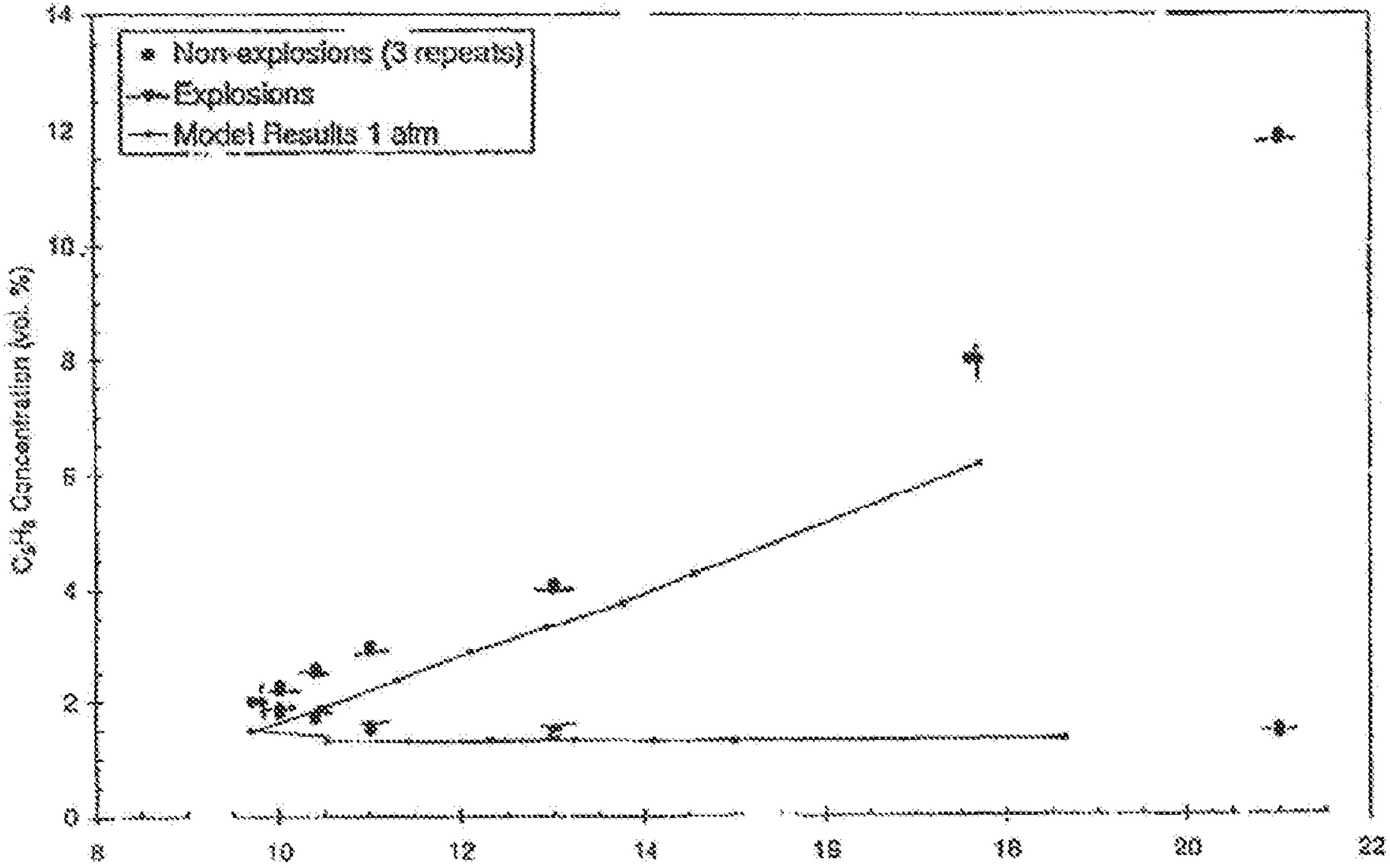

Non-explosions (3 repeats)
Explosions
Model Results 1 atm
C_6H_6 Concentration (vol. %)
O_2 Concentration (vol. %)
0
2
4
6
8
10
12
14
8
10
12
14
16
18
20
22

Figure 86A

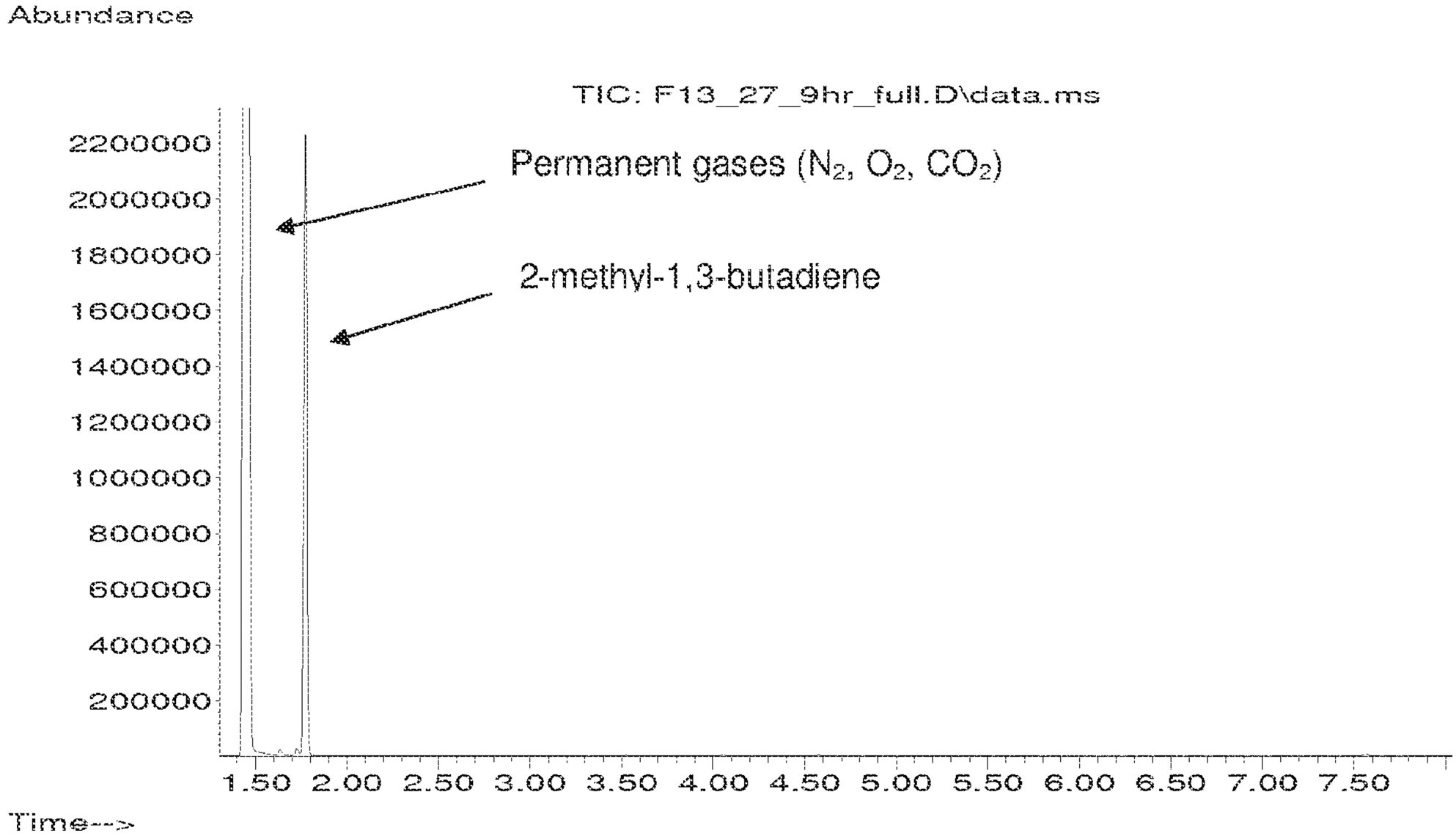

Abundance
TIC: F13_27_9hr_full.D\data.ms
2200000
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
Permanent gases (N_2, O_2, CO_2)
2-methyl-1,3-buladiene
1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50 7.00 7.50
Time-->

Figure 86B

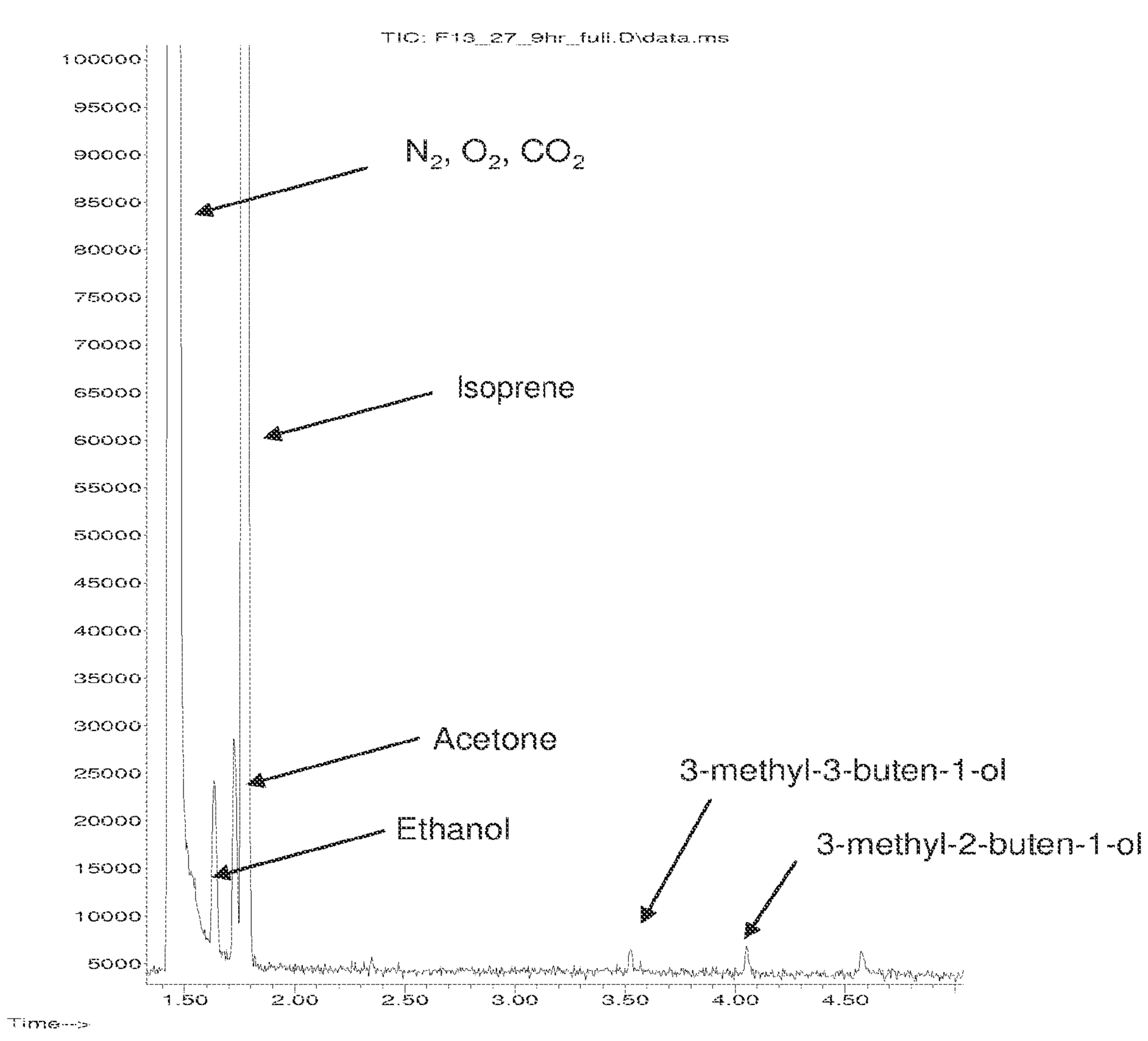
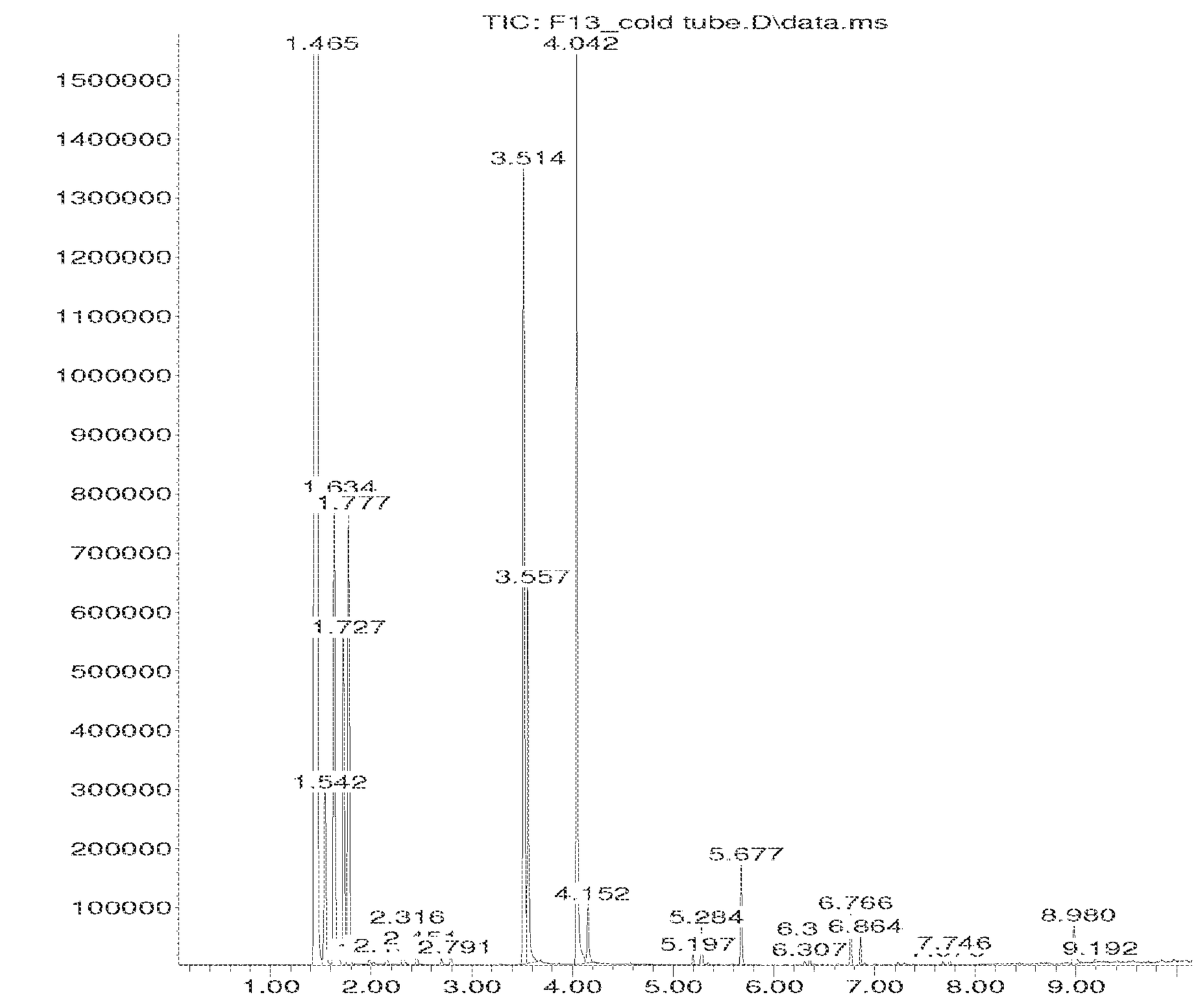

Abundance
TIC: F13_27_9hr_full.D\data.ms
100000
95000
90000
85000
80000
75000
70000
65000
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
N_2, O_2, CO_2
Isoprene
Acetone
Ethanol
3-methyl-3-buten-1-ol
3-methyl-2-buten-1-ol
1.50
2.00
2.50
3.00
3.50
4.00
4.50
Time-->

Figure 87A


Abundance
TIC: F13_cold tube.D\data.ms
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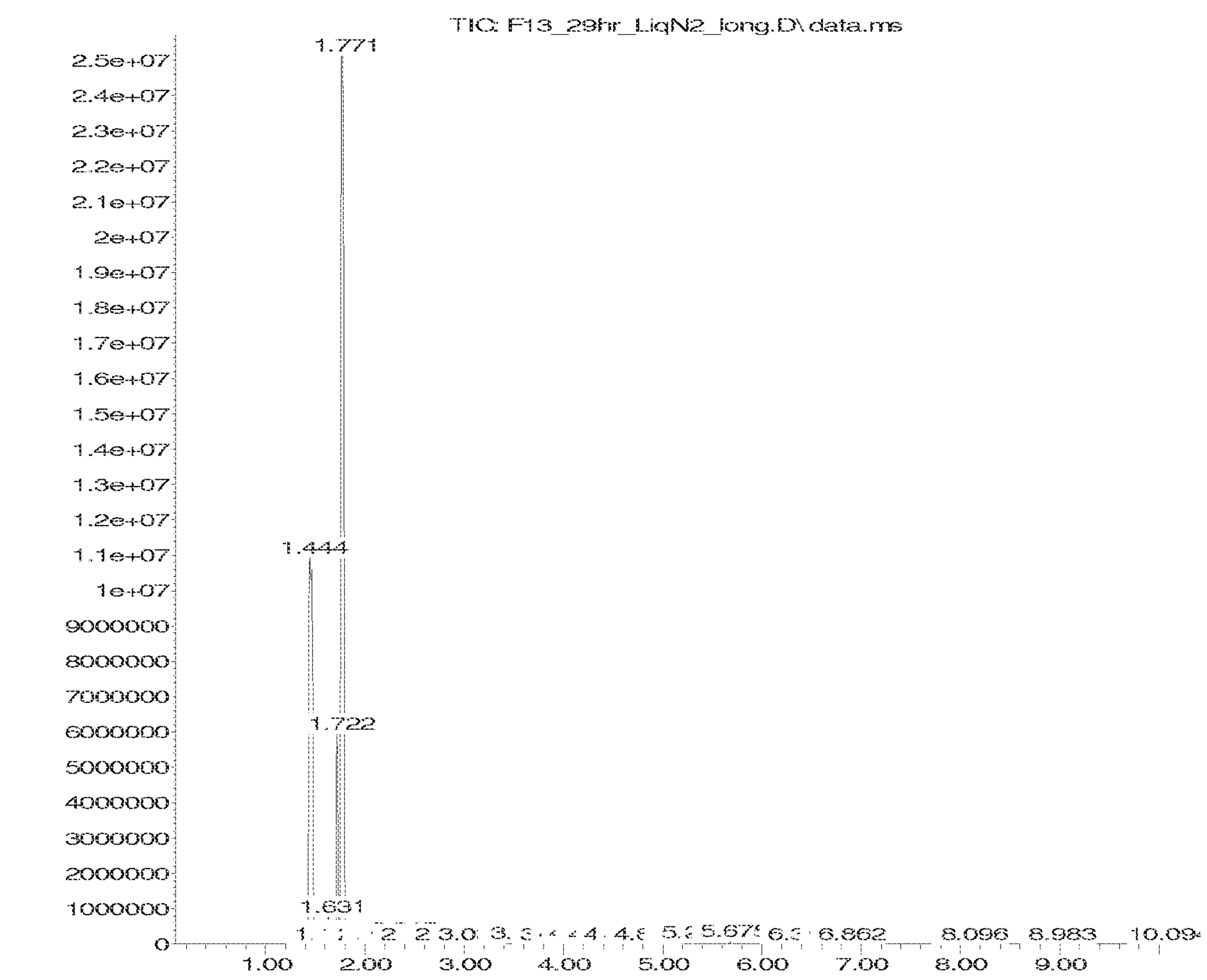
1400000
1300000
1200000
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
1.465
4.042
3.514
3.557
1.634
1.777
1.727
1.542
2.316
2.151
2.791
4.152
5.677
5.284
5.197
6.766
6.3
6.864
6.307
7.746
8.980
9.192
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Time-->

Figure 87B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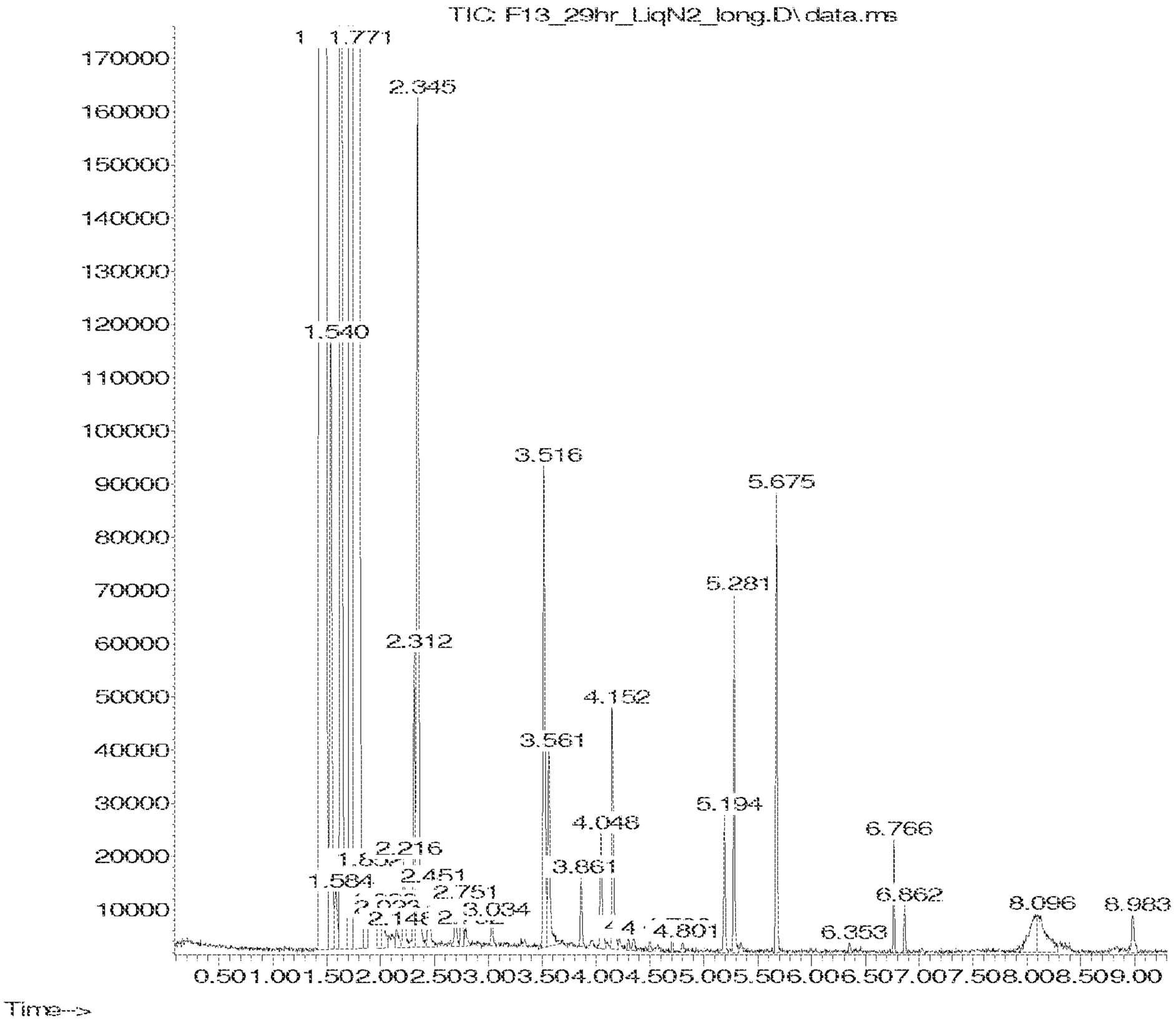
1.444
1.722
1.631
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Time-->

Figure 87C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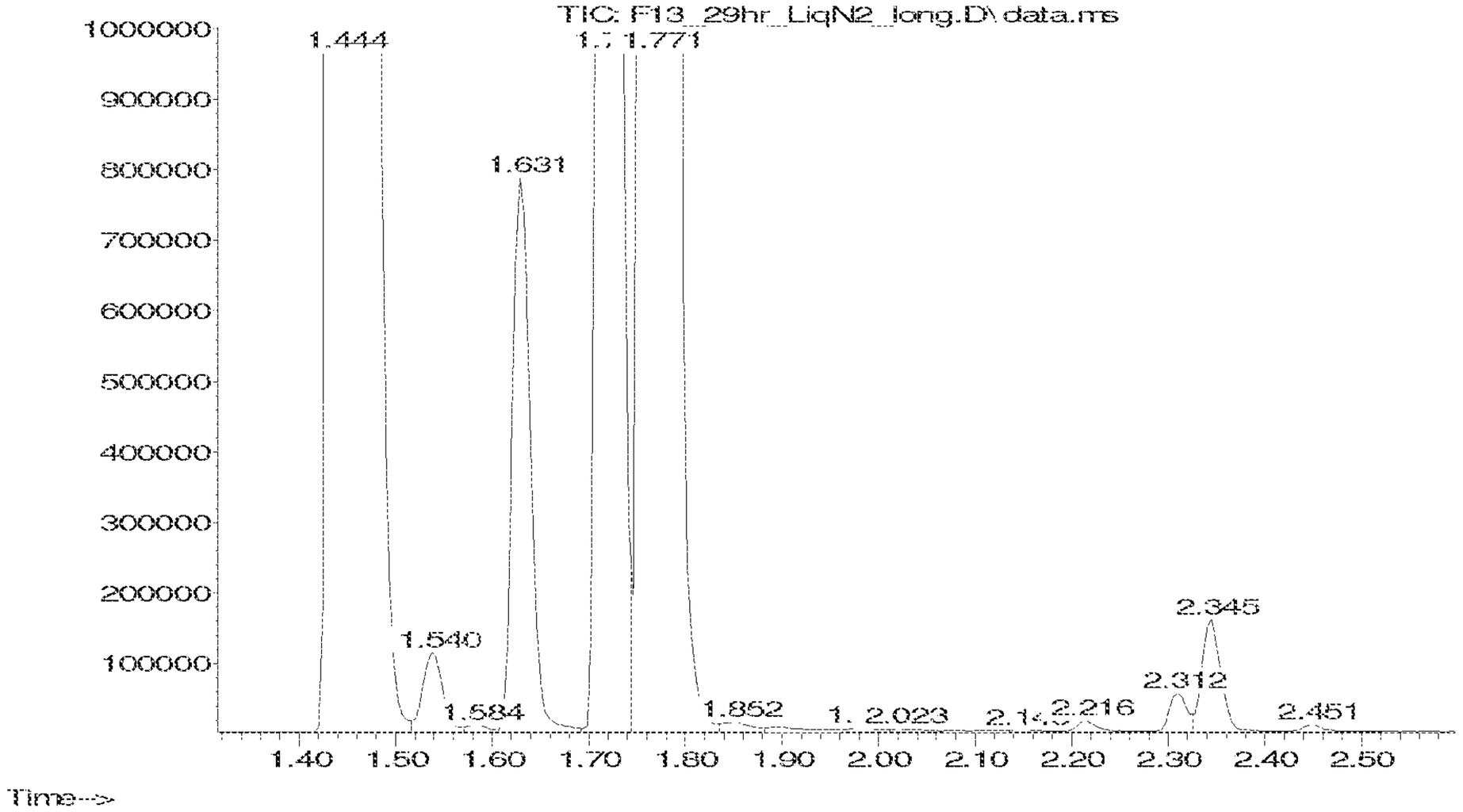
1.540
3.516
5.675
5.281
2.312
4.152
3.561
5.194
4.048
6.766
2.216
3.861
1.584
2.451
2.751
6.862
8.096
8.983
3.034
6.353
4.801
170000
160000
150000
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0.501.001.502.002.503.003.504.004.505.005.506.006.507.007.508.008.509.00
Time-->

Figure 87D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900000
800000
700000
600000
500000
400000
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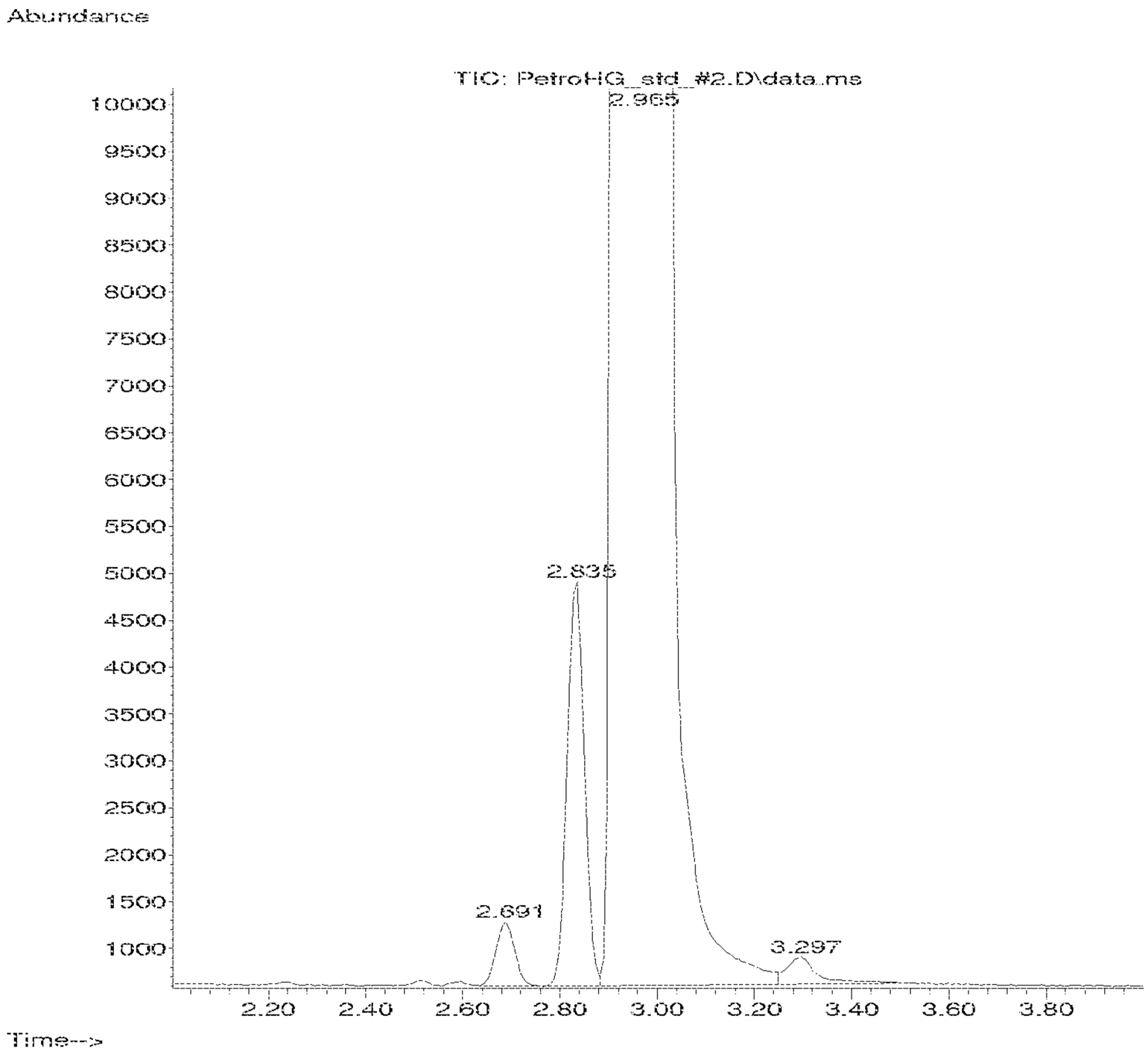
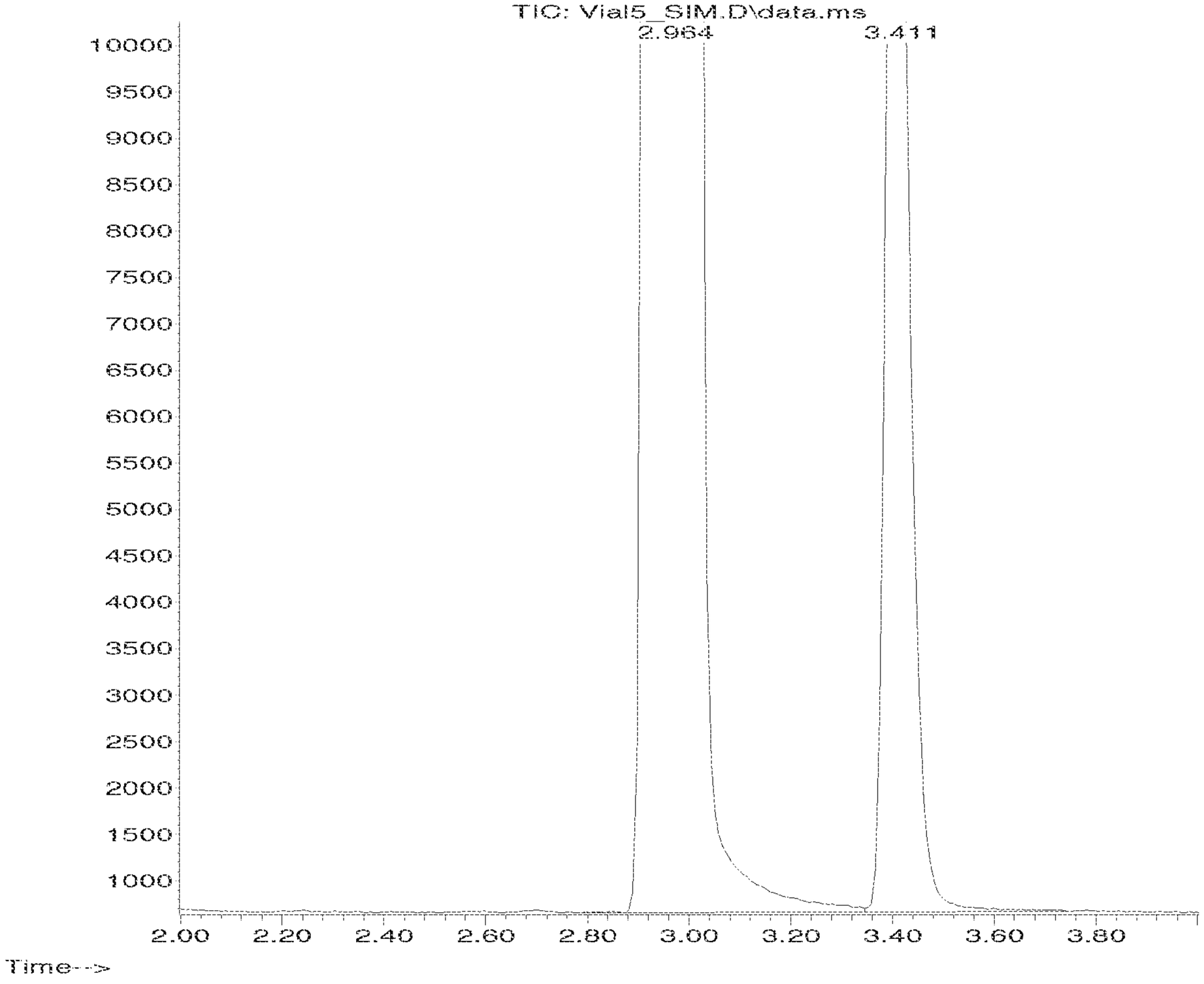
200000
100000
1.444
1.631
1.771
1.540
1.584
1.852
2.345
2.312
2.216
2.451
1.40
1.50
1.60
1.70
1.80
1.90
2.00
2.10
2.20
2.30
2.40
2.50
Time-->

Figure 88A



2-methyl-1,3-butadiene standard.

Figure 88B

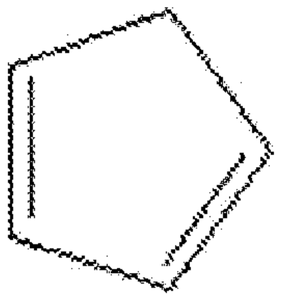


2-methyl-1,3-butadiene from recombinant *E. coli*

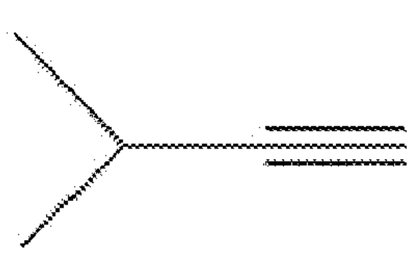
Figure 89

Fermentation Off-gas Composition
(E. coli BL21 (DE3) pTrcKudzu HGS w/ 3g/L yeast extract)
Conc (ug/L)
30
25
20
15
10
5
0
HG
3-MBA
0
10
20
30
EFT (hr)

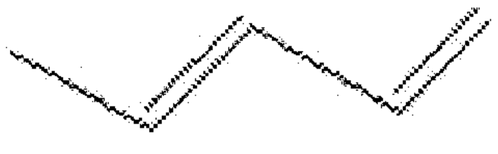
Figure 90



cyclopentadiene

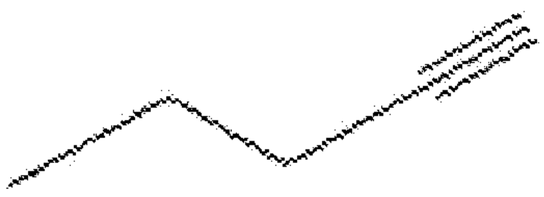


"isopryne" = 3-Me-1-butyne

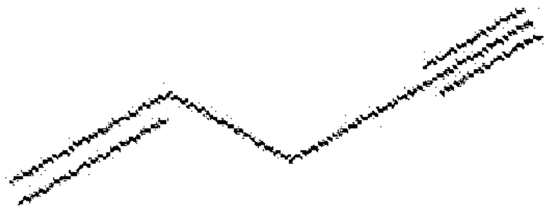


trans-piperylene

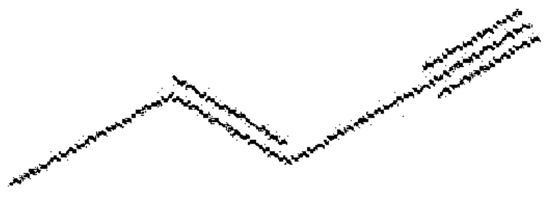
cis-piperylene



1-pentyne



pent-4-ene-1-yne



trans-pent-3-ene-1-yne

cis-pent-3-ene-1-yne

Figure 91

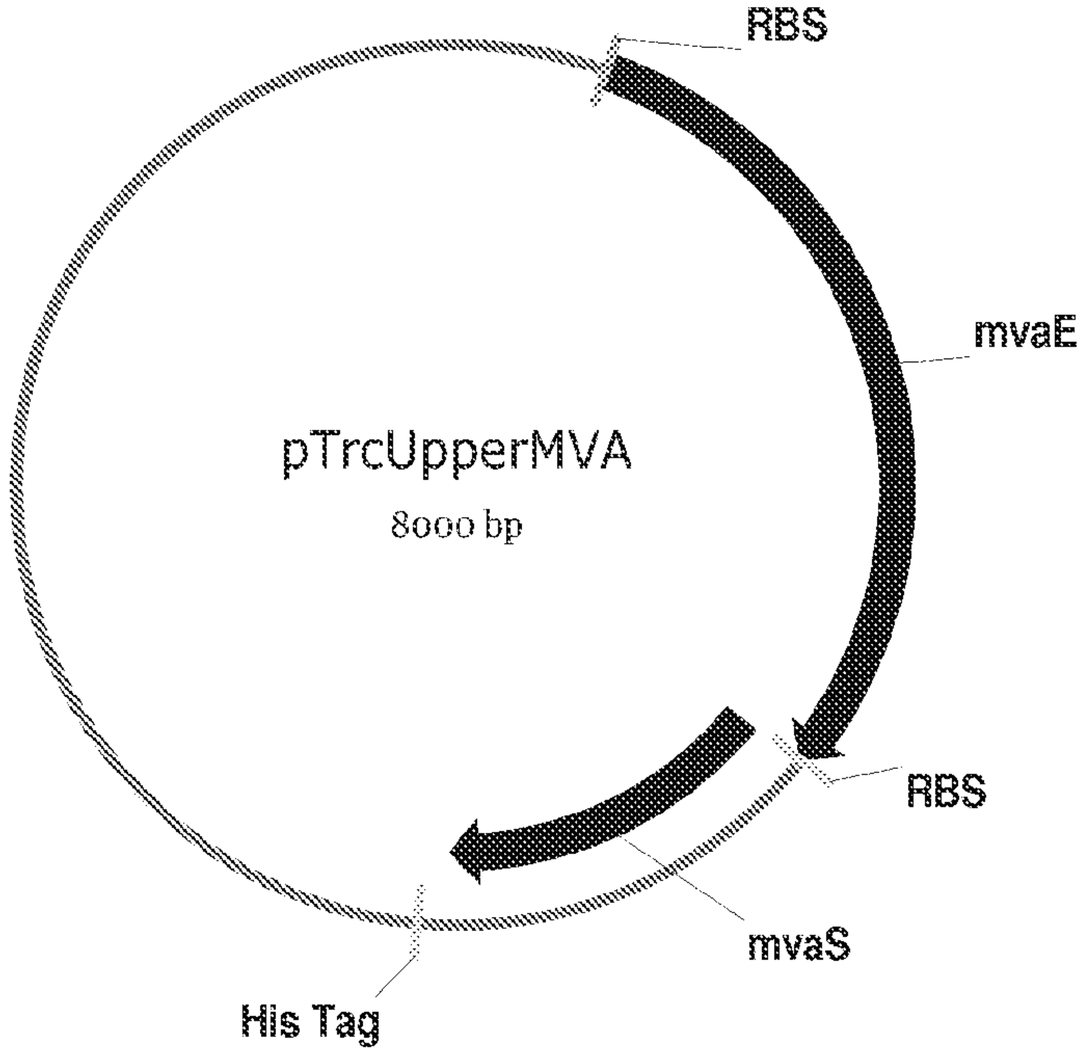


Figure 92A

1-

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Figure 92B

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Figure 92C

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Figure 93

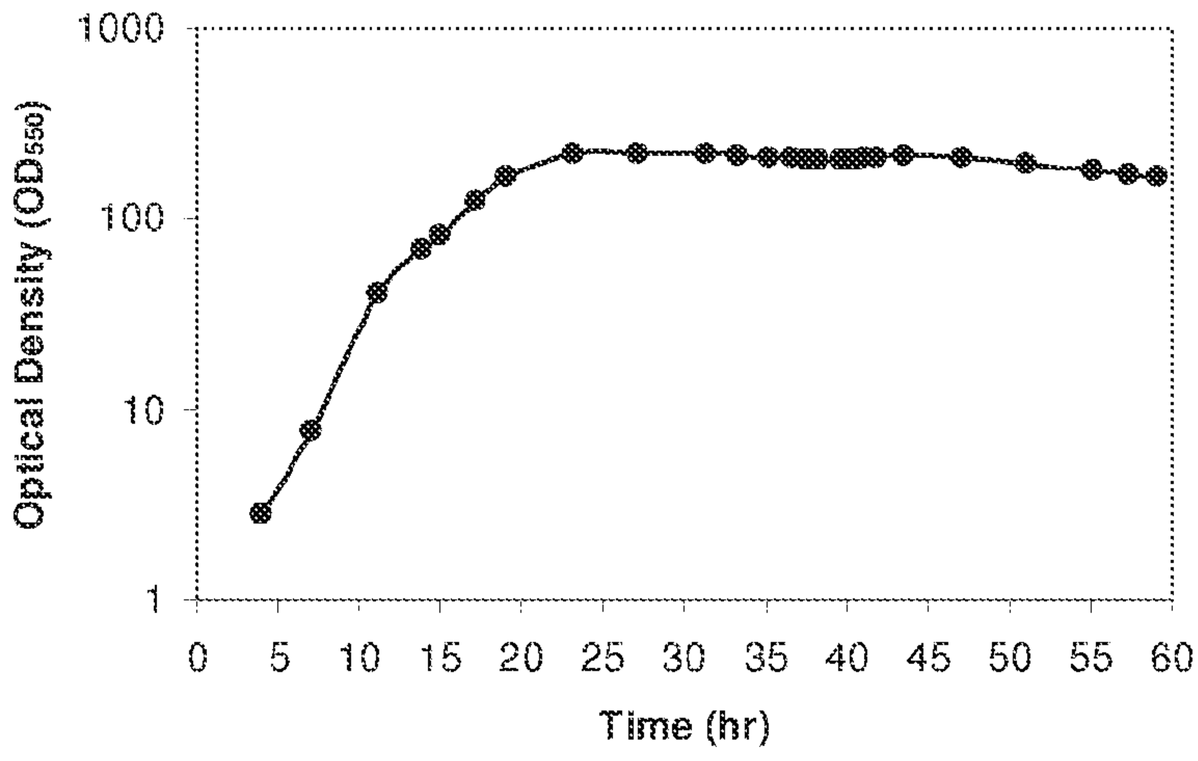

Figure 94

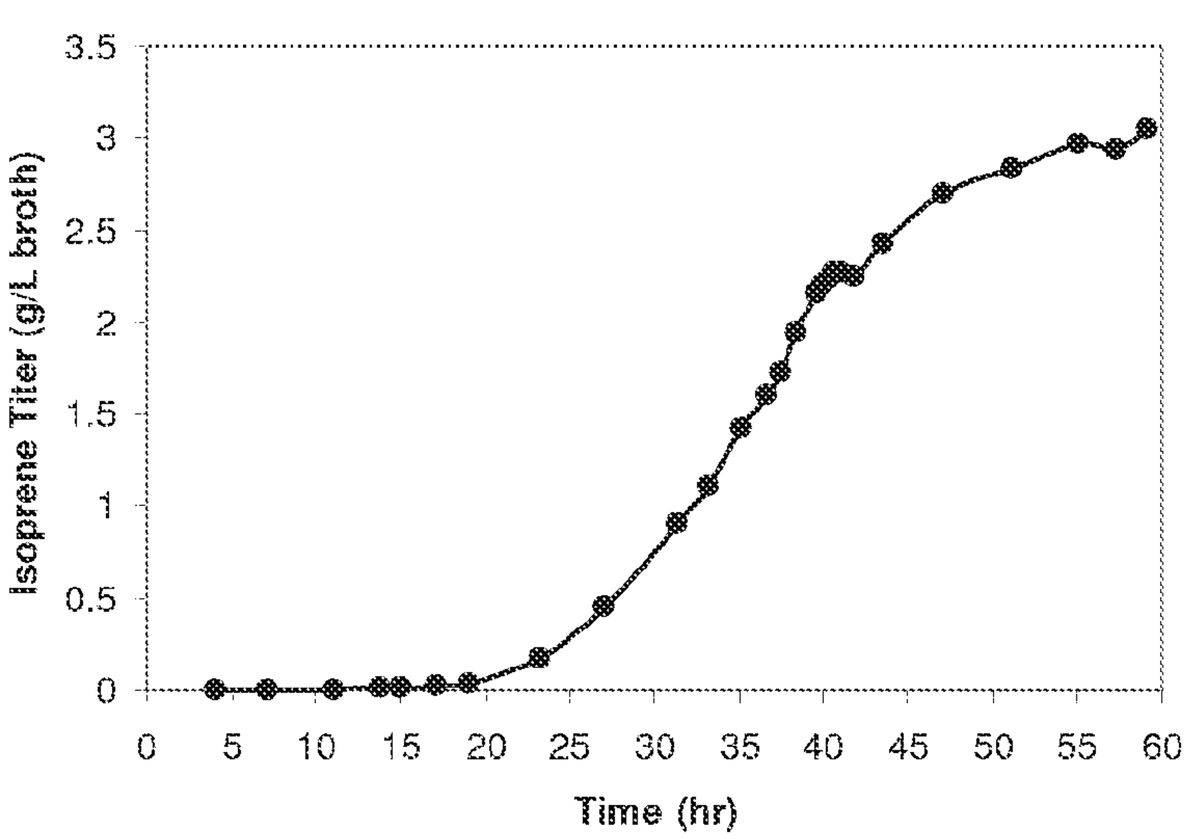

Figure 95

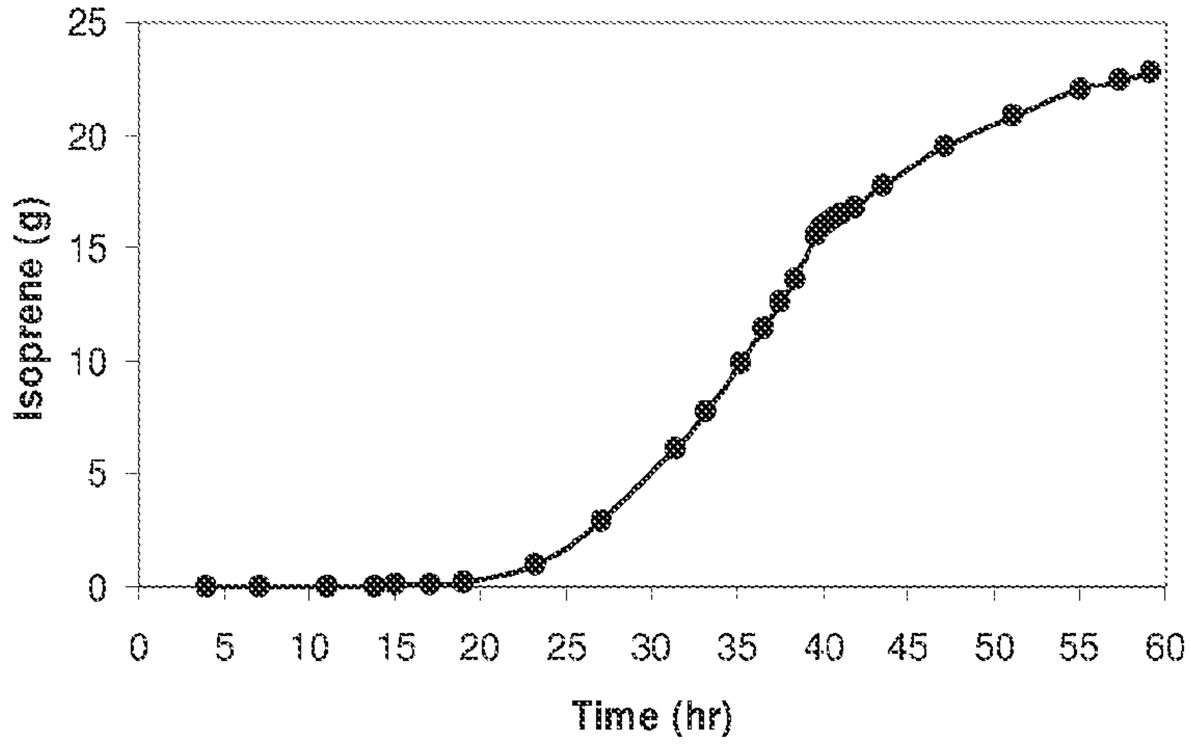

Figure 96A

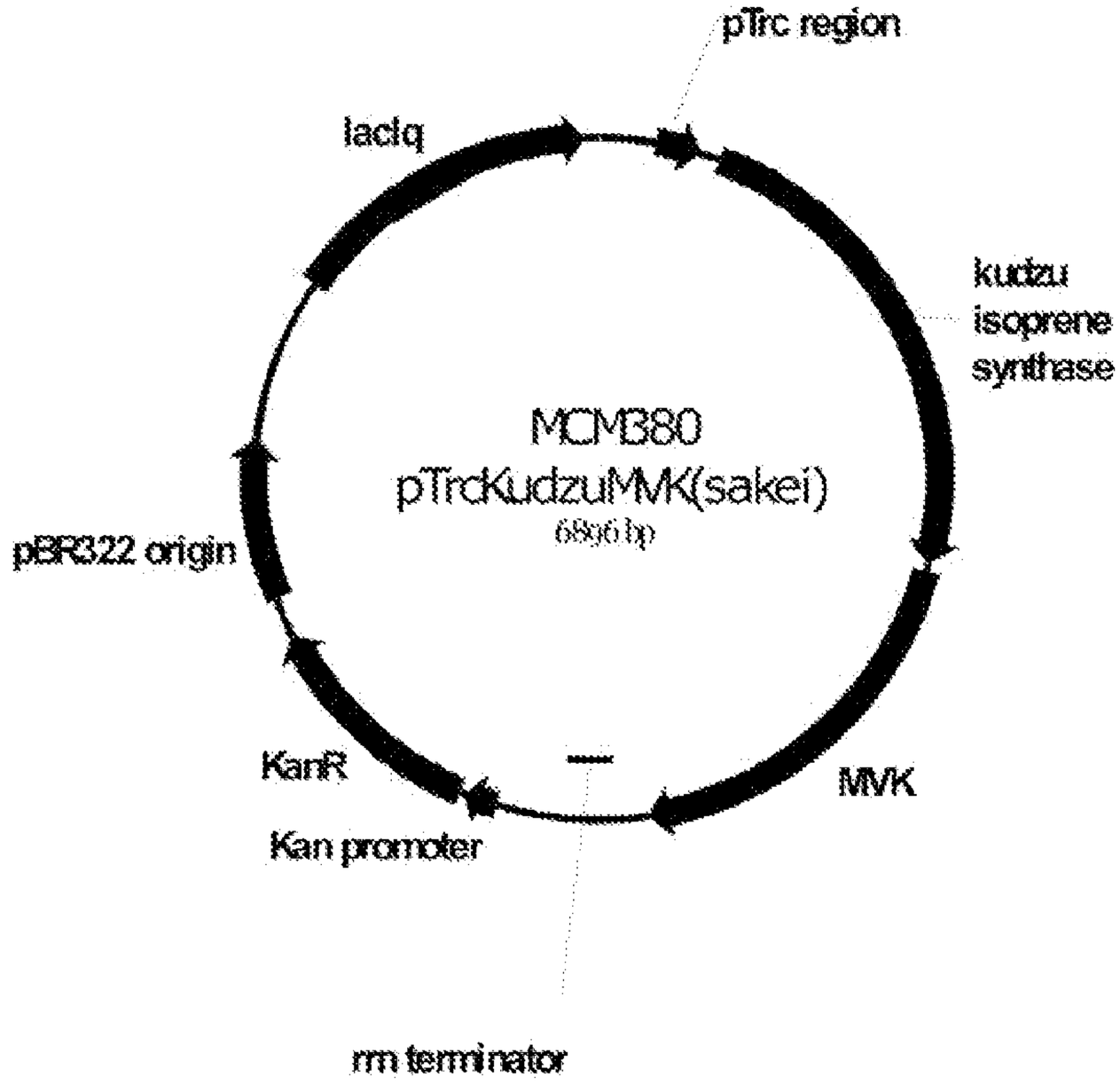
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Figure 96B

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Figure 96C

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Figure 97A

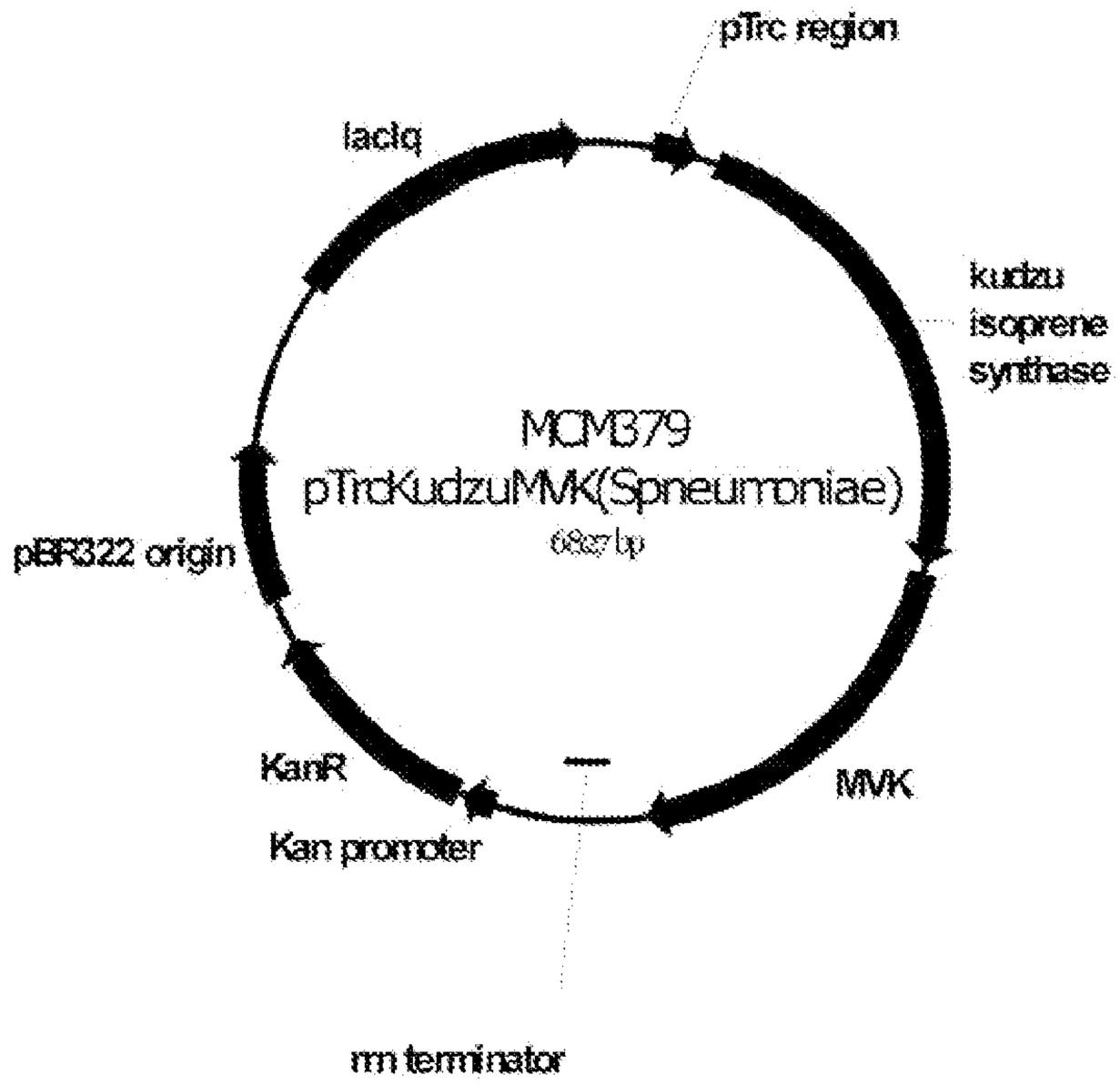
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Figure 97B

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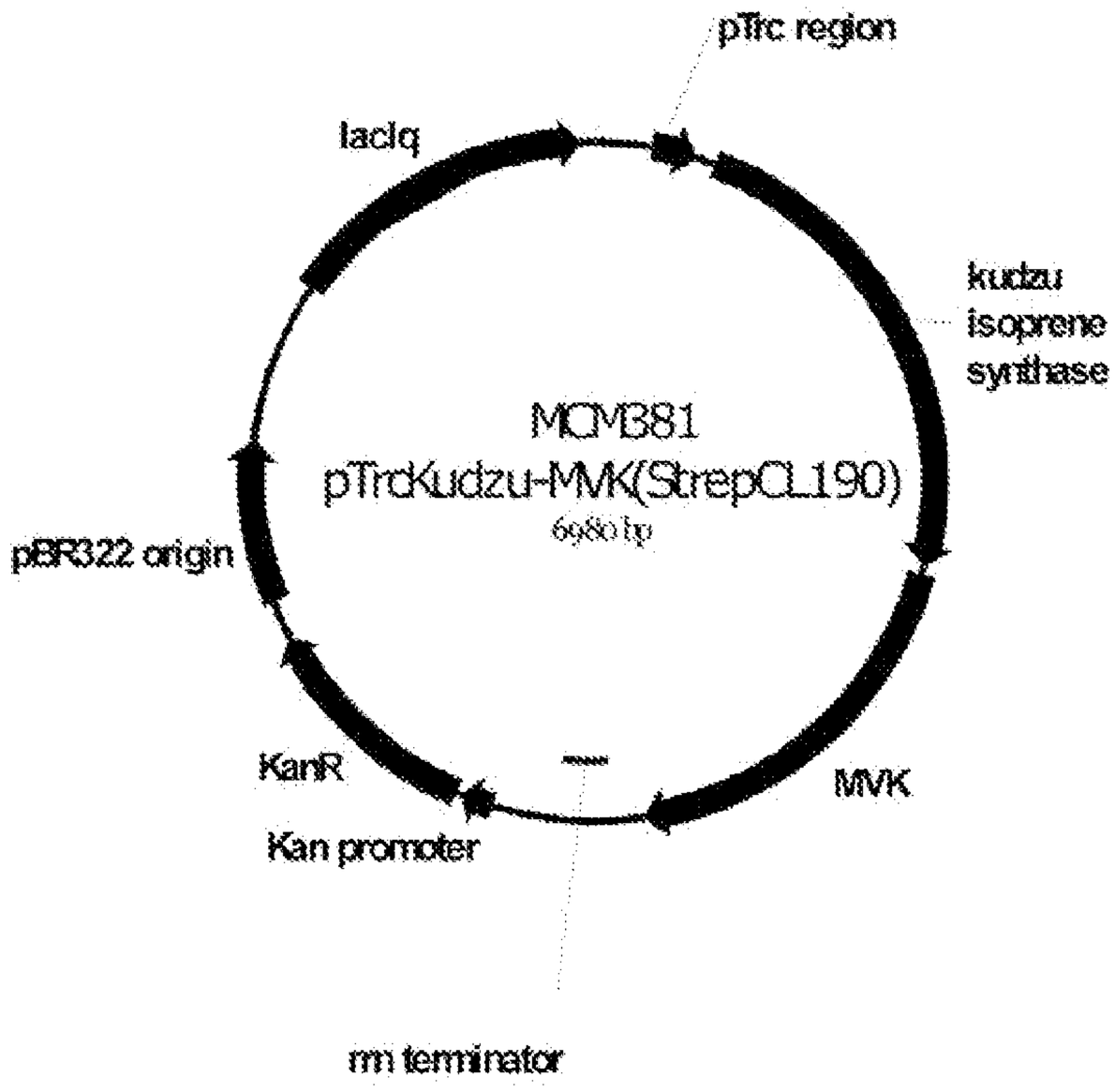
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Figure 98B

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Figure 98C

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Figure 99

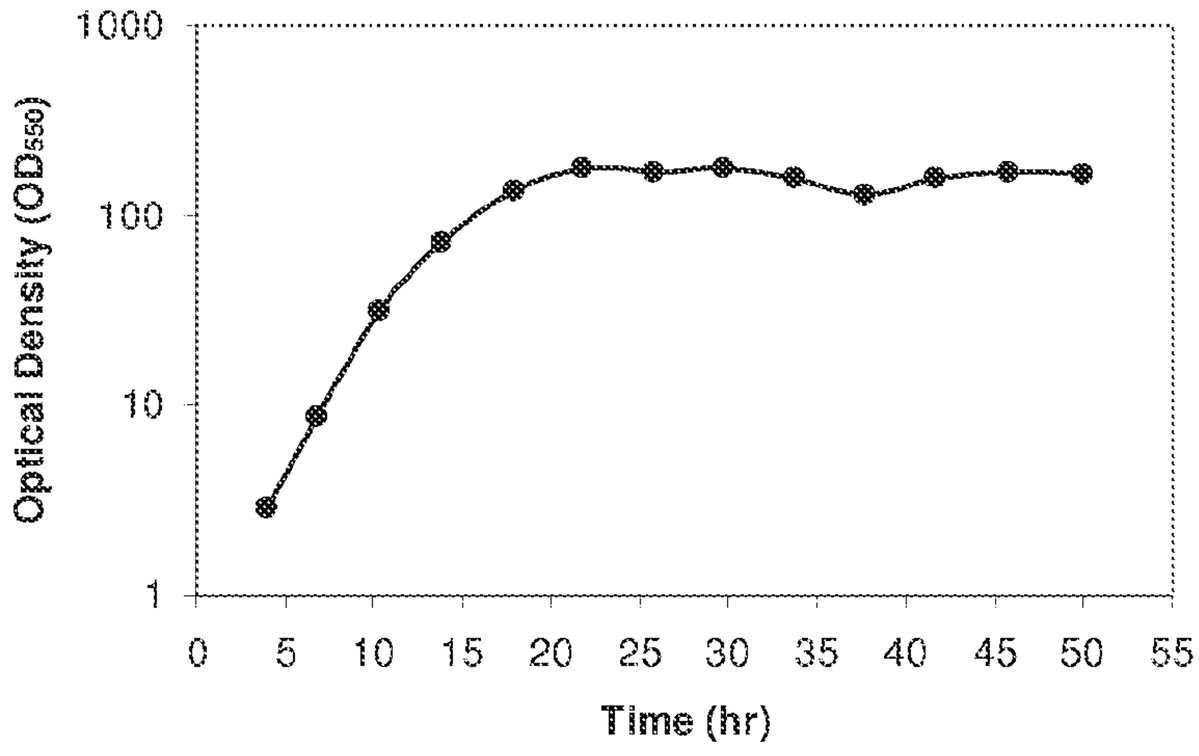


Figure 100

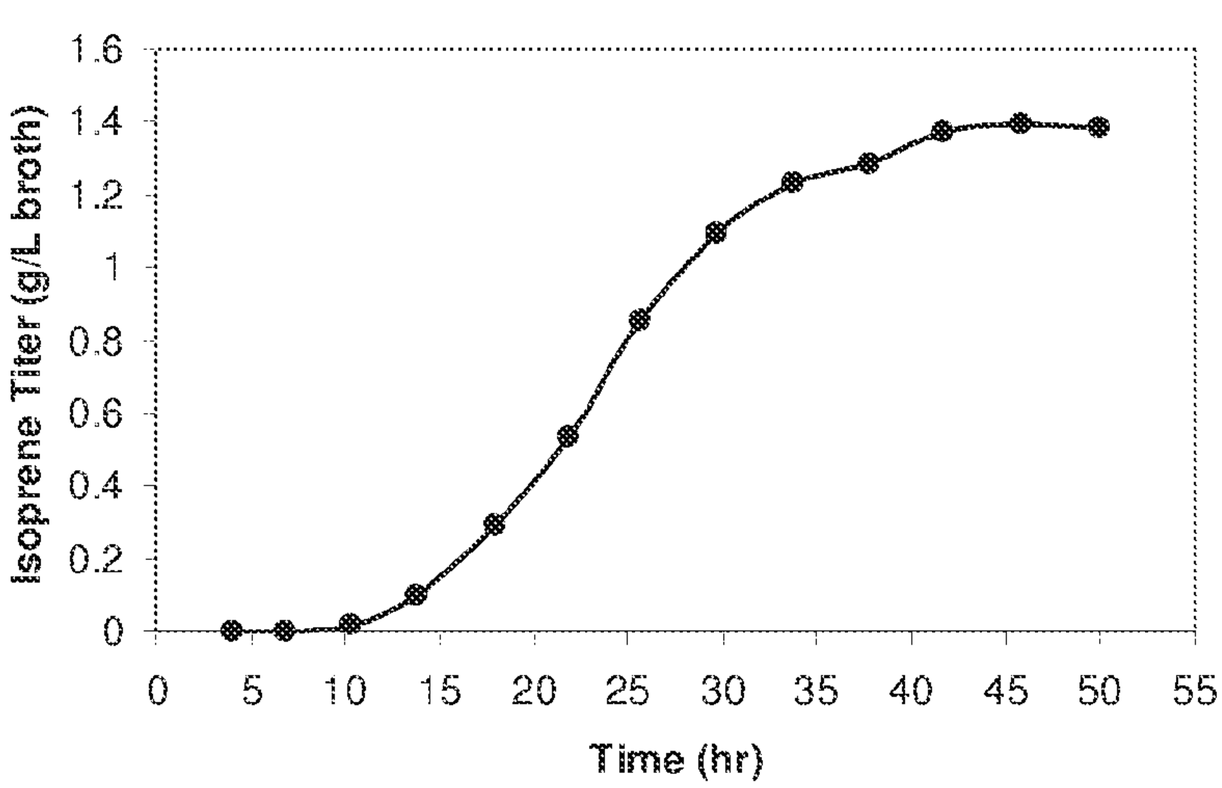
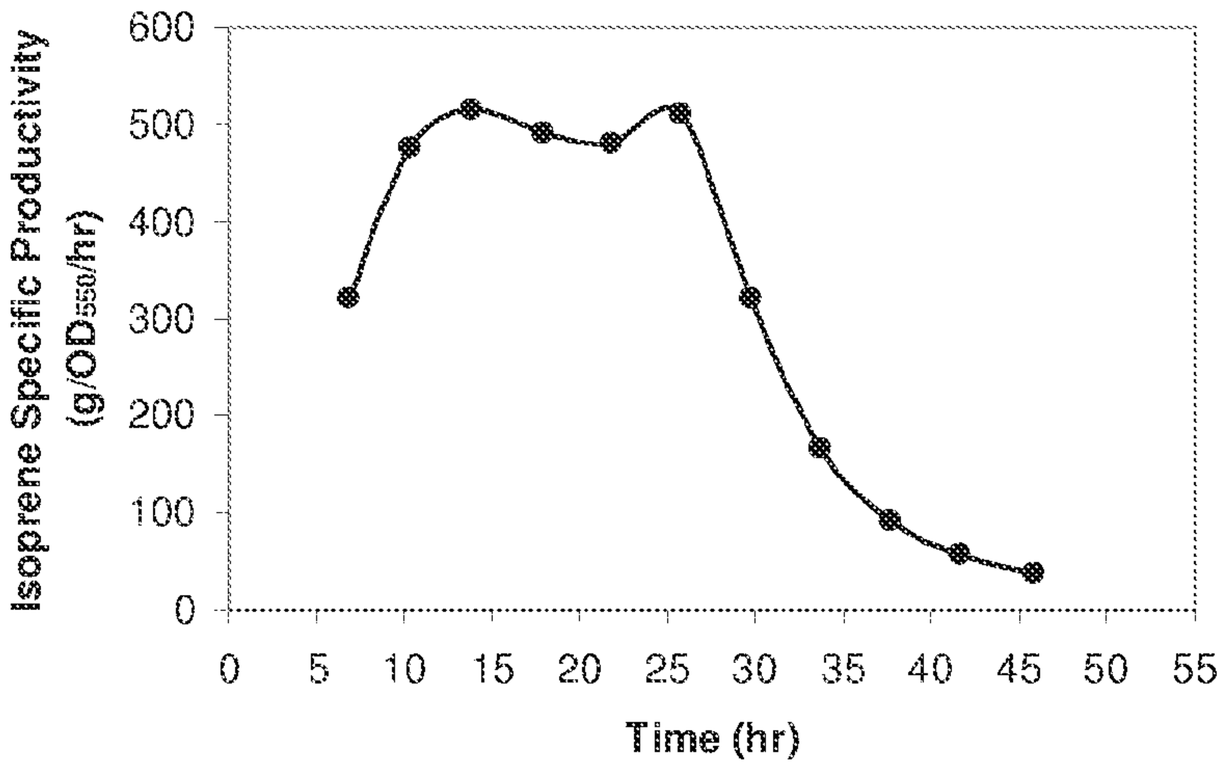


Figure 101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5
10
15
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25
30
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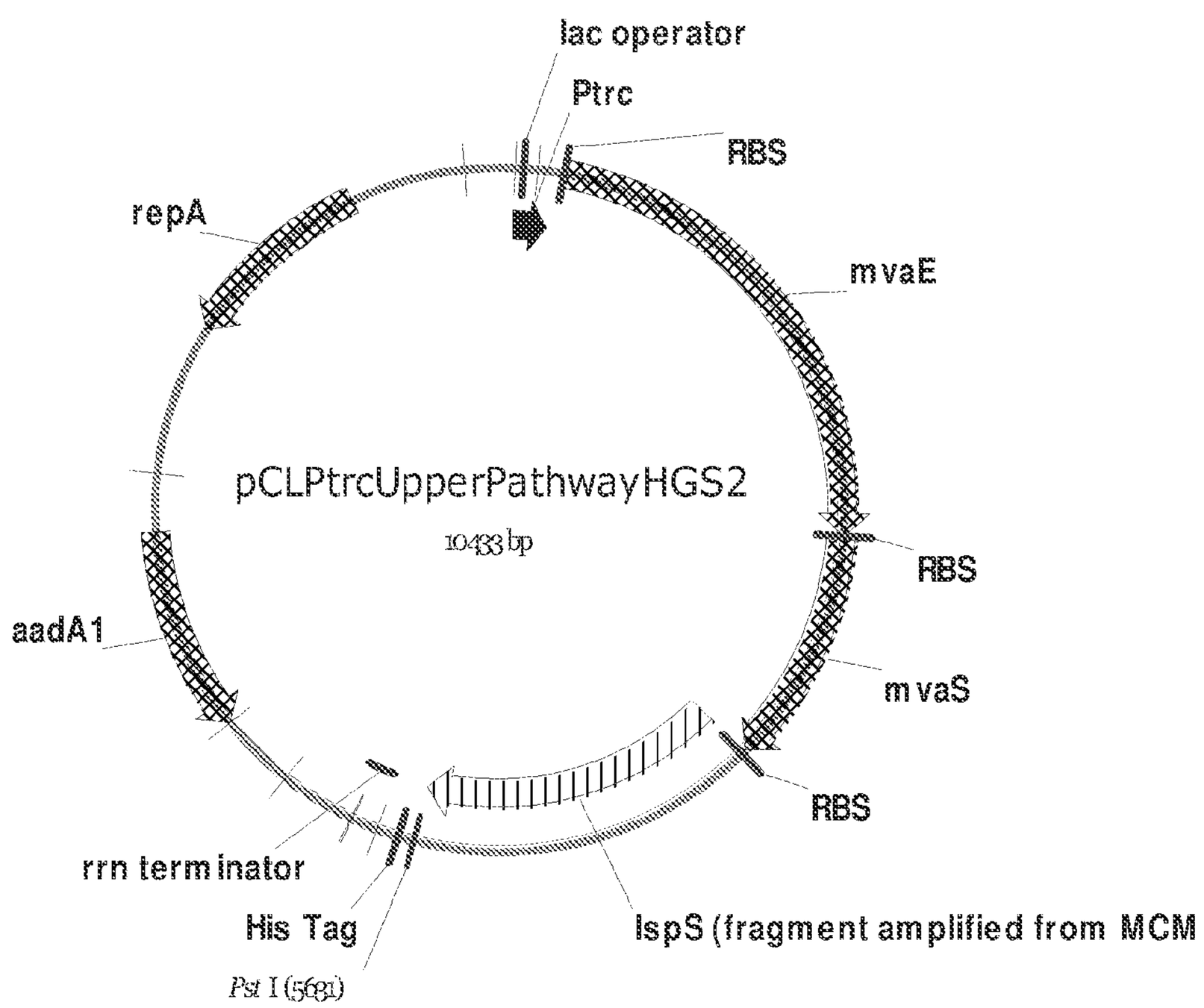
45
50
55
Time (hr)

Figure 102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RBS
rrn terminator
His Tag
IspS (fragment amplified from MCM
PstI (5681)

Figure 103A

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Figure 103B

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Figure 103C

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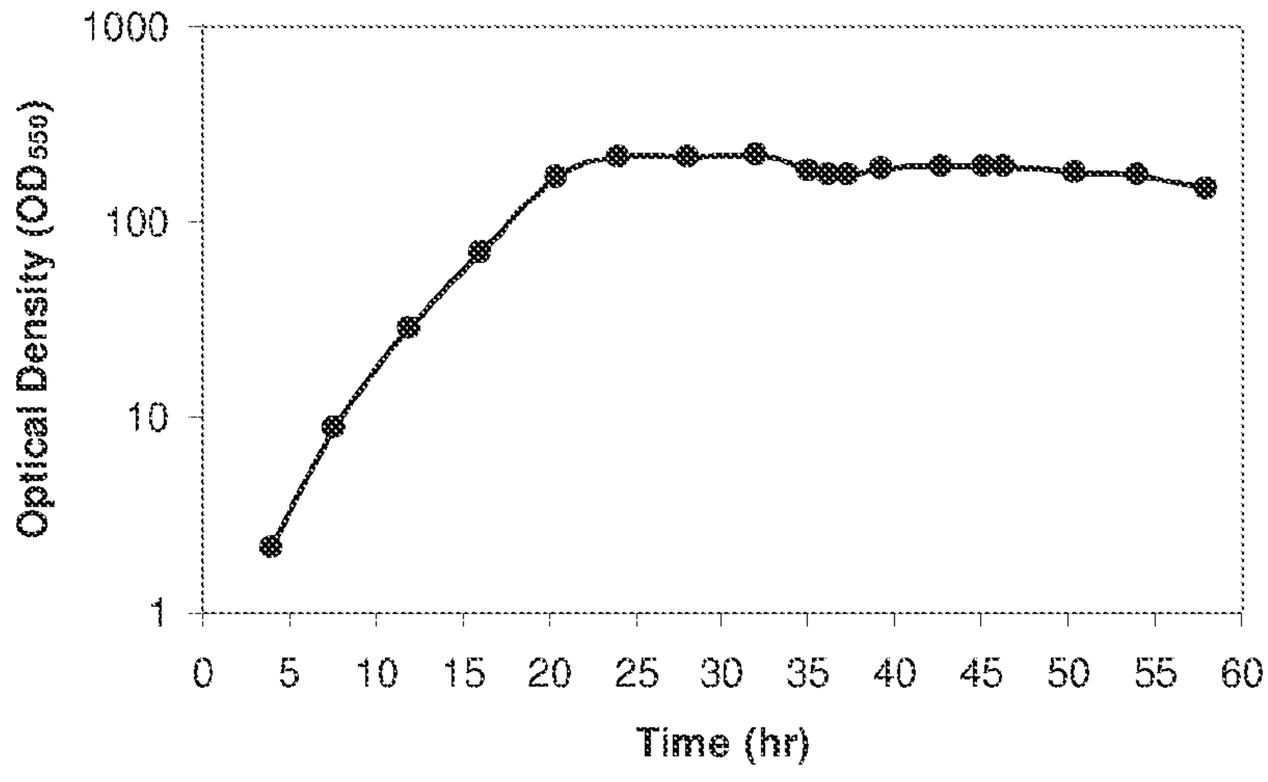

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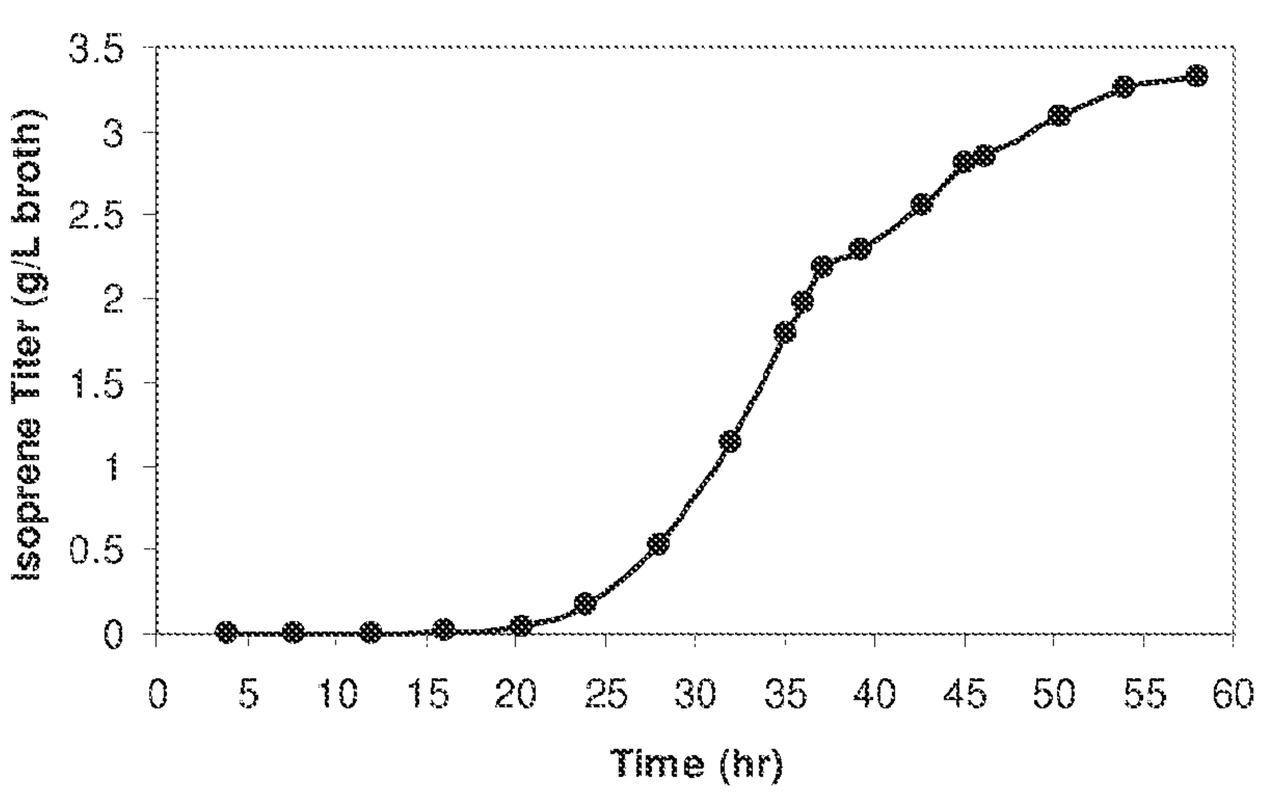

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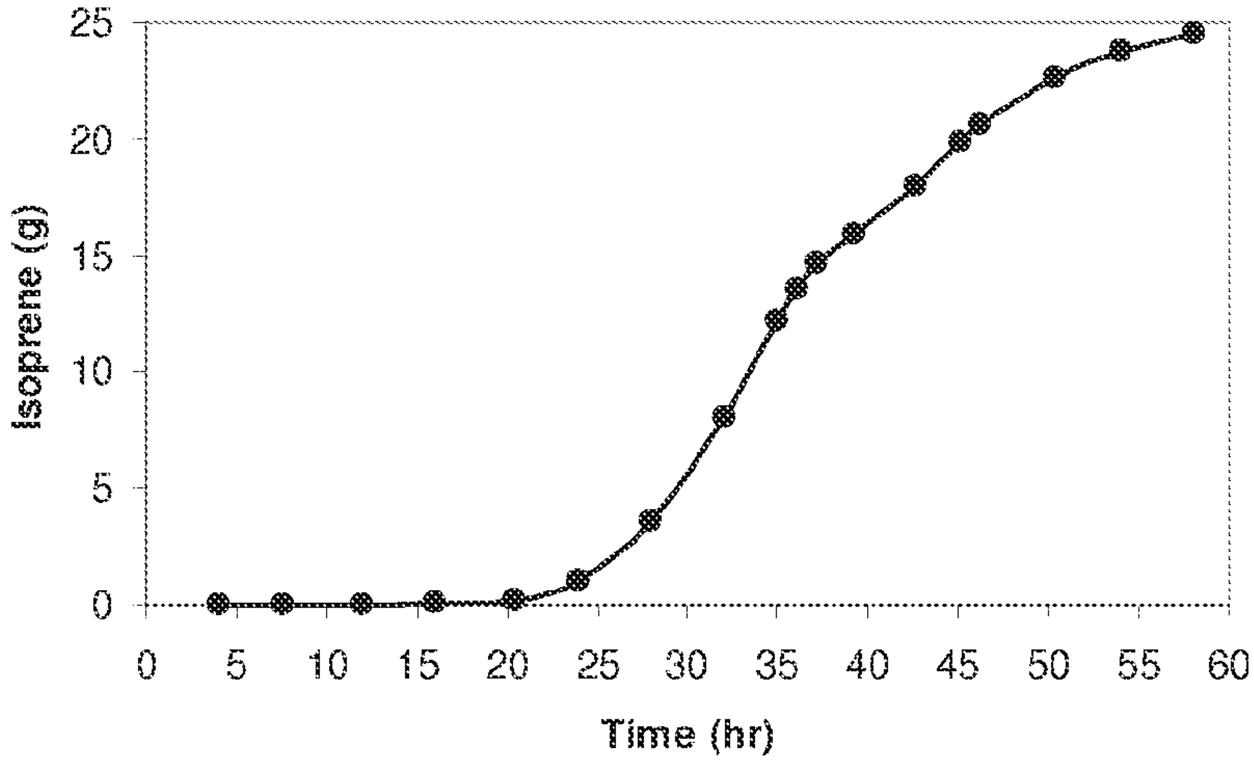

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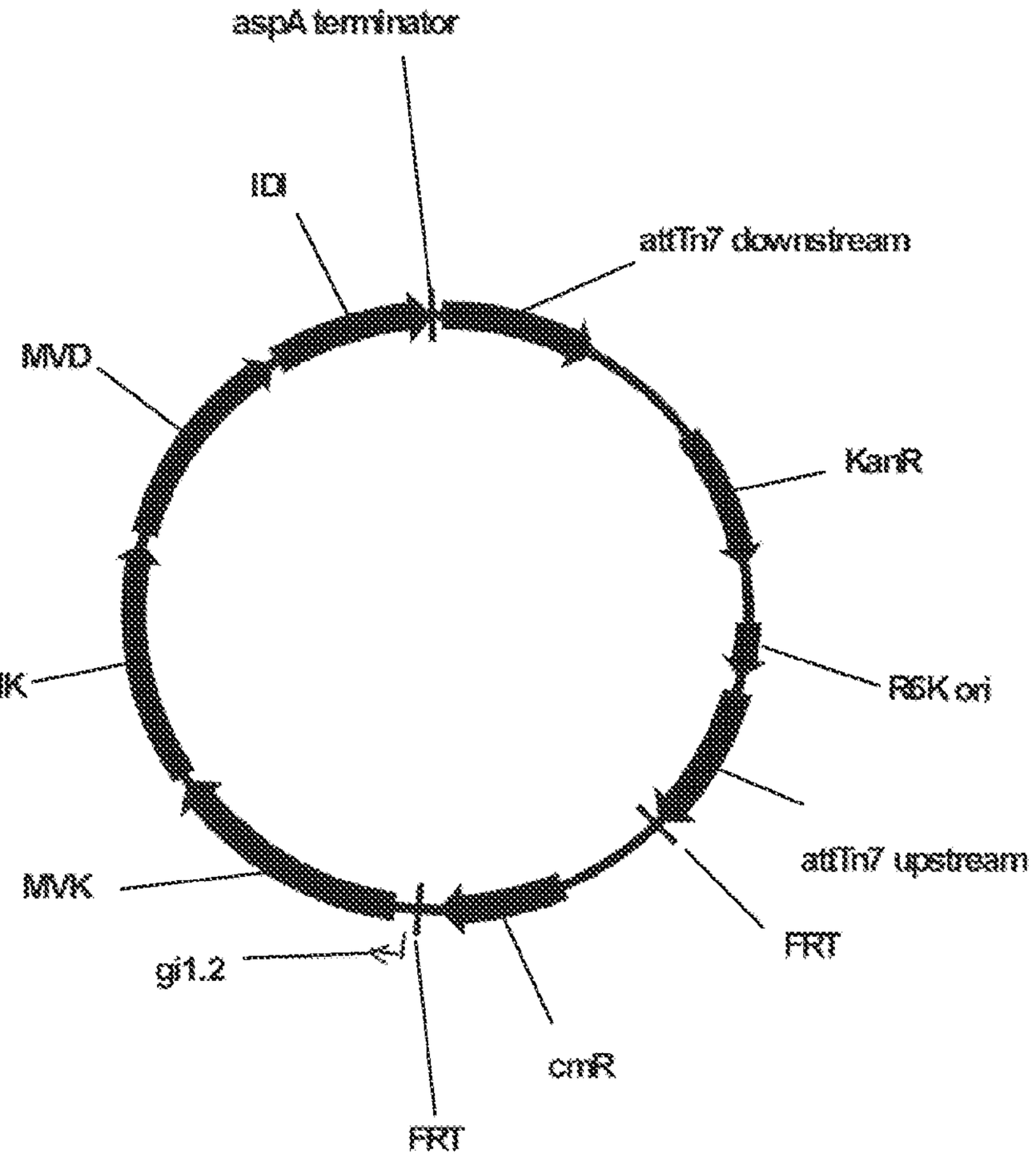

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Figure 108A

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Figure 108B

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Figure 108C

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Figure 109

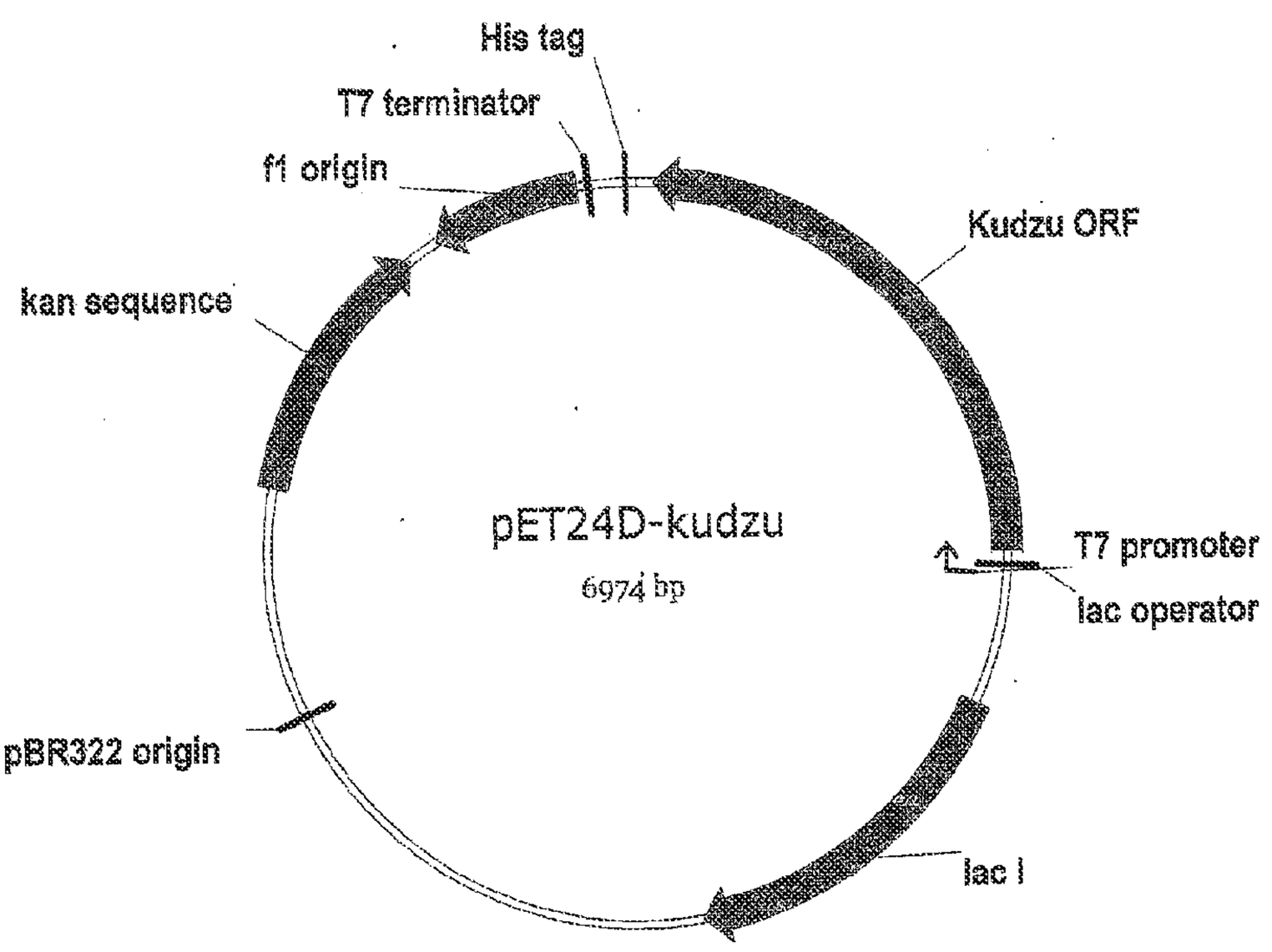
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Figure 110A

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Figure 110B

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Figure 111A

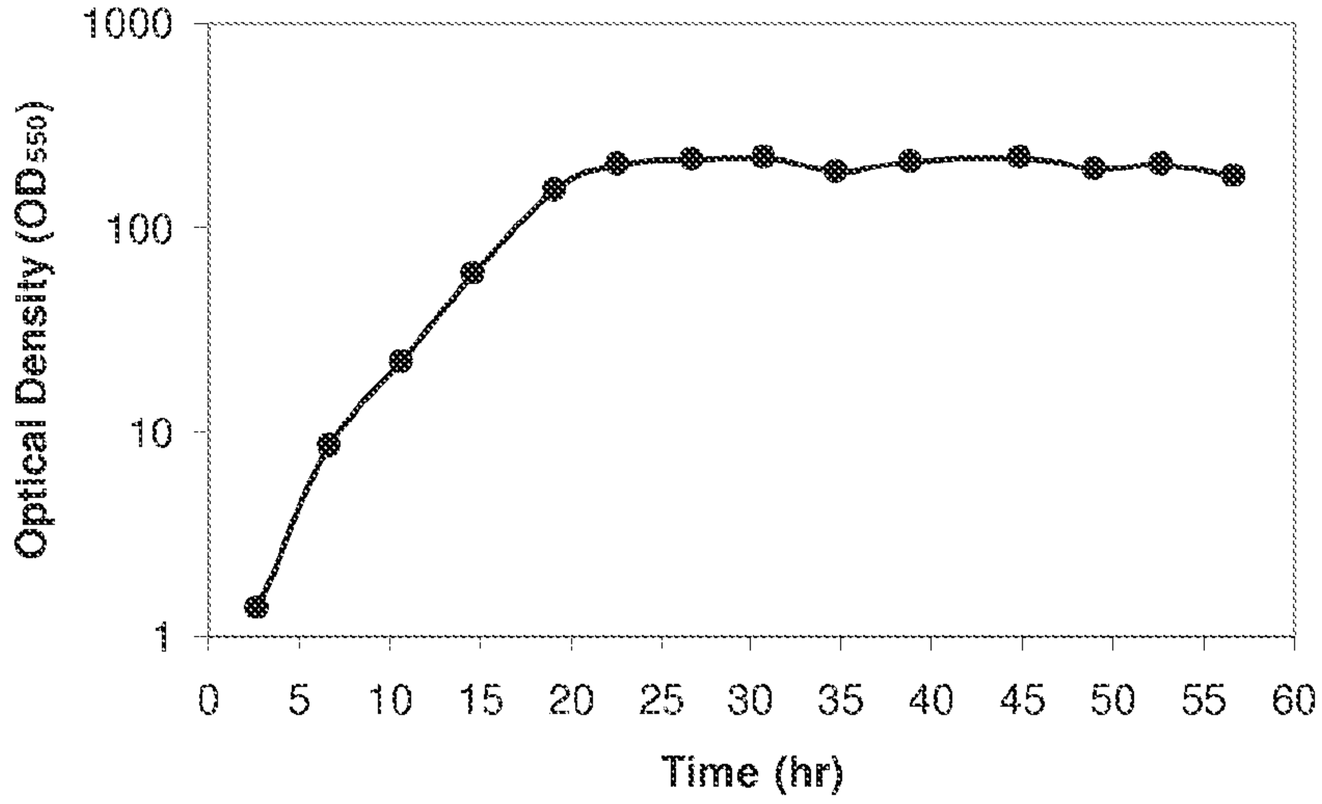


Figure 111B

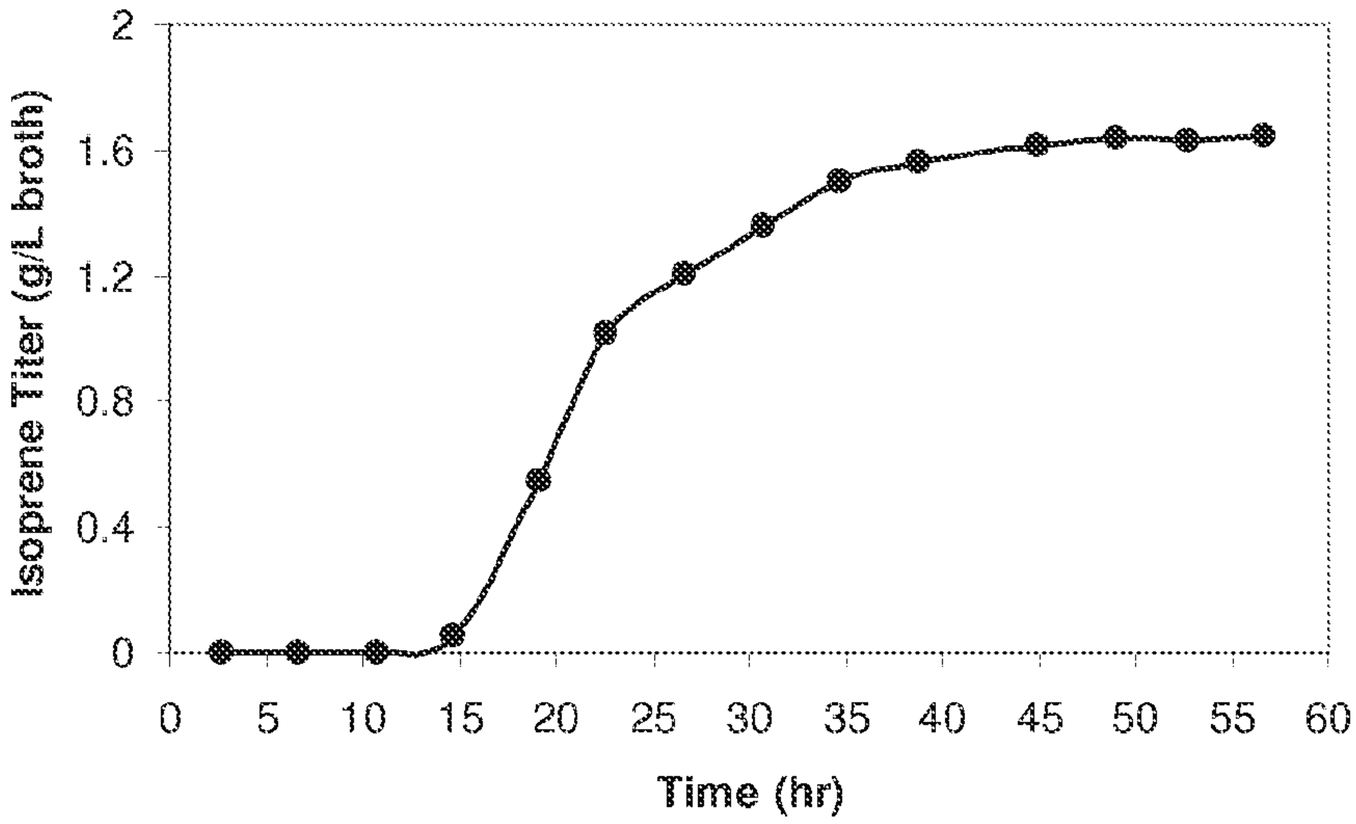
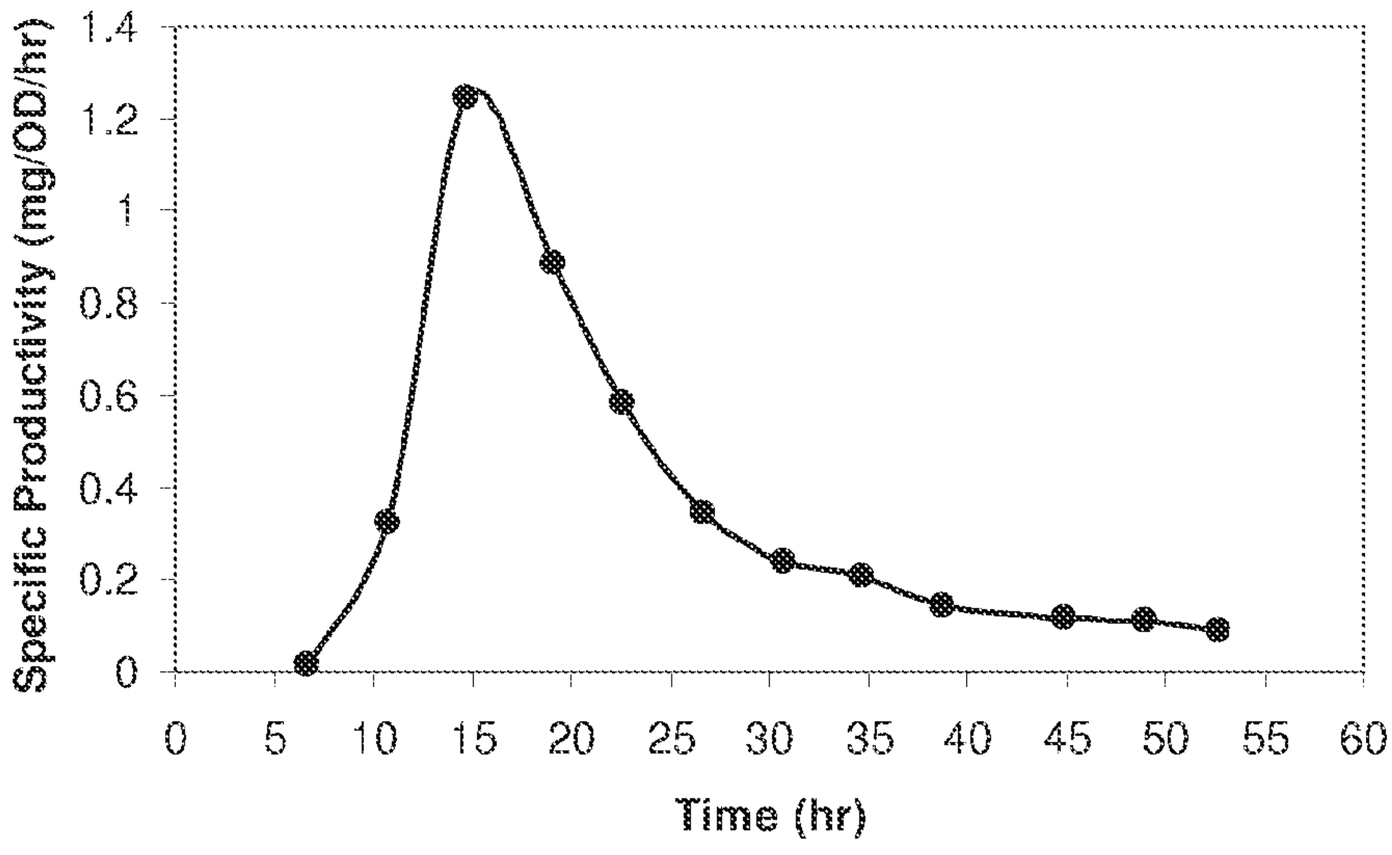


Figure 111C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0.8
0.6
0.4
0.2
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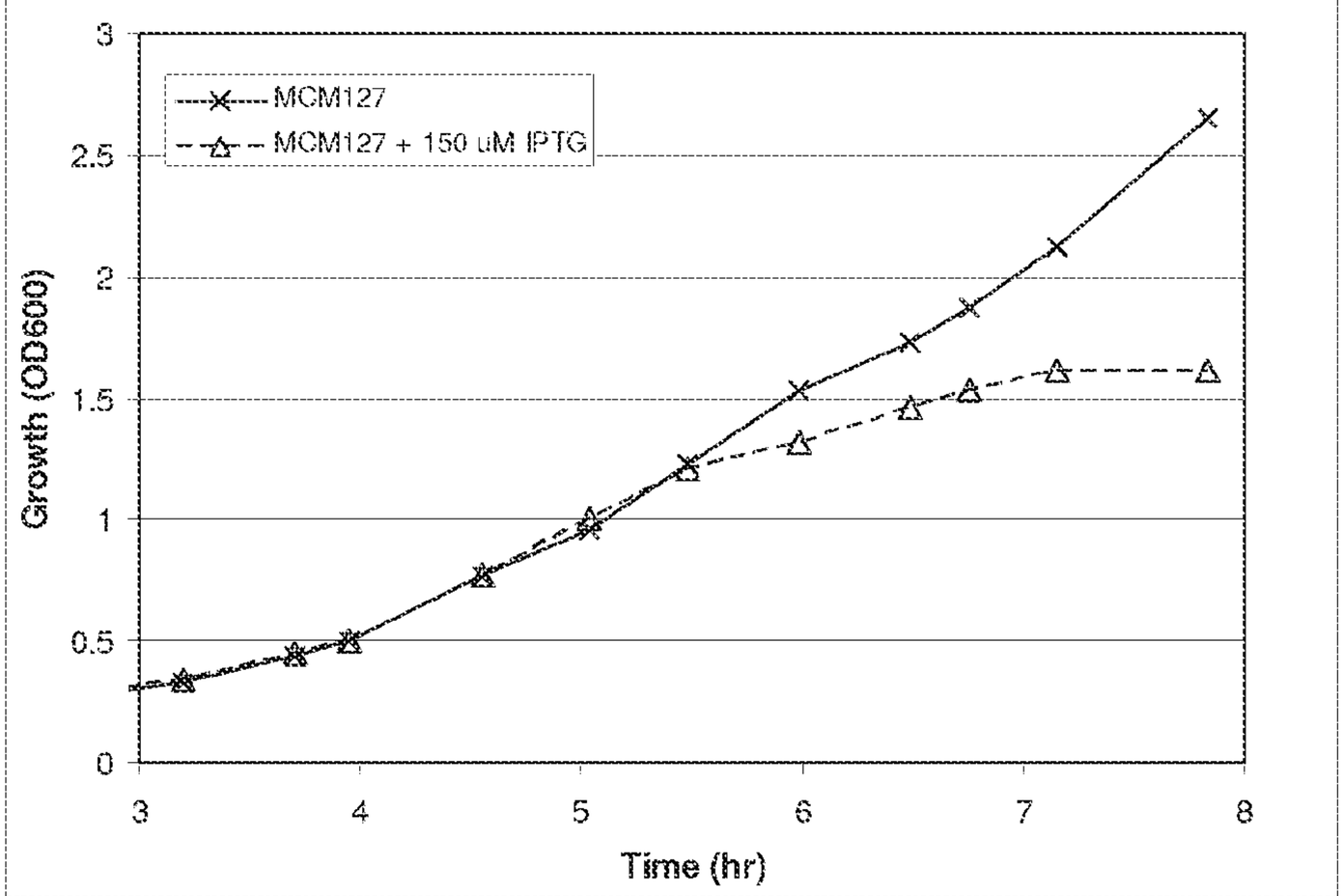
20
25
30
35
40
45
50
55
60
Time (hr)

Figure 112A-112B

A



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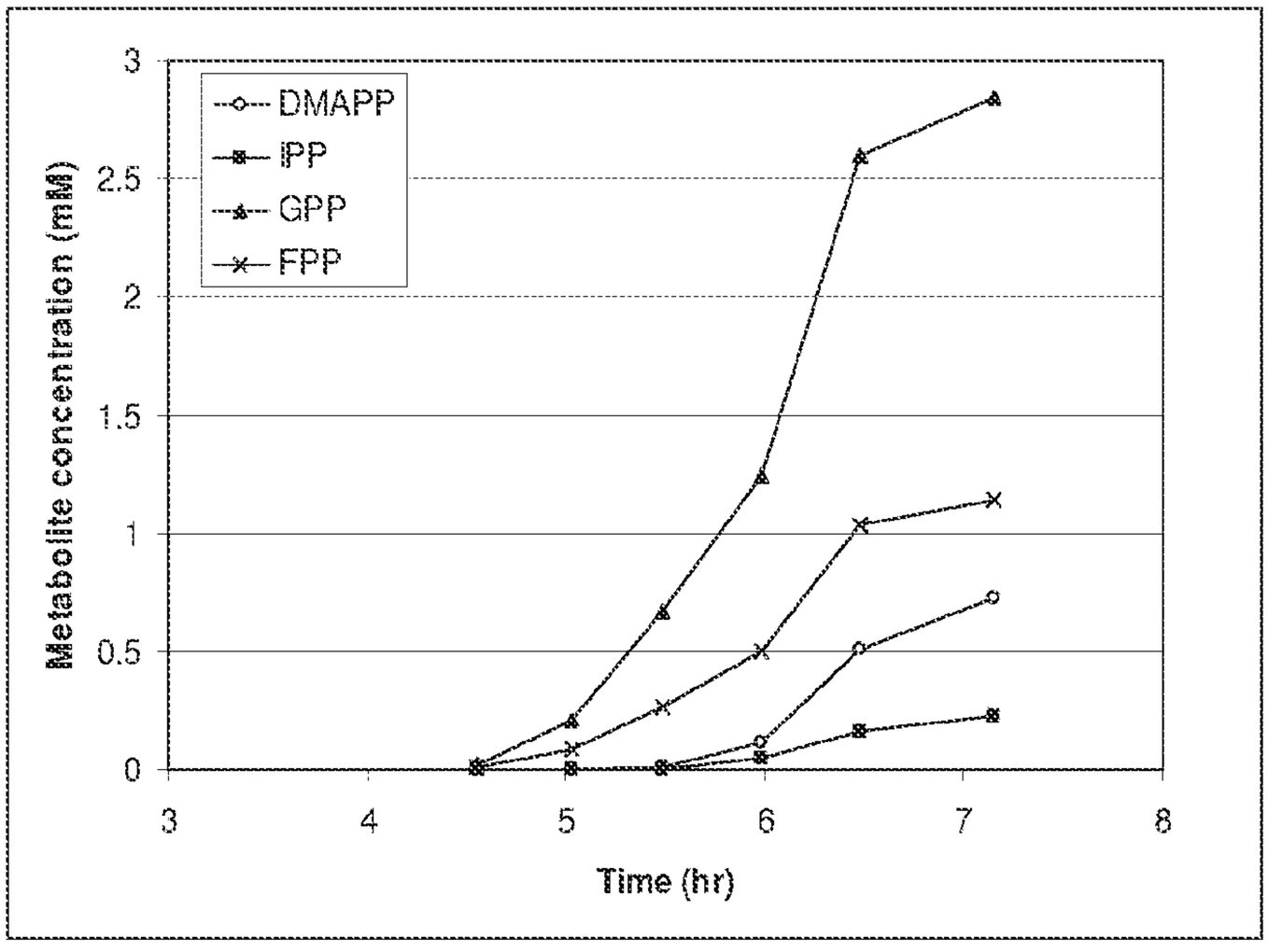
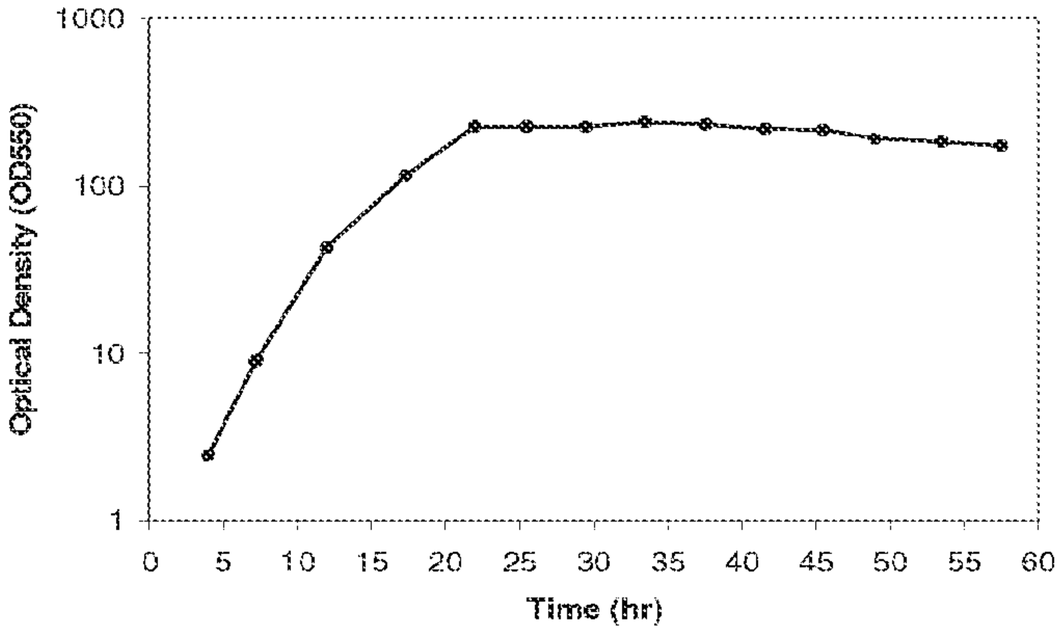


Figure 112C-112E

C



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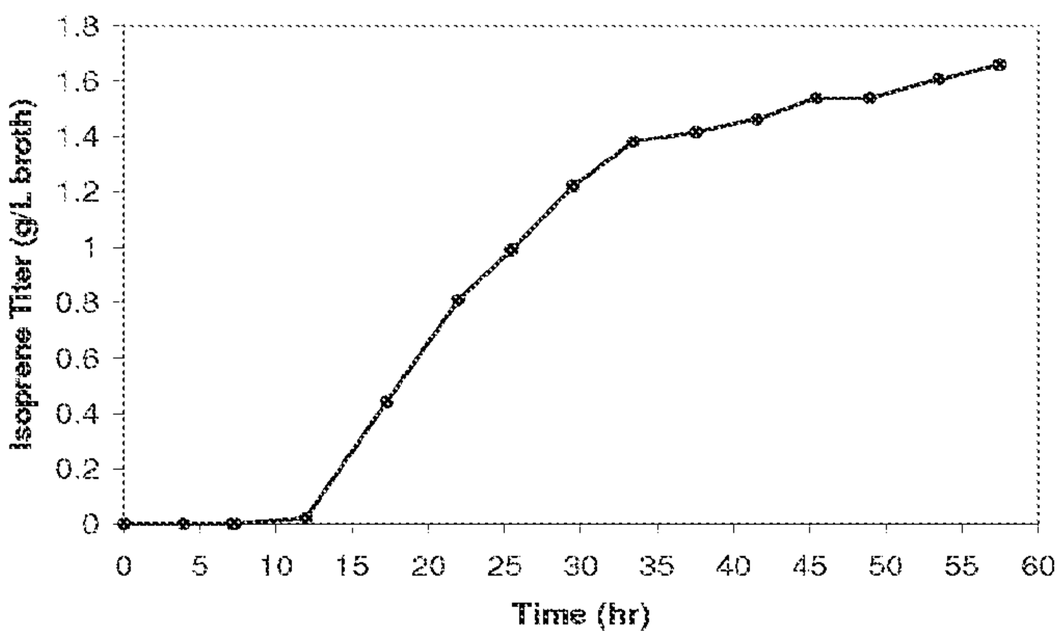

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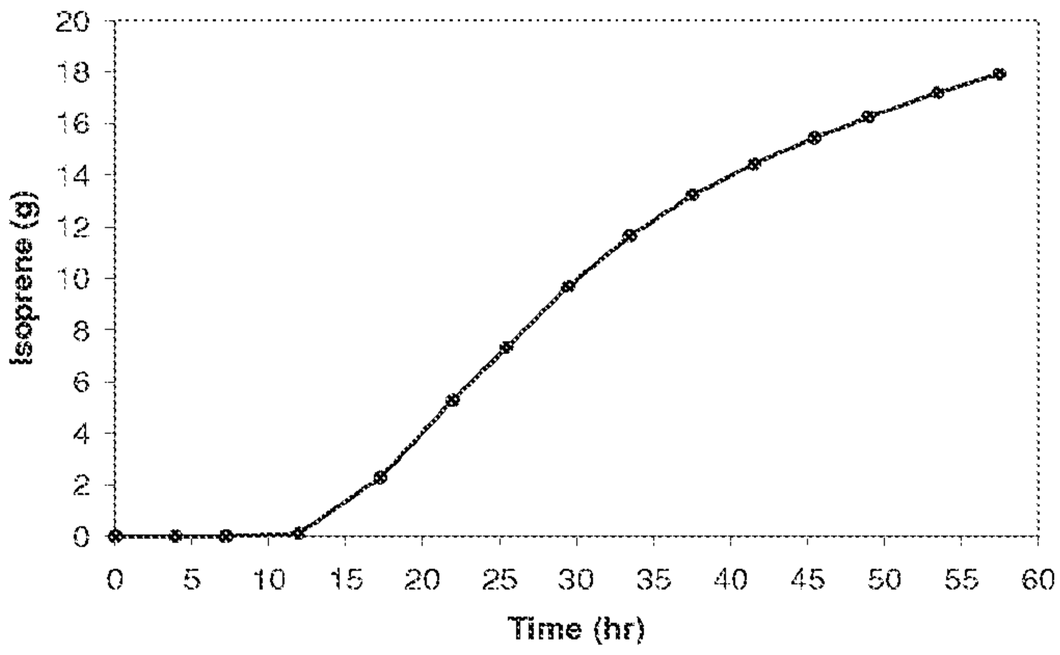
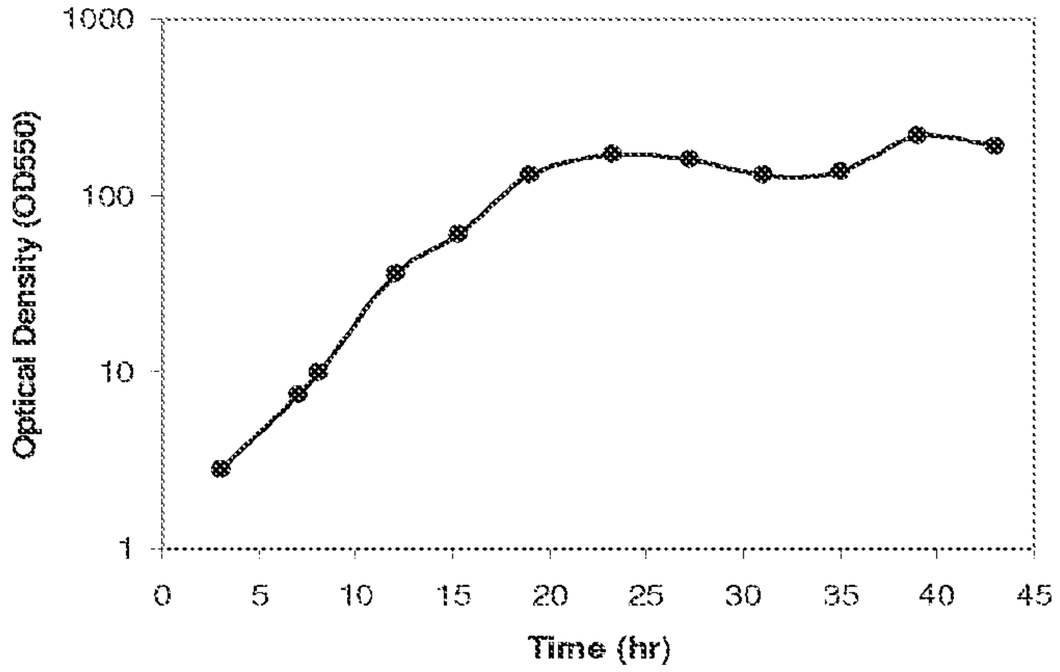


Figure 112F-112H

F



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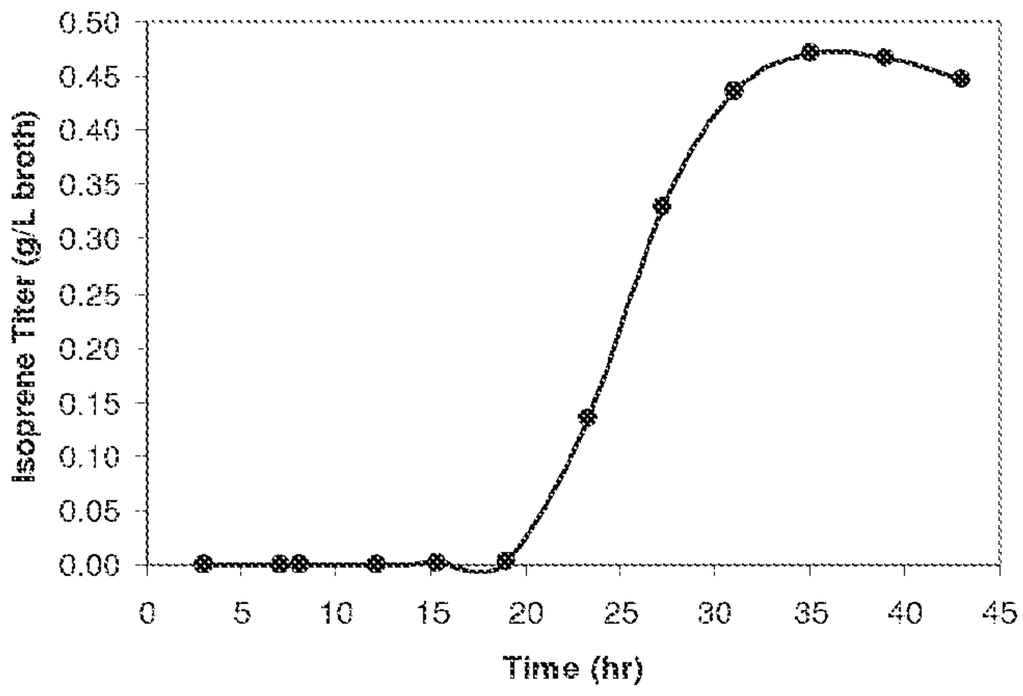

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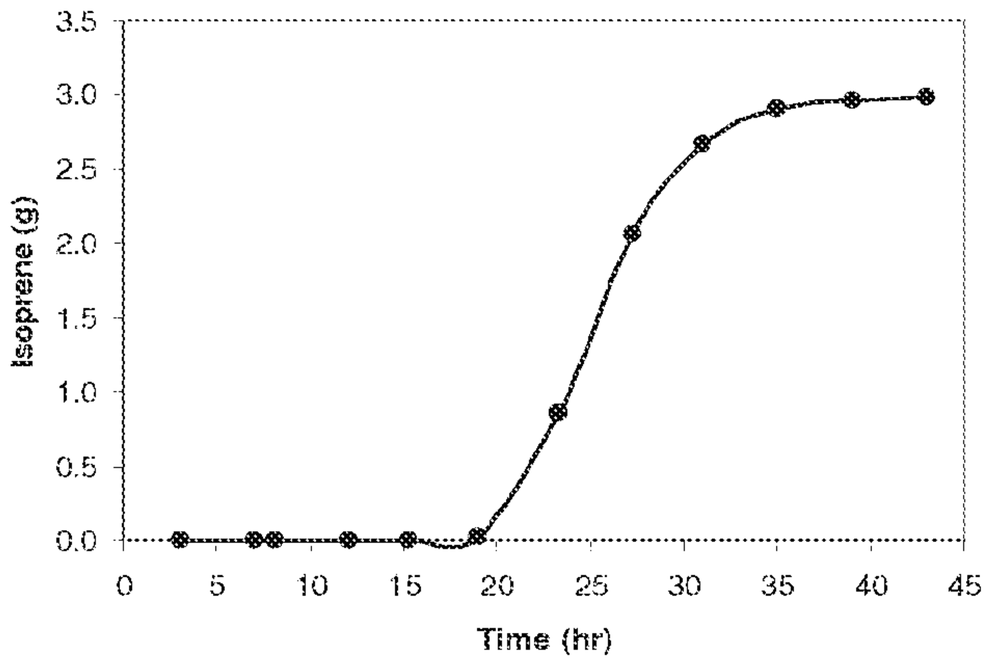
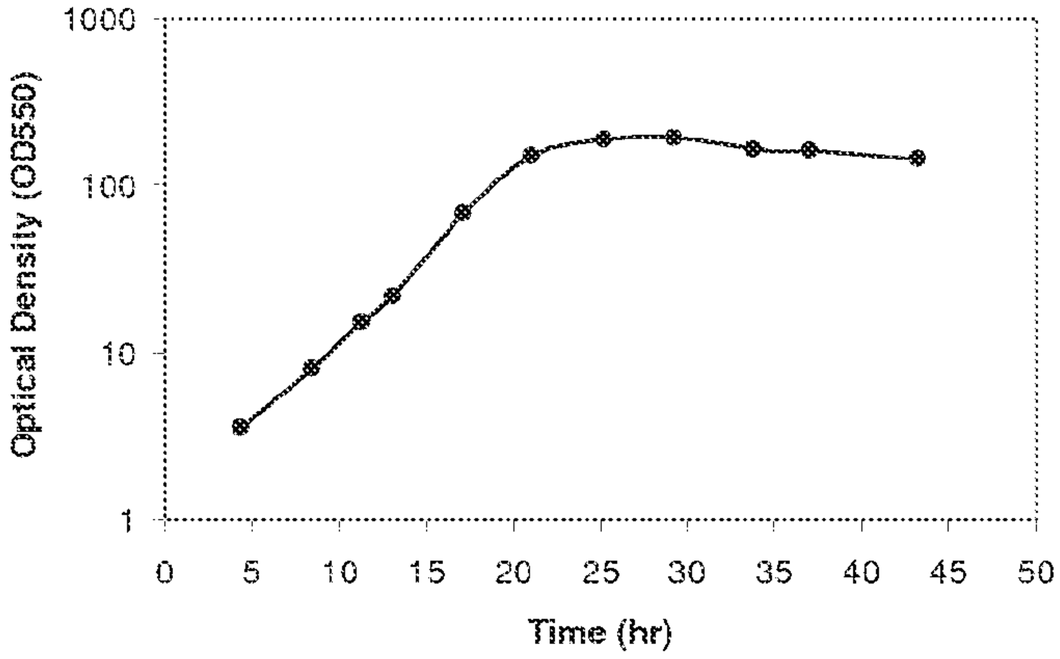


Figure 112I-112K

I



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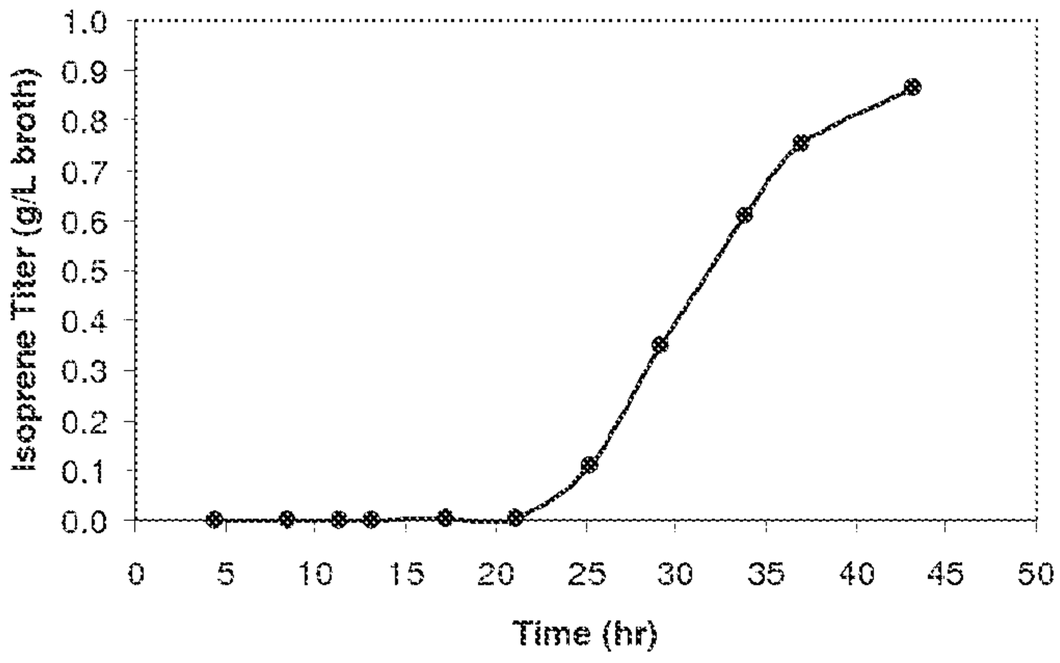

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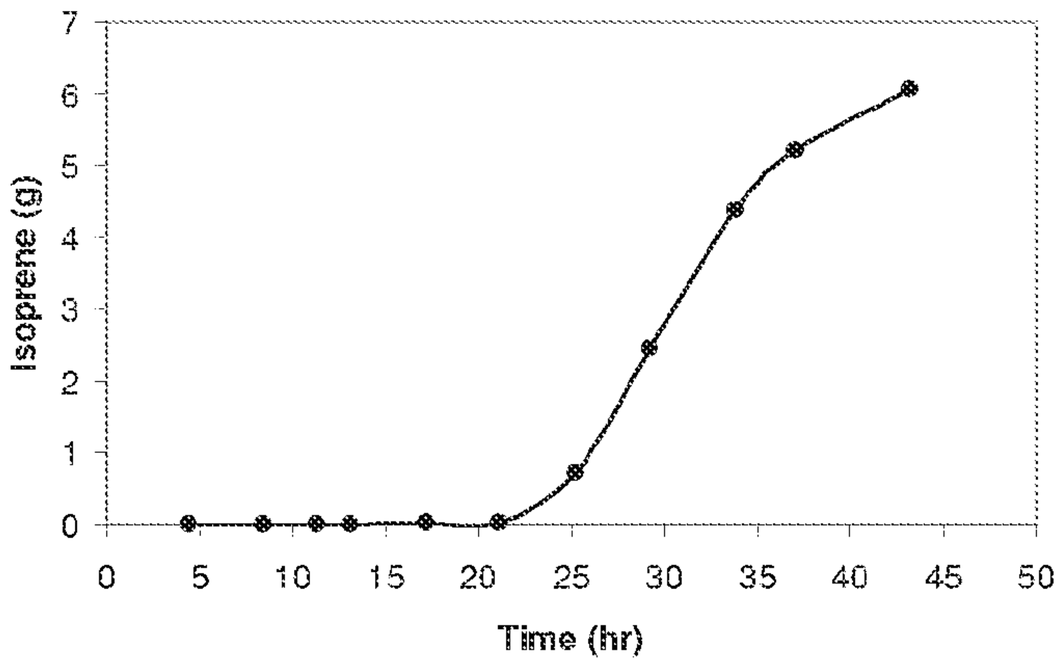

Figure 112L-112N

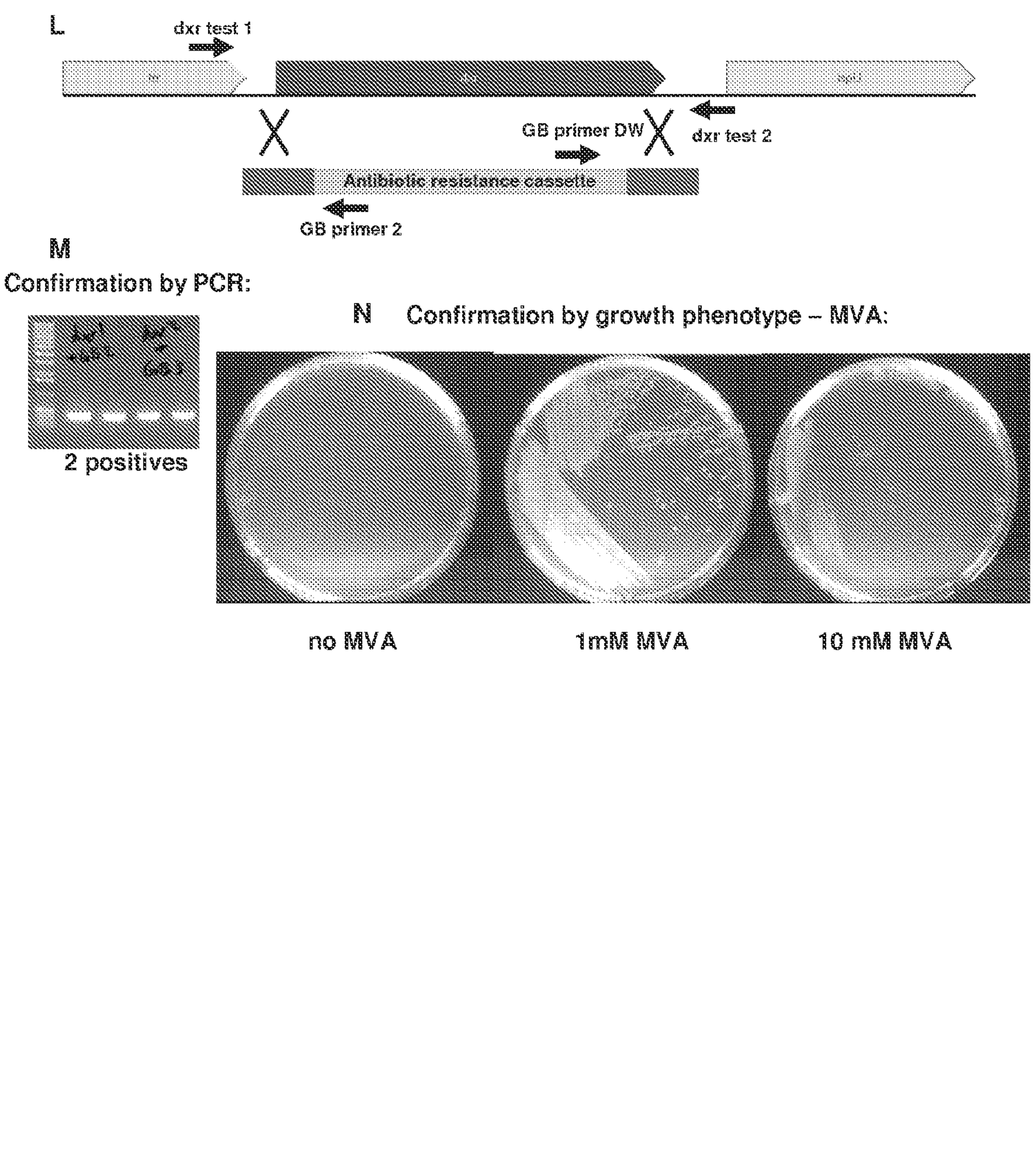

Figure 112O-112P

O

MCM63	Ptrc minus lacO forw	Tcatccggctcgtataatgtgtggtcacacaggaaacagcgccgctga (SEQ ID NO:139)
MCM64	Ptrc minus lacO rev	Tcagcggcgctgtttcctgtgtgaccacacattatacgagccggatga (SEQ ID NO:140)

P

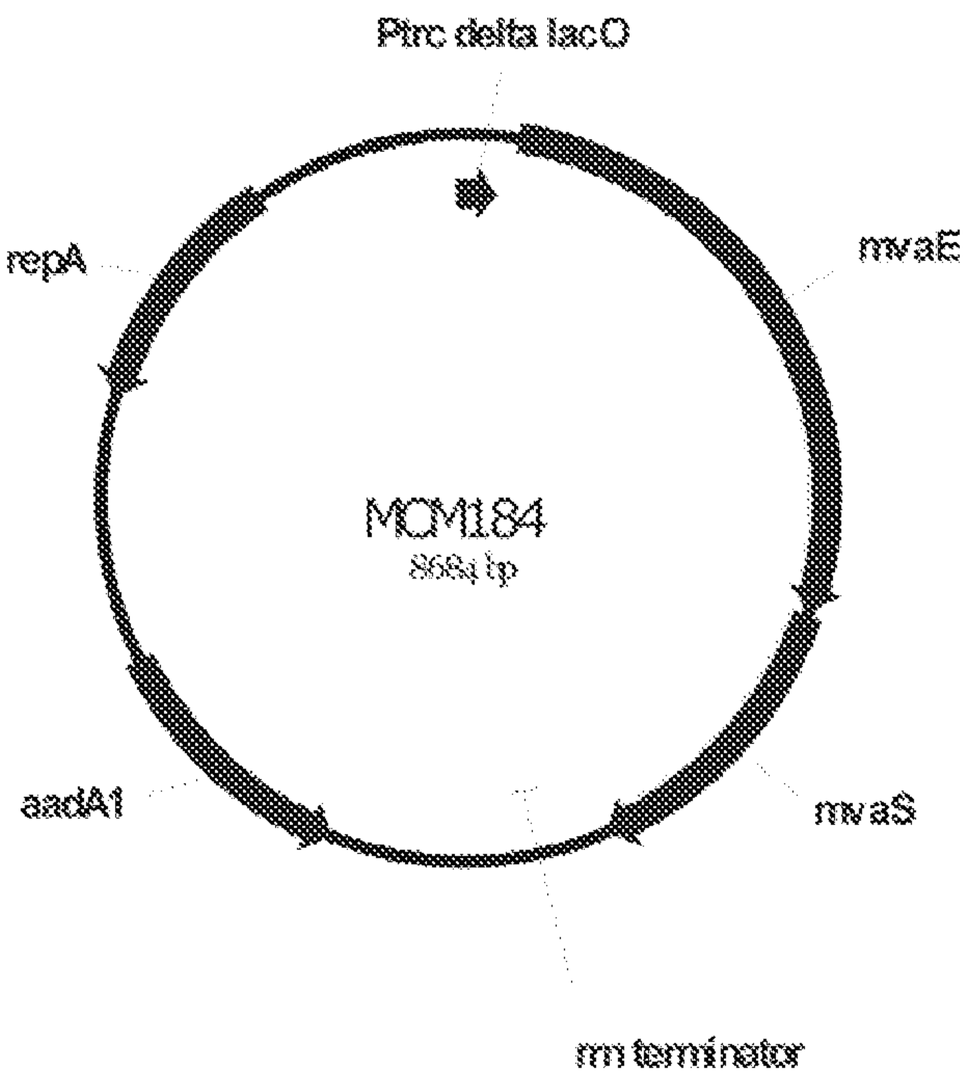

Figure 112Q

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Figure 112R

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Figure 112S

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ccgattcattaatgcagattctgaaatgagctgttgacaattaatcatccggctcgtataatgtgtgga (SEQ ID NO:141)

Figure 112T-112U

T

EL-976 : (+) PCR of KKDI insertion into pCL vector (upstream of promoter region)
CTTCTCAGGGCGTTTTATGGC (SEQ ID NO:142)

EL-977 : (-) PCR of KKDI insertion into pCL vector (anneals to MVK gene)
GTTGAGCTAACAACGGATCC (SEQ ID NO:143)

EL-978 : (+) sequence from yeast IDI towards terminator
GACTGTCAACCCAAACGTCAATG (SEQ ID NO :144)

U

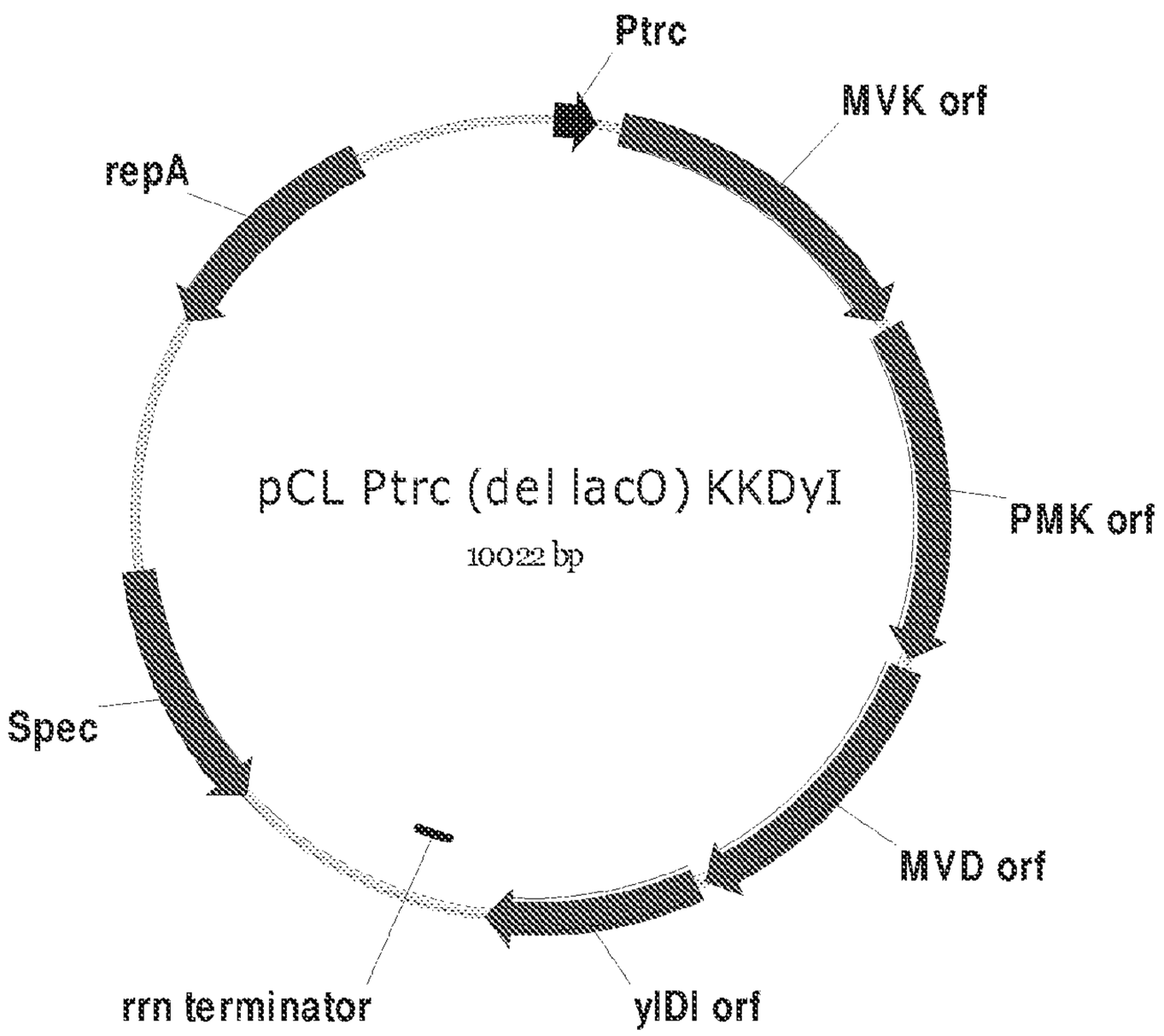

Figure 112V

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Figure 112W

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Figure 112X

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Figure 113A-113B

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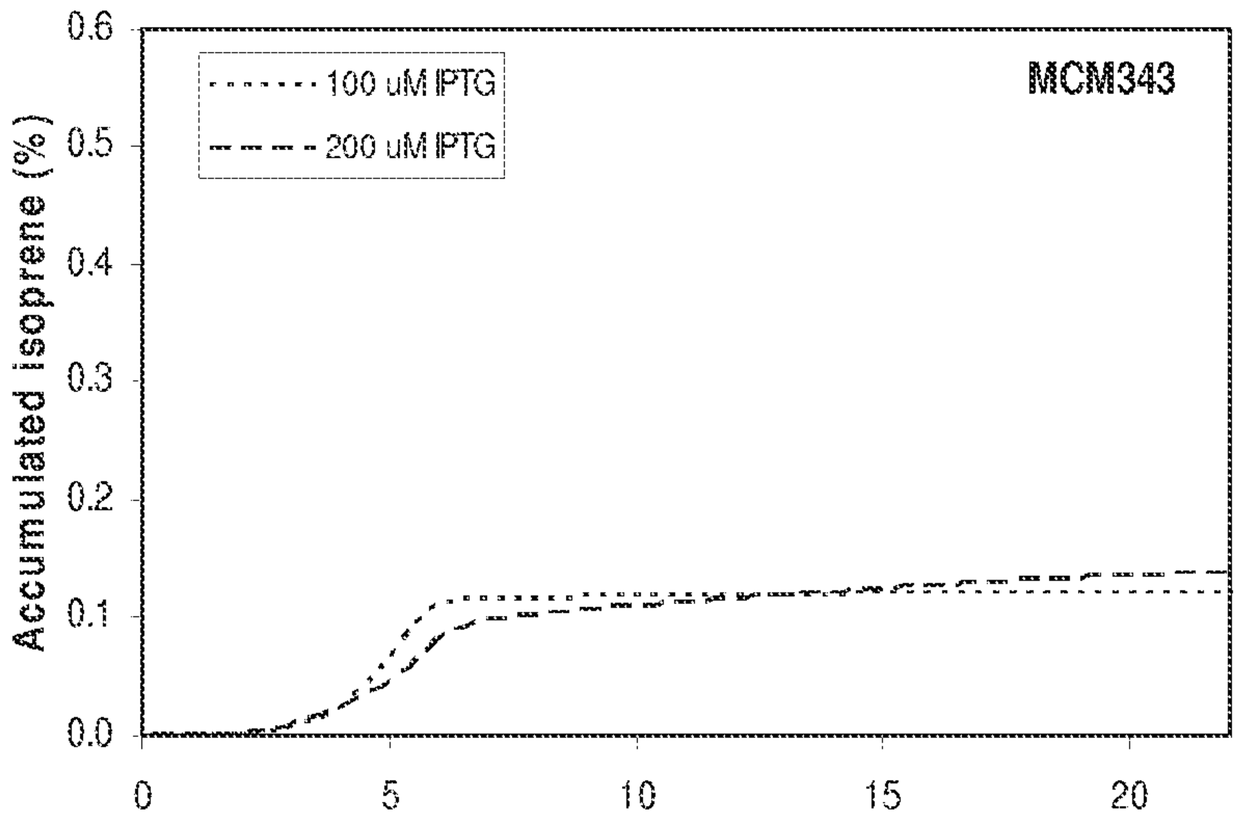

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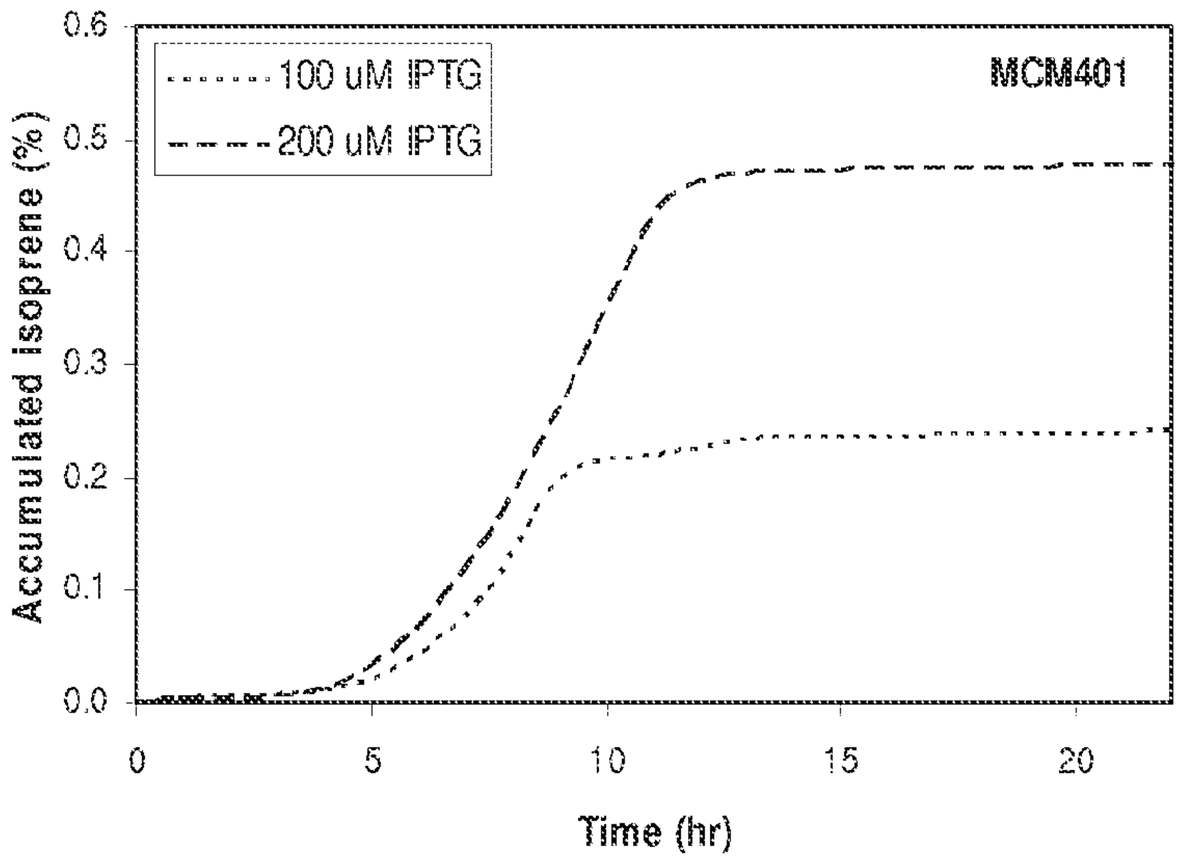

Figure 113C-113D

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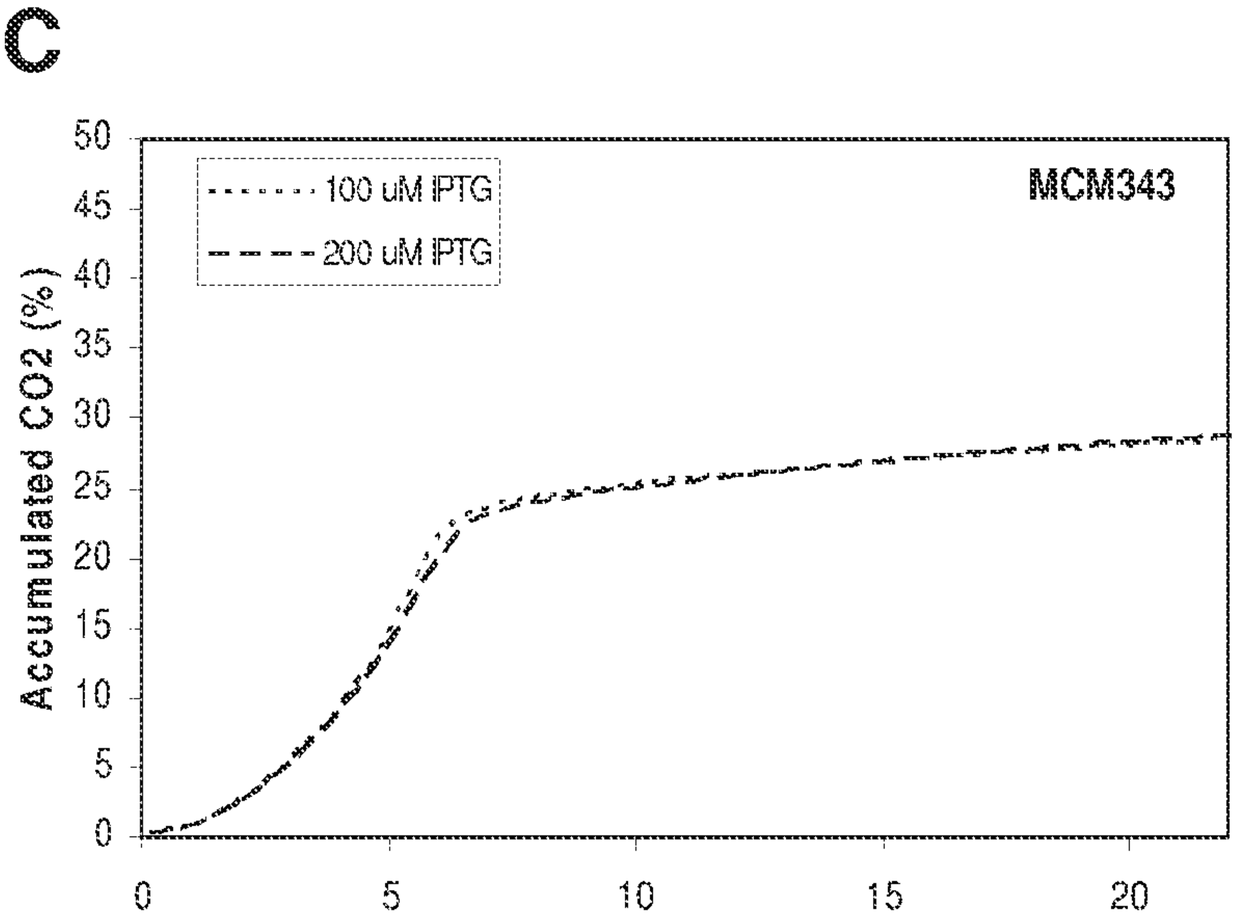

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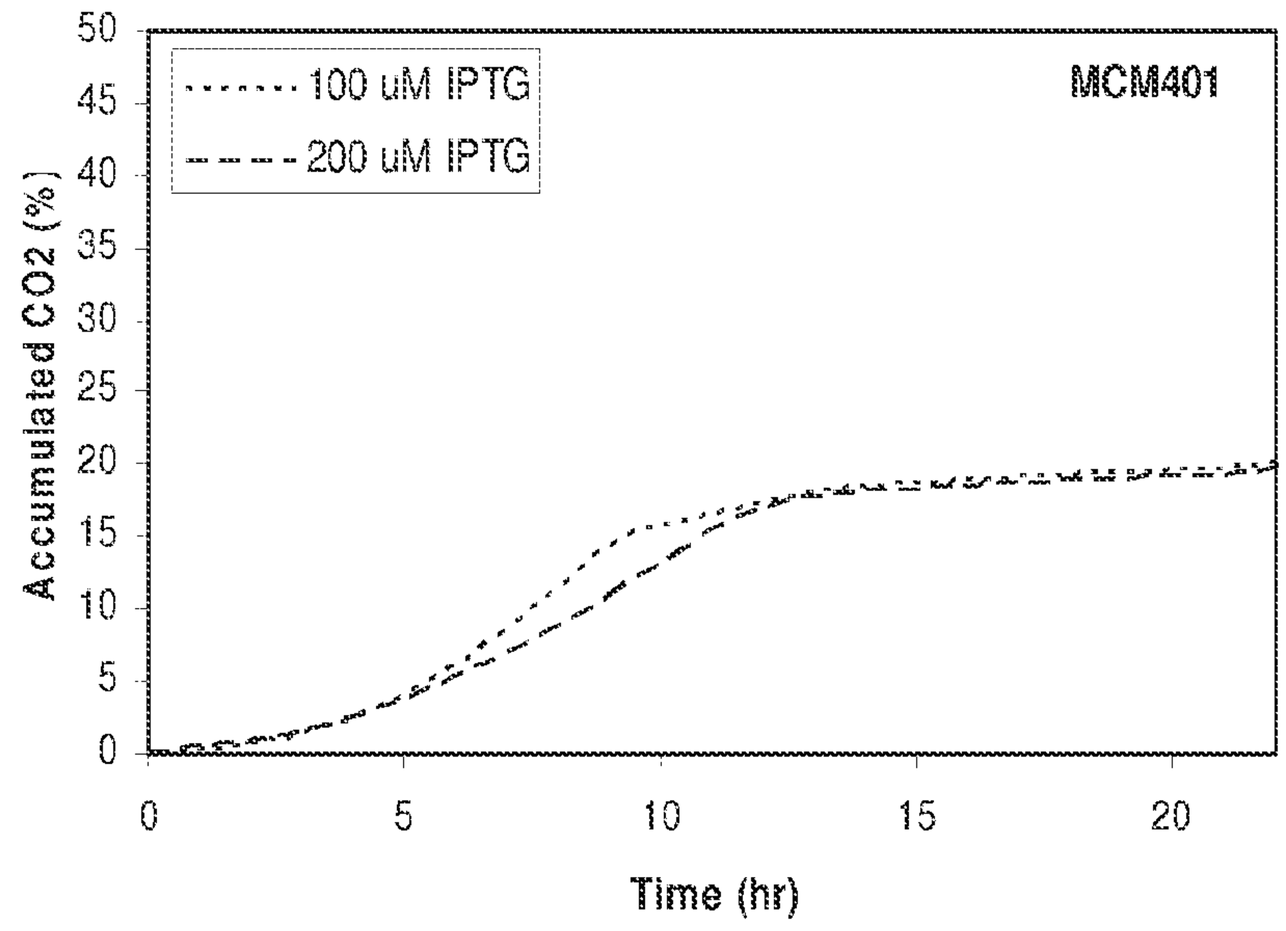

Figure 114

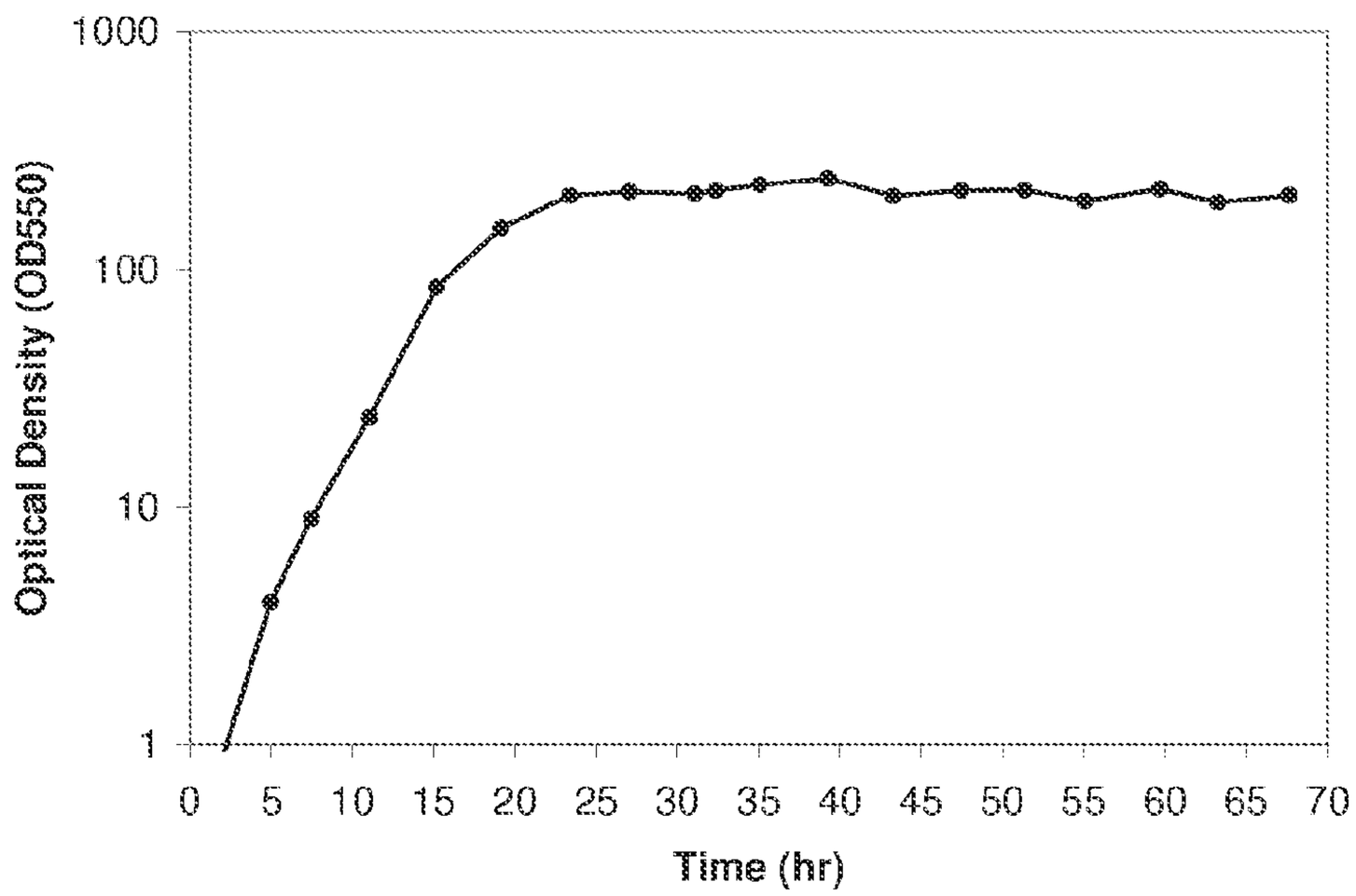

Figure 115

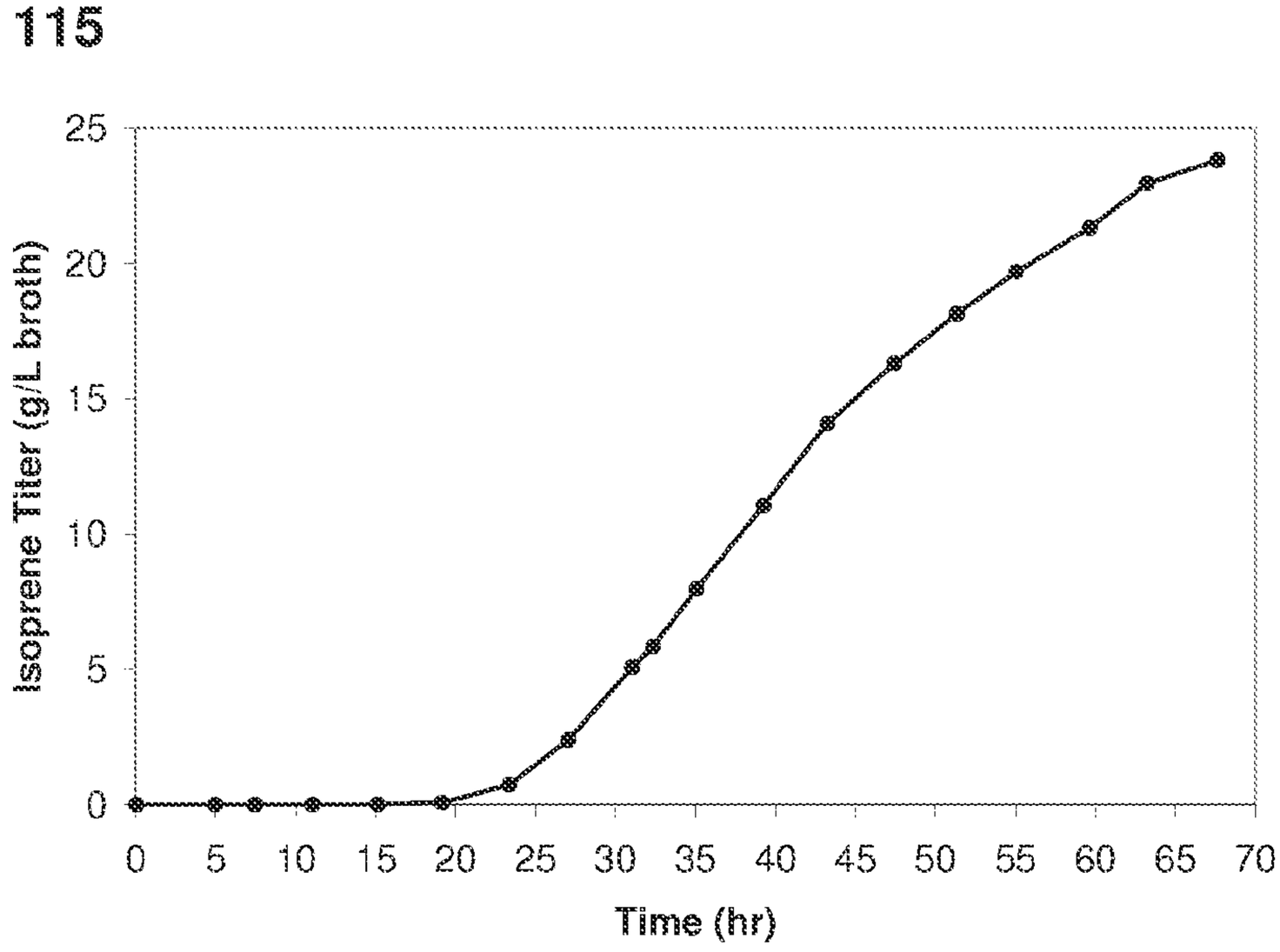

Figure 116

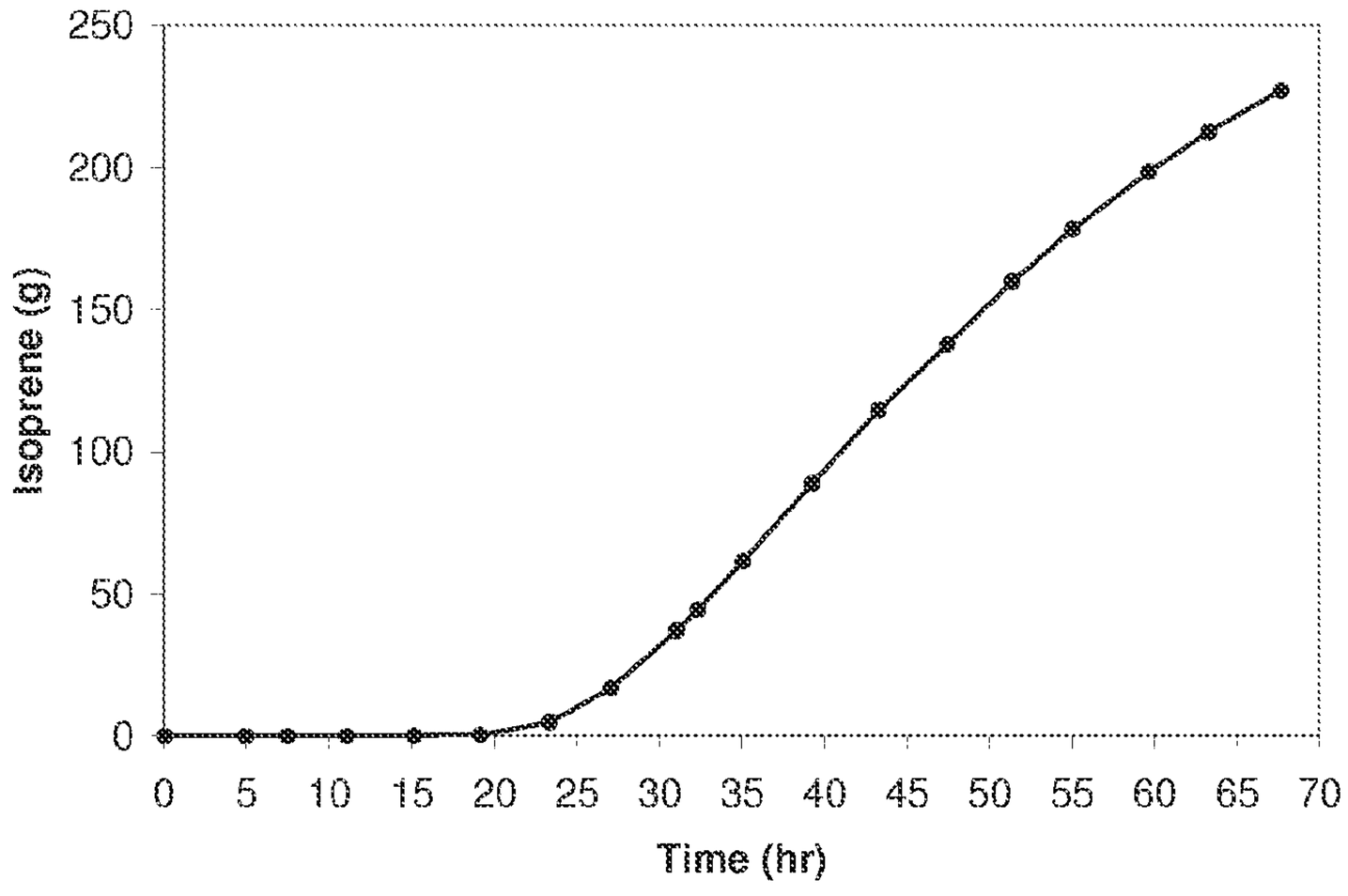

Figure 117

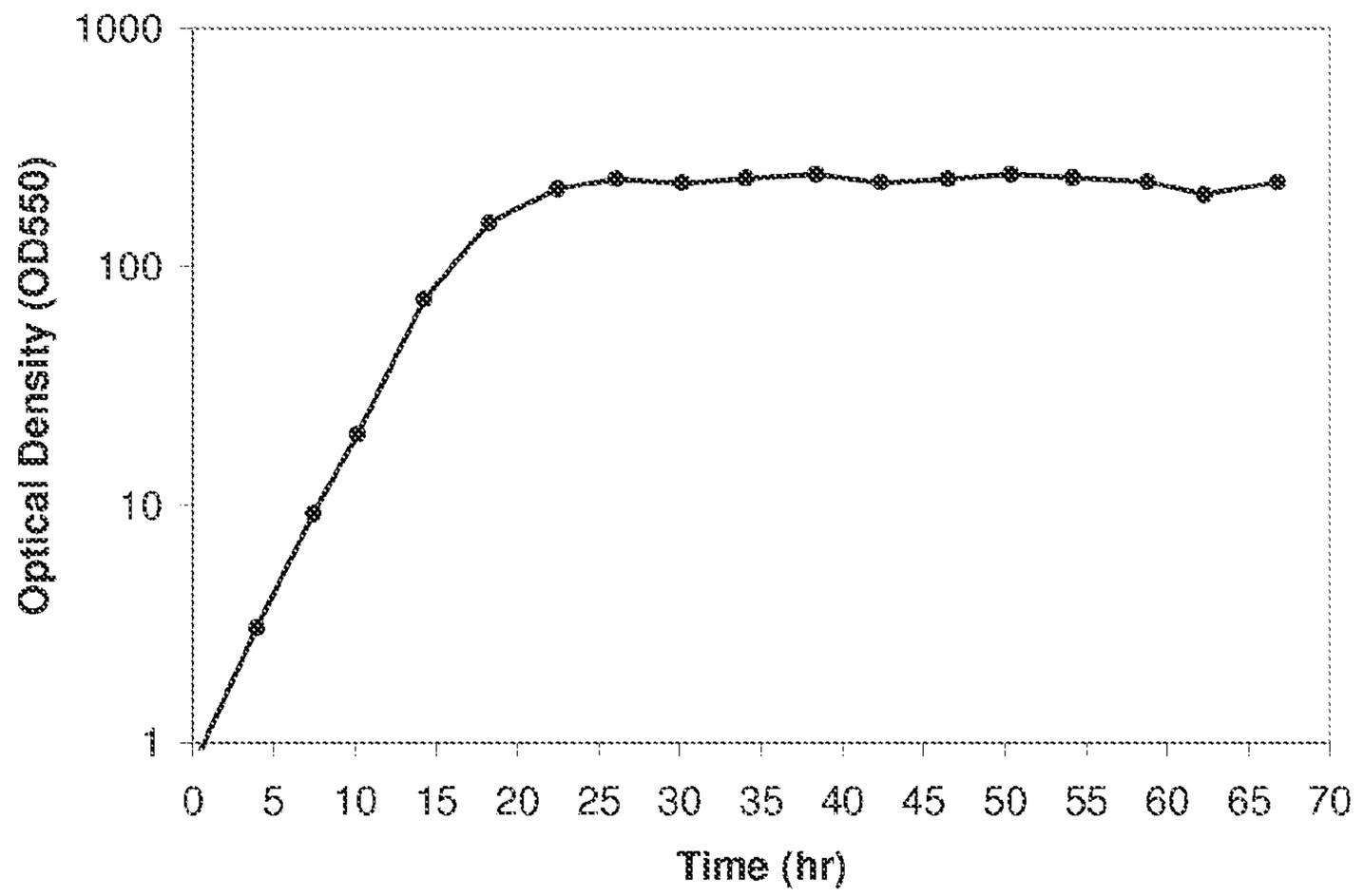

Figure 118

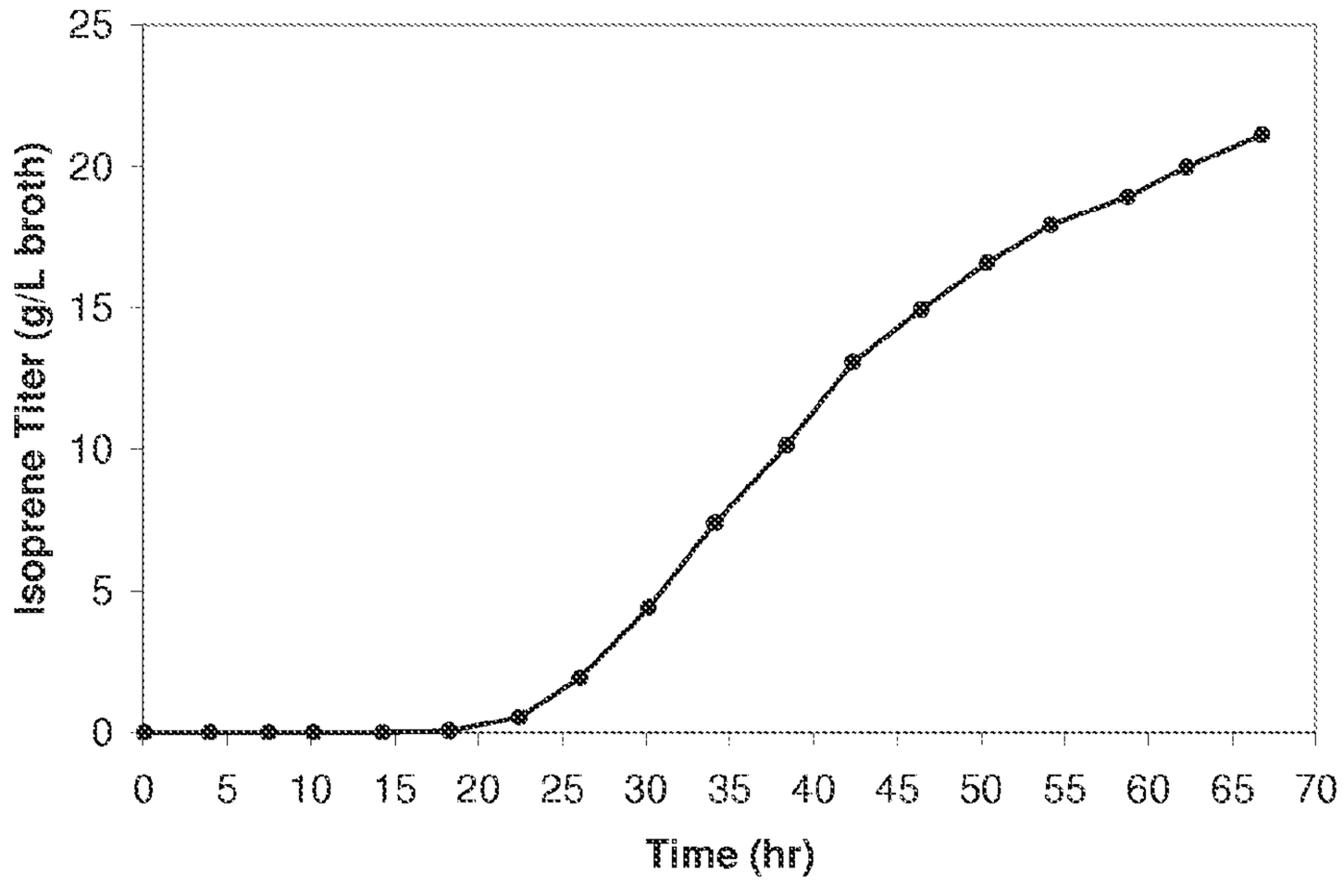

Figure 119

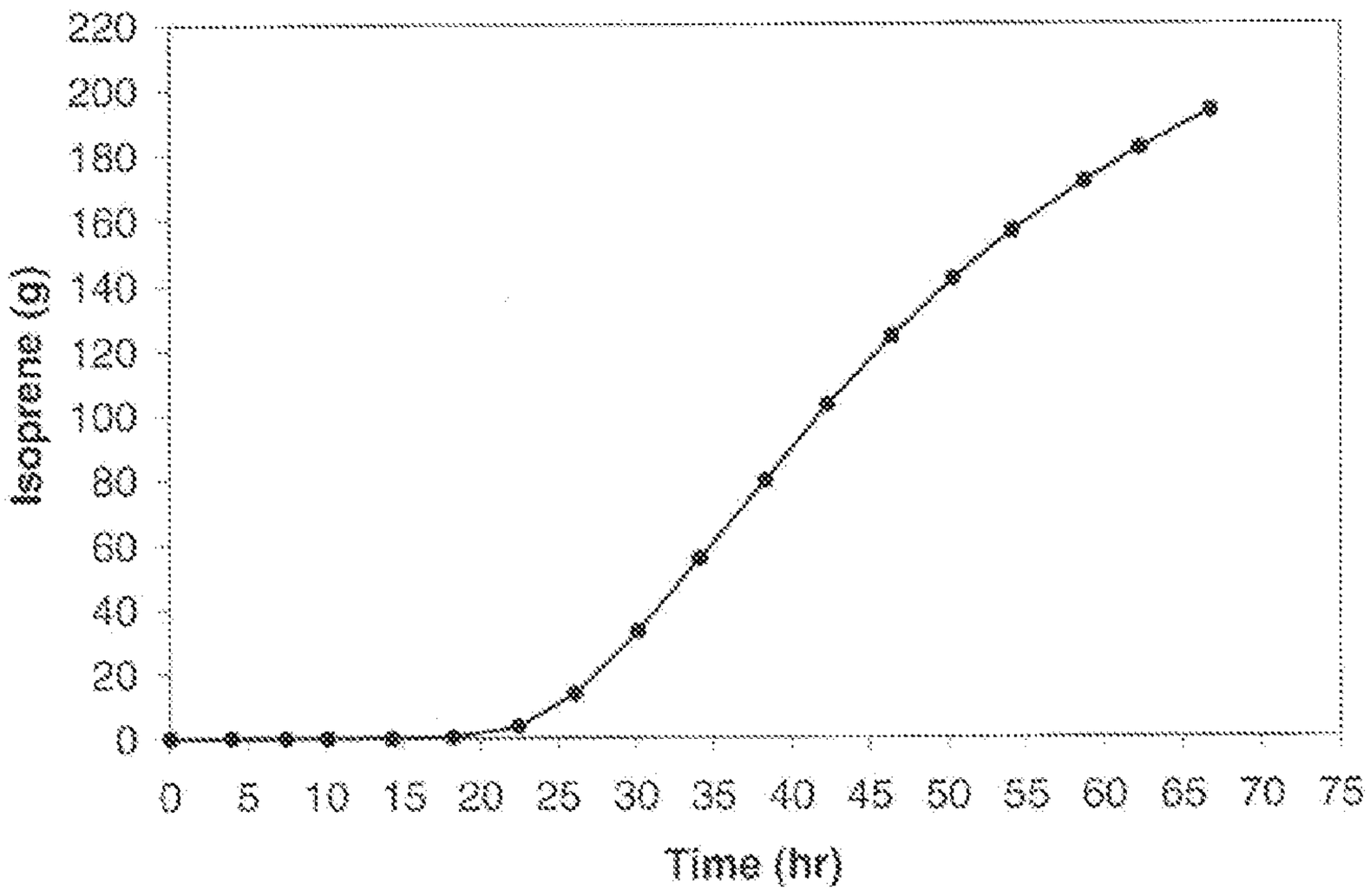

Figure 120

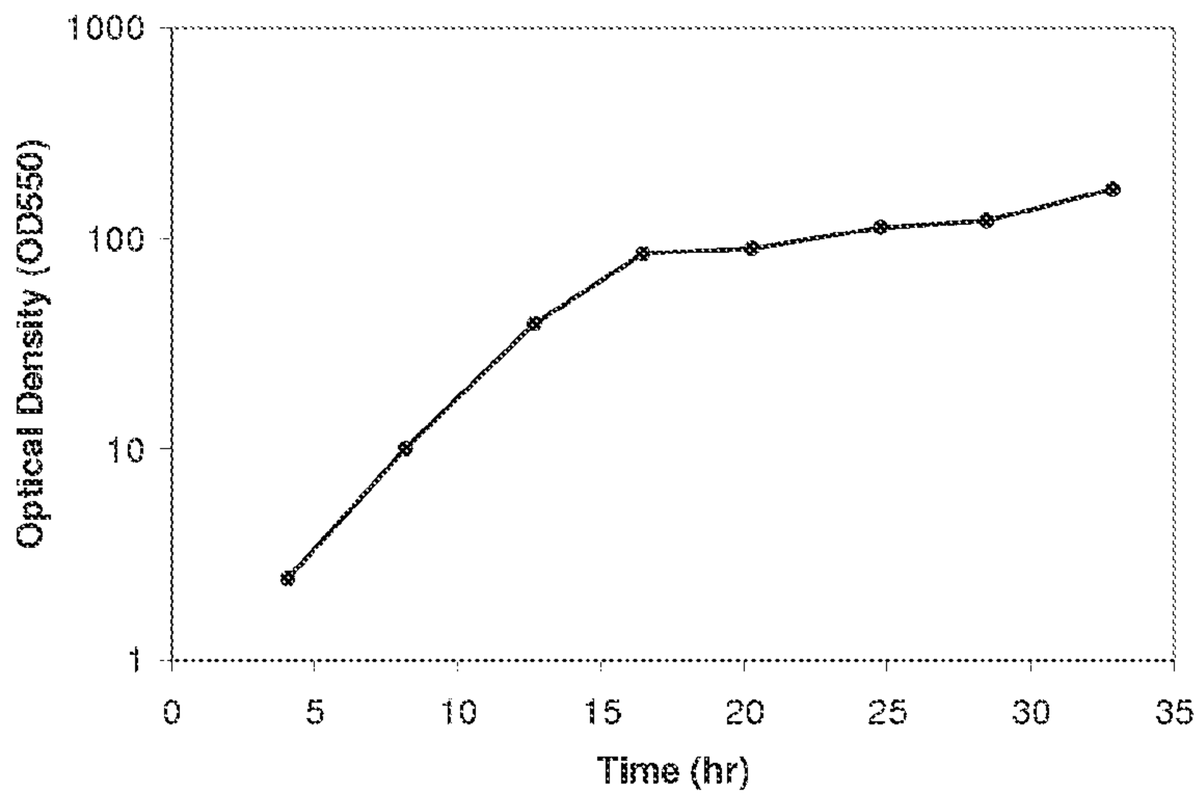

Figure 121

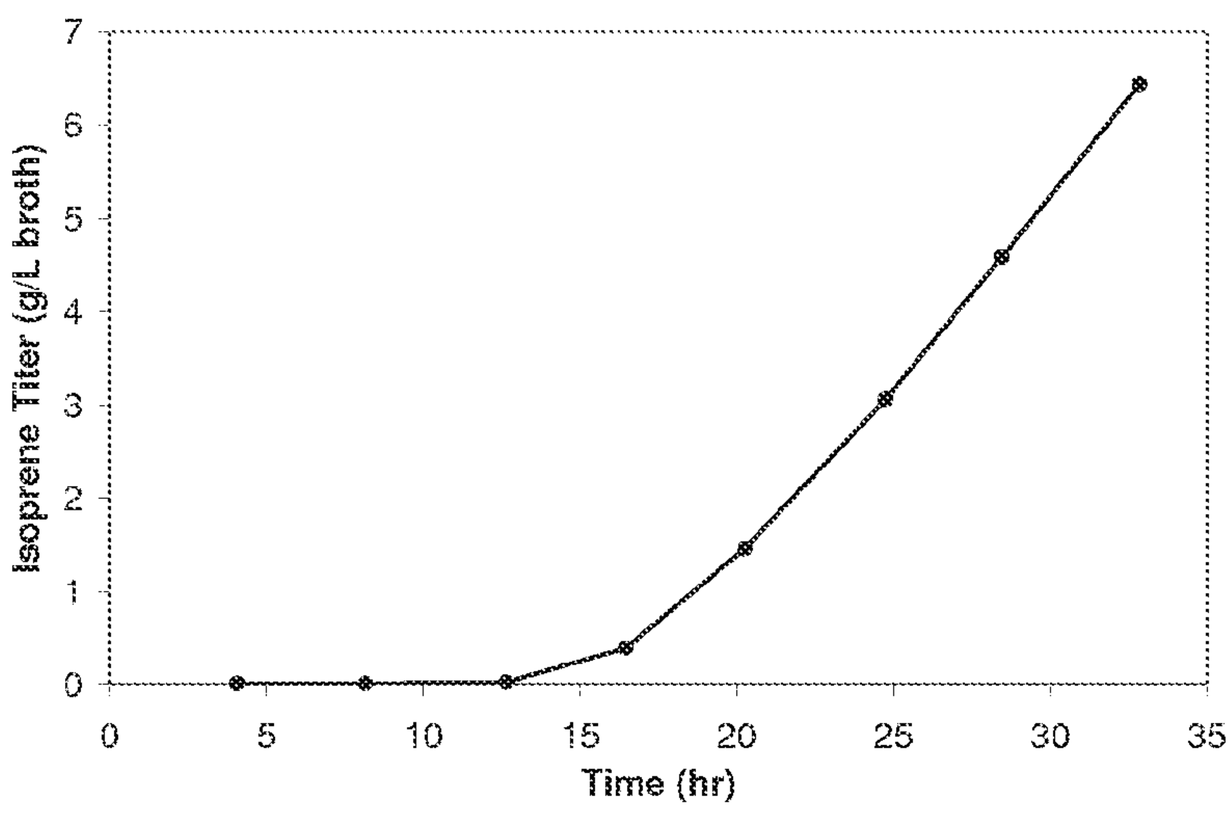

Figure 122

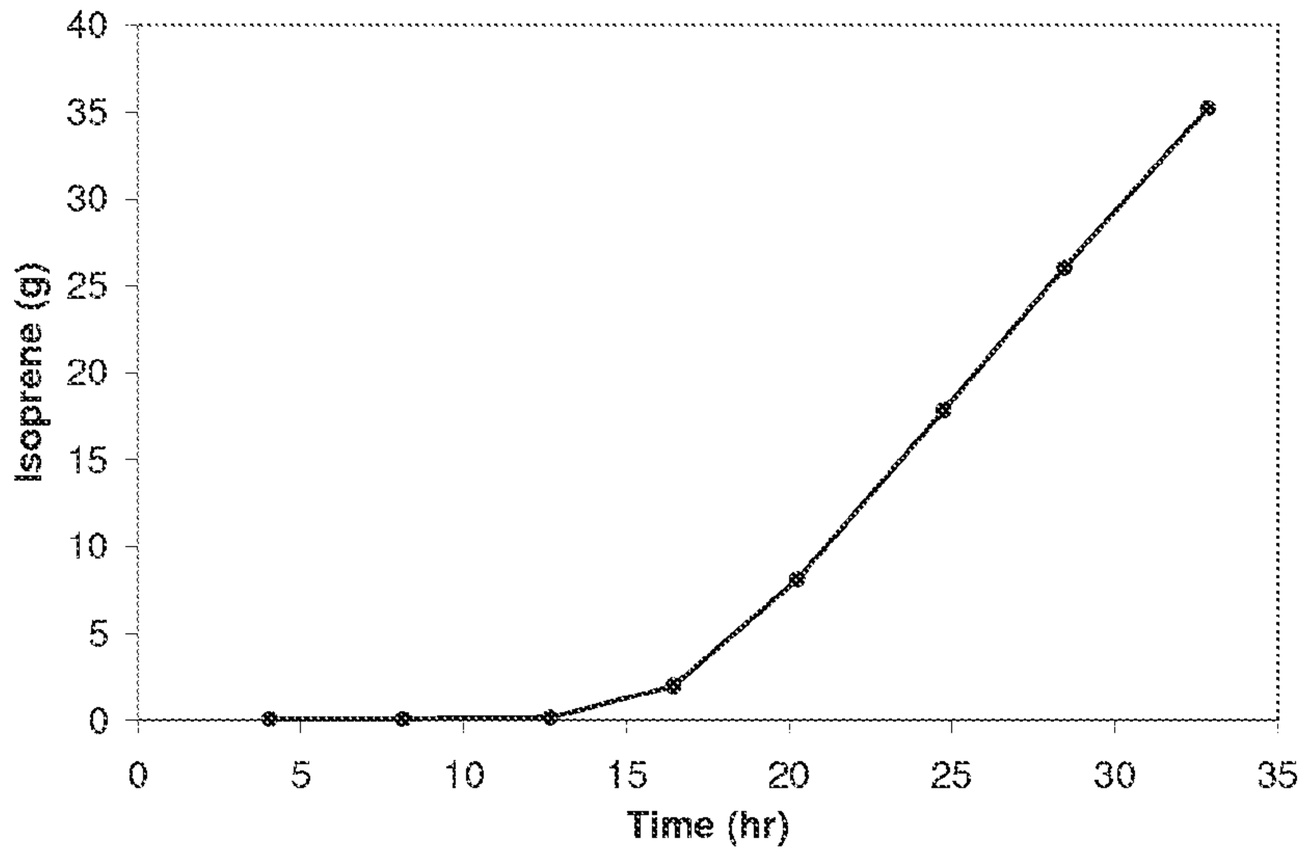

Figure 123

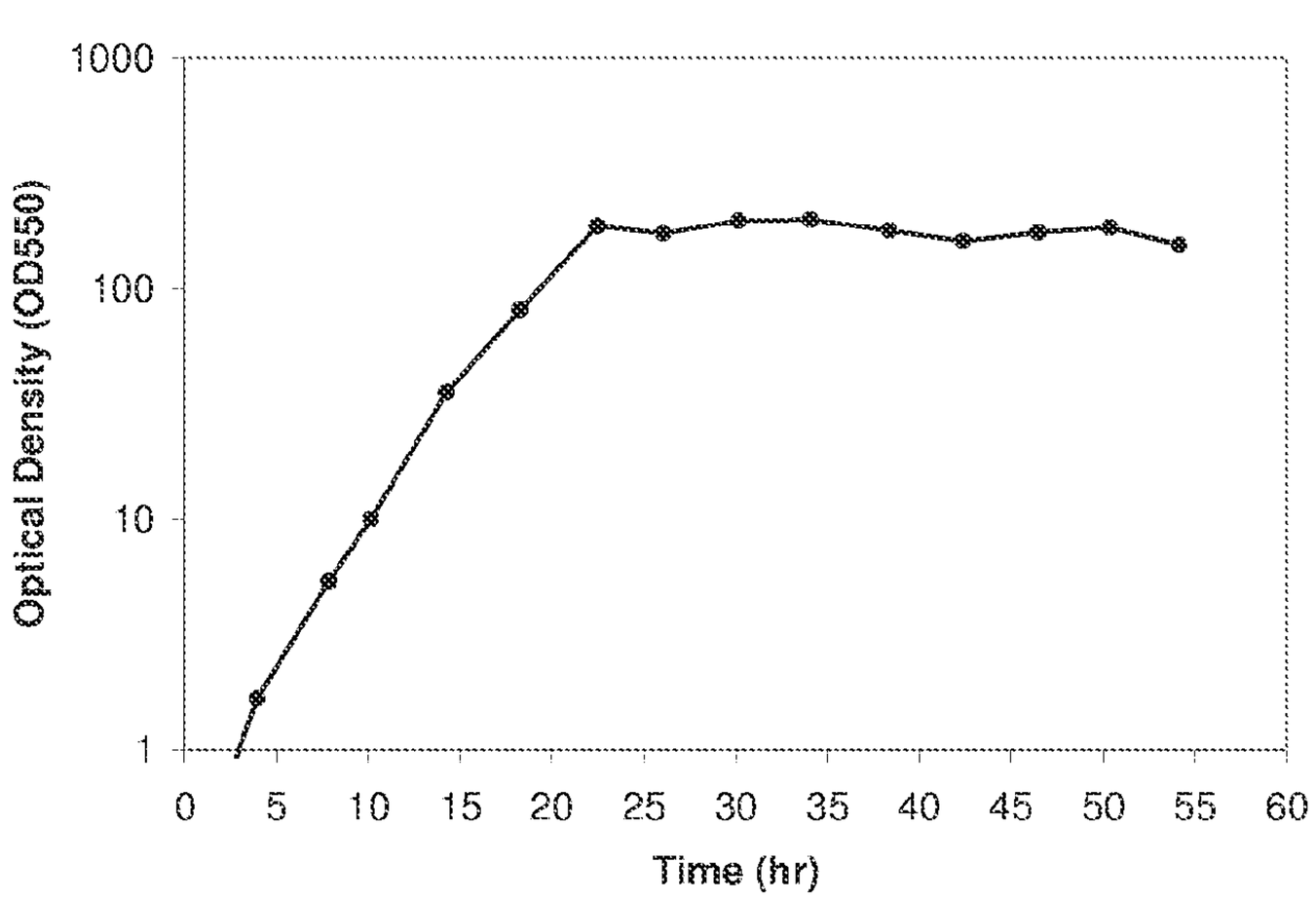

Figure 124

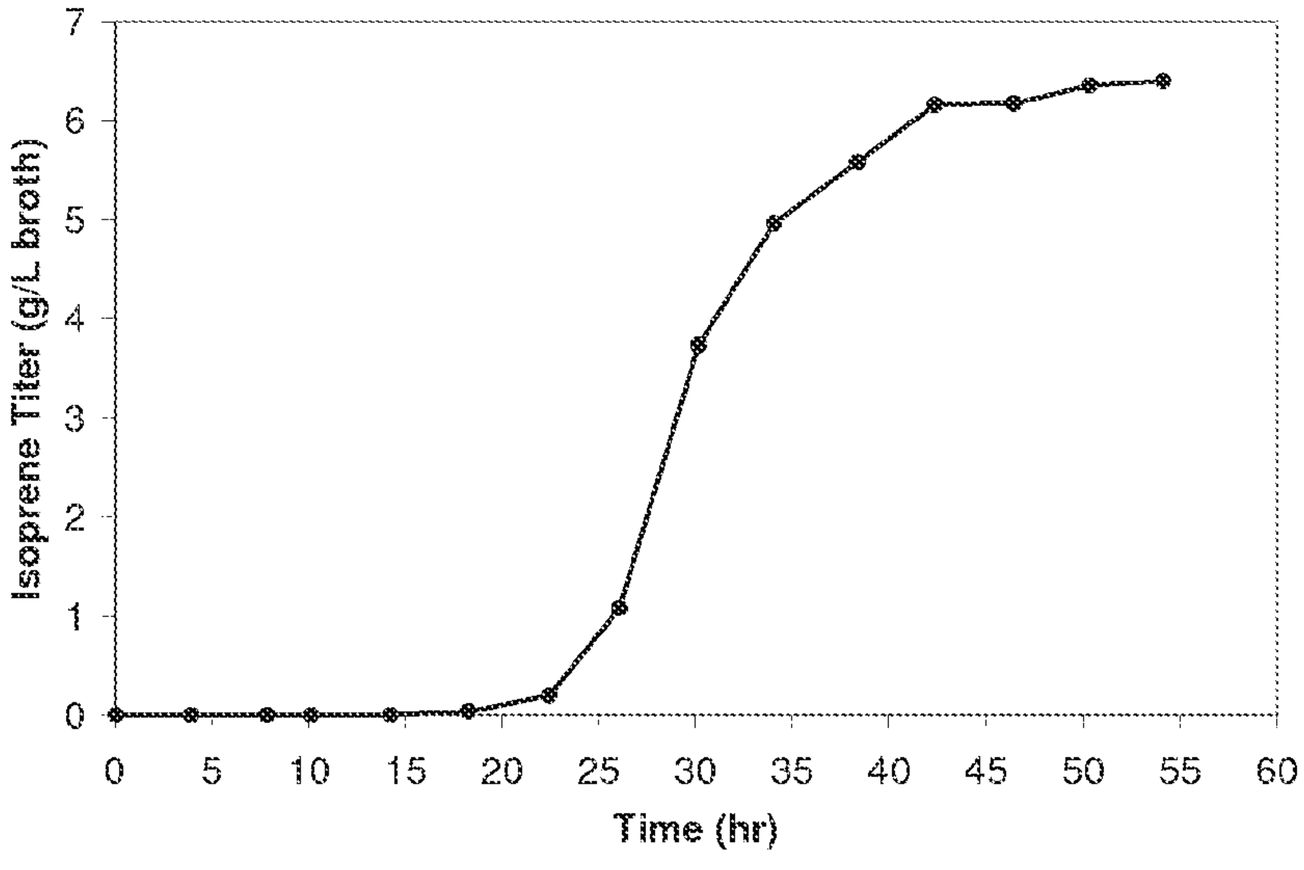

Figure 125

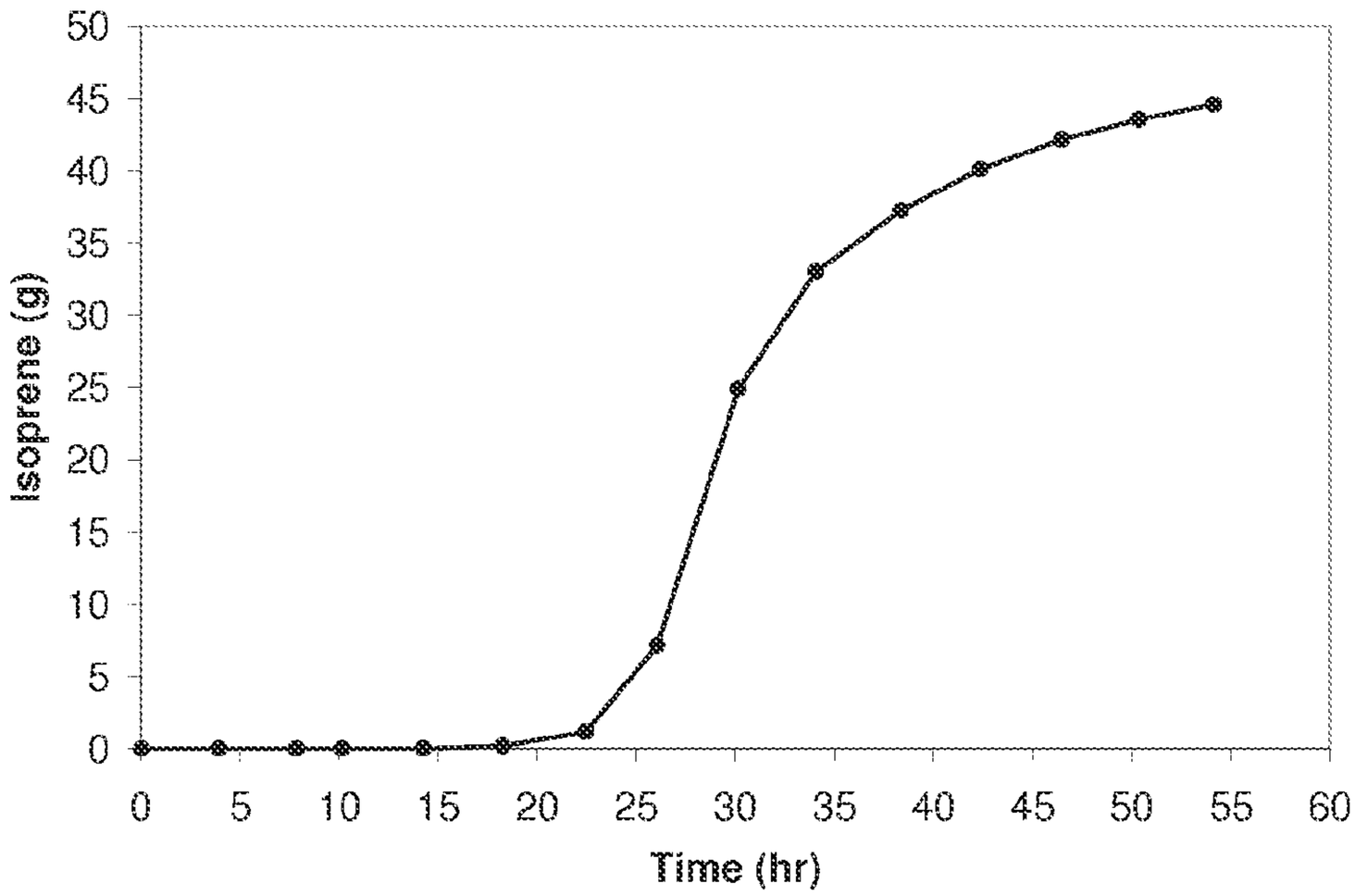

Figure 126A

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Figure 126B

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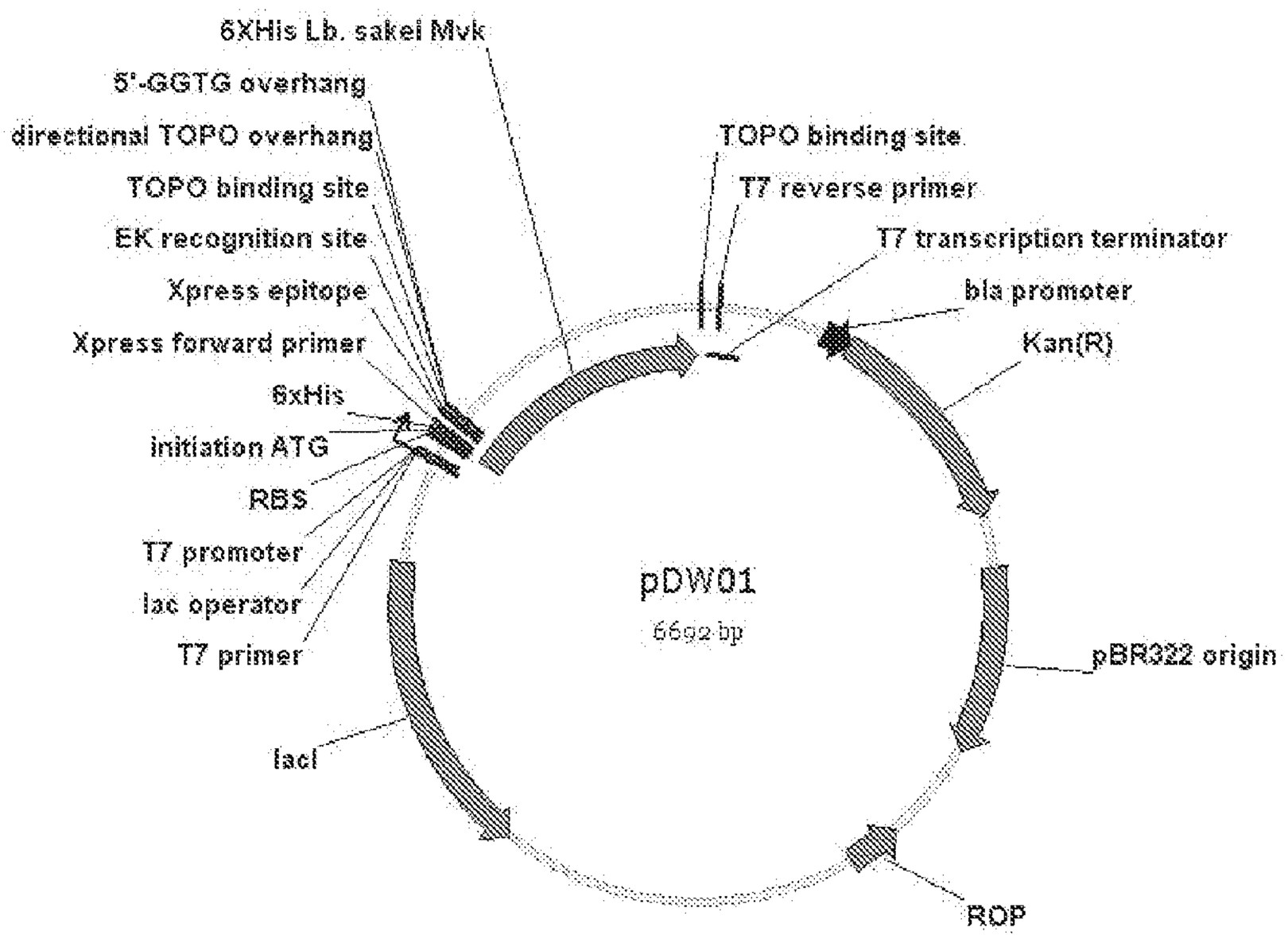
Figure 127A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T7 promoter
lac operator
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Figure 127B

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Figure 127C

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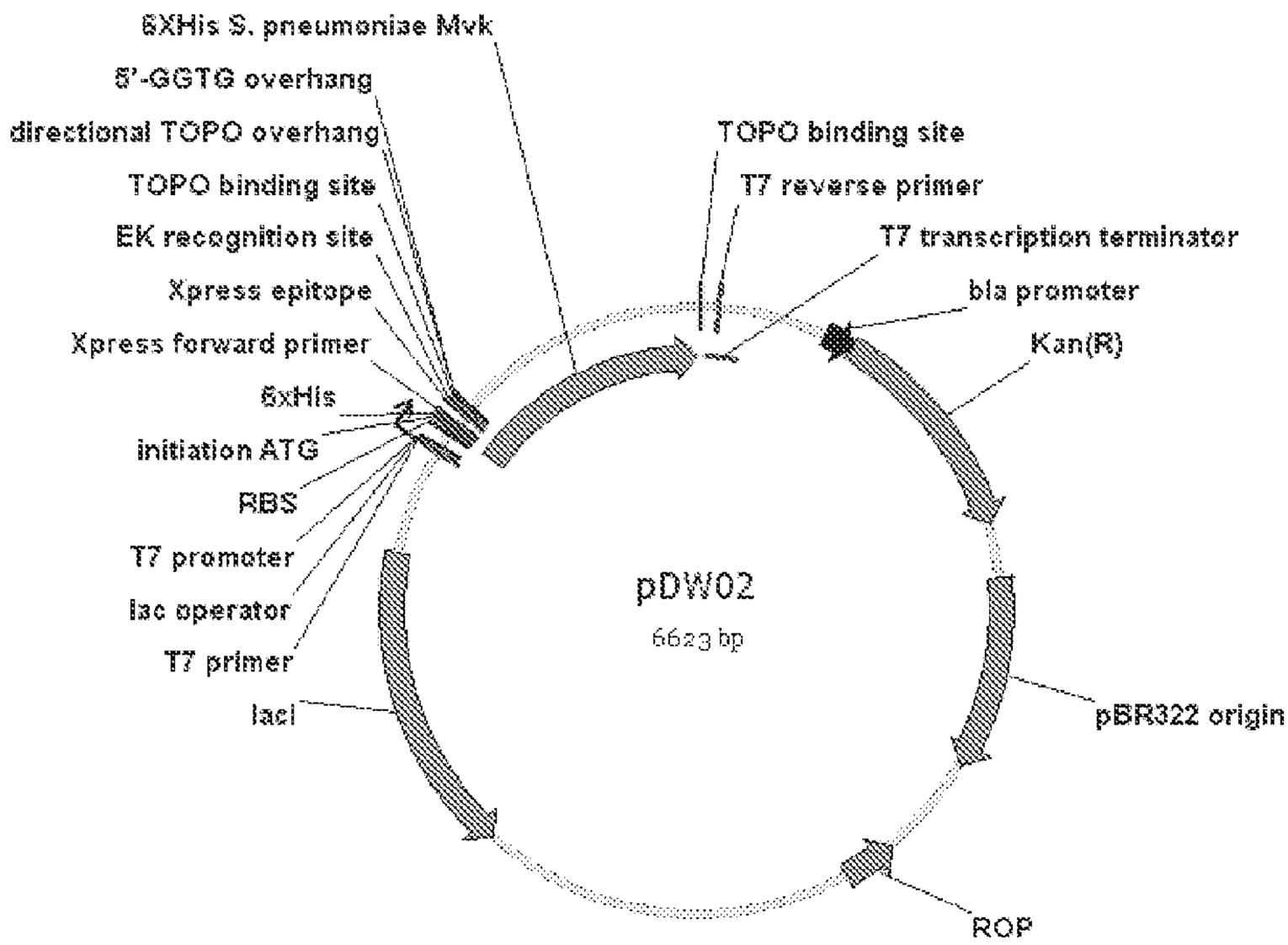
Figure 128A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T7 promoter
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bla promoter
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Figure 128B

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Figure 128C

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Figure 129

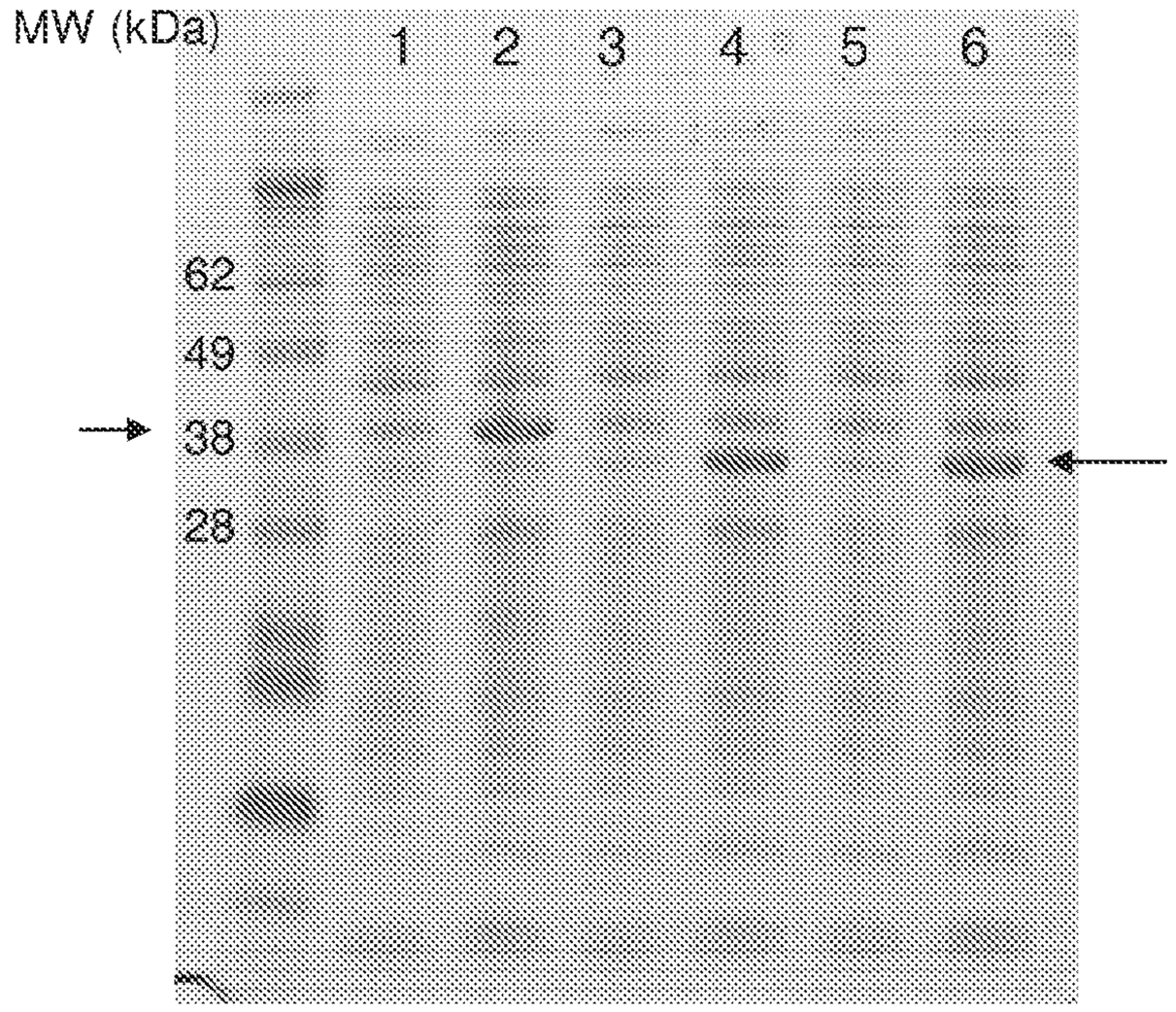


Figure 130

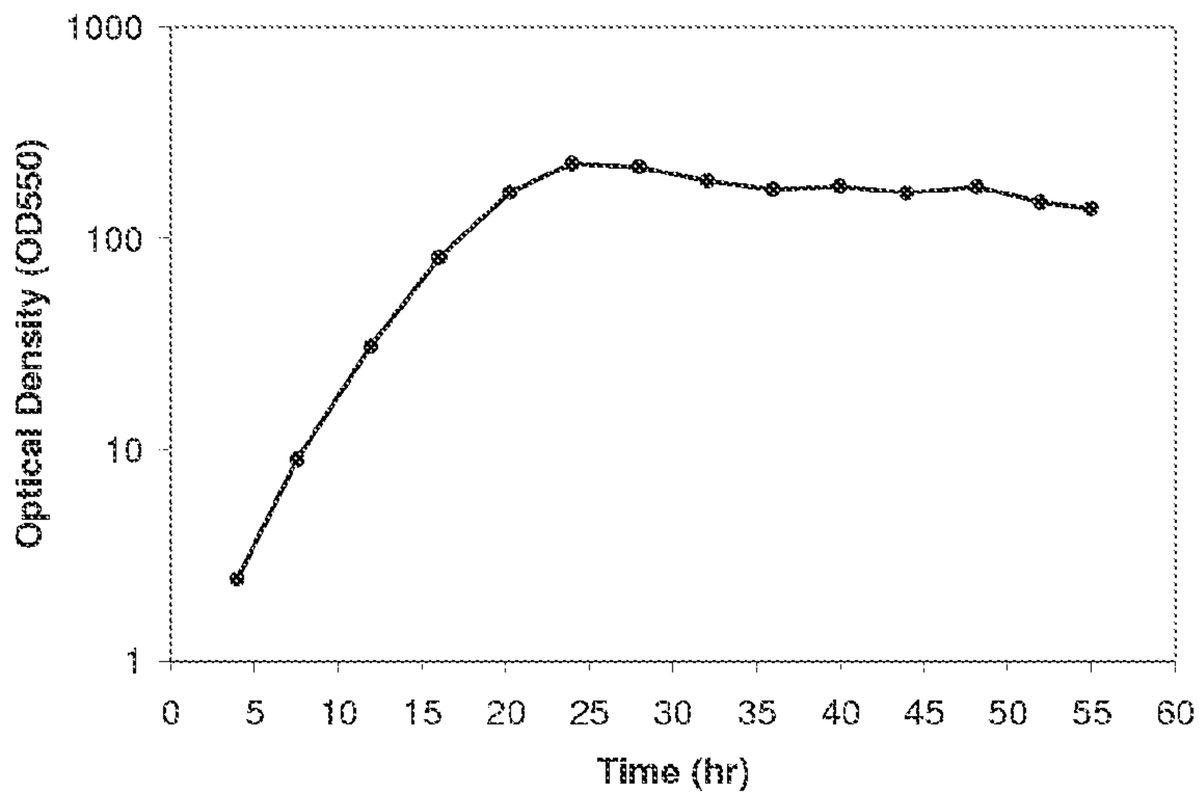
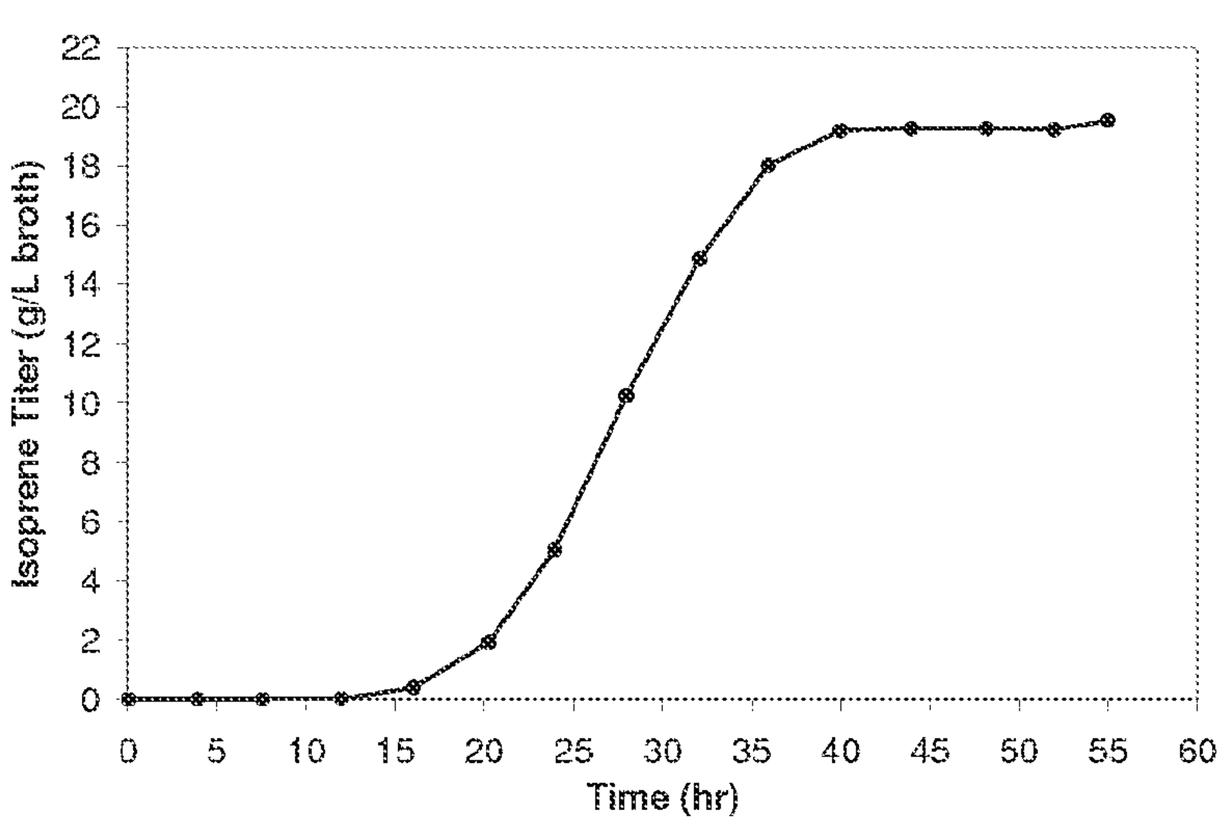


Figure 131



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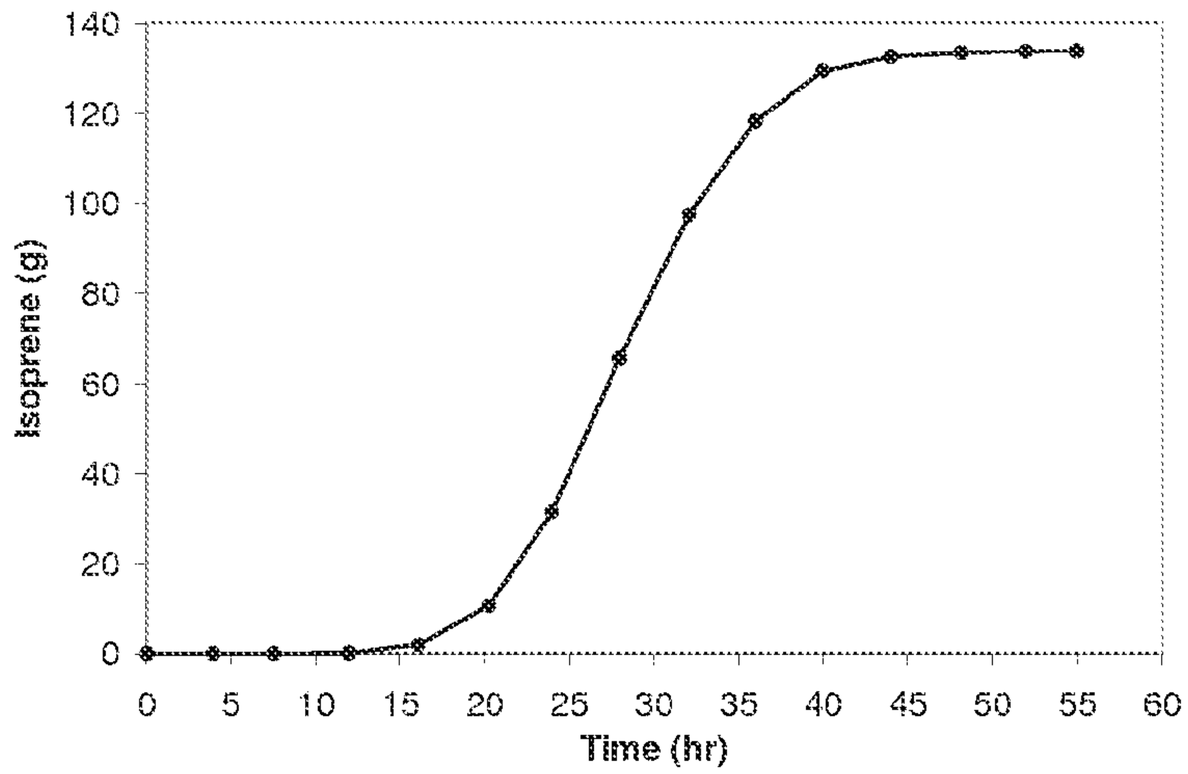


Figure 133

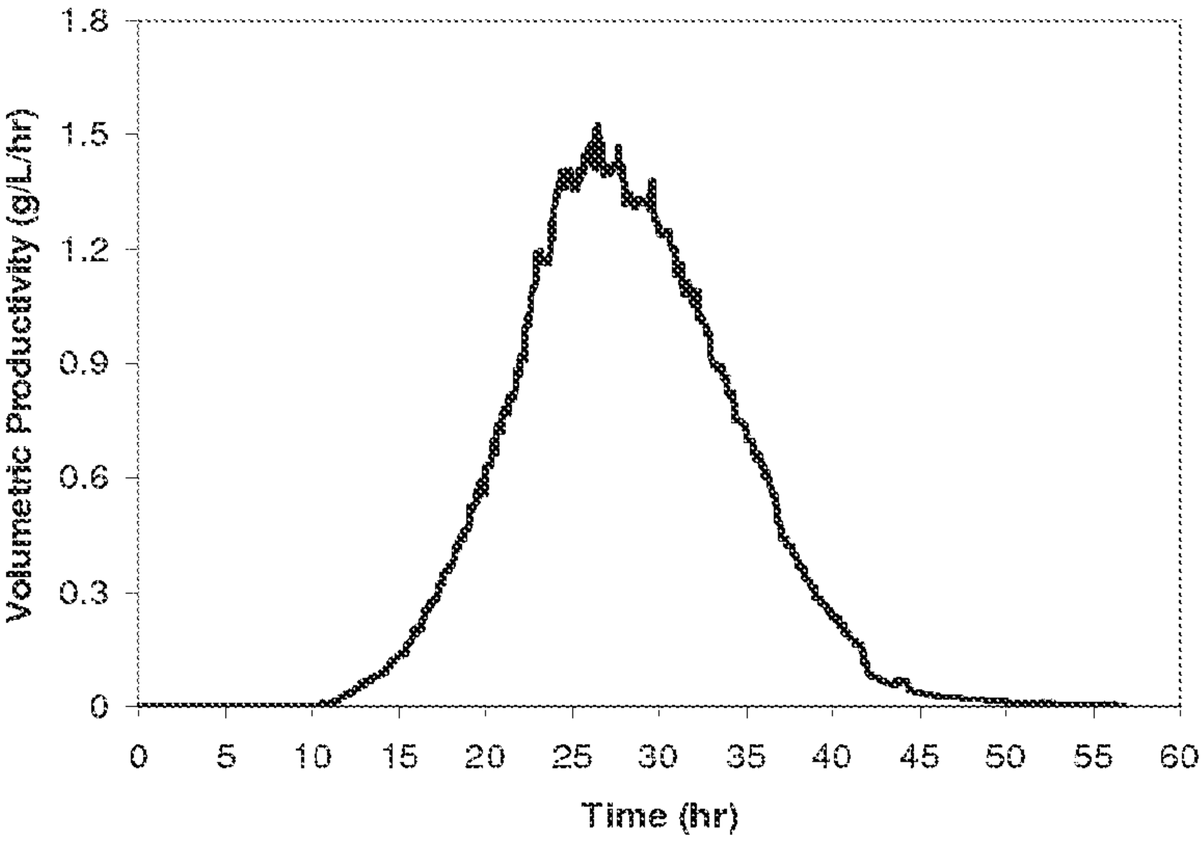


Figure 134

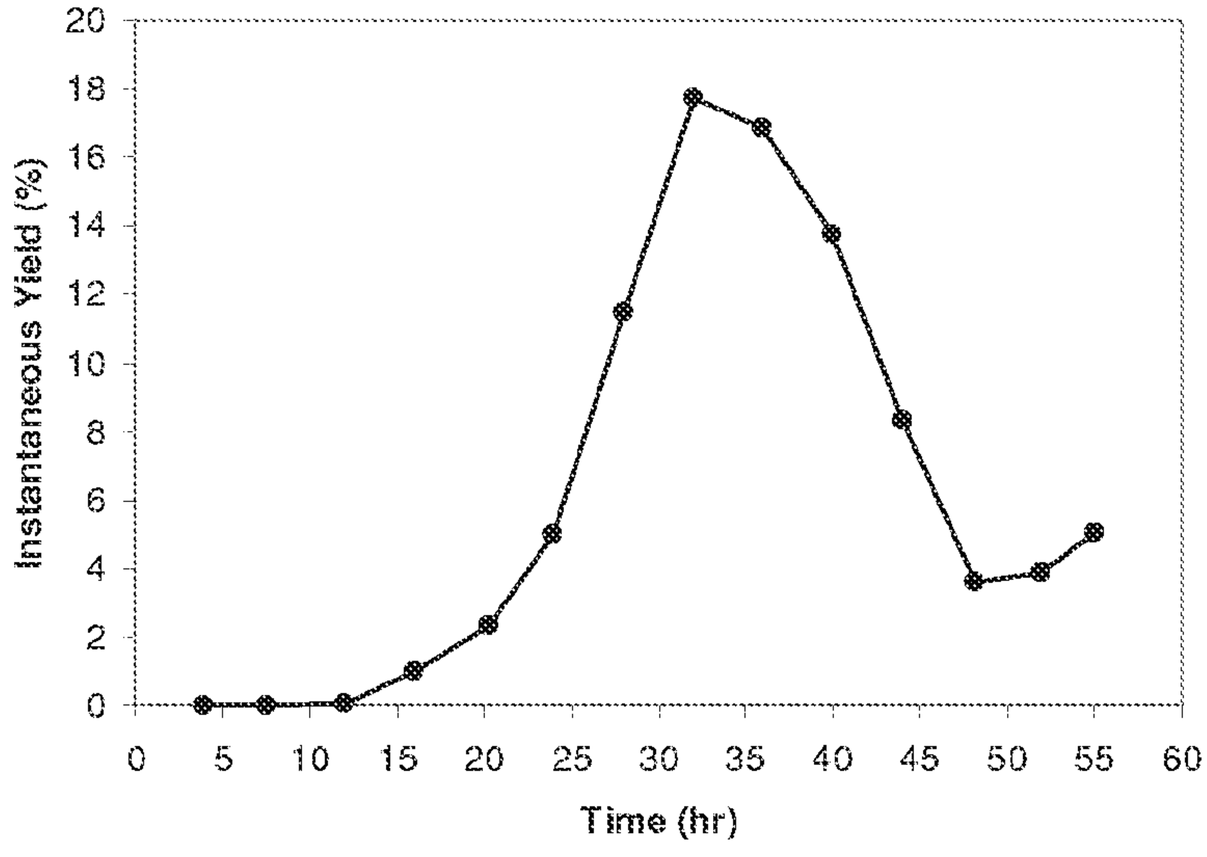


Figure 135

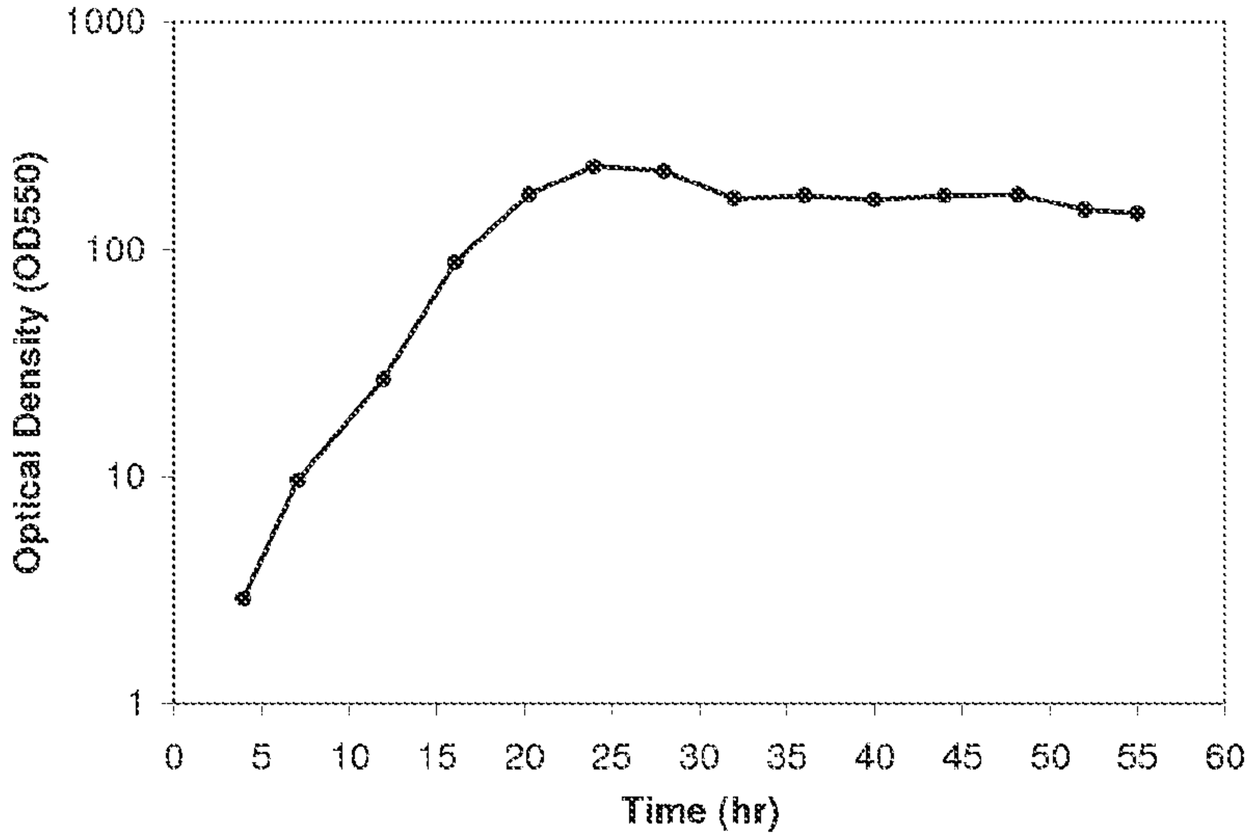
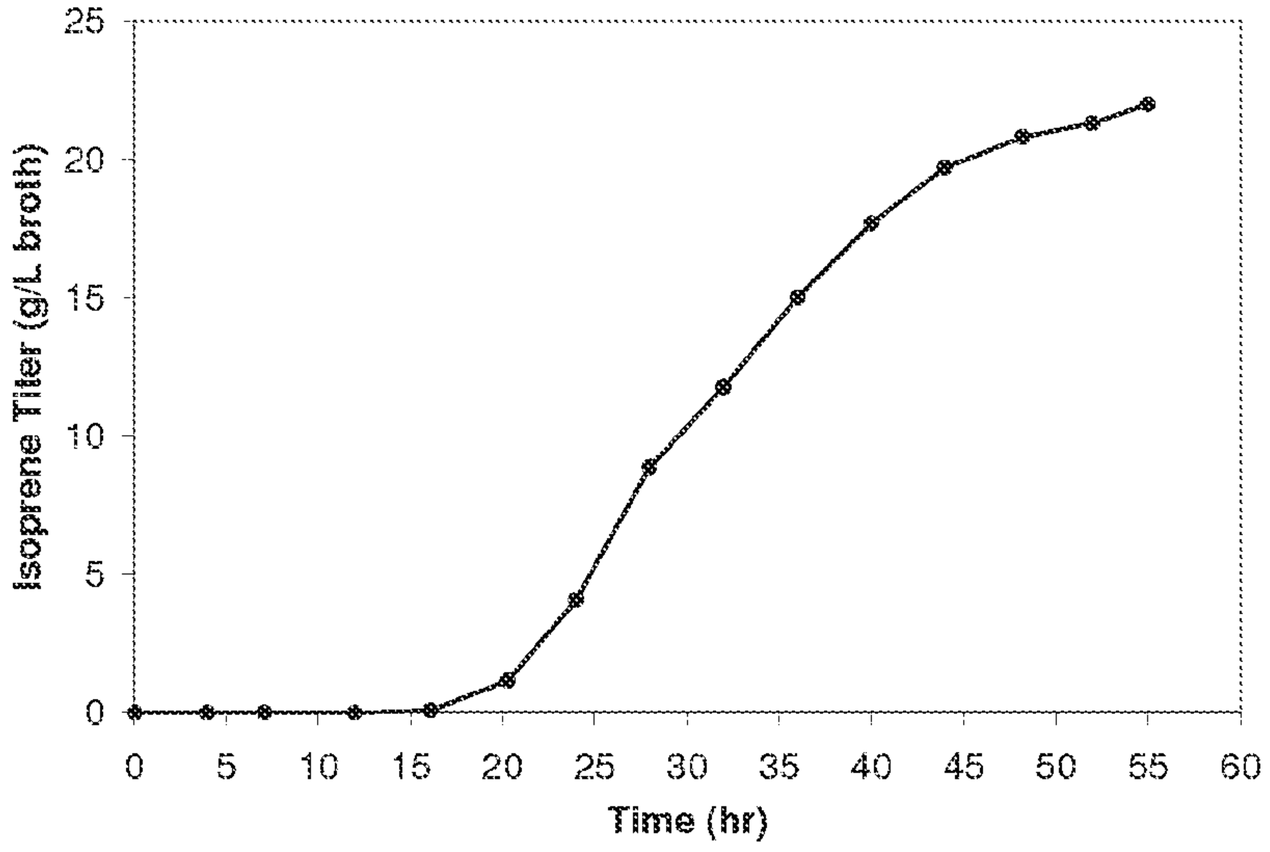


Figure 136



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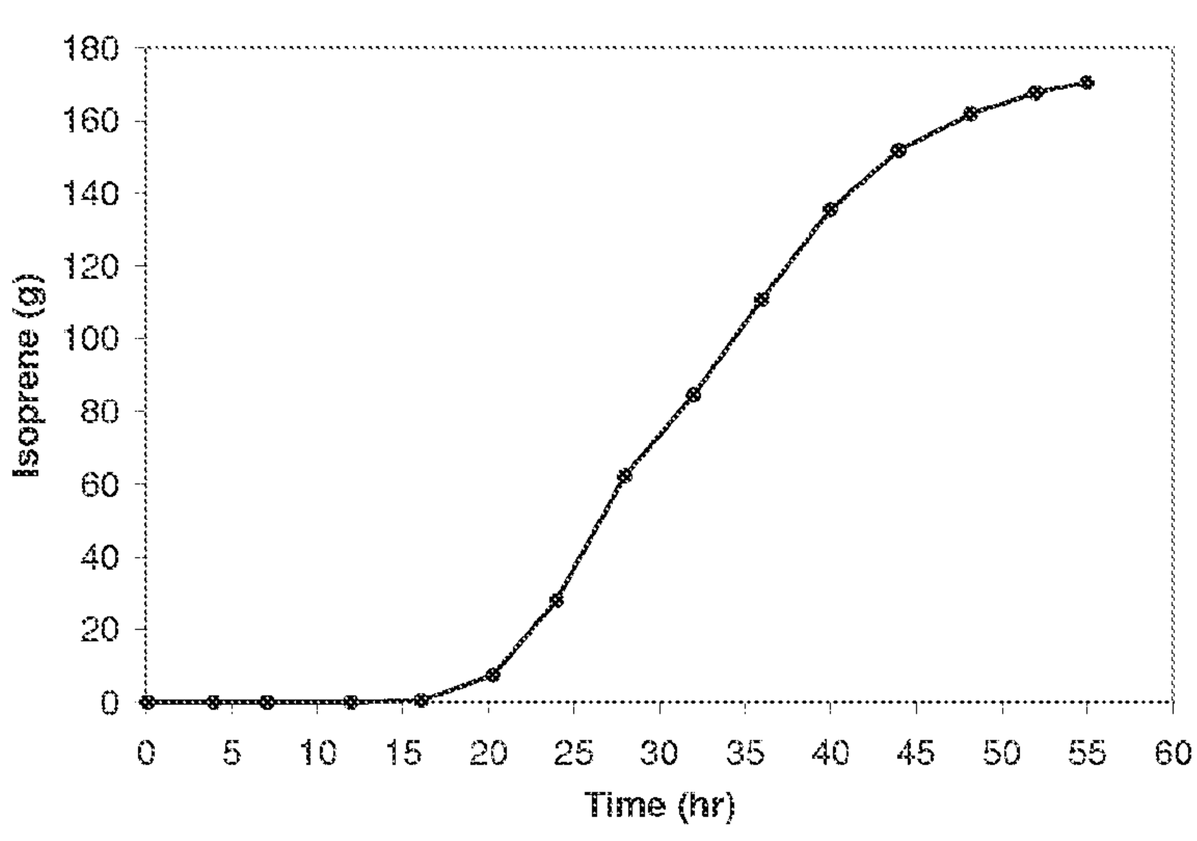

Figure 138A

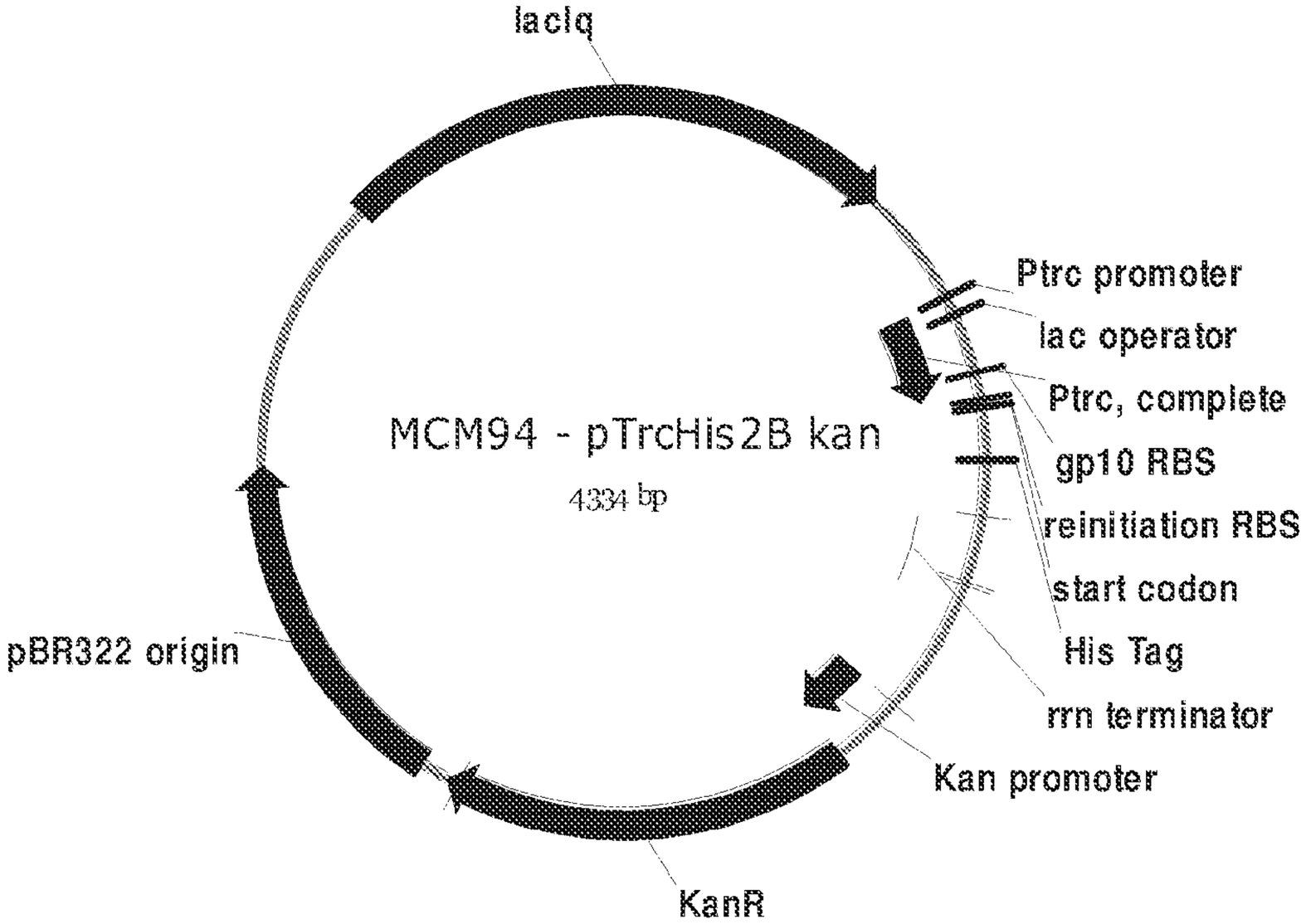
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Figure 138B

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Figure 138C

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Figure 139

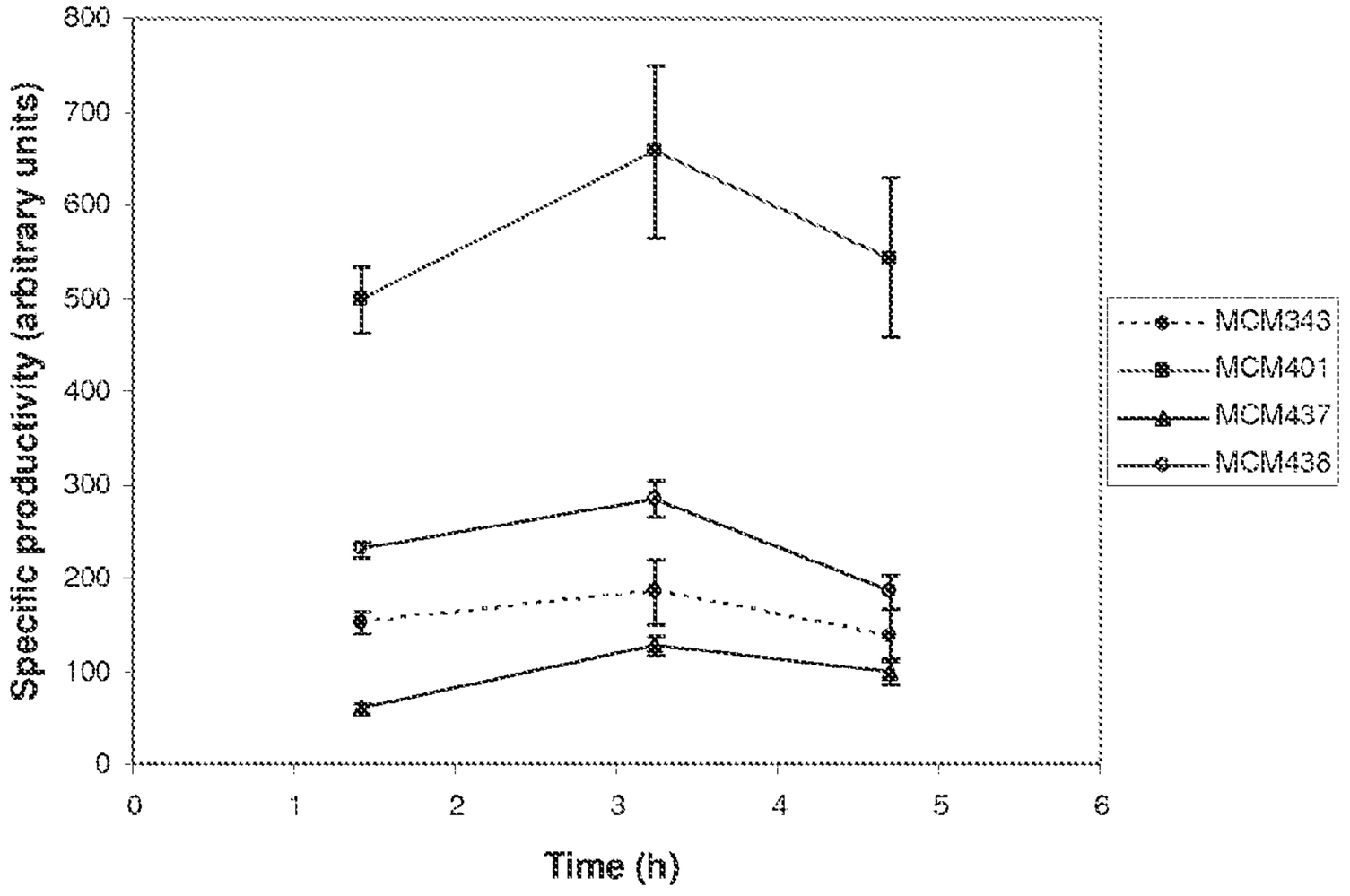
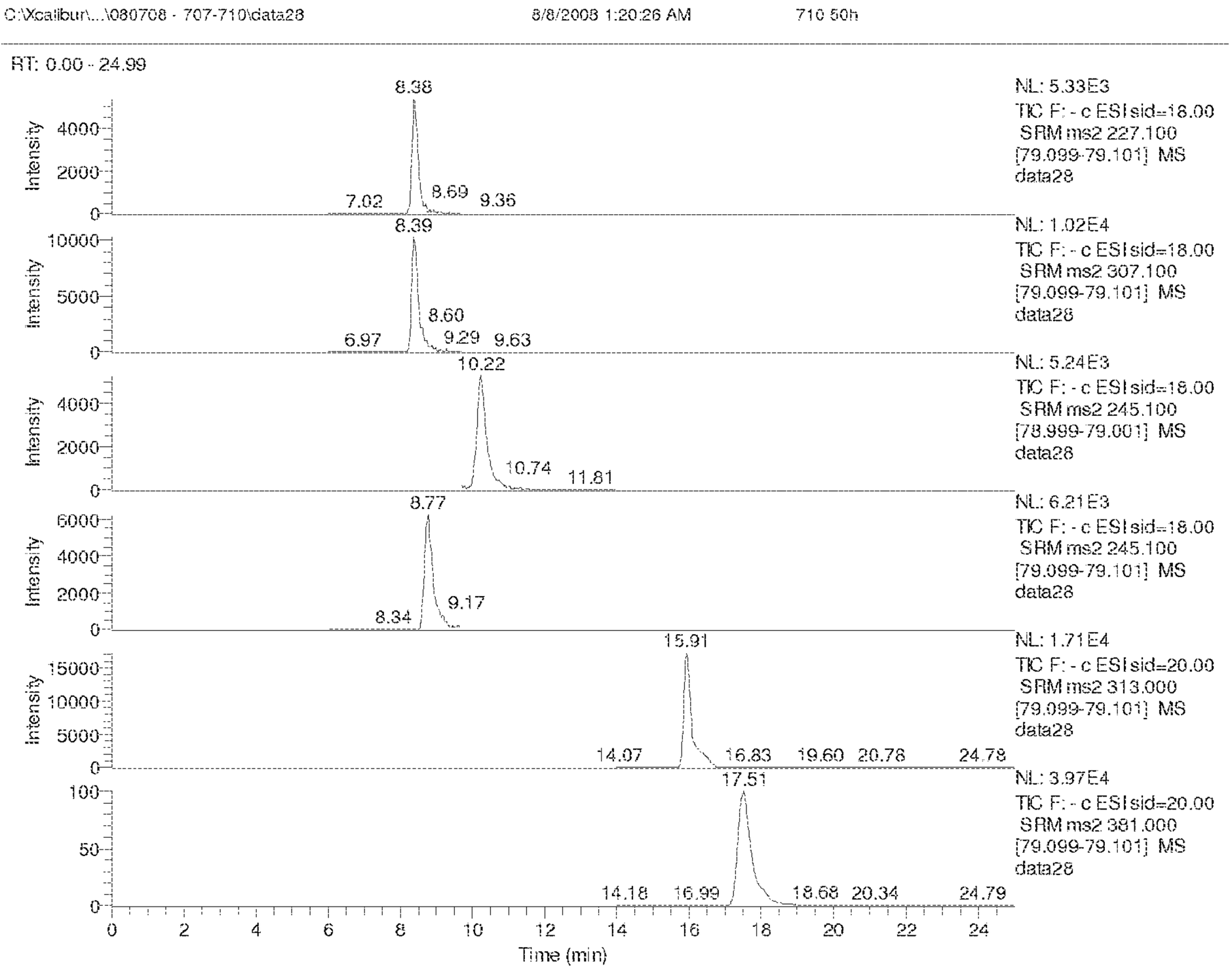

Specific productivity (arbitrary units)
0
100
200
300
400
500
600
700
800
0
1
2
3
4
5
6
Time (h)
MCM343
MCM401
MCM437
MCM438

Figure 140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710 50h
RT: 0.00 - 24.99
8.38
7.02
8.69
9.36
NL: 5.33E3
TIC F: - c ESI sid=18.00 SRM ms2 227.100 [79.099-79.101] MS data28
8.39
6.97
8.60
9.29
9.63
NL: 1.02E4
TIC F: - c ESI sid=18.00 SRM ms2 307.100 [79.099-79.101] MS data28
10.22
10.74
11.81
NL: 5.24E3
TIC F: - c ESI sid=18.00 SRM ms2 245.100 [78.999-79.001] MS data28
8.77
8.34
9.17
NL: 6.21E3
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14.07
16.83
19.60
20.78
24.78
NL: 1.71E4
TIC F: - c ESI sid=20.00 SRM ms2 313.000 [79.099-79.101] MS data28
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14.18
16.99
18.68
20.34
24.79
NL: 3.97E4
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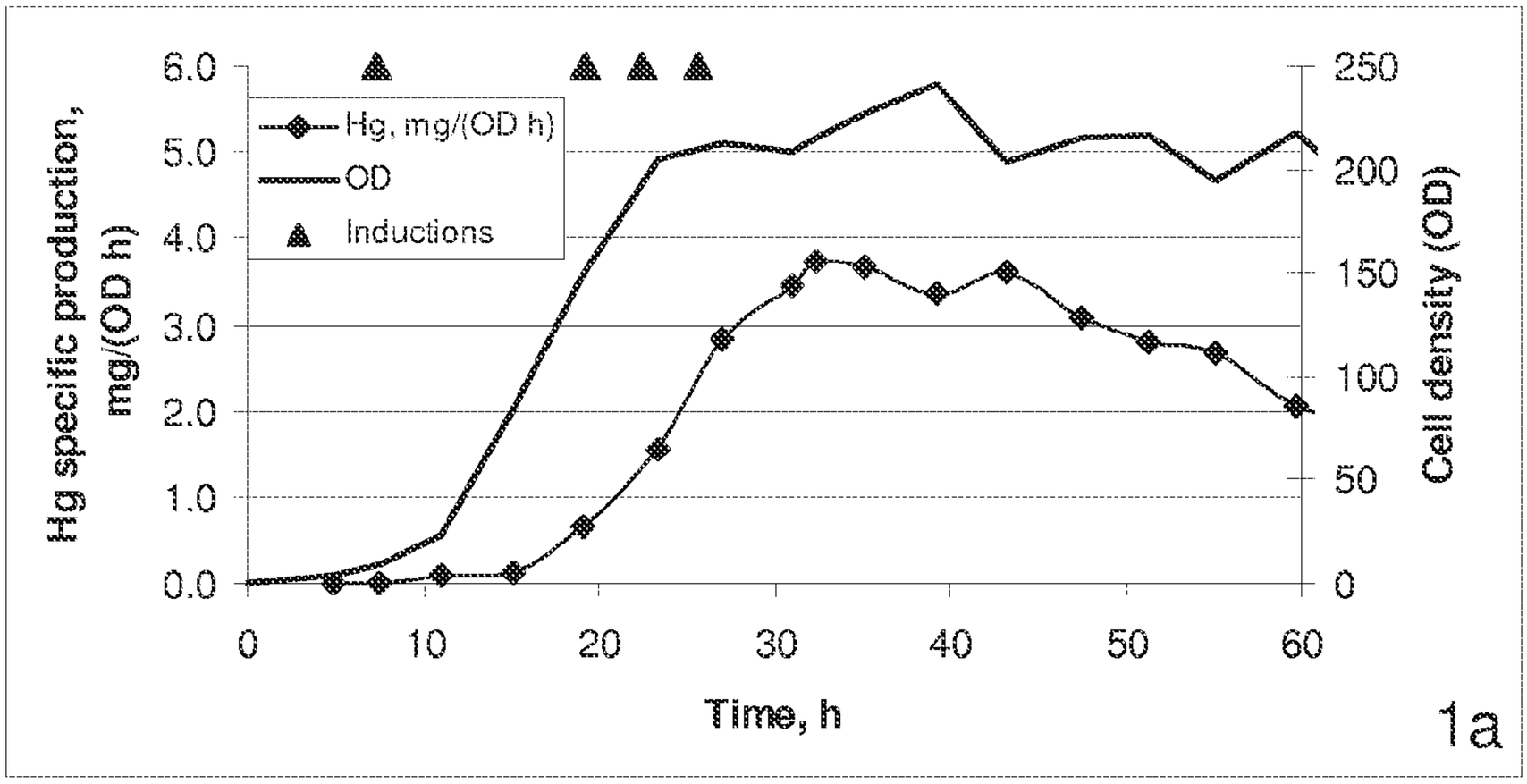
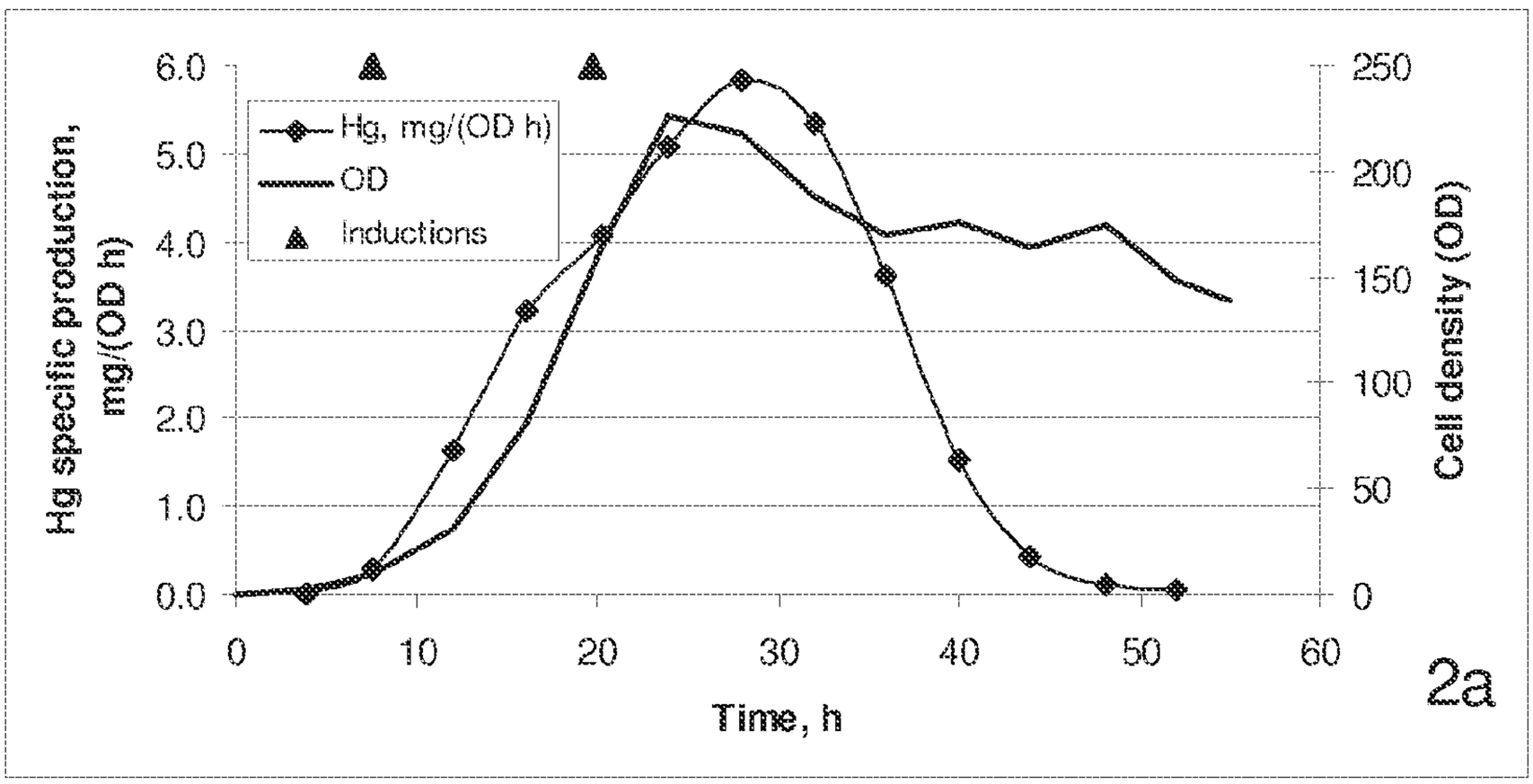
Intensity
Time (min)

Figures 141A-141B

A

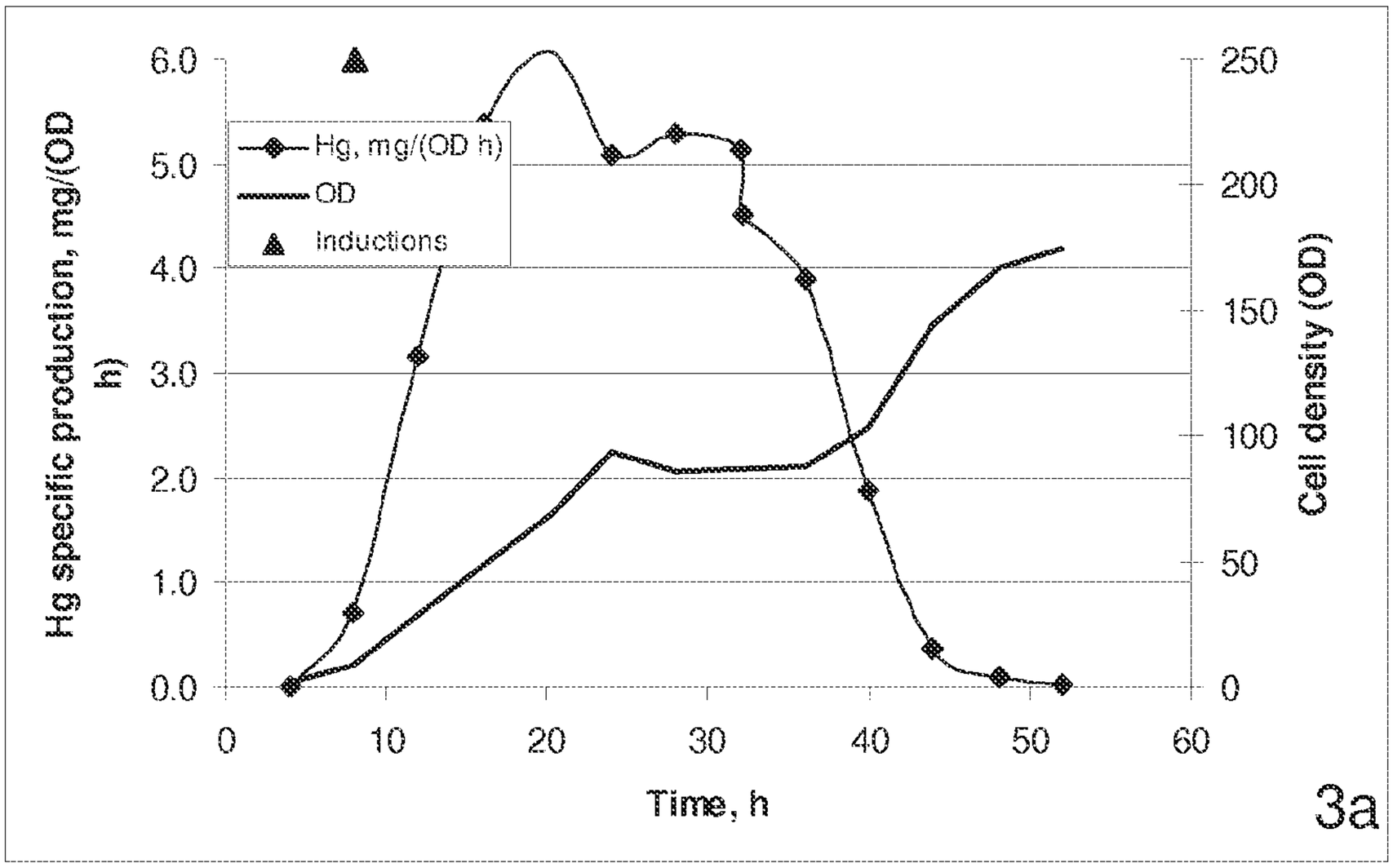


B

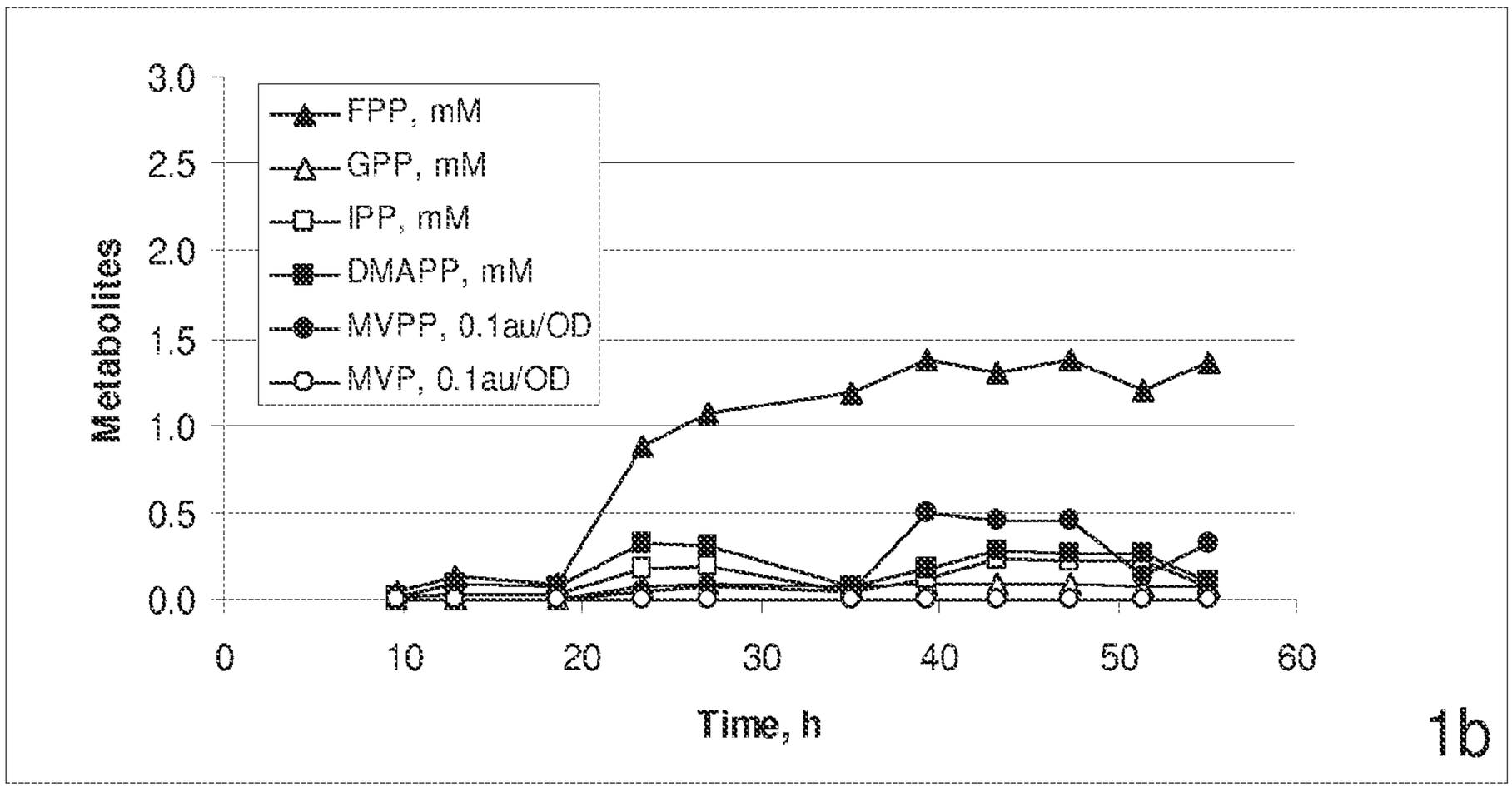


Figures 141C-141D

C

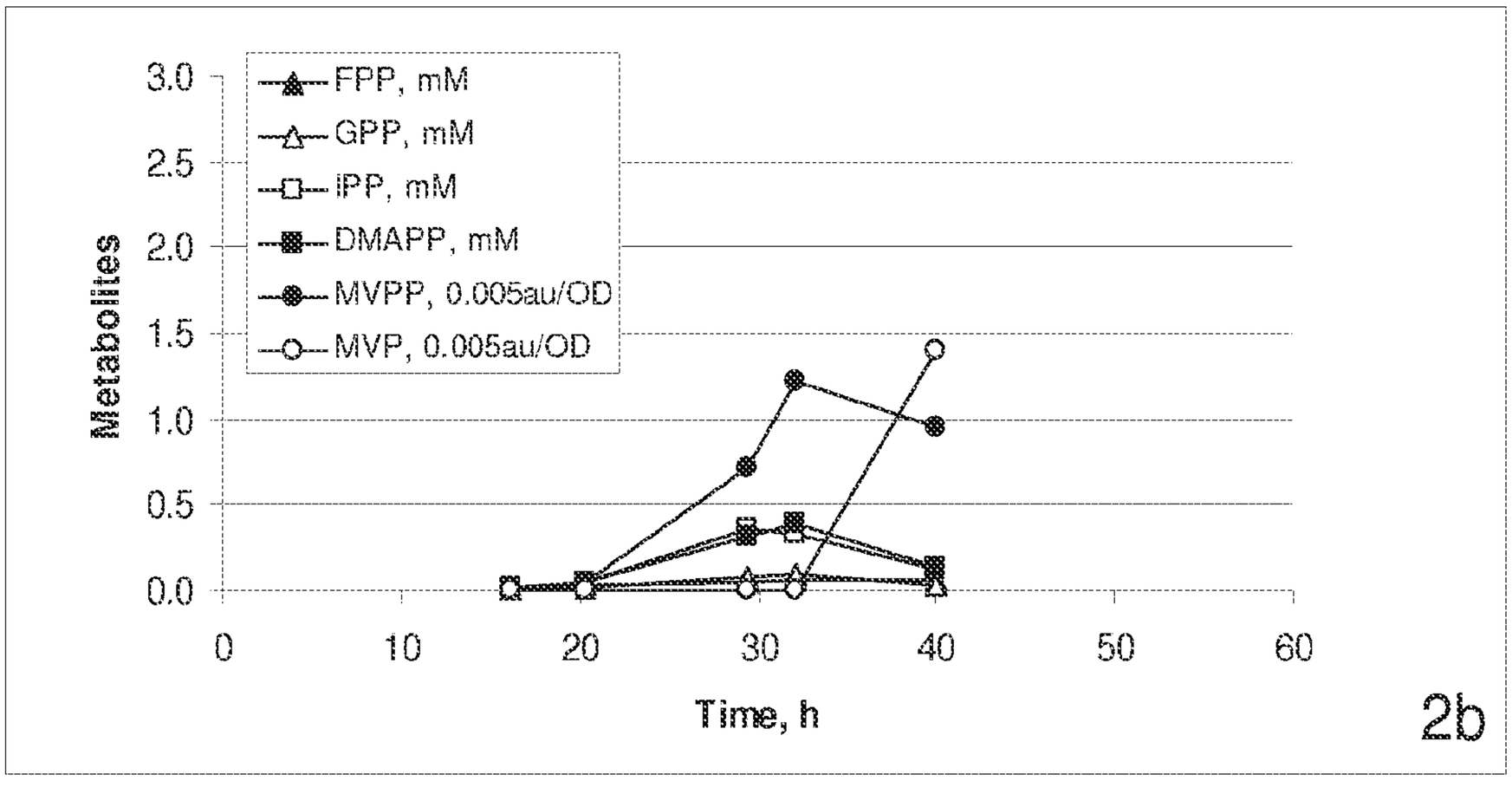


D

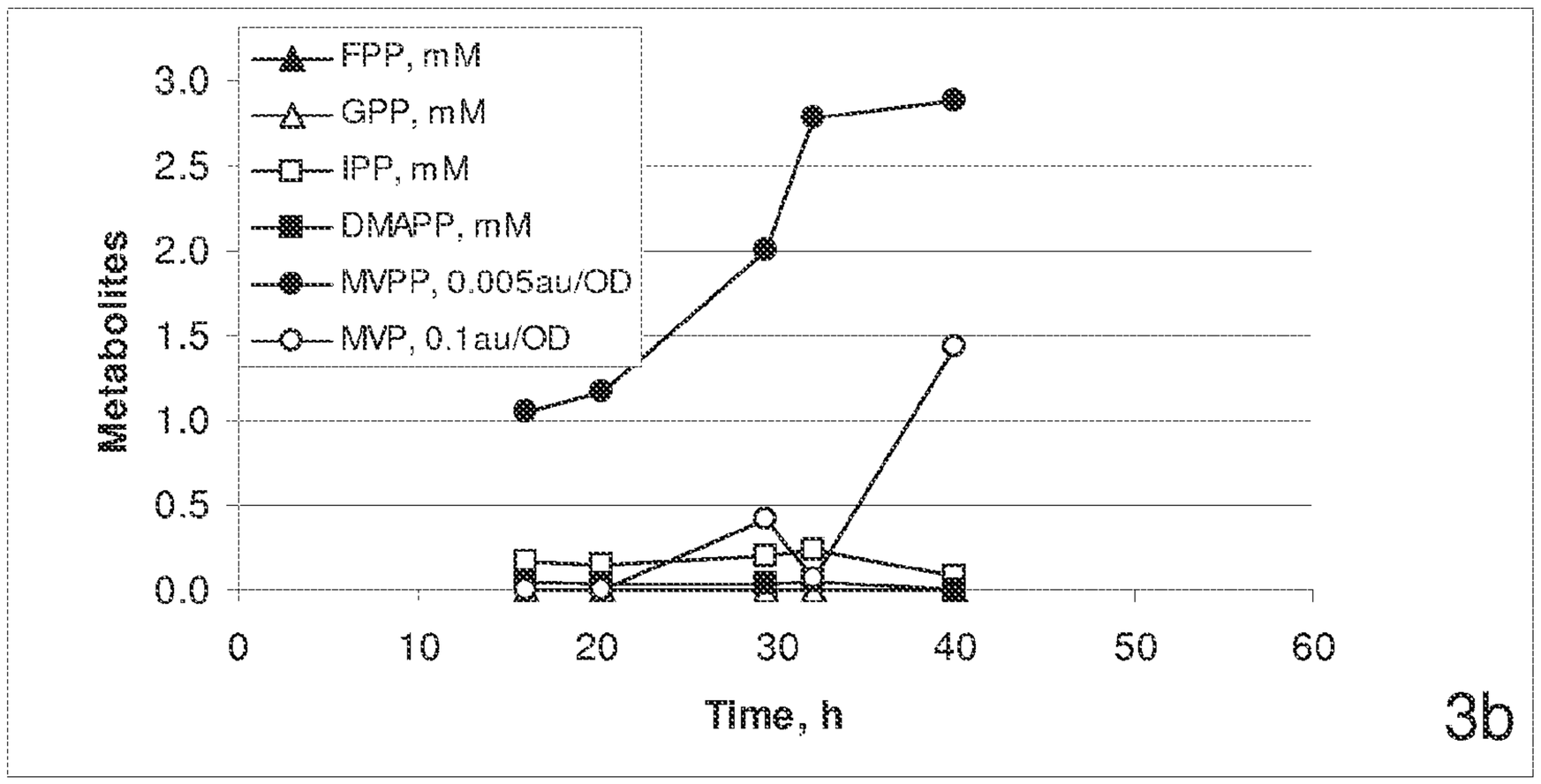


Figures 141E-141F

E

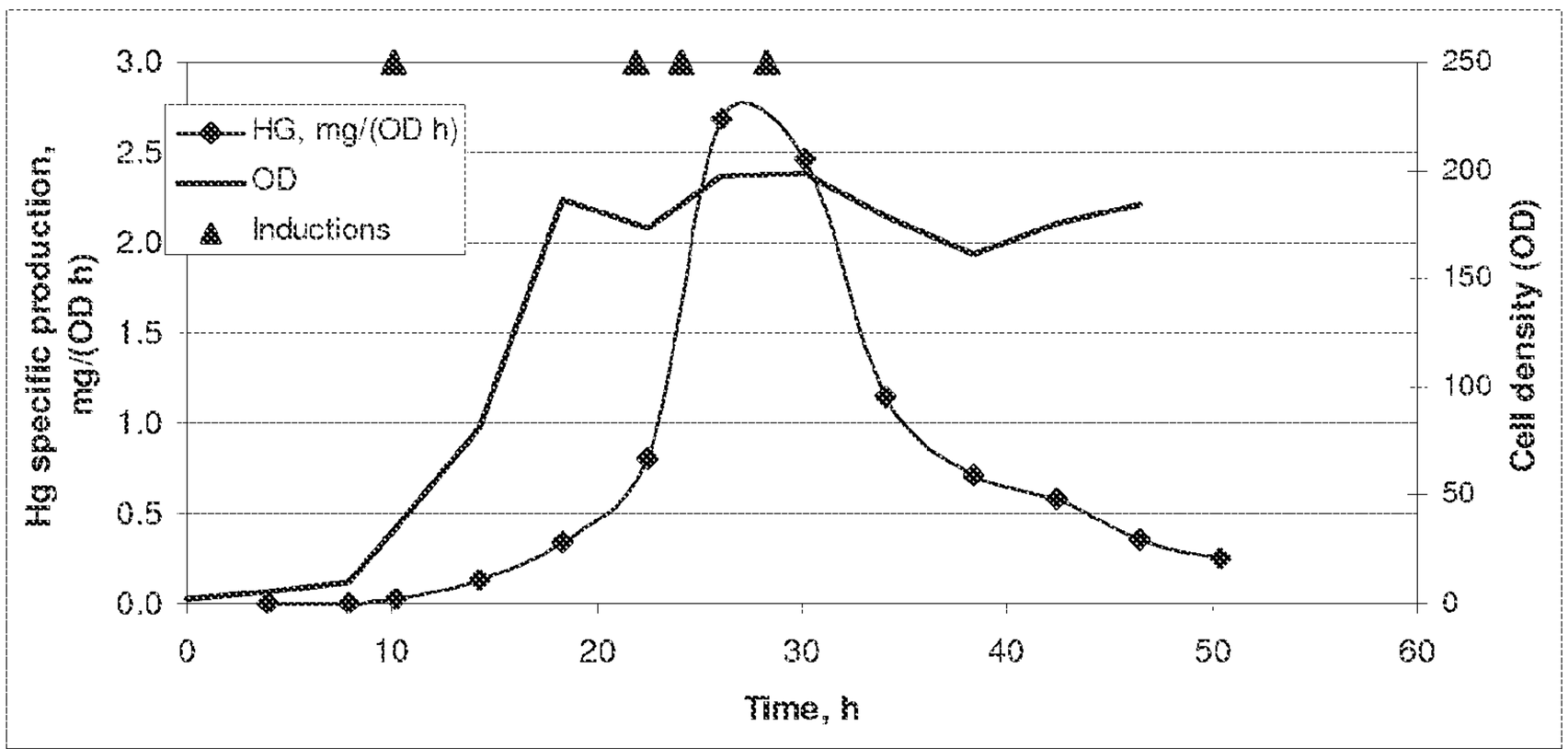


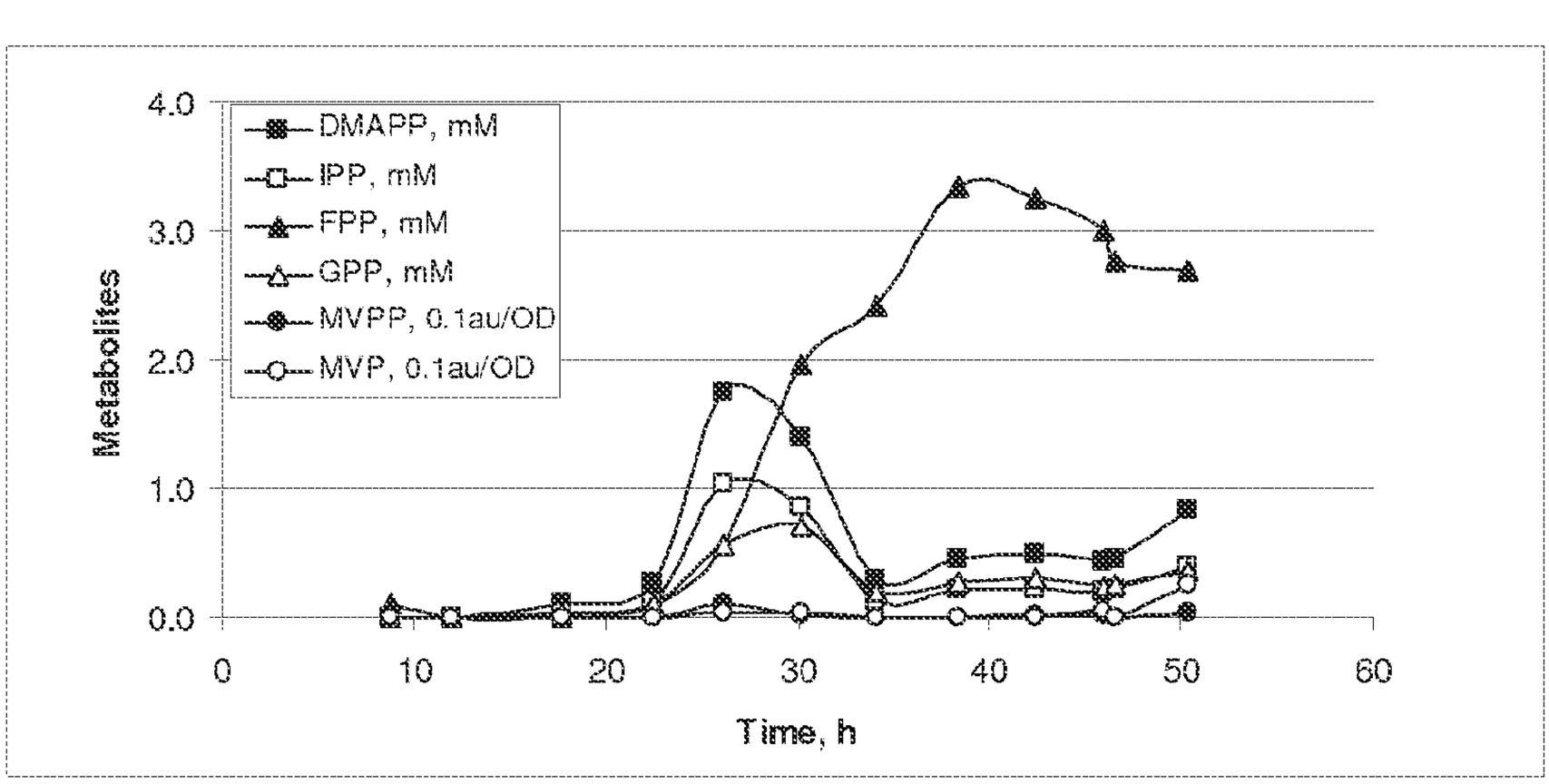
F



Figures 142A-142B

A

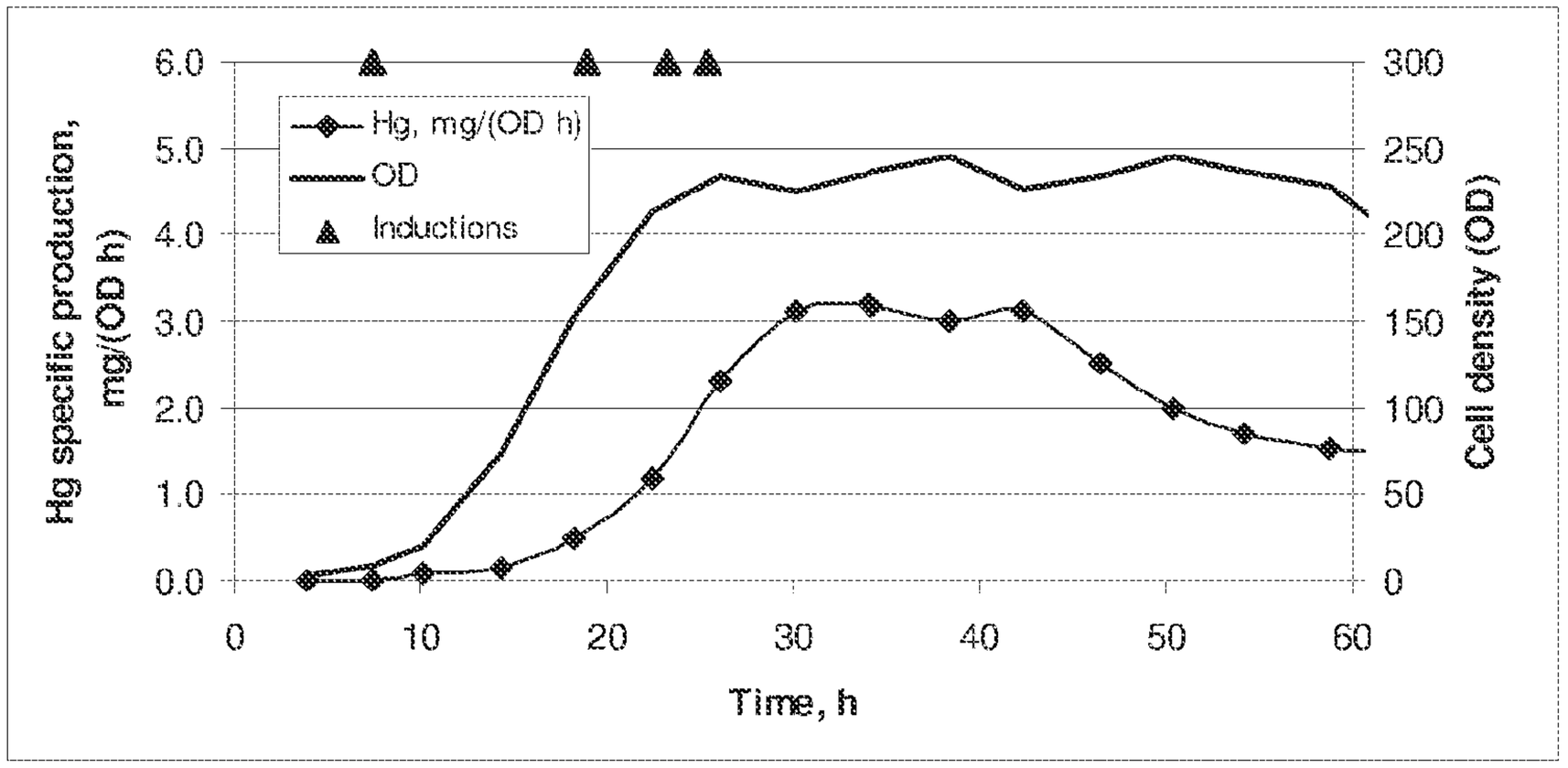


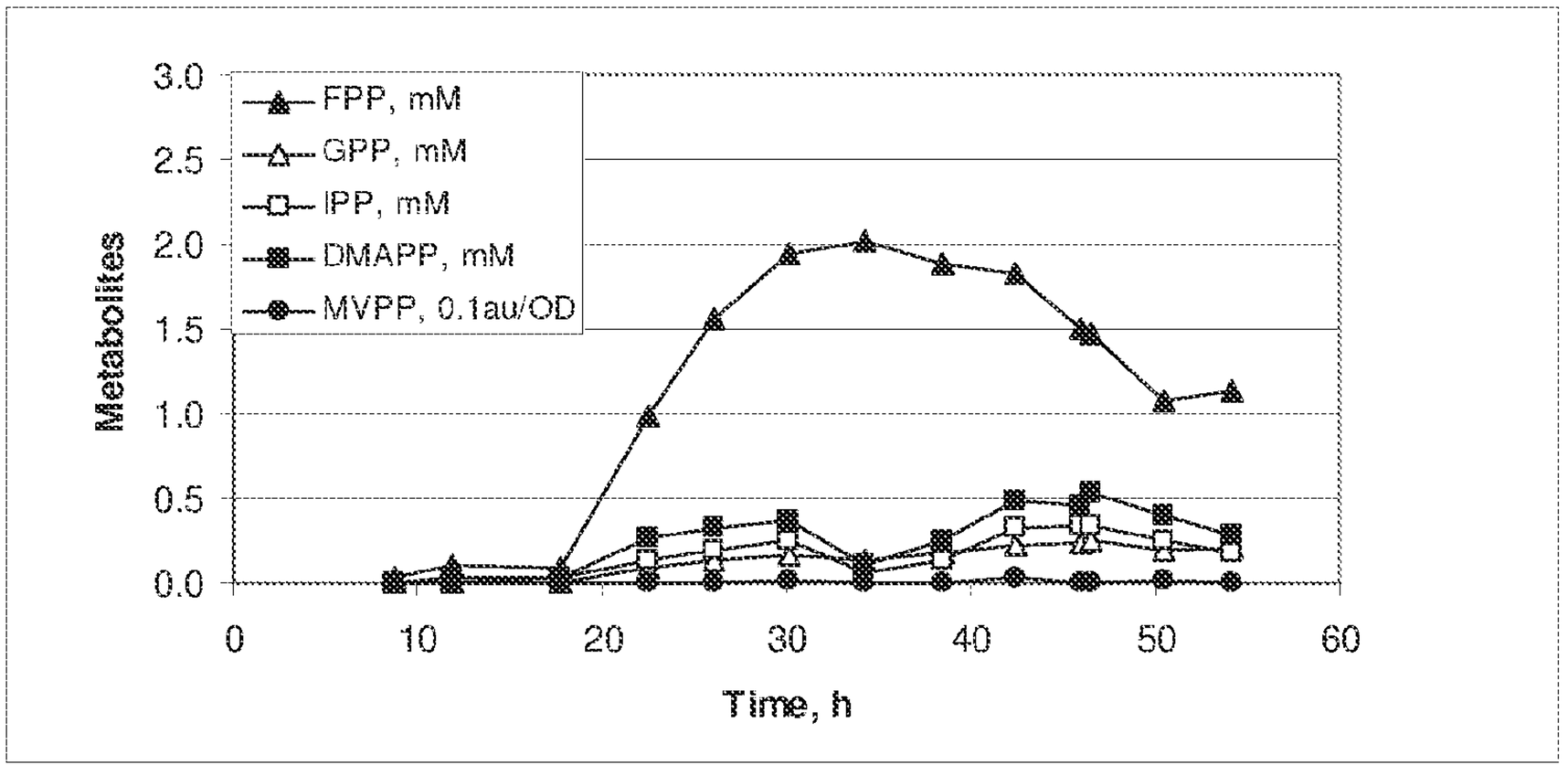


B

Figures 143A-143B

A

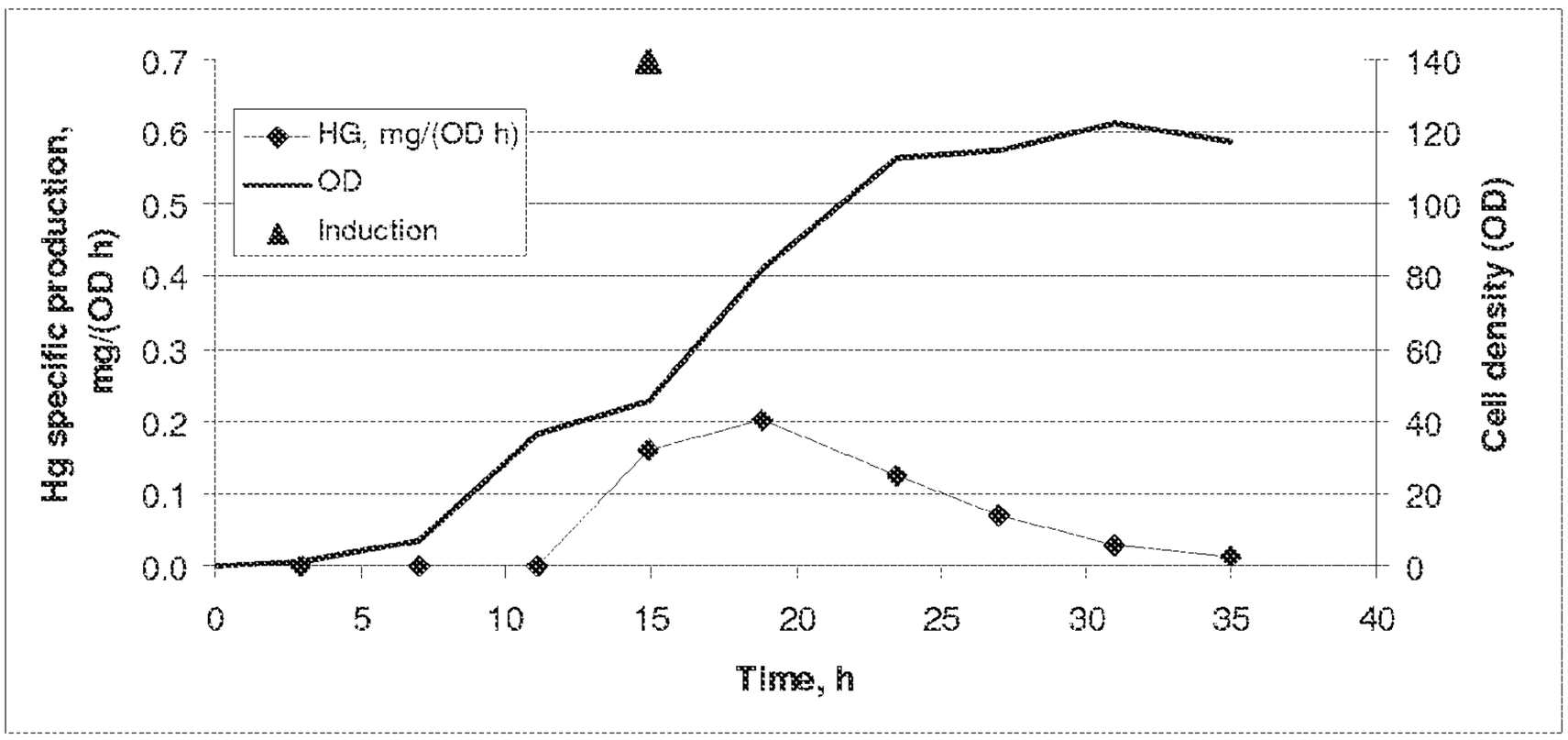


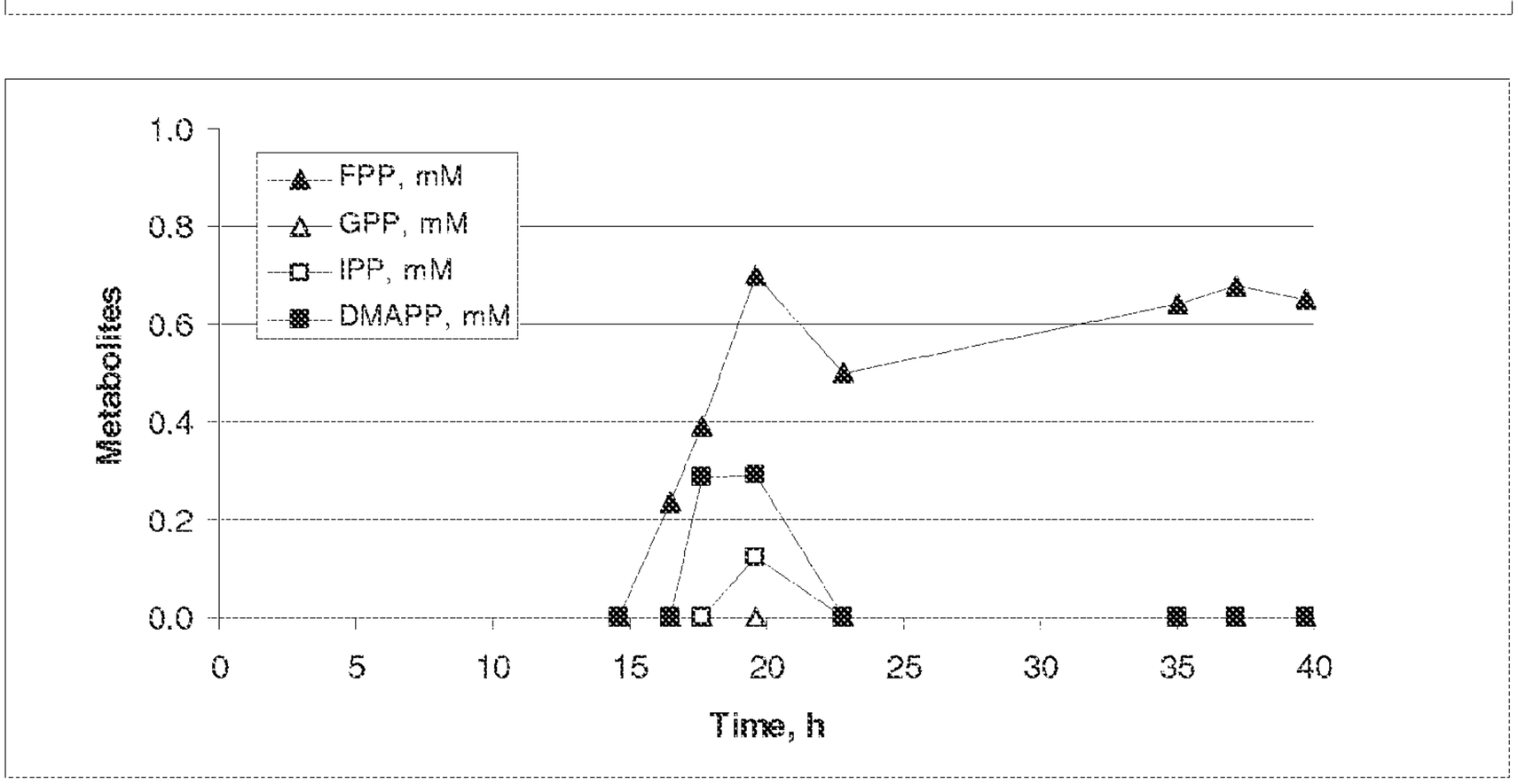


B

Figures 144A-144B

A





B

Figure 145

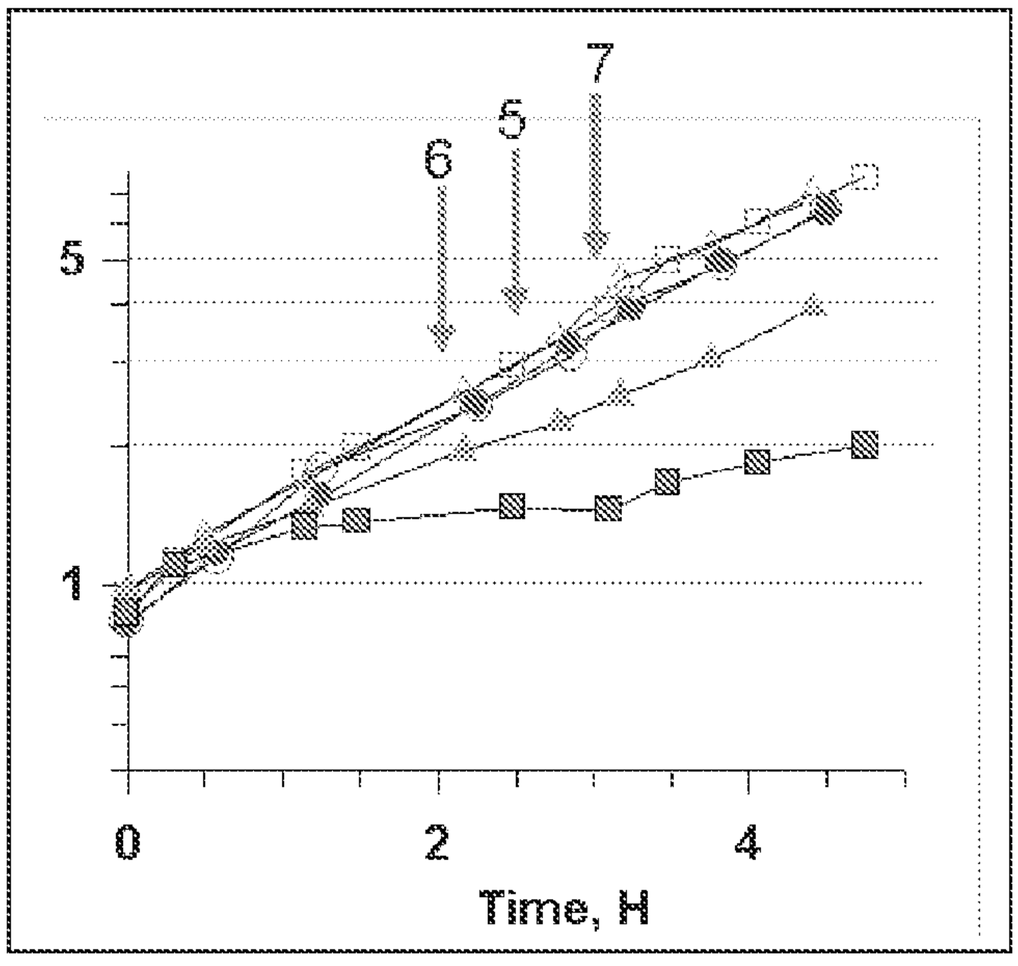


Figure 146

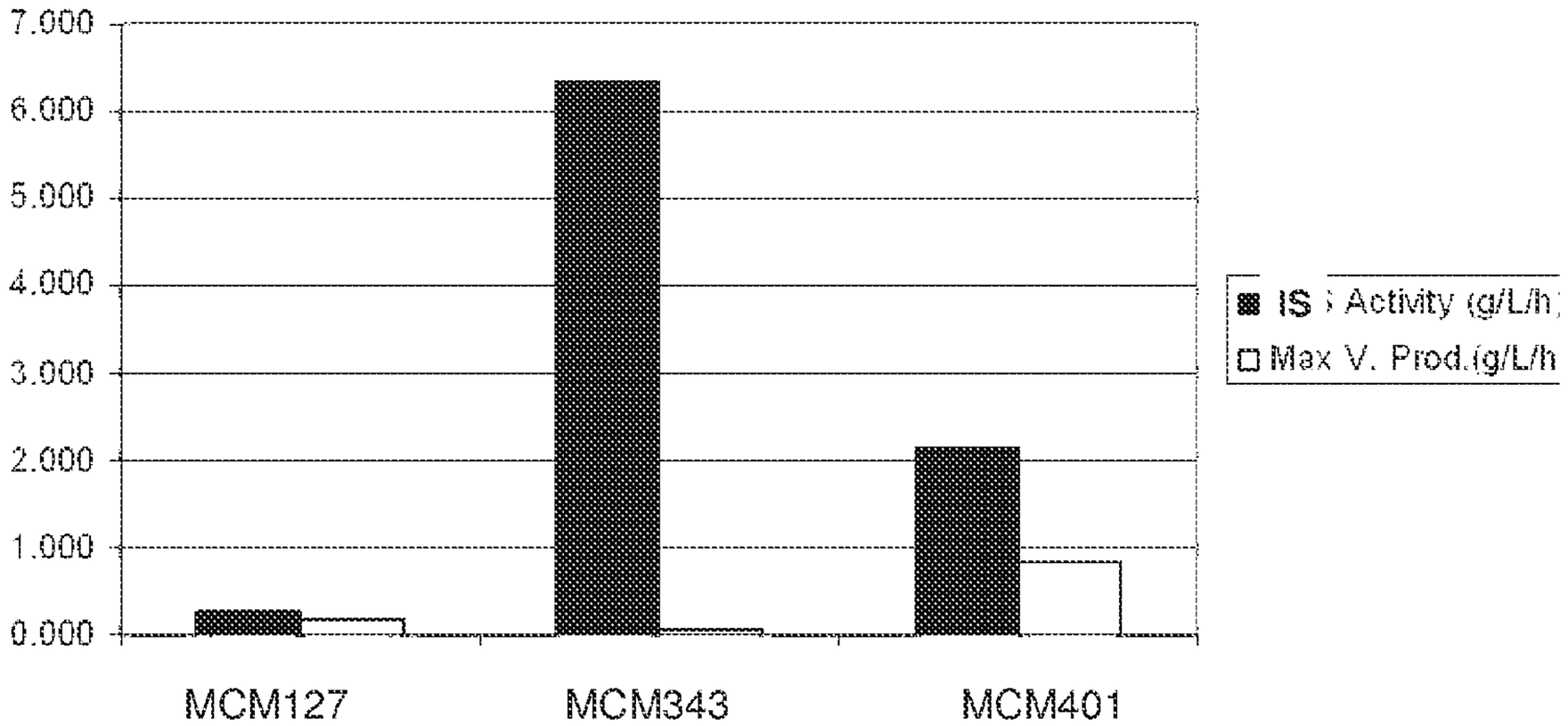

7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
MCM127
MCM343
MCM401
Max V. Prod.(g/L/h

Figure 19A

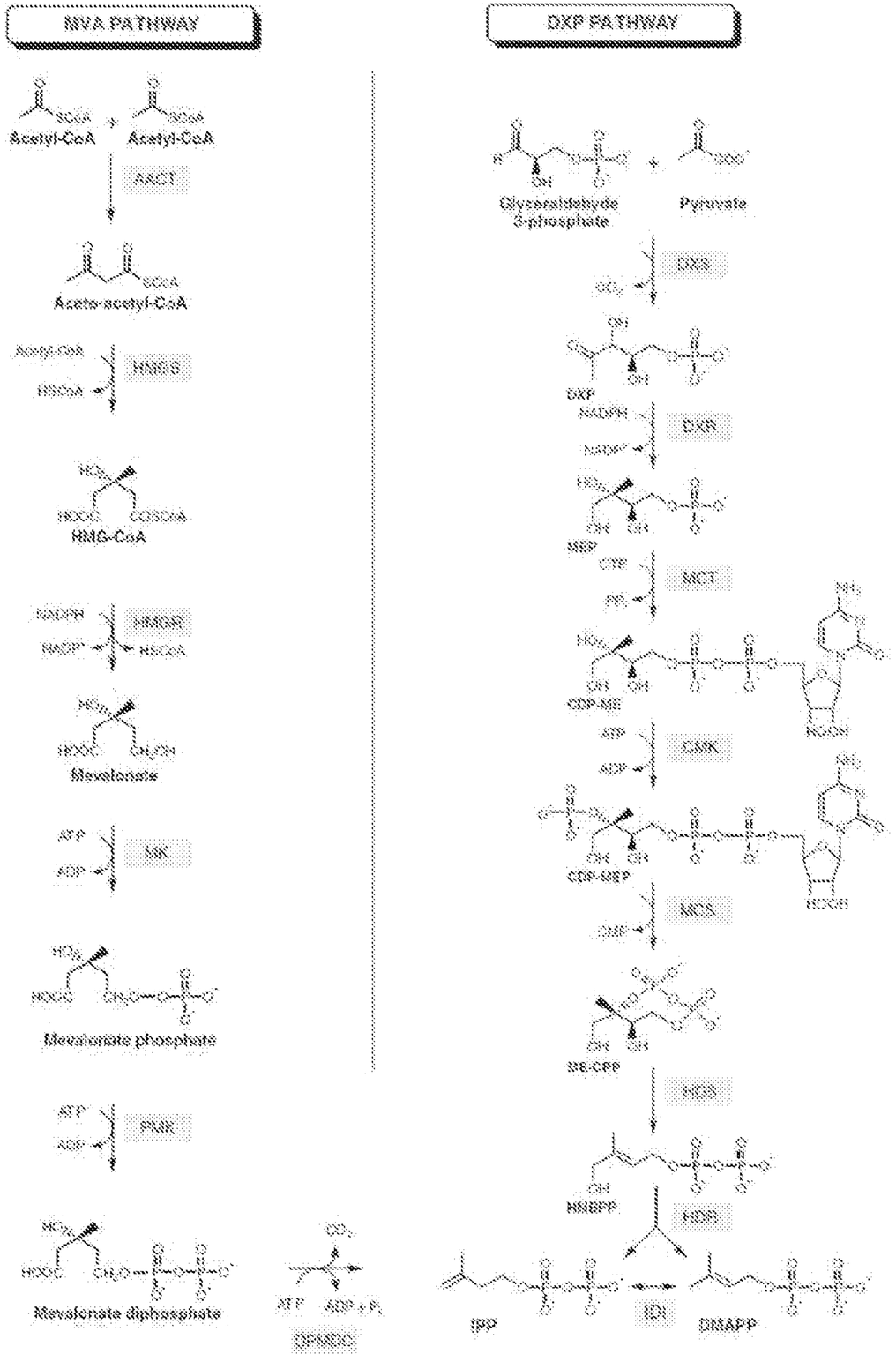


Figure 19B