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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2012 (01.11.2012)

(10) International Publication Number
WO 2012/149491 A2

(51) International Patent Classification:
C12N 1/21 (2006.01) *C12N 9/88* (2006.01)
C12N 9/00 (2006.01) *C12P 5/00* (2006.01)
C12N 9/10 (2006.01) *C12P 7/02* (2006.01)
C12N 9/12 (2006.01) *C12P 7/04* (2006.01)
C12N 9/18 (2006.01) *C12P 7/42* (2006.01)

(21) International Application Number:
PCT/US2012/035687

(22) International Filing Date:
27 April 2012 (27.04.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/481,121 29 April 2011 (29.04.2011) US

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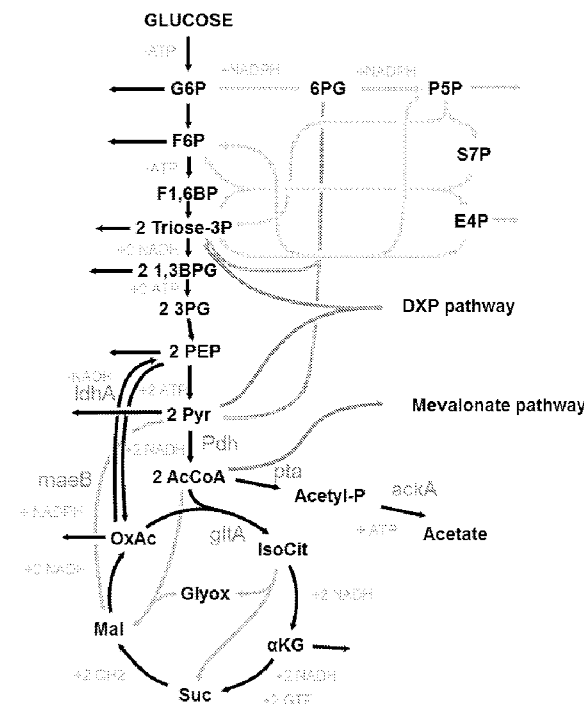
(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,

[Continued on next page]

(54) Title: RECOMBINANT MICROORGANISMS FOR ENHANCED PRODUCTION OF MEVALONATE, ISOPRENE, AND ISOPRENOIDS

FIGURE 5:



(57) Abstract: The invention features compositions and methods for the increased production of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids in microorganisms by engineering a microorganism for increased carbon flux towards mevalonate production in the following enzymatic pathways: (a) citrate synthase, (b) phosphotransacetylase, (c) acetate kinase, (d) lactate dehydrogenase, (e) malic enzyme, and (f) pyruvate dehydrogenase such that one of more of the enzyme activity is modulated. In addition, production of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids can be further enhanced by the heterologous expression of the *mvaE* and *mvaS* genes (such as, but not limited to, *mvaE* and *mvaS* genes from the organisms *Listeria grayi DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2*, and *Enterococcus casseliflavus*).

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MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Published:

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RECOMBINANT MICROORGANISMS FOR ENHANCED PRODUCTION OF MEVALONATE, ISOPRENE, AND ISOPRENOIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 61/481,121 filed April 29, 2011, the disclosure of which is incorporated by reference herein in its entirety

FIELD OF THE INVENTION

[0002] This disclosure relates to compositions and methods for the increased production of mevalonate, isoprene, isoprenoids and isoprenoid precursor molecules in recombinant microorganisms, as well as methods for producing and using the same.

BACKGROUND OF THE INVENTION

[0003] *R*-Mevalonate is an intermediate of the mevalonate-dependent biosynthetic pathway that converts acetyl-CoA to isopentenyl diphosphate and dimethylallyl diphosphate. The conversion of acetyl-CoA to mevalonate can be catalyzed by the thiolase, HMG-CoA synthase and the HMG-CoA reductase activities of the upper mevalonate-dependent biosynthetic pathway (MVA pathway). Based on molar conversion of glucose to acetyl-CoA via glycolysis, the theoretical mass yield for the production of mevalonate using the upper MVA pathway enzymes thiolase, HMG-CoA synthase and the HMG-CoA reductase is 54.8%.

[0004] Commercially, mevalonate has been used as an additive in cosmetics, for the production of biodegradable polymers, and can have value as a chiral building block for the synthesis of other chemicals.

[0005] The products of the mevalonate-dependent pathway are isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are precursors to isoprene as well as isoprenoids. Isoprene (2-methyl-1,3-butadiene) is the monomer of natural rubber and also a common structural motif to an immense variety of other naturally occurring compounds, collectively termed the isoprenoids. Isoprene is additionally the critical starting material for a variety of synthetic polymers, most notably synthetic rubbers.

[0006] Isoprenoids are compounds derived from the isoprenoid precursor molecules IPP and DMAPP. Over 29,000 isoprenoid compounds have been identified and new isoprenoids are being discovered each year. Isoprenoids can be isolated from natural products, such as microorganisms and species of plants that use isoprenoid precursor molecules as a basic building block to form the relatively complex structures of isoprenoids. Isoprenoids are vital to most living organisms and cells, providing a means to maintain cellular membrane fluidity and electron transport. In nature, isoprenoids function in roles as diverse as natural pesticides in plants to contributing to the scents associated with cinnamon, cloves, and ginger. Moreover, the pharmaceutical and chemical communities use isoprenoids as pharmaceuticals, nutraceuticals, flavoring agents, and agricultural pest control agents. Given their importance in biological systems and usefulness in a broad range of applications, isoprenoids have been the focus of much attention by scientists.

[0007] Conventional means for obtaining mevalonate and isoprenoids include extraction from biological materials (*e.g.*, plants, microbes, and animals) and partial or total organic synthesis in the laboratory. Such means, however, have generally proven to be unsatisfactory. In particular for isoprenoids, given the often times complex nature of their molecular structure, organic synthesis is impractical given that several steps are usually required to obtain the desired product. Additionally, these chemical synthesis steps can involve the use of toxic solvents as can extraction of isoprenoids from biological materials. Moreover, these extraction and purification methods usually result in a relatively low yield of the desired isoprenoid, as biological materials typically contain only minute amounts of these molecules. Unfortunately, the difficulty involved in obtaining relatively large amounts of isoprenoids has limited their practical use.

[0008] Recent developments in the production of isoprene, isoprenoid precursor molecules, and isoprenoids disclose methods for the production of isoprene and isoprenoids at rates, titers, and purities that can be sufficient to meet the demands of robust commercial processes (see, for example, International Patent Application Publication No. WO 2009/076676 A2 and U.S. Patent No. 7,915,026); however, improvements to increase the production of isoprene and isoprenoids and to increase yields of the same are still needed.

[0009] Such improvements are provided herein by the disclosure of compositions and methods to increase production of mevalonate as an intermediate of the mevalonate-dependent

biosynthetic pathway as well as to increase production of molecules derived from mevalonate, such as isoprene, isoprenoid precursors, and/or isoprenoids.

[0010] Throughout this specification, various patents, patent applications and other types of publications (*e.g.*, journal articles) are referenced. The disclosure of all patents, patent applications, and publications cited herein are hereby incorporated by reference in their entirety for all purposes.

SUMMARY OF THE INVENTION

[0011] The invention provided herein discloses, *inter alia*, compositions and methods for the increased production of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids in microorganisms by using specific gene manipulations in recombinant microorganisms which result in increased carbon flux towards mevalonate production.

[0012] Accodingly, in one aspect, provided herein are recombinant cells capable of increased production of isoprene wherein the cells are engineered for increased carbon flux towards isoprene production such that the activity of one or more enzymes from the group consisting of: citrate synthase, phosphotransacetylase, acetate kinase, lactate dehydrogenase, malate dehydrogenase, pyruvate dehydrogenase, phosphogluconolactonase (PGL), and phosphoenolpyruvate carboxylase is modulated, and wherein said cells further comprise one or more nucleic acids encoding one or more mevalonate (MVA) pathway polypeptides and one or more heterologous nucleic acids encoding an isoprene synthase polypeptide; and wherein said cells produce increased amounts of isoprene compared to isoprene-producing cells that have not been engineered for increased carbon flux towards isoprene. In some aspects, the one or more nucleic acids encoding MVA pathway polypeptides are from the upper MVA pathway, wherein the upper MVA pathway nucleic acids are selected from the group consisting of AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase nucleic acids. In some aspects, said one or more nucleic acids encoding an upper mevalonate (MVA) pathway polypeptide is an *mvaE* gene and an *mvaS* gene. In some aspects, the *mvaE* gene and the *mvaS* gene is selected from the group consisting of: (a) an *mvaE* gene and an *mvaS* gene from *L. grayi*; (b) an *mvaE* gene and an *mvaS* gene from *E. faecium*; (c) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*; and (e) an *mvaE* gene and an *mvaS* gene from *E. faecalis*. In some aspects, the one or more nucleic acids encoding MVA pathway

polypeptides are from the lower MVA pathway, wherein the lower MVA pathway nucleic acids are selected from the group consisting of MVK, PMK, and MVD nucleic acids. In some aspects, the MVK is selected from the group consisting of *M. mazei* mevalonate kinase, *M. burtonii* mevalonate kinase polypeptide, *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, *Streptomyces* mevalonate kinase polypeptide, and *Streptomyces* CL190 mevalonate kinase polypeptide. In some aspects, the cells further comprise one or more heterologous nucleic acids encoding one or DXP pathway polypeptides. In some aspects, the isoprene synthase polypeptide is a polypeptide from *Pueraria* or *Populus* or a hybrid, *Populus alba* x *Populus tremula* or variant thereof. In some aspects, 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. In some aspects, the activity of citrate synthase is modulated by decreasing the activity of an endogenous citrate synthase gene. In some aspects, the activity of citrate synthase is modulated by chromosomal replacement of an endogenous citrate synthase gene with a transgene encoding an NADH-insensitive citrate synthase. In some aspects, the transgene encoding an NADH-insensitive citrate synthase is derived from *Bacillus subtilis.* In some aspects, the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter. In some aspects of any of the aspects provided herein, decreasing the activity of citrate synthase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have decreased expression of citrate synthase. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of phosphotransacetylase and/or acetate kinase. In some aspects, the activity of phosphotransacetylase and/or acetate kinase is modulated by attenuating the activity of an endogenous phosphotransacetylase gene and/or an endogenous acetate kinase gene. In some aspects, endogenous phosphotransacetylase and/or endogenous acetate kinase gene expression is attenuated by deletion of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene. In some aspects of any of the aspects provided herein, the cells produces decreased amounts of acetate in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene

expression. In some aspects of any of the aspects provided herein, attenuating the activity of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of lactate dehydrogenase. In some aspects, the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene. In some aspects, endogenous lactate dehydrogenase gene expression is attenuated by deletion of the endogenous lactate dehydrogenase gene. In some aspects of any of the aspects provided herein, the cells produces decreased amounts of lactate in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression. In some aspects of any of the aspects provided herein, attenuating the activity of the endogenous lactate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of NADP-dependent malate dehydrogenase. In some aspects, the activity of NADP-dependent malate dehydrogenase is modulated by increasing the activity of an NADP-dependent malate dehydrogenase gene. In some aspects, the NADP-dependent malate dehydrogenase gene is an endogenous gene. In some aspects, expression of the endogenous NADP-dependent malate dehydrogenase gene is increased by replacing the endogenous NADP-dependent malate dehydrogenase gene promoter with a synthetic constitutively expressing promoter. In some aspects of any of the aspects provided herein, the cells further comprise a heterologous nucleic acid encoding an NADP-dependent malate dehydrogenase polypeptide. In some aspects of any of the aspects provided herein, the cells produces increased amounts of pyruvate in comparison to microorganisms that do not have increased expression of an NADP-dependent malate dehydrogenase gene. In some aspects of any of the aspects provided herein, increasing the activity of an NADP-dependent malate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have increased NADP-dependent malate dehydrogenase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of pyruvate dehydrogenase. In some aspects, the activity of pyruvate dehydrogenase is modulated by increasing the activity of one or

more genes of the pyruvate dehydrogenase complex consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. In some aspects of any of the aspects provided herein, the activity of the pyruvate dehydrogenase complex is modulated by attenuating the activity of an endogenous pyruvate dehydrogenase complex repressor gene. In some aspects, the one or more genes of the pyruvate dehydrogenase complex are endogenous genes. In some aspects, expression of the one or more endogenous genes of the pyruvate dehydrogenase complex is increased by replacing one or more endogenous gene promoters with one or more synthetic constitutively expressing promoters. In some aspects of any of the aspects provided herein, the cells further comprise one or more heterologous nucleic acids encoding one or more polypeptides from the group consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. In some aspects, the activity of an endogenous pyruvate dehydrogenase complex repressor is attenuated by deletion of the endogenous pyruvate dehydrogenase complex repressor gene. In some aspects of any of the aspects provided herein, the cells produce increased amounts of acetyl Co-A in comparison to microorganisms wherein the activity of pyruvate dehydrogenase is not modulated. In some aspects of any of the aspects provided herein, modulating the activity of pyruvate dehydrogenase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have modulated pyruvate dehydrogenase expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of phosphogluconolactonase (PGL). In some aspects, the activity of PGL is modulated by attenuating the activity of an endogenous PGL gene. In some aspects, the activity of PGL is attenuated by replacing the endogenous PGL gene promoter with a synthetic constitutively low expressing promoter. In some aspects, the activity of an endogenous PGL is attenuated by deletion of the endogenous PGL gene. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of phosphoenolpyruvate carboxylase. In some aspects, the activity of phosphoenolpyruvate carboxylase is modulated by attenuating the activity of an endogenous phosphoenolpyruvate carboxylase gene. In some aspects, the activity of phosphoenolpyruvate carboxylase is attenuated by replacing the endogenous phosphoenolpyruvate carboxylase gene promoter with a synthetic constitutively low expressing promoter. In some aspects, the activity of an endogenous phosphoenolpyruvate carboxylase is attenuated by deletion of the endogenous phosphoenolpyruvate carboxylase gene. In some

aspects, the cells further comprise one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide.

[0013] In other aspects, provided herein are methods for producing isoprene, comprising: (a) culturing any of the cells provided by any of the aspects disclosed herein under suitable culture conditions for production of isoprene; and (b) producing the isoprene. In another embodiment, the method further comprises recovering the isoprene.

[0014] In another aspect, provided herein are recombinant cells capable of increased production of mevalonate wherein the cells are engineered for increased carbon flux towards mevalonate, production such that the activity of one or more enzymes from the group consisting of citrate synthase, phosphotransacetylase, acetate kinase, lactate dehydrogenase, malate dehydrogenase, pyruvate dehydrogenase, phosphogluconolactonase, and phosphoenolpyruvate carboxylase is modulated and wherein said cells further comprise one or more nucleic acids encoding one or more upper mevalonate (MVA) pathway polypeptides; and wherein the cells produce increased amounts of mevalonate compared to mevalonate-producing cells that have not been engineered for increased carbon flux towards mevalonate. In some aspects, said one or more nucleic acids encoding an upper mevalonate (MVA) pathway polypeptide is an *mvaE* gene and an *mvaS* gene. In some aspects, the *mvaE* gene and the *mvaS* gene is selected from the group consisting of: (a) an *mvaE* gene and an *mvaS* gene from *L. grayi*; (b) an *mvaE* gene and an *mvaS* gene from *E. faecium*; (c) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*; and (e) an *mvaE* gene and an *mvaS* gene from *E. faecalis*. In some aspects, the activity of citrate synthase is modulated by decreasing the activity of an endogenous citrate synthase gene. In some aspects, the activity of citrate synthase is modulated by chromosomal replacement of an endogenous citrate synthase gene with a transgene encoding an NADH-insensitive citrate synthase. In some aspects, the transgene encoding an NADH-insensitive citrate synthase is derived from *Bacillus subtilis.* In some aspects, the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter. In some aspects of any of the aspects provided herein, decreasing the activity of citrate synthase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have decreased expression of citrate synthase. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of

phosphotransacetylase and/or acetate kinase. In some aspects, the activity of phosphotransacetylase and/or acetate kinase is modulated by attenuating the activity of an endogenous phosphotransacetylase gene and/or an endogenous acetate kinase gene. In some aspects, endogenous phosphotransacetylase and/or endogenous acetate kinase gene expression is attenuated by deletion of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene. In some aspects of any of the aspects provided herein, the cells produces decreased amounts of acetate in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression. In some aspects of any of the aspects provided herein, attenuating the activity of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of lactate dehydrogenase. In some aspects, the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene. In some aspects, endogenous lactate dehydrogenase gene expression is attenuated by deletion of the endogenous lactate dehydrogenase gene. In some aspects of any of the aspects provided herein, the cells produces decreased amounts of lactate in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression. In some aspects of any of the aspects provided herein, attenuating the activity of the endogenous lactate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of NADP-dependent malate dehydrogenase. In some aspects, the activity of NADP-dependent malate dehydrogenase is modulated by increasing the activity of an NADP-dependent malate dehydrogenase gene. In some aspects, the NADP-dependent malate dehydrogenase gene is an endogenous gene. In some aspects, expression of the endogenous NADP-dependent malate dehydrogenase gene is increased by replacing the endogenous NADP-dependent malate dehydrogenase gene promoter with a synthetic constitutively expressing promoter. In some aspects of any of the aspects provided herein, the cells further comprises a heterologous nucleic acid encoding an NADP-dependent malate dehydrogenase polypeptide. In some aspects of any of the aspects provided herein,

wherein the cells produces increased amounts of pyruvate in comparison to microorganisms that do not have increased expression of an NADP-dependent malate dehydrogenase gene. In some aspects of any of the aspects provided herein, wherein increasing the activity of an NADP-dependent malate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have increased NADP-dependent malate dehydrogenase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of pyruvate dehydrogenase. In some aspects, the activity of pyruvate dehydrogenase is modulated by increasing the activity of one or more genes of the pyruvate dehydrogenase complex consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. In some aspects of any of the aspects provided herein, the activity of the pyruvate dehydrogenase complex is modulated by attenuating the activity of an endogenous pyruvate dehydrogenase complex repressor gene. In some aspects, the one or more genes of the pyruvate dehydrogenase complex are endogenous genes. In some aspects, expression of the one or more endogenous genes of the pyruvate dehydrogenase complex is increased by replacing one or more endogenous gene promoters with one or more synthetic constitutively expressing promoters. In some aspects of any of the aspects provided herein, the cells further comprises one or more heterologous nucleic acids encoding one or more polypeptides from the group consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. In some aspects, the activity of an endogenous pyruvate dehydrogenase complex repressor is attenuated by deletion of the endogenous pyruvate dehydrogenase complex repressor gene. In some aspects of any of the aspects provided herein, the cells produce increased amounts of acetyl Co-A in comparison to microorganisms wherein the activity of pyruvate dehydrogenase is not modulated. In some aspects of any of the aspects provided herein, modulating the activity of pyruvate dehydrogenase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have modulated pyruvate dehydrogenase expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of phosphogluconolactonase (PGL). In some aspects, the activity of PGL is modulated by attenuating the activity of an endogenous PGL gene. In some aspects, the activity of PGL is attenuated by replacing the endogenous PGL gene promoter with a synthetic constitutively low expressing promoter. In some aspects, the activity of an endogenous PGL is attenuated by deletion of the endogenous PGL gene. In some aspects, carbon flux is directed

towards mevalonate production by modulating the activity of phosphoenolpyruvate carboxylase. In some aspects, the activity of phosphoenolpyruvate carboxylase is modulated by attenuating the activity of an endogenous phosphoenolpyruvate carboxylase gene. In some aspects, the activity of phosphoenolpyruvate carboxylase is attenuating by replacing the endogenous phosphoenolpyruvate carboxylase gene promoter with a synthetic constitutively low expressing promoter. In some aspects, the activity of an endogenous phosphoenolpyruvate carboxylase is attenuated by deletion of the endogenous phosphoenolpyruvate carboxylase gene. In some aspects of any of the aspects provided herein, mevalonate production is increased compared to microorganisms that are not grown under conditions of tri-carboxylic acid (TCA) cycle activity, wherein metabolic carbon flux in the cells is directed towards mevalonate production by modulating the activity of one or more enzymes from the group consisting of (a) citrate synthase, (b) phosphotransacetylase and/or acetate kinase, (c) lactate dehydrogenase, (d) malate dehydrogenase, and (e) pyruvate decarboxylase complex. In some aspects of any of the aspects provided herein, the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter, the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene, and the activity of acetate kinase is modulated by attenuating the activity of an endogenous acetate kinase gene.

[0015] In some aspects, provided herein are methods for producing mevalonate, comprising: (a) culturing any of the cells provided by any of the aspects disclosed herein under suitable culture conditions for production of isoprene; and (b) producing the mevalonate. In one embodiment, the method further comprises recovering the mevalonate.

[0016] In yet other aspects, provided herein are recombinant cells capable of increased production of isoprenoids wherein the cells are engineered for increased carbon flux towards mevalonate, production such that the activity of one or more enzymes from the group consisting of: citrate synthase, phosphotransacetylase, acetate kinase, lactate dehydrogenase, malate dehydrogenase, pyruvate dehydrogenase, phosphogluconolactonase (PGL), and phosphoenolpyruvate carboxylase is modulated and wherein said cells further comprise (i) one or more nucleic acids encoding one or more mevalonate (MVA) pathway polypeptides and (ii) one or more nucleic acids encoding polyprenyl pyrophosphate synthases; and wherein the cells produce increased amounts of isoprenoids compared to isoprenoid-producing cells that have not

been engineered for increased carbon flux towards mevalonate. In some aspects, the one or more nucleic acids encoding MVA pathway polypeptides are from the upper MVA pathway, wherein the upper MVA pathway nucleic acids are selected from the group consisting of AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase nucleic acids. In some aspects, said one or more nucleic acids encoding an upper mevalonate (MVA) pathway polypeptide is an *mvaE* gene and an *mvaS* gene. In some aspects, the *mvaE* gene and the *mvaS* gene is selected from the group consisting of: (a) an *mvaE* gene and an *mvaS* gene from *L. grayi*; (b) an *mvaE* gene and an *mvaS* gene from *E. faecium*; (c) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*; and (e) an *mvaE* gene and an *mvaS* gene from *E. faecalis*. In some aspects, the one or more nucleic acids encoding MVA pathway polypeptides are from the lower MVA pathway, wherein the lower MVA pathway nucleic acids are selected from the group consisting of MVK, PMK, and, MVD nucleic acids. In some aspects, the MVK is selected from the group consisting of *M. mazei* mevalonate kinase, *M. burtonii* mevalonate kinase polypeptide, *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, *Streptomyces* mevalonate kinase polypeptide, and *Streptomyces* CL190 mevalonate kinase polypeptide. In some aspects, the cells further comprise one or more heterologous nucleic acids encoding one or DXP pathway polypeptides. In some aspects, 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. In some aspects, the activity of citrate synthase is modulated by decreasing the activity of an endogenous citrate synthase gene. In some aspects, the activity of citrate synthase is modulated by chromosomal replacement of an endogenous citrate synthase gene with a transgene encoding an NADH-insensitive citrate synthase. In some aspects, the transgene encoding an NADH-insensitive citrate synthase is derived from *Bacillus subtilis*. In some aspects, the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter. In some aspects of any of the aspects provided herein, decreasing the activity of citrate synthase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have decreased expression of citrate synthase. In some aspects, carbon flux is directed towards mevalonate

production by modulating the activity of phosphotransacetylase and/or acetate kinase. In some aspects, the activity of phosphotransacetylase and/or acetate kinase is modulated by attenuating the activity of an endogenous phosphotransacetylase gene and/or an endogenous acetate kinase gene. In some aspects, endogenous phosphotransacetylase and/or endogenous acetate kinase gene expression is attenuated by deletion of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene. In some aspects of any of the aspects provided herein, the cells produces decreased amounts of acetate in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression. In some aspects of any of the aspects provided herein, attenuating the activity of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of lactate dehydrogenase. In some aspects, the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene. In some aspects, endogenous lactate dehydrogenase gene expression is attenuated by deletion of the endogenous lactate dehydrogenase gene. In some aspects of any of the aspects provided herein, the cells produces decreased amounts of lactate in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression. In some aspects of any of the aspects provided herein, attenuating the activity of the endogenous lactate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of NADP-dependent malate dehydrogenase. In some aspects, the activity of NADP-dependent malate dehydrogenase is modulated by increasing the activity of an NADP-dependent malate dehydrogenase gene. In some aspects, the NADP-dependent malate dehydrogenase gene is an endogenous gene. In some aspects, expression of the endogenous NADP-dependent malate dehydrogenase gene is increased by replacing the endogenous NADP-dependent malate dehydrogenase gene promoter with a synthetic constitutively expressing promoter. In some aspects of any of the aspects provided herein, the cells further comprise a heterologous nucleic acid encoding an NADP-dependent malate dehydrogenase polypeptide. In some aspects of any of the aspects provided herein, the

cells produces increased amounts of pyruvate in comparison to microorganisms that do not have increased expression of an NADP-dependent malate dehydrogenase gene. In some aspects of any of the aspects provided herein, increasing the activity of an NADP-dependent malate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have increased NADP-dependent malate dehydrogenase gene expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of pyruvate dehydrogenase. In some aspects, the activity of pyruvate dehydrogenase is modulated by increasing the activity of one or more genes of the pyruvate dehydrogenase complex consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. In some aspects of any of the aspects provided herein, the activity of the pyruvate dehydrogenase complex is modulated by attenuating the activity of an endogenous pyruvate dehydrogenase complex repressor gene. In some aspects, the one or more genes of the pyruvate dehydrogenase complex are endogenous genes. In some aspects, expression of the one or more endogenous genes of the pyruvate dehydrogenase complex is increased by replacing one or more endogenous gene promoters with one or more synthetic constitutively expressing promoters. In some aspects of any of the aspects provided herein, the cells further comprises one or more heterologous nucleic acids encoding one or more polypeptides from the group consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. In some aspects, the activity of an endogenous pyruvate dehydrogenase complex repressor is attenuated by deletion of the endogenous pyruvate dehydrogenase complex repressor gene. In some aspects of any of the aspects provided herein, the cell produces increased amounts of acetyl Co-A in comparison to microorganisms wherein the activity of pyruvate dehydrogenase is not modulated. In some aspects of any of the aspects provided herein, modulating the activity of pyruvate dehydrogenase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have modulated pyruvate dehydrogenase expression. In some aspects, carbon flux is directed towards mevalonate production by modulating the activity of phosphogluconolactonase (PGL). In some aspects, the activity of PGL is modulated by attenuating the activity of an endogenous PGL gene. In some aspects, the activity of PGL is decreased by replacing the endogenous PGL gene promoter with a synthetic constitutively low expressing promoter. In some aspects, the activity of an endogenous PGL is attenuated by deletion of the endogenous PGL gene. In some aspects, carbon flux is directed towards

mevalonate production by modulating the activity of phosphoenolpyruvate carboxylase. In some aspects, the activity of phosphoenolpyruvate carboxylase is modulated by attenuating the activity of an endogenous phosphoenolpyruvate carboxylase gene. In some aspects, the activity of phosphoenolpyruvate carboxylase is decreased by replacing the endogenous phosphoenolpyruvate carboxylase gene promoter with a synthetic constitutively low expressing promoter. In some aspects, the activity of an endogenous phosphoenolpyruvate carboxylase is attenuated by deletion of the endogenous phosphoenolpyruvate carboxylase gene. In some aspects of any of the aspects provided herein, wherein the isoprenoid is selected from group consisting of monoterpenes, diterpenes, triterpenes, tetraterpenes, sequiterpenes, and polyterpenes.In some aspects, the isoprenoid is a sesquiterpene. In some aspects, the isoprenoid is selected from the group consisting of abietadiene, amorphadiene, carene, α-farnesene, β-farnesene, farnesol, geraniol, geranylgeraniol, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, β-pinene, sabinene, γ-terpinene, terpindene and valencene. In some aspects, the cells further comprise one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide.

[0017] In other aspects, provided herein is a method for producing isoprenoids, comprising: (a) culturing any of the cells provided by any of the aspects disclosed herein under suitable culture conditions for production of isoprenoids; and (b) producing the isoprenoids. In one aspect, the method further comprises recovering the isoprenoids.

[0018] In one aspect, the invention provides a recombinant microorganism, or progeny thereof, comprising cells engineered for increased carbon flux towards mevalonate production wherein the activity of one or more enzymes from the group consisting of: (a) citrate synthase, (b) phosphotransacetylase; (c) acetate kinase; (d) lactate dehydrogenase; (e) NADP-dependent malic enzyme, and; (f) pyruvate dehydrogenase is modulated. In any of aspects herein, the cells can further comprise an *mvaE* gene and an *mvaS* gene (such as an *mvaE* gene and an *mvaS* gene selected from the group consisting of: (a) an *mvaE* gene and an *mvaS* gene from *L. grayi*; (b) an *mvaE* gene and an *mvaS* gene from *E. faecium*; (c) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*; and (e) an *mvaE* gene and an *mvaS* gene from *E. faecalis*).

[0019] In any of the aspects herein, the invention provides a recombinant microorganism, or progeny thereof, wherein increased carbon flux is directed towards mevalonate production by

modulating the activity of citrate synthase. In some aspects, the activity of citrate synthase is modulated by decreasing the activity of an endogenous citrate synthase gene. In some aspects, the activity of citrate synthase is modulated by chromosomal replacement of an endogenous citrate synthase gene with a transgene encoding an NADH-insensitive citrate synthase. In some aspects, the transgene encoding an NADH-insensitive citrate synthase is derived from *Bacillus subtilis*. In some aspects, the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter. In any of the aspects herein, decreasing the activity of citrate synthase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have decreased expression of citrate synthase.

[0020] In any of the aspects herein, the invention provides a recombinant microorganism, or progeny thereof, wherein increased carbon flux is directed towards mevalonate production by modulating the activity of phosphotransacetylase and/or acetate kinase. In some aspects, the activity of phosphotransacetylase and/or acetate kinase is modulated by attenuating the activity of an endogenous phosphotransacetylase gene and/or an endogenous acetate kinase gene. In one aspect, endogenous phosphotransacetylase and/or endogenous acetate kinase gene expression is attenuated by deletion of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene. In any of the aspects herein, the recombinant microorganism produces decreased amounts of acetate in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression. In any of the aspects herein, attenuating the activity of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression.

[0021] In any of the aspects herein, the invention provides a recombinant microorganism, or progeny thereof, wherein increased carbon flux is directed towards mevalonate production by modulating the activity of lactate dehydrogenase. In some aspects, the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene. In some aspects, endogenous lactate dehydrogenase gene expression is attenuated by deletion of the endogenous lactate dehydrogenase gene. In any of the aspects herein, the recombinant microorganism produces decreased amounts of lactate in comparison to

microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression. In any of the aspects herein, attenuating the activity of the endogenous lactate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression.

[0022] In any of the aspects herein, the invention provides a recombinant microorganism, or progeny thereof, wherein increased carbon flux is directed towards mevalonate production by modulating the activity of NADP-dependent malic enzyme. In some aspects, the activity of NADP-dependent malic enzyme is modulated by increasing the activity of an NADP-dependent malic enzyme gene. In some aspects, the NADP-dependent malic enzyme gene is an endogenous gene. In some aspects, expression of the endogenous NADP-dependent malic enzyme gene is increased by replacing the endogenous NADP-dependent malic enzyme gene promoter with a synthetic constitutively expressing promoter. In some aspects, the recombinant microorganism further comprises a heterologous nucleic acid encoding an NADP-dependent malic enzyme polypeptide. In any of the aspects herein, the recombinant microorganism produces increased amounts of pyruvate in comparison to microorganisms that do not have increased expression of an NADP-dependent malic enzyme gene. In any of the aspects herein, increasing the activity of an NADP-dependent malic enzyme gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have increased NADP-dependent malic enzyme gene expression.

[0023] In any of the aspects herein, the invention provides a recombinant microorganism, or progeny thereof, wherein increased carbon flux is directed towards mevalonate production by modulating the activity of pyruvate dehydrogenase. In some aspects, the activity of pyruvate dehydrogenase is modulated by increasing the activity of one or more genes of the pyruvate dehydrogenase complex consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. In any of the aspects herein, the activity of the pyruvate dehydrogenase complex is modulated by attenuating the activity of an endogenous pyruvate dehydrogenase complex repressor gene. In some aspects, the one or more genes of the pyruvate dehydrogenase complex are endogenous genes. In some aspects, expression of the one or more endogenous genes of the pyruvate dehydrogenase complex is increased by replacing one or more endogenous gene promoters with one or more synthetic constitutively expressing

promoters. In some aspects, the recombinant microorganism further comprises one or more heterologous nucleic acids encoding one or more polypeptides from the group consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. In some aspects, the activity of an endogenous pyruvate dehydrogenase complex repressor is attenuated by deletion of the endogenous pyruvate dehydrogenase complex repressor gene. In any of the aspects herein, the recombinant microorganism produces increased amounts of acetyl Co-A in comparison to microorganisms wherein the activity of pyruvate dehydrogenase is not modulated. In any of the aspect herein, modulating the activity of pyruvate dehydrogenase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have modulated pyruvate dehydrogenase expression.

[0024] In any of the aspects herein, the invention provides a recombinant microorganism wherein mevalonate production is increased compared to microorganisms that have not been engineered in one or more enzymes from the group consisting of (a) citrate synthase, (b) phosphotransacetylase and/or acetate kinase, (c) lactate dehydrogenase, (d) NADP-dependent malic enzyme, and (e) pyruvate decarboxylase complex for increase of carbon flux toward mevalonate production.

[0025] In any of the aspects herein, the invention provides a recombinant microorganism wherein mevalonate production is increased compared to microorganisms that are not grown under conditions of tri-carboxylic acid (TCA) cycle activity, wherein metabolic carbon flux in the recombinant microorganism is directed towards mevalonate production by modulating the activity of one or more enzymes from the group consisting of (a) citrate synthase, (b) phosphotransacetylase and/or acetate kinase, (c) lactate dehydrogenase, (d) malic enzyme, and (e) pyruvate decarboxylase complex.

[0026] In any of the aspects herein, the invention provides a recombinant microorganism wherein the recombinant microorganism is selected from the group consisting of yeast, bacteria, filamentous fungi, algae, and cyanobacteria. In some aspects, the recombinant microorganism is *E. coli*. In some aspects, the recombinant microorganism is a yeast.

[0027] In any of the aspects herein, the invention provides a recombinant microorganism wherein the activity of citrate synthase is modulated by replacing the endogenous citrate

synthase gene promoter with a synthetic constitutively low expressing promoter, the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene, and the activity of acetate kinase is modulated by attenuating the activity of an endogenous acetate kinase gene.

[0028] In another aspect, the invention provides for methods of producing mevalonate using any of the recombinant microorganisms described herein.

[0029] In another aspect, the invention provides for methods of producing isoprene using any of the recombinant microorganisms described herein.

[0030] In another aspect, the invention provides for methods of producing isoprenoid precursors using any of the recombinant microorganisms described herein.

[0031] In another aspect, the invention provides for methods of producing isoprenoids using any of the recombinant microorganisms described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] **Figure 1** depicts a graph showing mass yield of mevalonate from glucose. Error bars represent one standard deviation of two replicates.

[0033] **Figure 2** depicts a plasmid map of pDW34.

[0034] **Figure 3** depicts a Western blot where MvaE from strain DW326 is visualized. Lane 1 –Benchmark marker, 2-0.4 ug of purified MvaE, 3-7, Lysate samples from strain DW326 induced with 0, 25, 50, 100, 200 uM IPTG.

[0035] **Figure 4** depicts a SDS-PAGE gel stained with Safestain containing: Lane 1- Benchmark marker, 2-15- His-tag mediated purification of MvaE protein fractions eluted from a nickel column.

[0036] **Figure 5** depicts central metabolism of *E. coli*. The enzymes citrate synthase (*gltA*), phosphotransacetylase (*pta*), acetate kinase (*ackA*), lactate dehydrogenase (*ldhA*), NADP+-dependent malic enzyme (*maeB*) and the pyruvate dehydrogenase complex (*Pdh*) are shown.

[0037] **Figure 6** depicts citrate synthase activity of strains MD09-314, CMP451, CMP452 and CMP453.

[0038] **Figure 7** depicts growth curve of strains CMP694, CMP678, CMP680, CMP736, and CMP832. 100 uM IPTG were added at t=2. Absorbance at 600 nm is plotted as a function of time (EFT=Elapsed Fermentation Time (h)).

[0039] **Figure 8** depicts concentration of mevalonate (g/L) as obtained from 10 g/L glucose after shake flask fermentation of strains CMP694, CMP832, CMP678, CMP680, and CMP736.

[0040] **Figure 9** depicts genome local context of the gene *maeB* in *E. coli* BL21.

[0041] **Figure 10** depicts genome local context of the Pyruvate dehydrogenase complex (pdhR-aceEF-lpd) in *E. coli* K-12.

[0042] **Figure 11** depicts insertion of a PL.6 promoter upstream of the *aceE* gene.

[0043] **Figure 12** depicts construction design for the insertion of a PL.6 promoter upstream of the *aceE* gene.

[0044] **Figure 13** depicts growth curve of strains CMP678, MD10-555, CMP711, and CMP729. 100 uM IPTG were added at t=2. Absorbance at 600 nm is plotted as a function of time (EFT=Elapsed Fermentation Time (h)).

[0045] **Figure 14** depicts concentration of mevalonate (g/L) as obtained from 10 g/L glucose after shake flask fermentation of strains CMP678, MD1-555, CMP711 and CMP729.

[0046] **Figure 15** depicts yield of isoprene on glucose of 1.2 *gltA* strain (open squares) compared to the parental strain (closed diamonds) in the 15-L fermentation over time. Strains were run under the same conditions. Overall yield was calculated using the following formula: %wt Yield on glucose = Isoprene total(t)/[(Feed Wt(0)-Feed Wt(t)+50)*0.57)], where 0.57 is the wt% of glucose in the glucose feed solution and 50 is the grams of this feed batched into the fermentor at t=0. (20100278: strain CMP457 (open squares); GI1.2gltA20100131: strain MD09-317 (black diamonds) wt gltA).

[0047] **Figure 16** depicts titer of 1.2 *gltA* strain (open squares) compared to the parental strain (closed diamonds) in the 15-L fermentation over time. Strains were run under the same conditions. (20100278: strain CMP457 (open squares); GI1.2gltA 20100131: strain MD09-317 (black diamonds) wt gltA).

[0048] **Figure 17** depicts volumetric productivity of 1.2 gltA strain (open squares) compared to the parental strain (closed diamonds) in the 15-L fermentation over time. Strains were run under the same conditions. (20100278: strain CMP457 (open squares); GI1.2gltA20100131: strain MD09-317 (black diamonds) wt gltA).

[0049] **Figure 18** depicts cell productivity index for isoprene of 1.2 gltA strain (open squares) compared parental strain (closed diamonds) in the 15-L fermentation over time. Strains were run under the same conditions. (20100278: strain CMP457 (open squares); GI1.2gltA20100131: strain MD09-317 (black diamonds) wt gltA). Cell Performance Index (CPI): g isoprene/Avg. gDCW = [HG Total/[OD*Ferm Wt/1.05)/2.7] where 1.05 is the assumed fermentation broth specific gravity (kg/L), and 2.7 has units of OD*L/gDCW.

[0050] **Figure 19** depicts yield of mevalonate on glucose of 1.2 gltA strain (open squares) compared parental (closed diamonds) in the 15-L fermentation over time. Strains were run under the same conditions. (20100916: strain CMP678 (open squares); GI1.2gltA (20100154: strain MCM1002 (black diamonds) wt gltA). Overall yield was calculated using the following formula: %wt Yield on glucose = Mevalonate total(t)/[(Feed Wt(0)-Feed Wt(t)+50)*0.59)], where 0.59 is the weight fraction of glucose in the glucose feed solution and 50 is the grams of this feed batched into the fermentor at t=0.

[0051] **Figure 20** depicts Cell Productivity Index (CPI) of mevalonate on glucose of 1.2 gltA strain (open squares) compared parental (closed diamonds) in the 15-L fermentation over time. The dry cell weight was not directly measured in these experiments, rather, an experimentally determine optical density to dry cell weight conversion factor was used. This OD to DCW factor was generated in a similar *E.coli* BL21 host in the same media formulation. Strains were run under the same conditions. (20100916: strain CMP678 (open squares); GI1.2gltA (20100154: strain MCM1002 (black diamonds) wt gltA).

[0052] **Figure 21** depicts Volumetric Productivity of mevalonate 1.2 gltA strain (open squares) compared parental (closed diamonds) in the 15-L fermentation over time. Strains were run under the same conditions. (20100916: strain CMP678 (open squares); GI1.2gltA (20100154: strain MCM1002 (black diamonds) wt gltA).

[0053] **Figure 22** depicts mevalonate broth titer in 1.2 gltA strain (open squares) compared parental (closed diamonds) in the 15-L fermentation over time. Strains were run under the same conditions. (20100916: strain CMP678 (open squares); GI1.2gltA (20100154: strain MCM1002 (black diamonds) wt gltA).

[0054] **Figure 23** depicts Cell Productivity Index (CPI) of mevalonate on glucose of 1.2 gltA strain (open squares) compared parental (closed diamonds) in the 15-L fermentation over time. The dry cell weight was not directly measured in these experiments, rather, an experimentally determine optical density to dry cell weight conversion factor was used. This OD to DCW factor was generated in a similar *E.coli* BL21 host in the same media formulation. Strains in the chart below were run under the same conditions. (20100967: strain CMP680 (open triangles) ackA, pta-, ldh-GI1.2gltA; 20100916: strain CMP678 (open squares) GI1.2gltA; 20100154: strain MCM1002 (black diamonds) wt gltA).

[0055] **Figure 24** depicts yield of mevalonate on glucose of the “triple” host (CMP680, open triangles) is better at 36hrs compared to the 1.2 gltA strain (CMP678, open squares) and both are much improved over the parental (MCM1002, closed diamonds) in the 15-L fermentation. The CMP680 strain is notable for its high initial yield. Strains were run under the same conditions. (20100967: strain CMP680 (open triangles) ackA, pta-, ldh-GI1.2gltA; 20100916: strain CMP678 (open squares) GI1.2gltA; 20100154: strain MCM1002 (black diamonds) wt gltA).

[0056] **Figure 25** depicts Specific productivity of mevalonate reported in terms of milligrams mevalonate per liter of fermentor broth per hour per unit of optical density (absorbance at 550nm). The “triple” host (CMP680, open triangles) shows a much higher specific productivity from 0 to 20hrs EFT compared to the 1.2 gltA strain (CMP678, open squares) and both are improved over the parental (MCM1002, closed diamonds) in the 15-L fermentation. Strains were run under the same conditions. (20100967: strain CMP680 (open triangles) ackA, pta-, ldh-GI1.2gltA; 20100916: strain CMP678 (open squares) GI1.2gltA; 20100154: strain MCM1002 (black diamonds) wt gltA).

[0057] **Figure 26** depicts Volumetric productivity of mevalonate reported in terms of grams mevalonate per liter of fermentor broth per hour of elapsed fermentation time. The "triple" host (CMP680, open triangles) shows a lower volumetric productivity compared to the 1.2 gltA strain (CMP678, open squares) but both are improved over the parental (MCM1002, closed diamonds) in the 15-L fermentation. Strains were run under the same conditions. (20100967: strain CMP680 (open triangles) ackA, pta-, ldh-GI1.2gltA; 20100916: strain CMP678 (open squares) GI1.2gltA; 20100154: strain MCM1002 (black diamonds) wt gltA).

[0058] **Figure 27** depicts Mevalonate broth titer (or mevalonate broth concentration) was higher in the 1.2 gltA strain (CMP678, open squares) compared to the "triple" host (CMP680, open triangles). Both were higher than the parental control (MCM1002, closed diamonds) in the 15-L fermentation over time. Strains were run under the same conditions. (20100967: strain CMP680 (open triangles) ackA, pta-, ldh-GI1.2gltA; 20100916: strain CMP678 (open squares) GI1.2gltA; 20100154: strain MCM1002 (black diamonds) wt gltA).

[0059] **Figure 28** depicts solubility of *M. burtonii* and *M. mazei* mevalonate kinases (MVK) in MCM1666 and MCM1669 strains respectively.

[0060] **Figure 29** depicts growth and isoprene productivity in engineered *E. coli* strains expressing *M. burtonii* mevalonate kinase (A) or *M. mazei* mevalonate kinase (B) on the *E. coli* chromosome at small scale.

[0061] **Figure 30** depicts expression of *M. mazei* and *M. burtonii* mevalonate kinases in *E. coli* 15-L fermentations.

[0062] **Figure 31** depicts yield of isoprene on glucose achieved in each15-L fermentation over time. %wt Yield on glucose = Isoprene total (t)/[(Feed Wt(0)-Feed Wt(t)+83.5)*0.59)], where 0.59 is the wt% of glucose in the glucose feed solution and 83.5 is the grams of this feed batched into the fermentor at t=0. Each feed had its weight % measured independently. DW708: mazei MVK on plasmid and chromosome (closed diamonds); DW708: mazei MVK on plasmid and chromosome (open diamonds); MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles).

[0063] **Figure 32** depicts instantaneous yield of isoprene on glucose achieved in each15-L fermentation over time. Isoprene Instantaneous yield was calculated using the formula: Isoprene

Inst. yield (g/g%) = Isoprene produced (t_1-t_0)/consumed glucose (t_1-t_0)*100. DW708: mazei MVK on plasmid and chromosome (closed diamonds); DW708: mazei MVK on plasmid and chromosome (open diamonds); MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles).

[0064] **Figure 33** depicts cell productivity index (CPI) achieved in each15-L fermentation over time. Cell Productivity Index (CPI) was calculated using the following formula: CPI = total grams Isoprene / total grams dry cell weight. DW708: mazei MVK on plasmid and chromosome (closed diamonds); DW708: mazei MVK on plasmid and chromosome (open diamonds); MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles).

[0065] **Figure 34** depicts volumetric productivity achieved in each15-L fermentation over time. Volumetric Productivity was calculated using the following formula: $[\sum HGER(t)/1000*68.117)]/[t-t_0]$,where the summation is from t_0 to t. Tank turnaround time is not factored in. DW708: mazei MVK on plasmid and chromosome (closed diamonds); DW708: mazei MVK on plasmid and chromosome (open diamonds); MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles).

[0066] **Figure 35** depicts specific productivity achieved in each15-L fermentation over time. Specific Productivity was calculated using the following formula: **Specific productivity (mg/L/hr/OD)** = HgER*68.117g/mol/OD. HgER is the Isoprene Evolution Rate in (mmol/L/hr). OD = optical density = Absorbance at 550nm * dilution factor in water. DW708: mazei MVK on plasmid and chromosome (closed diamonds); DW708: mazei MVK on plasmid and chromosome (open diamonds); MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles).

[0067] **Figure 36** depicts yield of isoprene on glucose achieved in each15-L fermentation over time. %wt Yield on glucose = Isoprene total (t)/[(Feed Wt(0)-Feed Wt(t)+83.5)*0.59)], where 0.59 is the wt% of glucose in the glucose feed solution and 83.5 is the grams of this feed batched into the fermentor at t=0. Each feed had its weight % measured independently. MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on

chromosome only (open triangles); MCM2126: mazei MVK on chromosome only (closed squares); MCM2127: mazei MVK on chromosome only (stars).

[0068] **Figure 37** depicts instantaneous yield of isoprene on glucose achieved in each15-L fermentation over time. Isoprene Instantaneous yield was calculated using the formula: Isoprene Inst. yield (g/g%) = Isoprene produced (t_1-t_0)/consumed glucose (t_1-t_0)*100. MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles); MCM2126: mazei MVK on chromosome only (closed squares); MCM2127: mazei MVK on chromosome only (stars).

[0069] **Figure 38** depicts cell productivity index (CPI) achieved in each15-L fermentation over time. Cell Productivity Index (CPI) was calculated using the following formula: CPI = total grams Isoprene / total grams dry cell weight. MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles); MCM2126: mazei MVK on chromosome only (closed squares); MCM2127: mazei MVK on chromosome only (stars).

[0070] **Figure 39** depicts volumetric productivity achieved in each15-L fermentation over time. Volumetric Productivity was calculated using the following formula: [ΣHGER(t)/1000*68.117)]/[t-t_0],where the summation is from t_0 to t. Tank turnaround time is not factored in. MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles); MCM2126: mazei MVK on chromosome only (closed squares); MCM2127: mazei MVK on chromosome only (stars).

[0071] **Figure 40** depicts specific productivity achieved in each15-L fermentation over time. Specific Productivity was calculated using the following formula: Specific productivity (mg/L/hr/OD) = HgER*68.117g/mol/OD. HgER is the Isoprene Evolution Rate in (mmol/L/hr). OD = optical density = Absorbance at 550nm * dilution factor in water. MCM2125: burtonii MVK on chromosome only (closed triangles); MCM2125: burtonii MVK on chromosome only (open triangles); MCM2126: mazei MVK on chromosome only (closed squares); MCM2127: mazei MVK on chromosome only (stars).

[0072] **Figure 41** depicts yield of isoprene on glucose achieved in each15-L fermentation over time. CMP1082 (pgl+) is depicted by open triangles and CMP1136 (pgl-) is depicted by closed squares.

[0073] **Figure 42** depicts instantaneous yield of isoprene on glucose achieved in each15-L fermentation over time. CMP1082 (pgl+) is depicted by open triangles and CMP1136 (pgl-) is depicted by closed squares.

[0074] **Figure 43** depicts Cell Productivity Index (CPI) achieved in each15-L fermentation over time. CMP1082 (pgl+) is depicted by open triangles and CMP1136 (pgl-) is depicted by closed squares.

[0075] **Figure 44** depicts volumetric productivity achieved in each15-L fermentation over time. CMP1082 (pgl+) is depicted by open triangles and CMP1136 (pgl-) is depicted by closed squares.

[0076] **Figure 45** depicts specific productivity achieved in each15-L fermentation over time. CMP1082 (pgl+) is depicted by open triangles and CMP1136 (pgl-) is depicted by closed squares.

[0077] **Figure 46** depicts OD600 of isoprene-producing cultures as a function of time, in a 250-mL Erlenmeyer shake flask, filled with 25 mL of medium, and incubated at 34 °C and 200 rpm.

[0078] **Figure 47** depicts specific productivity (in arbitrary units) of isoprene-producing cultures as a function of OD. Cultures were incubated in a 250-mL Erlenmeyer shake flask, filled with 25 mL of medium, at 34 °C and 200 rpm.

[0079] **Figure 48** depicts yield of isoprene on glucose achieved in each15-L fermentation over time. %wt Yield on glucose = Isoprene total (t)/[(Feed Wt(0)-Feed Wt(t)+83.5)*0.59)], where 0.59 is the wt% of glucose in the glucose feed solution and 83.5 is the grams of this feed batched into the fermentor at t=0. Each feed had its weight % measured independently. CMP1136: wild type ppc promoter (open triangles); CMP1237: GI1.2ppc promoter (closed squares).

[0080] **Figure 49** depicts instantaneous yield of isoprene on glucose achieved in each15-L fermentation over time. Isoprene Instantaneous yield was calculated using the formula: Isoprene

Inst. yield (g/g%) = Isoprene produced (t_1-t_0)/consumed glucose (t_1-t_0)*100. CMP1136: wild type ppc promoter (open triangles); CMP1237: GI1.2ppc promoter (closed squares).

[0081] **Figure 50** depicts cell productivity index (CPI) achieved in each15-L fermentation over time. Cell Productivity Index (CPI) was calculated using the following formula: CPI = total grams Isoprene / total grams dry cell weight. CMP1136: wild type ppc promoter (open triangles); CMP1237: GI1.2ppc promoter (closed squares).

[0082] **Figure 51** depicts volumetric productivity achieved in each15-L fermentation over time. Volumetric Productivity was calculated using the following formula: [$\sum$HGER(t)/1000*68.117)]/[t-t_0],where the summation is from t_0 to t. Tank turnaround time is not factored in. CMP1136: wild type ppc promoter (open triangles); CMP1237: GI1.2ppc promoter (closed squares).

[0083] **Figure 52** depicts specific productivity achieved in each15-L fermentation over time. Specific Productivity was calculated using the following formula: Specific productivity (mg/L/hr/OD) = HgER*68.117g/mol/OD. HgER is the Isoprene Evolution Rate in (mmol/L/hr). OD = optical density = Absorbance at 550nm * dilution factor in water. CMP1136: wild type ppc promoter (open triangles); CMP1237: GI1.2ppc promoter (closed squares).

[0084] **Figure 53** depicts genome organization of *E. coli* MG1655 around FNR (source: GenBank U00096)

[0085] **Figure 54** depicts OD600 of isoprene-producing cultures as a function of time, in a 250-mL Erlenmeyer shake flask, filled with 25 mL of medium, and incubated at 34 °C and 200 rpm.

[0086] **Figure 55** depicts specific productivity (in arbitrary units) of isoprene-producing cultures as a function of OD. Cultures were incubated in a 250-mL Erlenmeyer shake flask, filled with 25 mL of medium, at 34 °C and 200 rpm.

[0087] **Figure 56** depicts OD600 of isoprene-producing cultures as a function of time, in a 250-mL Erlenmeyer shake flask, filled with 25 mL of medium, and incubated at 34 °C and 50 rpm.

[0088] **Figure 57** depicts specific productivity (in arbitrary units) of isoprene-producing cultures as a function of OD. Cultures were incubated in a 250-mL Erlenmeyer shake flask, filled with 25 mL of medium, at 34 °C and 50 rpm.

[0089] **Figure 58** depicts OD600 of isoprene-producing cultures as a function of time.

[0090] **Figure 59** depicts specific productivity (in arbitrary units) of isoprene-producing cultures as a function of time.

[0091] **Figure 60** depicts RNA hybridization signal on the array form CMP457 (producing isoprene) vs MCM1020 (control strain).

[0092] **Figure 61** depicts OD600 of cultures as a function of time.

[0093] **Figure 62** depicts specific productivity (in arbitrary units) of isoprene-producing cultures as a function of time.

[0094] **Figure 63**depicts a map of plasmid pMCM1666-pET24-His-TEV-bMVK.

[0095] **Figure 64** depicts a map of plasmid pMCM1669-pET24-His-TEV-mMVK(GO).

[0096] **Figure 65** depicts a schematic of the two cistron MVK construct.

[0097] **Figure 66** depicts a map of plasmid pMCM2020-pTrcAlba-bMVK.

[0098] **Figure 67** depicts a map of plasmidpMCM2095- pTrcAlba-mMVK(del).

[0099] **Figure 68** depicts OD600 of cultures as a function of time.

[0100] **Figure 69** depicts specific productivity (in arbitrary units) of isoprene-producing cultures as a function of time.

DETAILED DESCRIPTION

[0101] The invention provides, *inter alia,* compositions and methods for the increased production of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids in recombinant microorganisms that have been engineered for increased carbon flux towards mevalonate production. In one aspect, the invention provides, *inter alia,* recombinant

microorganisms, or progeny thereof, comprising cells engineered for increased carbon flux towards mevalonate production wherein the activity of one or more enzymes or proteins from the group consisting of: (a) citrate synthase, (b) phosphotransacetylase; (c) acetate kinase; (d) lactate dehydrogenase; (e) NADP-dependent malic enzyme; (f) pyruvate dehydrogenase; (g) 6-phosphogluconolactonase; (h) phosphoenolpyruvate carboxylase; (i) the inhibitor of RssB activity during magnesium starvation protein; (j) the acrA component of the multidrug efflux pump acrAB-TolC; and (k) the fumarate and nitrate reduction sRNA (FNR) is modulated. In one aspect, the recombinant microorganisms disclosed herein are cells that have been engineered to heterologously express nucleic acids encoding one or more upper MVA pathway polypeptides. In another aspect, the recombinant microorganisms are cells (such as bacterial cells) that have been engineered to heterologously express polypeptides encoded by the *mvaE* and *mvaS* genes (such as *mvaE* and *mvaS* genes from the microorganisms *Listeria grayi, Enterococcus faecium, Enterococcus gallinarum, Enterococcus casseliflavus* and/or *Enterococcus faecalis*). Any progeny of the recombinant microorganism is contemplated to be within the scope of the invention as well.

[0102] The mevalonate-dependent biosynthetic pathway is particularly important for the production of the isoprenoid precursor molecules dimethylallyl diphosphate (DMAPP) and isopentenyl pyrophosphate (IPP). The enzymes of the upper mevalonate pathway convert acetyl CoA, produced from glucose, into mevalonate via three enzymatic reactions. Together, upper MVA pathway genes (for example, the *mvaE* and *mvaS* genes, such as the *mvaE* and *mvaS* from the above-mentioned bacterial species) encode polypeptides that possess the enzymatic activities of the upper mevalonate pathway. Without being bound to theory, it is believed that increasing the efficiency and productivity of these three enzymatic activities in the upper mevalonate-dependent biosynthetic pathway will substantially increase intracellular concentrations of mevalonate and, consequently, of downstream isoprenoid precursor molecules such as DMAPP and IPP. The increased yield of mevalonate production by these strains is therefore advantageous for commercial applications.

[0103] As detailed herein, the enzymatic pathways that include citrate synthase, phosphotransacetylase, acetate kinase, lactate dehydrogenase, malic enzyme and/or pyruvate dehydrogenase can be modulated to increase or decrease the activity of enzymes in these pathways such that more carbon flux is directed toward mevalonate production. Other factors,

the modulation of which can increase carbon flux towards mevalonate in cells, can include 6-phosphogluconolactonase, phosphoenolpyruvate carboxylase, the inhibitor of RssB activity during magnesium starvation protein, the acrA component of the multidrug efflux pump acrAB-TolC, and the fumarate and nitrate reduction sRNA. This, in turn, can lead to more substrate for the production of isoprene, isoprenoid precursors, and isoprenoids. The compositions and methods of the present application, therefore, represent an improvement over what has previously been practiced in the art, both in the number of strains of microorganisms available for increased production of mevalonate, isoprene, isoprenoid precursor molecules, and isoprenoids as well as in the amount of these compounds (*e.g.,* mevalonate) produced by those cells (such as bacterial cells).

General Techniques

[0104] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, "*Molecular Cloning: A Laboratory Manual*", second edition (Sambrook et al., 1989); "*Oligonucleotide Synthesis*" (M. J. Gait, ed., 1984); "*Animal Cell Culture*" (R. I. Freshney, ed., 1987); "*Methods in Enzymology*" (Academic Press, Inc.); "*Current Protocols in Molecular Biology*" (F. M. Ausubel et al., eds., 1987, and periodic updates); "*PCR: The Polymerase Chain Reaction*", (Mullis et al., eds., 1994). Singleton et al., *Dictionary of Microbiology and Molecular Biology* 2nd ed., J. Wiley & Sons (New York, N.Y. 1994), and March, *Advanced Organic Chemistry Reactions, Mechanisms and Structure* 4th ed., John Wiley & Sons (New York, N.Y. 1992), provide one skilled in the art with a general guide to many of the terms used in the present application.

Definitions

[0105] The terms "complete mevalonate (MVA) pathway" or "entire mevalonate (MVA) pathway" refer to the cellular metabolic pathway which converts acetyl Co-A into dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) and which is catalyzed by the enzymes acetoacetyl-CoenzymeA synthase (*e.g.,* thiolase), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, mevalonate kinase (MVK), phosphomevalonate kinase (PMK), diphosphomevalonate decarboxylase (MVD), and isopentenyl diphosphate isomerase (IDI).

[0106] As used herein, the terms "upper mevalonate pathway" or "upper MVA pathway" refer to the series of reactions in cells catalyzed by the enzymes acetoacetyl-CoenzymeA synthase (*e.g.,* thiolase), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase, and 3-hydroxy-3-methylglutaryl-Coenzyme A reductase.

[0107] The terms "lower mevalonate pathway" or "lower MVA pathway" refer to the series of reactions in cells catalyzed by the enzymes mevalonate kinase (MVK), phosphomevalonate kinase (PMK), diphosphomevalonate decarboxylase (MVD), and isopentenyl diphosphate isomerase (IDI).

[0108] The term "isoprene" refers to 2-methyl-1,3-butadiene (CAS# 78-79-5). It can be the direct and final volatile C5 hydrocarbon product from the elimination of pyrophosphate from 3,3-dimethylallyl diphosphate (DMAPP). It may not involve the linking or polymerization of IPP molecules to DMAPP molecules. The term "isoprene" is not generally intended to be limited to its method of production unless indicated otherwise herein.

[0109] As used herein, the term "polypeptides" includes polypeptides, proteins, peptides, fragments of polypeptides, and fusion polypeptides.

[0110] 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.

[0111] By "heterologous polypeptide" is meant a polypeptide encoded by a nucleic acid sequence derived from a different organism, species, or strain than the host cell. In some embodiments, a heterologous polypeptide is not identical to a wild-type polypeptide that is found in the same host cell in nature.

[0112] As used herein, a "nucleic acid" refers to two or more deoxyribonucleotides and/or ribonucleotides covalently joined together in either single or double-stranded form.

[0113] By "recombinant nucleic acid" is meant a nucleic acid of interest that is free of one or more nucleic acids (*e.g.*, genes) which, in the genome occurring in nature of the organism from which the nucleic acid of interest is derived, flank the nucleic acid of interest. The term therefore

includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA, a genomic DNA fragment, or a cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences.

[0114] By "heterologous nucleic acid" is meant a nucleic acid sequence derived from a different organism, species or strain than the host cell. In some embodiments, the heterologous nucleic acid is not identical to a wild-type nucleic acid that is found in the same host cell in nature. For example, a nucleic acid encoded by the *mvaE* and *mvaS* genes (such as, but not limited to, the *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum,* and *E. casseliflavus*) transformed in or integrated into the chromosome of *E. coli* is a heterologous nucleic acid.

[0115] 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 expression control sequence can be "native" or heterologous. A native expression control sequence is derived from the same organism, species, or strain as the gene being expressed. A heterologous expression control sequence is derived from a different organism, species, or strain as the gene being expressed. An "inducible promoter" is a promoter that is active under environmental or developmental regulation.

[0116] By "operably linked" is meant a functional linkage between a nucleic acid expression control sequence (such as a promoter) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

[0117] As used herein, the terms "minimal medium" or "minimal media" refer to growth medium containing the minimum nutrients possible for cell growth, generally without the presence of amino acids. Minimal medium typically contains: (1) a carbon source for microorganism (*e.g.,* bacterial) growth; (2) various salts, which can vary among microorganism (*e.g.,* bacterial) species and growing conditions; and (3) water. The carbon source can vary significantly, from simple sugars like glucose to more complex hydrolysates of other biomass,

such as yeast extract, as discussed in more detail below. The salts generally provide essential elements such as magnesium, nitrogen, phosphorus, and sulfur to allow the cells to synthesize proteins and nucleic acids. Minimal medium can also be supplemented with selective agents, such as antibiotics, to select for the maintenance of certain plasmids and the like. For example, if a microorganism is resistant to a certain antibiotic, such as ampicillin or tetracycline, then that antibiotic can be added to the medium in order to prevent cells lacking the resistance from growing. Medium can be supplemented with other compounds as necessary to select for desired physiological or biochemical characteristics, such as particular amino acids and the like.

[0118] As used herein, the term “isoprenoid” refers to a large and diverse class of naturally-occurring class of organic compounds composed of two or more units of hydrocarbons, with each unit consisting of five carbon atoms arranged in a specific pattern. As used herein, “isoprene” is expressly excluded from the definition of “isoprenoid.”

[0119] As used herein, the term “terpenoid” refers to a large and diverse class of organic molecules derived from five-carbon isoprenoid units assembled and modified in a variety of ways and classified in groups based on the number of isoprenoid units used in group members. Hemiterpenoids have one isoprenoid unit. Monoterpenoids have two isoprenoid units. Sesquiterpenoids have three isoprenoid units. Diterpenoids have four isoprene units. Sesterterpenoids have five isoprenoid units. Triterpenoids have six isoprenoid units. Tetraterpenoids have eight isoprenoid units. Polyterpenoids have more than eight isoprenoid units.

[0120] As used herein, “isoprenoid precursor” refers to any molecule that is used by organisms in the biosynthesis of terpenoids or isoprenoids. Non-limiting examples of isoprenoid precursor molecules include, *e.g.*, isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP).

[0121] As used herein, the term “mass yield” refers to the mass of the product produced by the cells (such as bacterial cells) divided by the mass of the glucose consumed by the cells (such as bacterial cells) multiplied by 100.

[0122] By "specific productivity," it is meant the mass of the product produced by the cells (such as bacterial cells) divided by the product of the time for production, the cell density, and the volume of the culture.

[0123] By "titer," it is meant the mass of the product produced by the cells (such as bacterial cells) divided by the volume of the culture.

[0124] As used herein, the term "cell productivity index (CPI)" refers to the mass of the product produced by the cells (such as bacterial cells) divided by the mass of the cells (such as bacterial cells) produced in the culture.

[0125] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains.

[0126] As used herein, the singular terms "a," "an," and "the" include the plural reference unless the context clearly indicates otherwise.

[0127] It is intended that every maximum numerical limitation given throughout this specification includes every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

Recombinant cells (such as bacterial cells) capable of increased production of mevalonate

[0128] The mevalonate-dependent biosynthetic pathway (MVA pathway) is a key metabolic pathway present in all higher eukaryotes and certain bacteria. In addition to being important for the production of molecules used in processes as diverse as protein prenylation, cell membrane maintenance, protein anchoring, and N-glycosylation, the mevalonate pathway provides a major source of the isoprenoid precursor molecules DMAPP and IPP, which serve as the basis for the biosynthesis of terpenes, terpenoids, isoprenoids, and isoprene.

[0129] In the upper portion of the MVA pathway, acetyl Co-A produced during cellular metabolism is converted to mevalonate via the actions of polypeptides having thiolase, HMG-CoA reductase, and HMG-CoA synthase enzymatic activity. First, acetyl Co-A is converted to acetoacetyl CoA via the action of a thiolase. Next, acetoacetyl CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the enzymatic action of HMG-CoA synthase. This Co-A derivative is reduced to mevalonate by HMG-CoA reductase, which is the rate-limiting step of the mevalonate pathway of isoprenoid production. Mevalonate is then converted into mevalonate-5-phosphate via the action of mevalonate kinase which is subsequently transformed into mevalonate-5-pyrophosphate by the enzymatic activity of phosphomevalonate kinase. Finally, IPP is formed from mevalonate-5-pyrophosphate by the activity of the enzyme mevalonate-5-pyrophosphate decarboxylase.

[0130] In some aspects, modulation of the any of the enzymes referred to herein can affect the expression (e.g., transcription or translation), production, post-translational modification or any other function of the enzyme. In some embodiments, the function of the enzyme (e.g., catalytic ability) in recombinant cells is increased or decreased as compared to a cell that has not been engineered for such modulation. In one embodiment, the function of the enzyme (e.g. activity) is increased as compared to a cell that has not been engineered. In another embodiment, the function of the enzyme (e.g. activity) is decreased as compared to a cell that has not been engineered.

Citrate Synthase Pathway

[0131] Citrate synthase catalyzes the condensation of oxaloacetate and acetyl-CoA to form citrate, a metabolite of the Tricarboxylic acid (TCA) cycle (Ner, S. et al. 1983. *Biochemistry*, 22: 5243-5249; Bhayana, V. and Duckworth, H. 1984. *Biochemistry* 23: 2900-2905) (**Figure 5**). In *E. coli*, this enzyme, encoded by *gltA*, behaves like a trimer of dimeric subunits. The hexameric form allows the enzyme to be allosterically regulated by NADH. This enzyme has been widely studied (Wiegand, G., and Remington, S. 1986. *Annual Rev. Biophysics Biophys. Chem.*15: 97-117; Duckworth et al. 1987. *Biochem Soc Symp*. 54:83-92; Stockell, D. et al. 2003. J. Biol. Chem. 278: 35435-43; Maurus, R. et al. 2003. *Biochemistry*. 42:5555-5565). To avoid allosteric inhibition by NADH, replacement by or supplementation with the *Bacillus subtilis* NADH-insensitive citrate synthase has been considered (Underwood et al. 2002. *Appl. Environ. Microbiol.* 68:1071-1081; Sanchez et al. 2005. *Met. Eng*. 7:229-239).

[0132] The reaction catalyzed by citrate synthase is directly competing with the thiolase catalyzing the first step of the mevalonate pathway, as they both have acetyl-CoA as a substrate (Hedl et al. 2002. *J. Bact.* 184:2116-2122). Therefore, one of skill in the art can modulate citrate synthase expression (*e.g.*, decrease enzyme activity) to allow more carbon to flux into the mevalonate pathway, thereby increasing the eventual production of mevalonate, isoprene and isoprenoids. Decrease of citrate synthase activity can be any amount of reduction of specific activity or total activity as compared to when no manipulation has been effectuated. In some instances, the decrease of enzyme activity is decreased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%. In some aspects, the activity of citrate synthase is modulated by decreasing the activity of an endogenous citrate synthase gene. This can be accomplished by chromosomal replacement of an endogenous citrate synthase gene with a transgene encoding an NADH-insensitive citrate synthase or by using a transgene encoding an NADH-insensitive citrate synthase that is derived from *Bacillus subtilis.* The activity of citrate synthase can also be modulated (*e.g.*, decreased) by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter. The decrease of the activity of citrate synthase can result in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have decreased expression of citrate synthase.

Pathways involving Phosphotransacetylase and/or Acetate Kinase

[0133] Phosphotransacetylase (*pta*) (Shimizu et al. 1969. Biochim. Biophys. Acta 191: 550-558) catalyzes the reversible conversion between acetyl-CoA and acetylphosphate (acetyl-P), while acetate kinase (ackA) (Kakuda, H. et al. 1994. J. Biochem. 11:916-922) uses acetyl-P to form acetate. These genes can be transcribed as an operon in *E. coli*. Together, they catalyze the dissimilation of acetate, with the release of ATP. Thus, one of skill in the art can increase the amount of available acetyl Co-A by attenuating the activity of phosphotransacetylase gene (*e.g.*, the endogenous phosphotransacetylase gene) and/or an acetate kinase gene (*e.g.*, the endogenous acetate kinase gene). One way of achieving attenuation is by deleting phosphotransacetylase (pta) and/or acetate kinase (ackA). This can be accomplished by replacing one or both genes with a chloramphenicol cassette followed by looping out of the cassette. Acetate is produced by *E. coli* for a variety of reasons (Wolfe, A. 2005. Microb. Mol. Biol. Rev. 69:12-50). Without

being bound by theory, since ackA-pta use acetyl-CoA, deleting those genes might allow carbon not to be diverted into acetate and to increase the yield of mevalonate, isoprene or isoprenoids.

[0134] In some aspects, the recombinant microorganism produces decreased amounts of acetate in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression. Decrease in the amount of acetate produced can be measured by routine assays known to one of skill in the art. The amount of acetate reduction is at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% as compared when no molecular manipulations are done.

[0135] The activity of phosphotransacetylase (pta) and/or acetate kinase (ackA) can also be decreased by other molecular manipulation of the enzymes. The decrease of enzyme activity can be any amount of reduction of specific activity or total activity as compared to when no manipulation has been effectuated. In some instances, the decrease of enzyme activity is decreased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%.

[0136] In some cases, attenuating the activity of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression.

Pathways Involving Lactate Dehydrogenase

[0137] In *E. coli*, D-Lactate is produced from pyruvate through the enzyme lactate dehydrogenase (ldhA – **Figure 5**) (Bunch, P. et al. 1997. Microbiol. 143:187-195). Production of lactate is accompanied with oxidation of NADH, hence lactate is produced when oxygen is limited and cannot accommodate all the reducing equivalents. Thus, production of lactate could be a source for carbon consumption. As such, to improve carbon flow through to mevalonate production (and isoprene, isoprenoid precursor and isoprenoids production, if desired), one of

skill in the art can modulate the activity of lactate dehydrogenase, such as by decreasing the activity of the enzyme.

[0138] Accordingly, in one aspect, the activity of lactate dehydrogenase can be modulated by attenuating the activity of an endogenous lactate dehydrogenase gene. Such attenuation can be achieved by deletion of the endogenous lactate dehydrogenase gene. Other ways of attenuating the activity of lactate dehydrogenase gene known to one of skill in the art may also be used. By manipulating the pathway that involves lactate dehydrogenase, the recombinant microorganism produces decreased amounts of lactate in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression. Decrease in the amount of lactate produced can be measured by routine assays known to one of skill in the art. The amount of lactate reduction is at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% as compared when no molecular manipulations are done.

[0139] The activity of lactate dehydrogenase can also be decreased by other molecular manipulations of the enzyme. The decrease of enzyme activity can be any amount of reduction of specific activity or total activity as compared to when no manipulation has been effectuated. In some instances, the decrease of enzyme activity is decreased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%.

[0140] Accordingly, in some cases, attenuation of the activity of the endogenous lactate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression.

Pathways Involving Malic enzyme

[0141] Malic enzyme (in *E. coli sfcA* and *maeB*) is an anaplerotic enzyme that catalyzes the conversion of malate into pyruvate (using NAD+ or NADP+) by the equation below:

$$\text{(S)-malate} + \text{NAD(P)}^+ \rightleftharpoons \text{pyruvate} + CO_2 + \text{NAD(P)H}$$

[0142] Thus, the two substrates of this enzyme are (S)-malate and $NAD(P)^+$, whereas its 3 products are pyruvate, CO_2, and NADPH.

[0143] Expression of the NADP-dependent malic enzyme (maeB – **Figure 5**) (Iwikura, M. et al. 1979. J. Biochem. 85: 1355-1365) can help increase mevalonate, isoprene, isoprenoid precursors and isoprenoids yield by 1) bringing carbon from the TCA cycle back to pyruvate, direct precursor of acetyl-CoA, itself direct precursor of the mevalonate pathway and 2) producing extra NADPH which could be used in the HMG-CoA reductase reaction (Oh, MK et al. (2002) *J. Biol. Chem.* 277: 13175-13183; Bologna, F. et al. (2007) J. Bact. 189:5937-5946).

[0144] As such, more starting substrate (pyruvate or acetyl-CoA) for the downstream production of mevalonate, isoprene, isoprenoid precursors and isoprenoids can be achieved by modulating, such as increasing, the activity and/or expression of malic enzyme. The NADP-dependent malic enzyme gene can be an endogenous gene. One non-limiting way to accomplish this is by replacing the endogenous NADP-dependent malic enzyme gene promoter with a synthetic constitutively expressing promoter. Another non-limiting way to increase enzyme activity is by using one or more heterologous nucleic acids encoding an NADP-dependent malic enzyme polypeptide. One of skill in the art can monitor the expression of maeB RNA during fermentation or culturing using readily available molecular biology techniques.

[0145] Accordingly, in some embodiments, the recombinant microorganism produces increased amounts of pyruvate in comparison to microorganisms that do not have increased expression of an NADP-dependent malic enzyme gene. In some aspects, increasing the activity of an NADP-dependent malic enzyme gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have increased NADP-dependent malic enzyme gene expression.

[0146] Increase in the amount of pyruvate produced can be measured by routine assays known to one of skill in the art. The amount of pyruvate increase can be at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% as compared when no molecular manipulations are done.

[0147] The activity of malic enzyme can also be increased by other molecular manipulations of the enzyme. The increase of enzyme activity can be any amount of increase of specific activity or total activity as compared to when no manipulation has been effectuated. In some instances, the increase of enzyme activity is at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%.

Pathways Involving Pyruvate Dehydrogenase Complex

[0148] The pyruvate dehydrogenase complex, which catalyzes the decarboxylation of pyruvate into acetyl-CoA, is composed of the proteins encoded by the genes aceE, aceF and lpdA. Transcription of those genes is regulated by several regulators. Thus, one of skill in the art can increase acetyl-CoA by modulating the activity of the pyruvate dehydrogenase complex. Modulation can be to increase the activity and/or expression (*e.g.*, constant expression) of the pyruvate dehydrogenase complex. This can be accomplished by different ways, for example, by placing a strong constitutive promoter, like PL.6 (aattcatataaaaaacatacagataaccatctgcggtgataaattatctctggcggtgttgacataaataccactggcggtgatactgagcacatcagcaggacgcactgaccaccatgaaggtg - lambda promoter, GenBank NC_001416), in front of the operon or using one or more synthetic constitutively expressing promoters.

[0149] Accordingly, in one aspect, the activity of pyruvate dehydrogenase is modulated by increasing the activity of one or more genes of the pyruvate dehydrogenase complex consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase. It is understood that any one, two or three of these genes can be manipulated for increasing activity of pyruvate dehydrogenase. In another aspect, the activity of the pyruvate dehydrogenase complex can be modulated by attenuating the activity of an endogenous pyruvate dehydrogenase complex repressor gene, further detailed below. The activity of an endogenous pyruvate dehydrogenase complex repressor can be attenuated by deletion of the endogenous pyruvate dehydrogenase complex repressor gene.

[0150] In some cases, one or more genes of the pyruvate dehydrogenase complex are endogenous genes. Another way to increase the activity of the pyruvate dehydrogenase complex is by introducing into the microorganism one or more heterologous nucleic acids encoding one

or more polypeptides from the group consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase.

[0151] By using any of these methods, the recombinant microorganism can produce increased amounts of acetyl Co-A in comparison to microorganisms wherein the activity of pyruvate dehydrogenase is not modulated. Modulating the activity of pyruvate dehydrogenase can result in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have modulated pyruvate dehydrogenase expression.

Combinations of Mutations

[0152] It is understood that for any of the enzymes and/or enzyme pathways described herein, molecular manipulations that modulate any combination (two, three, four, five or six) of the enzymes and/or enzyme pathways described herein is expressly contemplated. For ease of the recitation of the combinations, citrate synthase (*gltA*) is designated as A, phosphotransacetylase (*ptaB*) is designated as B, acetate kinase (*ackA*) is designated as C, lactate dehydrogenase (*ldhA*) is designated as D, malic enzyme (*sfcA* or *maeB*) is designated as E, pyruvate decarboxylase (*aceE*, *aceF*, and/or *lpdA*) is designated as F, 6-phosphogluconolactonase *(ybhE)* is designated as G, and phosphoenolpyruvate carboxylase (*ppl*) is designated as H. As discussed above, aceE, aceF, and/or lpdA enzymes of the pyruvate decarboxylase complex can be used singly, or two of three enzymes, or three of three enzymes for increasing pyruvate decarboxylase activity.

[0153] Accordingly, for combinations of any two of the enzymes A-H, non-limiting combinations that can be used are: AB, AC, AD, AE, AF, AG, AH, BC, BD, BE, BF, BG, BH, CD, CE, CF, CG, CH, DE, DF, DG, DH, EF, EG, EH, and GH. For combinations of any three of the enzymes A-H, non-limiting combinations that can be used are: ABC, ABD, ABE, ABF, ABG, ABH, BCD, BCE, BCF, BCG, BCH, CDE, CDF, CDG, CDH, DEF, DEH, ACD, ACE, ACF, ACG, ACH, ADE, ADF, ADG, ADH, AEF, AEG, AEH, BDE, BDF, BDG, BDH, BEF, BEG, BEH, CEF, CEG, CEH, CFG, CFH, and CGH. For combinations of any four of the enzymes A-H, non-limiting combinations that can be used are: ABCD, ABCE, ABCF, ABCG, ABCH, ABDE, ABDF, ABDG, ABDH, ABEF, ABEG, ABEH, BCDE, BCDF, BCDG, BCDH, CDEF, CDEG, CDEH, ACDE, ACDF, ACDG, ACDH, ACEF, ACEG, ACEH, BCEF, BDEF, BGEF, BHEF, ADEF. For combinations of any five of the enzymes A-H, non-limiting combinations that can be used are: ABCDE, ABCDF, ABCDG, ABCDH, ABDEF, ABDEG,

ABDEH, BCDEF, BCDEG, BCDEH, ACDEF, ACDEG, ACEDH, ABCEF, ABCEG, and ABCEH. For combinations of any six of the enzymes A-H, non-limiting combinations that can be used are: ABCDEF, ABCDEG, ABCDEH, BCDEFG, BCDEFH, and CDEFGH. For combinations of any seven of the enzymes A-H, non-limiting combinations that can be used are: ABCDEFG, ABCDEFH, BCDEFGH. In another aspect, all eight enzyme combinations are used ABCDEFGH.

[0154] Accordingly, the recombinant microorganism as described herein can achieve increased mevalonate production that is increased compared to microorganisms that are not grown under conditions of tri-carboxylic acid (TCA) cycle activity, wherein metabolic carbon flux in the recombinant microorganism is directed towards mevalonate production by modulating the activity of one or more enzymes from the group consisting of (a) citrate synthase, (b) phosphotransacetylase and/or acetate kinase, (c) lactate dehydrogenase, (d) malic enzyme, and (e) pyruvate decarboxylase complex.

Other Regulators and Factors for Increased Production

[0155] Other molecular manipulations can be used to increase the flow of carbon towards mevalonate production. One method is to reduce, decrease or eliminate the effects of negative regulators for pathways that feed into the mevalonate pathway. For example, in some cases, the genes *aceEF-lpdA* are in an operon, with a fourth gene upstream *pdhR*. pdhR is a negative regulator of the transcription of its operon. In the absence of pyruvate, it binds its target promoter and represses transcription. It also regulates ndh and cyoABCD in the same way (Ogasawara, H. et al. 2007. *J. Bact.* 189:5534-5541). In one aspect, deletion of pdhR regulator can improve the supply of pyruvate, and hence the production of mevalonate, isoprene, isoprenoid precursors, and isoprenoids.

[0156] In other aspects, the introduction of 6-phosphogluconolactonase (PGL) into microorganisms (such as various *E. coli* strains) which lack PGL can be used to improve production of mevalonate, isoprene, isoprenoid precursors, and isoprenoids. PGL may be introduced using chromosomal integration or extra-chromosomal vehicles, such as plasmids. In yet other aspects, PGL may be deleted from the genome of cells (for example, microorganisms, such as various *E. coli* strains) which express a PGL to improve production of mevalonate and/or isoprene. In another aspect, a heterologous nucleic acid encoding a PGL polypeptide can

be expressed in a cell which does not endogenously express PGL. In some aspects, deletion of PGL results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, such as any values in between these percentages, higher percent yield of isoprene in comparison to microorganisms that express PGL. In other aspects, deletion of PGL results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher instantaneous percent yield of isoprene in comparison to microorganisms that express PGL. In other aspects, deletion of PGL results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher cell productivity index for isoprene in comparison to microorganisms that express PGL. In other aspects, deletion of PGL results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher volumetric productivity of isoprene in comparison to microorganisms that express PGL. In other aspects, deletion of PGL results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher peak specific productivity of isoprene in comparison to microorganisms that express PGL. In some aspects the deletion of PGL results in peak specific productivity being maintained for a longer period of time in comparison to microorganisms that express PGL.

[0157] In another aspect, modulation of phosphoenolpyruvate carboxylase (*ppc* in *E. coli*) gene expression can be used to improve production of mevalonate, isoprene, isoprenoid precursors, and isoprenoids in any of the cells disclosed herein. In one aspect, the gene expression of phosphoenolpyruvate carboxylase can be decreased by replacing the promoter sequence of the *ppc* gene with another promoter that results in decreased *ppc* gene expression in comparison to wild type cells. In some aspects, *ppc* gene expression can be decreased by any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, in comparison to wild type cells. In some aspects, decreased expression of phosphoenolpyruvate carboxylase results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, such as any values in between these percentages, higher percent yield of isoprene in comparison to microorganisms that express phosphoenolpyruvate carboxylase at wild type levels. In other aspects, decreased expression of phosphoenolpyruvate carboxylase results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher

instantaneous percent yield of isoprene in comparison to microorganisms that express phosphoenolpyruvate carboxylase at wild type levels. In other aspects, decreased expression of phosphoenolpyruvate carboxylase results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher cell productivity index for isoprene in comparison to microorganisms that express phosphoenolpyruvate carboxylase at wild type levels. In other aspects, decreased expression of phosphoenolpyruvate carboxylase results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher volumetric productivity of isoprene in comparison to microorganisms that express phosphoenolpyruvate carboxylase at wild type levels. In other aspects, decreased expression of phosphoenolpyruvate carboxylase results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher peak specific productivity of isoprene in comparison to microorganisms that express phosphoenolpyruvate carboxylase at wild type levels. In some aspects decreased expression of phosphoenolpyruvate carboxylase results in peak specific productivity being maintained for a longer period of time in comparison to microorganisms that express phosphoenolpyruvate carboxylase at wild type levels.

[0158] In another aspect, modulation of the inhibitor of RssB activity during magnesium starvation (*iraM* in *E. coli*) gene expression can be used to improve production of mevalonate, isoprene, isoprenoid precursors, and isoprenoids in any of the cells disclosed herein. In one aspect, the gene expression of *iraM* can be increased by replacing the promoter sequence of the *iraM* gene with another promoter that results in increased *iraM* gene expression in comparison to wild type cells. In some aspects, *iraM* gene expression can be increased by any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, in comparison to wild type cells. In some aspects, increased expression of the *iraM* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, such as any values in between these percentages, higher percent yield of isoprene in comparison to microorganisms that express the *iraM* gene at wild type levels. In other aspects, increased expression of the *iraM* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher instantaneous percent yield of isoprene in comparison to microorganisms that express the *iraM* gene at wild type levels. In other aspects, increased

expression of the *iraM* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher cell productivity index for isoprene in comparison to microorganisms that express the *iraM* gene at wild type levels. In other aspects, increased expression of the *iraM* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher volumetric productivity of isoprene in comparison to microorganisms that express the *iraM* gene at wild type levels. In other aspects, increased expression of the *iraM* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher peak specific productivity of isoprene in comparison to microorganisms that express the *iraM* gene at wild type levels. In some aspects increased expression of the *iraM* gene results in peak specific productivity being maintained for a longer period of time in comparison to microorganisms that express the *iraM* gene at wild type levels.

[0159] In another aspect, modulation of the acrA component of the multidrug efflux pump acrAB-TolC (*acrA* in *E. coli*) gene expression can be used to improve production of mevalonate, isoprene, isoprenoid precursors, and isoprenoids in any of the cells disclosed herein. In one aspect, the gene expression of *acrA* can be decreased by replacing the promoter sequence of the *acrA* gene with another promoter that results in decreased *acrA* gene expression in comparison to wild type cells. In some aspects, *acrA* gene expression can be decreased by any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, in comparison to wild type cells. In another aspect, expression of *acrA* can be completely abolished, such as by deleting, the *acrA* gene in the genome of the cell, so that it no longer produces a functional acrA protein. In some aspects, deletion or decreased expression of the *acrA* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, such as any values in between these percentages, higher percent yield of isoprene in comparison to microorganisms that express the *acrA* gene at wild type levels. In other aspects, deletion or decreased expression of the *acrA* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher instantaneous percent yield of isoprene in comparison to microorganisms that express the *acrA* gene at wild type levels. In other aspects, deletion or decreased expression of the *acrA* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these

percentages, higher cell productivity index for isoprene in comparison to microorganisms that express the *acrA* gene at wild type levels. In other aspects, deletion or decreased expression of the *acrA* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher volumetric productivity of isoprene in comparison to microorganisms that express the *acrA* gene at wild type levels. In other aspects, deletion or decreased expression of the *acrA* gene results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher peak specific productivity of isoprene in comparison to microorganisms that express the *acrA* gene at wild type levels. In some aspects deletion or decreased expression of the *acrA* gene results in peak specific productivity being maintained for a longer period of time in comparison to microorganisms that express the *acrA* gene at wild type levels.

[0160] In another aspect, modulation of FNR DNA binding transcriptional regulator (FNR) gene expression can be used to improve production of mevalonate, isoprene, isoprenoid precursors, and isoprenoids in any of the cells disclosed herein. In one aspect, the gene expression of FNR can be increased by replacing the promoter sequence of the gene which encodes FNR with another promoter that results in increased FNR expression in comparison to wild type cells. In other aspects, a heterologous nucleic acid encoding FNR can be expressed in a cell that does not endogenously express FNR. In some aspects, FNR expression can be increased by any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, in comparison to wild type cells or cells that do not endogenously express FNR. In some aspects, increased FNR expression results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, such as any values in between these percentages, higher percent yield of isoprene in comparison to wild type cells or cells that do not endogenously express FNR. In other aspects, increased FNR expression results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher instantaneous percent yield of isoprene in comparison to in comparison to wild type cells or cells that do not endogenously express FNR. In other aspects, increased FNR expression results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher cell productivity index for isoprene in comparison to wild type cells or cells that do not endogenously express FNR. In other aspects, increased FNR expression results

in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher volumetric productivity of isoprene in comparison to wild type cells or cells that do not endogenously express FNR. In other aspects, increased FNR expression results in any of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, inclusive, including any values in between these percentages, higher peak specific productivity of isoprene in comparison to wild type cells or cells that do not endogenously express FNR. In some aspects increased FNR expression results in peak specific productivity being maintained for a longer period of time i in comparison to wild type cells or cells that do not endogenously express FNR.

[0161] In addition to the host cell (*e.g.*, microorganism) mutations for modulating various enzymatic pathways described herein that increase carbon flux towards mevalonate production, host cells expressing one or more copies of a heterologous nucleic acid encoding upper MVA pathway polypeptides can be used in conjunction with the host cell mutations to increase the production of desired end products, such as mevalonate, isoprene, isoprenoid precursors, and isoprenoids. In another embodiment, genes encoding *mvaE* and *mvaS* from various species can be used in conjunction with the host cell mutations to increase the production of desired end products, such as mevalonate, isoprene, isoprenoid precursors, and isoprenoids.

[0162] In addition, other enzymes from the upper and lower MVA pathway may be used as well as the *mvaE* and *mvaS* gene products. Non-limiting examples of MVA pathway polypeptides include acetyl-CoA acetyltransferase (AA-CoA thiolase) polypeptides, 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) polypeptides, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) polypeptides, mevalonate kinase (MVK) polypeptides, phosphomevalonate kinase (PMK) polypeptides, diphosphomevalonte decarboxylase (MVD) polypeptides, phosphomevalonate decarboxylase (PMDC) polypeptides, isopentenyl phosphate kinase (IPK) polypeptides, IDI polypeptides, and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more MVA pathway polypeptides. MVA pathway polypeptides can 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.

[0163] Non-limiting examples of MVA pathway polypeptides which can be used are described in International Patent Application Publication No. WO2009/076676; WO2010/003007 and WO2010/148150.

Genes encoding mvaE and mvaS polypeptides

[0164] In some microorganisms (such as, but not limited to, *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and *E. faecalis*), the *mvaE* gene encodes a polypeptide that possesses both thiolase and HMG-CoA reductase activities. In fact, the *mvaE* gene product represented the first bifunctional enzyme of IPP biosynthesis found in eubacteria and the first example of HMG-CoA reductase fused to another protein in nature (Hedl, et al., *J Bacteriol.* 2002 April; 184(8): 2116–2122). The *mvaS* gene, on the other hand, encodes a polypeptide having an HMG-CoA synthase activity. The *mvaE* and *mvaS* genes of a different bacterial species, *E. faecalis*, have been incorporated into *E. coli* strains previously to produce mevalonate (*see* US 2005/0287655 A1, the disclosure of which is incorporated by reference herein; Tabata, K. and Hashimoto,S.-I. *Biotechnology Letters* 26: 1487–1491, 2004).

[0165] Accordingly, cells (such as bacterial cells, *e.g.*, *E. coli*) can be engineered to express one or more *mvaE* and *mvaS* genes (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), to increase production, peak titer, and cell productivity of mevalonate. The one or more *mvaE* and *mvaS* genes can be expressed on a multicopy plasmid. The plasmid can be a high copy plasmid, a low copy plasmid, or a medium copy plasmid. Alternatively, the one or more *mvaE* and *mvaS* genes can be integrated into the host cell's chromosome. For both heterologous expression of the one or more *mvaE* and *mvaS* genes on a plasmid or as an integrated part of the host cell's chromosome, expression of the genes can be driven by either an inducible promoter or a constitutively expressing promoter. The promoter can be a strong driver of expression, it can be a weak driver of expression, or it can be a medium driver of expression of the one or more *mvaE* and *mvaS* genes.

[0166] Any genes encoding an upper MVA pathway polypeptide can be used in the present invention. In certain embodiments, various options of *mvaE* and *mvaS* genes (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) alone or in combination with one or more other *mvaE* and *mvaS* genes encoding proteins from the upper MVA pathway are contemplated within the scope of the invention. Thus, in certain aspects, any of the combinations of genes contemplated in **Table 1** can be expressed in cells (such as bacterial cells) in any of the ways described above.

Table 1: Options for expression of *mvaE* and *mvaS* genes in host cells contemplated for the present invention.

	L. grayi, mvaE	***E. faecium, mvaE***	***E. gallinarum, mvaE***	***E. casseliflavus, mvaE***	***E. faecalis, mvaE***
L. grayi, mvaS	*L. grayi, mvaE L. grayi, mvaS*	*E. faecium, mvaE L. grayi, mvaS*	*E. gallinarum, mvaE L. grayi, mvaS*	*E. casseliflavus, mvaE L. grayi, mvaS*	*E. faecalis, mvaE L. grayi, mvaS*
E. faecium, mvaS	*L. grayi, mvaE E. faecium, mvaS*	*E. faecium, mvaE E. faecium, mvaS*	*E. gallinarum, mvaE E. faecium, mvaS*	*E. casseliflavus, mvaE E. faecium, mvaS*	*E. faecalis, mvaE E. faecium, mvaS*
E. gallinarum, mvaS	*L. grayi, mvaE E. gallinarum, mvaS*	*E. faecium, mvaE E. gallinarum, mvaS*	*E. gallinarum, mvaE E. gallinarum, mvaS*	*E. casseliflavus, mvaE E. gallinarum, mvaS*	*E. faecalis, mvaE E. gallinarum, mvaS*
E. casseliflavus, mvaS	*L. grayi, mvaE E. casseliflavus, mvaS*	*E. faecium, mvaE E. casseliflavus, mvaS*	*E. gallinarum, mvaE E. casseliflavus, mvaS*	*E. casseliflavus, mvaE E. casseliflavus, mvaS*	*E. faecalis, mvaE E. casseliflavus, mvaS*
E. faecalis, mvaS	*L. grayi, mvaE E. faecalis, mvaS*	*E. faecium, mvaE E. faecalis, mvaS*	*E. gallinarum, mvaE E. faecalis, mvaS*	*E. casseliflavus, mvaE E. faecalis, mvaS*	*E. faecalis, mvaE E. faecalis, mvaS*

Exemplary mvaE polypeptides and nucleic acids

[0167] The *mvaE* gene encodes a polypeptide that possesses both thiolase and HMG-CoA reductase activities. The thiolase activity of the polypeptide encoded by the *mvaE* gene converts acetyl Co-A to acetoacetyl CoA whereas the HMG-CoA reductase enzymatic activity of the polypeptide converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate. Exemplary mvaE

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 that have at least one activity of a mvaE polypeptide.

[0168] Mutant mvaE polypeptides include those in which one or more amino acid residues have undergone an amino acid substitution while retaining mvaE polypeptide activity (*i.e.*, the ability to convert acetyl Co-A to acetoacetyl CoA as well as the ability to convert 3-hydroxy-3-methylglutaryl-CoA to mevalonate). The amino acid substitutions can be conservative or non-conservative and such substituted amino acid residues can or can not be one encoded by the genetic code. The standard twenty amino acid “alphabet” has been divided into chemical families based on similarity of their side chains. Those families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a chemically similar side chain (*i.e.*, replacing an amino acid having a basic side chain with another amino acid having a basic side chain). A “non-conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a chemically different side chain (*i.e.*, replacing an amino acid having a basic side chain with another amino acid having an aromatic side chain).

[0169] Amino acid substitutions in the mvaE polypeptide can be introduced to improve the functionality of the molecule. For example, amino acid substitutions that increase the binding affinity of the mvaE polypeptide for its substrate, or that improve its ability to convert acetyl Co-A to acetoacetyl CoA and/or the ability to convert 3-hydroxy-3-methylglutaryl-CoA to mevalonate can be introduced into the mvaE polypeptide. In some aspects, the mutant mvaE polypeptides contain one or more conservative amino acid substitutions.

[0170] In one aspect, mvaE proteins that are not degraded or less prone to degradation can be used for the production of mevalonate, isoprene, isoprenoid precursors, and/or isoprenoids. Examples of gene products of *mvaEs* that are not degraded or less prone to degradation which

can be used include, but are not limited to, those from the organisms *E. faecium*, *E. gallinarum*, *E. casseliflavus*, *E. faecalis,* and *L. grayi*. One of skill in the art can express mvaE protein in *E. coli* BL21 (DE3) and look for absence of fragments by any standard molecular biology techniques. For example, absence of fragments can be identified on Safestain stained SDS-PAGE gels following His-tag mediated purification or when expressed in mevalonate, isoprene or isoprenoid producing *E. coli* BL21 using the methods of detection described herein.

[0171] Standard methods, such as those described in Hedl et al., (*J Bacteriol.* 2002, April; 184(8): 2116–2122) can be used to determine whether a polypeptide has mvaE activity, by measuring acetoacetyl-CoA thiolase as well as HMG-CoA reductase activity. In an exemplary assay, acetoacetyl-CoA thiolase activity is measured by spectrophotometer to monitor the change in absorbance at 302 nm that accompanies the formation or thiolysis of acetoacetyl-CoA. Standard assay conditions for each reaction to determine synthesis of acetoacetyl-CoA, are 1 mM acetyl-CoA, 10 mM $MgCl_2$, 50 mM Tris, pH 10.5 and the reaction is initiated by addition of enzyme. Assays can employ a final volume of 200 µl. For the assay, 1 enzyme unit (eu) represents the synthesis or thiolysis in 1 min of 1 µmol of acetoacetyl-CoA. In another exemplary assay, of HMG-CoA reductase activity can be monitored by spectrophotometer by the appearance or disappearance of NADP(H) at 340 nm. Standard assay conditions for each reaction measured to show reductive deacylation of HMG-CoA to mevalonate are 0.4 mM NADPH, 1.0 mM (*R*,*S*)-HMG-CoA, 100 mM KCl, and 100 mM K_xPO_4, pH 6.5. Assays employ a final volume of 200 µl. Reactions are initiated by adding the enzyme. For the assay, 1 eu represents the turnover, in 1 min, of 1 µmol of NADP(H). This corresponds to the turnover of 0.5 µmol of HMG-CoA or mevalonate.

[0172] Alternatively, production of mevalonate in cells (such as bacterial cells) can be measured by, without limitation, gas chromatography (see U.S. Patent Application Publication No.: US 2005/0287655 A1) or HPLC (See U.S. Patent Application No.: 12/978,324). As an exemplary assay, cultures can be inoculated in shake tubes containing LB broth supplemented with one or more antibiotics and incubated for 14h at 34°C at 250 rpm. Next, cultures can be diluted into well plates containing TM3 media supplemented with 1% Glucose, 0.1% yeast extract, and 200 µM IPTG to final OD of 0.2. The plate are then sealed with a Breath Easier membrane (Diversified Biotech) and incubated at 34°C in a shaker/incubator at 600 rpm for 24 hours. 1 mL of each culture is then centrifuged at 3,000 x g for 5 min. Supernatant is then added

to 20% sulfuric acid and incubated on ice for 5 min. The mixture is then centrifuged for 5 min at 3000 x g and the supernatant was collected for HPLC analysis. The concentration of mevalonate in samples is determined by comparison to a standard curve of mevalonate (Sigma). The glucose concentration can additionally be measured by performing a glucose oxidase assay according to any method known in the art. Using HPLC, levels of mevalonate can be quantified by comparing the refractive index response of each sample versus a calibration curve generated by running various mevalonate containing solutions of known concentration.

[0173] Exemplary mvaE 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 mvaE polypeptide. Exemplary mvaE 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 mvaE nucleic acids include, for example, mvaE nucleic acids isolated from Listeria grayi DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2, and/or Enterococcus casseliflavus. The mvaE nucleic acid encoded by the Listeria grayi DSM 20601 mvaE gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85% sequence identity to SEQ ID NO:1. In another aspect, the mvaE nucleic acid encoded by the Listeria grayi DSM 20601 mvaE gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:1. The mvaE nucleic acid encoded by the Enterococcus faecium mvaE gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:3. In another aspect, the mvaE nucleic acid encoded by the Enterococcus faecium mvaE gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:3. The mvaE nucleic acid encoded by the Enterococcus gallinarum EG2 mvaE gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:5. In another aspect, the mvaE nucleic acid encoded by the Enterococcus gallinarum EG2 mvaE gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:5. The mvaE nucleic acid encoded by the Enterococcus casseliflavus mvaE gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:7. In another aspect, the mvaE nucleic acid encoded by the Enterococcus casseliflavus mvaE gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:7. In any of the

aspects herein, the upper MVA pathway polypeptides may be encoded by a nucleic acid with at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, or 80% sequence identity to any one of SEQ ID NOs:1-8. In any of the aspects herein, the upper MVA pathway polypeptides may be encoded by a nucleic acid with of any one of SEQ ID NOs:1-8.

[0174] Exemplary mvaE polypeptides include fragments of a polypeptide, peptide, or fusion polypeptide that has at least one activity of an mvaE polypeptide. Exemplary mvaE polypeptides and include naturally-occurring polypeptides from any of the source organisms described herein as well as mutant polypeptides derived from any of the source organisms described herein. Exemplary mvaE polypeptides include, for example, mvaE polypeptides isolated from Listeria grayi DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2, and/or Enterococcus casseliflavus. The mvaE polypeptide encoded by the Listeria grayi DSM 20601 mvaE gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85% sequence identity to SEQ ID NO:13. In another aspect, the mvaE polypeptide encoded by the Listeria grayi DSM 20601 mvaE gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:13. The mvaE polypeptide encoded by the Enterococcus faecium mvaE gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:15. In another aspect, the mvaE polypeptide encoded by the Enterococcus faecium mvaE gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:15. The mvaE polypeptide encoded by the Enterococcus gallinarum EG2 mvaE gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:11. In another aspect, the mvaE polypeptide encoded by the Enterococcus gallinarum EG2 mvaE gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:11. The mvaE polypeptide encoded by the Enterococcus casseliflavus mvaE gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:17. In another aspect, the mvaE polypeptide encoded by the Enterococcus casseliflavus mvaE gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:17. In any of the aspects herein, the upper MVA pathway polypeptides may be encoded by a polypeptide with at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, or 80% sequence identity to any one of SEQ ID NOs:11-18. In any

of the aspects herein, the upper MVA pathway polypeptides may be encoded by a polypeptide with any one of SEQ ID NOs:11-18.

[0175] The mvaE nucleic acid can be expressed in a cell (such as a bacterial cell) on a multicopy plasmid. The plasmid can be a high copy plasmid, a low copy plasmid, or a medium copy plasmid. Alternatively, the mvaE nucleic acid can be integrated into the host cell's chromosome. For both heterologous expression of an mvaE nucleic acid on a plasmid or as an integrated part of the host cell's chromosome, expression of the nucleic acid can be driven by either an inducible promoter or a constitutively expressing promoter. The promoter can be a strong driver of expression, it can be a weak driver of expression, or it can be a medium driver of expression of the mvaE nucleic acid.

Exemplary mvaS polypeptides and nucleic acids

[0176] The *mvaS* gene encodes a polypeptide that possesses HMG-CoA synthase activity. This polypeptide can convert acetoacetyl CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Exemplary mvaS 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 that have at least one activity of a mvaS polypeptide.

[0177] Mutant mvaS polypeptides include those in which one or more amino acid residues have undergone an amino acid substitution while retaining mvaS polypeptide activity (*i.e.*, the ability to convert acetoacetyl CoA to 3-hydroxy-3-methylglutaryl-CoA). Amino acid substitutions in the mvaS polypeptide can be introduced to improve the functionality of the molecule. For example, amino acid substitutions that increase the binding affinity of the mvaS polypeptide for its substrate, or that improve its ability to convert acetoacetyl CoA to 3-hydroxy-3-methylglutaryl-CoA can be introduced into the mvaS polypeptide. In some aspects, the mutant mvaS polypeptides contain one or more conservative amino acid substitutions.

[0178] Standard methods, such as those described in Quant et al. (*Biochem J.*, 1989, 262:159-164), can be used to determine whether a polypeptide has mvaS activity, by measuring HMG-CoA synthase activity. In an exemplary assay, HMG-CoA synthase activity can be assayed by spectrophotometrically measuring the disappearance of the enol form of acetoacetyl-CoA by monitoring the change of absorbance at 303 nm. A standard 1 ml assay system containing 5

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mm-Tris/HCl, pH 8.0, 10 mM-MgCl2 and 0.2 mM-dithiothreitol at 30 °C; 5 mM-acetyl phosphate, 10,M-acetoacetyl- CoA and 5 ul samples of extracts can be added, followed by simultaneous addition of acetyl-CoA (100 uM) and 10 units of PTA. HMG-CoA synthase activity is then measured as the difference in the rate before and after acetyl-CoA addition. The absorption coefficient of acetoacetyl-CoA under the conditions used (pH 8.0, 10 mM-MgCl2), is $12.2 \times 10^3 M^{-1} cm^{-1}$. By definition, 1 unit of enzyme activity causes 1 umol of acetoacetyl-CoA to be transformed per minute.

[0179] Alternatively, production of mevalonate in cells (such as bacterial cells) can be measured by, without limitation, gas chromatography (see U.S. Patent Application Publication No.: US 2005/0287655 A1) or HPLC (See U.S. Patent Application No.: 12/978,324). As an exemplary assay, cultures can be inoculated in shake tubes containing LB broth supplemented with one or more antibiotics and incubated for 14h at 34°C at 250 rpm. Next, cultures can be diluted into well plates containing TM3 media supplemented with 1% Glucose, 0.1% yeast extract, and 200 μM IPTG to final OD of 0.2. The plate are then sealed with a Breath Easier membrane (Diversified Biotech) and incubated at 34°C in a shaker/incubator at 600 rpm for 24 hours. 1 mL of each culture is then centrifuged at 3,000 x g for 5 min. Supernatant is then added to 20% sulfuric acid and incubated on ice for 5 min. The mixture is then centrifuged for 5 min at 3000 x g and the supernatant was collected for HPLC analysis. The concentration of mevalonate in samples is determined by comparison to a standard curve of mevalonate (Sigma). The glucose concentration can additionally be measured by performing a glucose oxidase assay according to any method known in the art. Using HPLC, levels of mevalonate can be quantified by comparing the refractive index response of each sample versus a calibration curve generated by running various mevonate containing solutions of known concentration.

[0180] Exemplary mvaS 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 mvaS polypeptide. Exemplary mvaS 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 mvaS nucleic acids include, for example, mvaS nucleic acids isolated from *Listeria grayi_DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2,* and/or *Enterococcus casseliflavus*. The mvaS nucleic acid encoded by the *Listeria grayi_DSM 20601*

mvaS gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:2. The mvaS nucleic acid encoded by the *Listeria grayi_DSM 20601 mvaS* gene can also have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:2. The mvaS nucleic acid encoded by the *Enterococcus faecium mvaS* gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:4. The mvaS nucleic acid encoded by the *Enterococcus faecium mvaS* gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:4. The mvaS nucleic acid encoded by the *Enterococcus gallinarum EG2 mvaS* gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:6. The mvaS nucleic acid encoded by the *Enterococcus gallinarum EG2 mvaS* gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:6. The mvaS nucleic acid encoded by the *Enterococcus casseliflavus mvaS* gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:8. The mvaS nucleic acid encoded by the *Enterococcus casseliflavus mvaS* gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:8.

[0181] Exemplary mvaS polypeptides include fragments of a polypeptide, peptide, or fusion polypeptide that has at least one activity of an mvaS polypeptide. Exemplary mvaS polypeptides include naturally-occurring polypeptides and polypeptides from any of the source organisms described herein as well as mutant polypeptides derived from any of the source organisms described herein. Exemplary mvaS polypeptides include, for example, mvaS polypeptides isolated from *Listeria grayi_DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2,* and/or *Enterococcus casseliflavus.* The mvaS polypeptide encoded by the *Listeria grayi_DSM 20601 mvaS* gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:14. The mvaS polypeptide encoded by the *Listeria grayi_DSM 20601 mvaS* gene can also have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:14. The mvaS polypeptide encoded by the *Enterococcus faecium mvaS* gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:16. The mvaS polypeptide encoded by the *Enterococcus faecium mvaS* gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:16. The mvaS polypeptide encoded by the *Enterococcus gallinarum EG2 mvaS* gene can have at least about

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99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:12. The mvaS polypeptide encoded by the *Enterococcus gallinarum EG2 mvaS* gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:12. The mvaS polypeptide encoded by the *Enterococcus casseliflavus mvaS* gene can have at least about 99%, 98%, 97%, 96%, 95%, 95%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, or 85% sequence identity to SEQ ID NO:18. The mvaS polypeptide encoded by the *Enterococcus casseliflavus mvaS* gene can have at least about 84%, 83%, 82%, 81%, or 80% sequence identity to SEQ ID NO:18.

[0182] The mvaS nucleic acid can be expressed in a cell (such as a bacterial cell) on a multicopy plasmid. The plasmid can be a high copy plasmid, a low copy plasmid, or a medium copy plasmid. Alternatively, the mvaS nucleic acid can be integrated into the host cell's chromosome. For both heterologous expression of an mvaS nucleic acid on a plasmid or as an integrated part of the host cell's chromosome, expression of the nucleic acid can be driven by either an inducible promoter or a constitutively expressing promoter. The promoter can be a strong driver of expression, it can be a weak driver of expression, or it can be a medium driver of expression of the mvaS nucleic acid.

Nucleic acids encoding acetoacetyl-CoA synthase polypeptides

[0183] In one aspect, any of the cells (such as bacterial cells) described herein can contain one or more heterologous nucleic acid(s) encoding an acetoacetyl-CoA synthase polypeptide. The acetoacetyl-CoA synthase gene (a.k.a. *nphT7*) is a gene encoding an enzyme having the activity of synthesizing acetoacetyl-CoA from malonyl-CoA and acetyl-CoA and having minimal activity (*e.g.*, no activity) of synthesizing acetoacetyl-CoA from two acetyl-CoA molecules. *See, e.g.*, Okamura et al., *PNAS* Vol 107, No. 25, pp. 11265-11270 (2010), the contents of which are expressly incorporated herein for teaching about *nphT7*. An acetoacetyl-CoA synthase gene from an actinomycete of the genus *Streptomyces CL190* strain was described in Japanese Patent Publication (Kokai) No. 2008-61506 A and U.S. Patent Application Publication No. 2010/0285549, the disclosure of each of which are incorporated by reference herein. Acetoacetyl-CoA synthase can also be referred to as acetyl CoA:malonyl CoA acyltransferase. A representative acetoacetyl-CoA synthase (or acetyl CoA:malonyl CoA acyltransferase) that can be used is Genbank AB540131.1.

In one aspect, acetoacetyl-CoA synthase of the present invention synthesizes acetoacetyl-CoA from malonyl-CoA and acetyl-CoA via an irreversible reaction. The use of acetoacetyl-CoA synthase to generate acetyl-CoA provides an additional advantage in that this reaction is irreversible while acetoacetyl-CoA thiolase enzyme's action of synthesizing acetoacetyl-CoA from two acetyl-CoA molecules is reversible. Consequently, the use of acetoacetyl-CoA synthase to synthesize acetoacetyl-CoA from malonyl-CoA and acetyl-CoA can result in significant improvement in productivity for isoprene compared with using thiolase to generate the end same product.

[0185] Furthermore, the use of acetoacetyl-CoA synthase to produce isoprene provides another advantage in that acetoacetyl-CoA synthase can convert malonyl CoA to acetyl CoA via decarboxylation of the malonyl CoA. Thus, stores of starting substrate are not limited by the starting amounts of acetyl CoA. The synthesis of acetoacetyl-CoA by acetoacetyl-CoA synthase can still occur when the starting substrate is only malonyl-CoA. In one aspect, the pool of starting malonyl-CoA is increased by using host strains that have more malonyl-CoA. Such increased pools can be naturally occurring or be engineered by molecular manipulation. *See*, for example Fowler, et al., *Applied and Environmental Microbiology*, Vol. 75, No. 18, pp. 5831-5839 (2009).

[0186] In any of the aspects or embodiments described herein, an enzyme that has the ability to synthesize acetoacetyl-CoA from malonyl-CoA and acetyl-CoA can be used. Non-limiting examples of such an enzyme are described herein. In certain embodiments described herein, an acetoacetyl-CoA synthase gene derived from an actinomycete of the genus *Streptomyces* having the activity of synthesizing acetoacetyl-CoA from malonyl-CoA and acetyl-CoA can be used.

[0187] An example of such an acetoacetyl-CoA synthase gene is the gene encoding a protein having the amino acid sequence of SEQ ID NO:19. Such a protein having the amino acid sequence of SEQ ID NO:19 corresponds to an acetoacetyl-CoA synthase having activity of synthesizing acetoacetyl-CoA from malonyl-CoA and acetyl-CoA and having no activity of synthesizing acetoacetyl-CoA from two acetyl-CoA molecules.

[0188] In one embodiment, the gene encoding a protein having the amino acid sequence of SEQ ID NO:19 can be obtained by a nucleic acid amplification method (*e.g.*, PCR) with the use of genomic DNA obtained from an actinomycete of the *Streptomyces* sp. CL190 strain as a template and a pair of primers that can be designed with reference to Japanese Patent Publication (Kokai) No. 2008-61506 A.

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[0189] As described herein, an acetoacetyl-CoA synthase gene for use in the present invention is not limited to a gene encoding a protein having the amino acid sequence of SEQ ID NO:19 from an actinomycete of the *Streptomyces sp. CL190* strain. Any gene encoding a protein having the ability to synthesize acetoacetyl-CoA from malonyl-CoA and acetyl-CoA and which does not synthesize acetoacetyl-CoA from two acetyl-CoA molecules can be used in the presently described methods. In certain embodiments, the acetoacetyl-CoA synthase gene can be a gene encoding a protein having an amino acid sequence with high similarity or substantially identical to the amino acid sequence of SEQ ID NO:19 and having the function of synthesizing acetoacetyl-CoA from malonyl-CoA and acetyl-CoA. The expression "highly similar" or "substantially identical" refers to, for example, at least about 80% identity, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, and at least about 99% identity. As used above, the identity value corresponds to the percentage of identity between amino acid residues in a different amino acid sequence and the amino acid sequence of SEQ ID NO:19, which is calculated by performing alignment of the amino acid sequence of SEQ ID NO:19 and the different amino acid sequence with the use of a program for searching for a sequence similarity.

[0190] In other embodiments, the acetoacetyl-CoA synthase gene may be a gene encoding a protein having an amino acid sequence derived from the amino acid sequence of SEQ ID NO:19 by substitution, deletion, addition, or insertion of 1 or more amino acid(s) and having the function of synthesizing acetoacetyl-CoA from malonyl-CoA and acetyl-CoA. Herein, the expression "more amino acids" refers to, for example, 2 to 30 amino acids, preferably 2 to 20 amino acids, more preferably 2 to 10 amino acids, and most preferably 2 to 5 amino acids.

[0191] In still other embodiments, the acetoacetyl-CoA synthase gene may consist of a polynucleotide capable of hybridizing to a portion or the entirety of a polynucleotide having a nucleotide sequence complementary to the nucleotide sequence encoding the amino acid sequence of SEQ ID NO:19 under stringent conditions and capable of encoding a protein having the function of synthesizing acetoacetyl-CoA from malonyl-CoA and acetyl-CoA. Herein, hybridization under stringent conditions corresponds to maintenance of binding under conditions of washing at 60 °C 2x SSC. Hybridization can be carried out by conventionally known methods such as the method described in J. Sambrook et al. Molecular Cloning, A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory (2001).

[0192] As described herein, a gene encoding an acetoacetyl-CoA synthase having an amino acid sequence that differs from the amino acid sequence of SEQ ID NO:19 can be isolated from potentially any organism, for example, an actinomycete that is not obtained from the *Streptomyces sp. CL190* strain. In addition, acetoacetyl-CoA synthase genes for use herein can be obtained by modifying a polynucleotide encoding the amino acid sequence of SEQ ID NO:19 by a method known in the art. Mutagenesis of a nucleotide sequence can be carried out by a known method such as the Kunkel method or the gapped duplex method or by a method similar to either thereof. For instance, mutagenesis may be carried out with the use of a mutagenesis kit (*e.g.*, product names; Mutant-K and Mutant-G (TAKARA Bio)) for site-specific mutagenesis, product name; an LA PCR in vitro Mutagenesis series kit (TAKARA Bio), and the like.

[0193] The activity of an acetoacetyl-CoA synthase having an amino acid sequence that differs from the amino acid sequence of SEQ ID NO:19 can be evaluated as described below. Specifically, a gene encoding a protein to be evaluated is first introduced into a host cell such that the gene can be expressed therein, followed by purification of the protein by a technique such as chromatography. Malonyl-CoA and acetyl-CoA are added as substrates to a buffer containing the obtained protein to be evaluated, followed by, for example, incubation at a desired temperature (*e.g.*, 10°C to 60°C). After the completion of reaction, the amount of substrate lost and/or the amount of product (acetoacetyl-CoA) produced are determined. Thus, it is possible to evaluate whether or not the protein being tested has the function of synthesizing acetoacetyl-CoA from malonyl-CoA and acetyl-CoA and to evaluate the degree of synthesis. In such case, it is possible to examine whether or not the protein has the activity of synthesizing acetoacetyl-CoA from two acetyl-CoA molecules by adding acetyl-CoA alone as a substrate to a buffer containing the obtained protein to be evaluated and determining the amount of substrate lost and/or the amount of product produced in a similar manner.

Exemplary host cells

One of skill in the art will recognize that expression vectors are designed to contain certain components which optimize gene expression for certain host strains. Such optimization

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components include, but are not limited to origin of replication, promoters, and enhancers. The vectors and components referenced herein are described for exemplary purposes and are not meant to narrow the scope of the invention.

[0195] Any microorganism or progeny thereof that can be used to heterologously express genes can be used for modulation of any of the genes described herein for increased production of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids (*e.g.,* citrate synthase, phosphotransacetylase, acetate kinase, lactate dehydrogenase, malic enzyme, pyruvate dehydrogenase, 6-phosphogluconolactonase, phosphoenolpyruvate carboxylase, the inhibitor of RssB activity during magnesium starvation protein, the acrA component of the multidrug efflux pump acrAB-TolC, and/or FNR. Also, any microorganism or progeny thereof that can be used to heterologously express genes can be used to express one or more heterologous nucleic acids encoding upper MVA pathway polypeptides. In some aspects, any microorganism or progeny thereof that can be used to heterologously express genes can be used to express one or more *mvaE* and *mvaS* genes (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). Bacteria cells, including gram positive or gram negative bacteria can be used to express any of the upper MVA pathway genes (such as *mvaE* and *mvaS* genes) described above. In particular, upper MVA pathway gene (such as *mvaE* and *mvaS* genes) can be expressed in any one of *P. citrea, 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, B. thuringiensis, S. albus, S. lividans, S. coelicolor, S. griseus, Pseudomonas sp.,* and *P. alcaligenes* cells. In some aspects, the host cell can be a *Lactobacilis spp.,* such as *Lactobacillus lactis* or a *Lactobacillus plantarum.*

[0196] There are numerous types of anaerobic cells that can be used as host cells in the compositions and methods of the present invention. In one aspect of the invention, the cells described in any of the compositions or methods described herein are obligate anaerobic cells and progeny thereof. Obligate anaerobes typically do not grow well, if at all, in conditions where oxygen is present. It is to be understood that a small amount of oxygen may be present, that is, there is some tolerance level that obligate anaerobes have for a low level of oxygen. In one aspect, obligate anaerobes engineered to produce mevalonate, isoprene, isoprenoid precursors, and/or isoprenoids can serve as host cells for any of the methods and/or compositions described

herein and are grown under substantially oxygen-free conditions, wherein the amount of oxygen present is not harmful to the growth, maintenance, and/or fermentation of the anaerobes.

[0197] In another aspect of the invention, the host cells described and/or used in any of the compositions or methods described herein are facultative anaerobic cells and progeny thereof. Facultative anaerobes can generate cellular ATP by aerobic respiration (*e.g.*, utilization of the TCA cycle) if oxygen is present. However, facultative anaerobes can also grow in the absence of oxygen. This is in contrast to obligate anaerobes which die or grow poorly in the presence of greater amounts of oxygen. In one aspect, therefore, facultative anaerobes can serve as host cells for any of the compositions and/or methods provided herein and can be engineered to produce mevalonate, isoprene, isoprenoid precursors, and/or isoprenoids. Facultative anaerobic host cells can be grown under substantially oxygen-free conditions, wherein the amount of oxygen present is not harmful to the growth, maintenance, and/or fermentation of the anaerobes, or can be alternatively grown in the presence of greater amounts of oxygen.

[0198] The host cell can additionally be a filamentous fungal cell and progeny thereof. (*See, e.g.,* Berka & Barnett, *Biotechnology Advances,* (1989), 7(2):127-154). In some aspects, the filamentous fungal cell can be any of *Trichoderma longibrachiatum, T. viride, T. koningii, T. harzianum*, *Penicillium sp., Humicola insolens, H. lanuginose, H. grisea*, *Chrysosporium sp.*, *C. lucknowense, Gliocladium sp., Aspergillus sp.*, such as *A. oryzae*, *A. niger*, *A sojae, A. japonicus*, *A. nidulans*, or *A. awamori*, *Fusarium sp.,* such as *F. roseum, F. graminum F. cerealis, F. oxysporuim*, or *F. venenatum, Neurospora sp.,*such as *N. crassa, Hypocrea sp., Mucor sp.,*such as *M. miehei, Rhizopus sp.* or *Emericella sp.* In some aspects, the fungus is *A. nidulans, A. awamori, A. oryzae, A. aculeatus, A. niger, A. japonicus, T. reesei, T. viride, F. oxysporum,* or *F. solani*. In certain embodiments, plasmids or plasmid components for use herein include those described in U.S. patent pub. No. US 2011/0045563.

[0199] The host cell can also be a yeast, such as *Saccharomyces sp., Schizosaccharomyces sp., Pichia sp.,* or Candida *sp.* In some aspects, the *Saccharomyces sp.* is *Saccharomyces cerevisiae* (See, *e.g.*, Romanos et al., *Yeast*, (1992), 8(6):423-488). In certain embodiments, plasmids or plasmid components for use herein include those described in U.S. pat. No, 7,659,097 and U.S. patent pub. No. US 2011/0045563.

[0200] The host cell can additionally be a species of algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates. (See, *e.g.*, Saunders & Warmbrodt, "*Gene Expression in Algae and Fungi, Including Yeast*," (1993), National Agricultural Library, Beltsville, MD). In certain embodiments, plasmids or plasmid components for use herein include those described in U.S. Patent Pub. No. US 2011/0045563. In some aspects, the host cell is a cyanobacterium, such as cyanobacterium classified into any of the following groups based on morphology: *Chlorococcales, Pleurocapsales, Oscillatoriales, Nostocales,* or *Stigonematales* (See, *e.g.*, Lindberg et al., Metab. Eng., (2010) 12(1):70-79). In certain embodiments, plasmids or plasmid components for use herein include those described in U.S. patent pub. No. US 2010/0297749; US 2009/0282545 and Intl. Pat. Appl. No. WO 2011/034863.

[0201] *E. coli* host cells that have been engineered to increase carbon flux to mevalonate can be used to express one or more upper MVA pathway polypeptides, such as any of the upper MVA pathway polypeptides described herein. In some aspects, *E. coli* host cells can be used to express one or more mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) in the compositions and methods described herein. In one aspect, the host cell is a recombinant cell of an *Escherichia coli* (*E. coli*) strain, or progeny thereof, capable of producing mevalonate that expresses one or more nucleic acids encoding upper MVA pathway polypeptides (*e.g.,* mvaE and mvaS polypeptides, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). The *E. coli* host cells (such as those cells that have been engineered to increase carbon flux to mevalonate as described herein) can produce mevalonate in amounts, peak titers, and cell productivities greater than that of the same cells lacking one or more heterologously expressed nucleic acids encoding upper MVA pathway polypeptides (*e.g.,* mvaE and mvaS polypeptides, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered to increase carbon flux to mevalonate. In addition, the one or more heterologously expressed nucleic acids encoding upper MVA pathway polypeptides in *E. coli* can be chromosomal copies (*e.g.*, integrated into the *E. coli* chromosome). In another aspect, the one or more heterologously expressed nucleic acids encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) in *E. coli* can be chromosomal

copies (*e.g.*, integrated into the *E. coli* chromosome). In other aspects, the *E. coli* cells are in culture.

Exemplary Cell Culture Media

[0202] As used herein, the terms "minimal medium" or "minimal media" refer to growth medium containing the minimum nutrients possible for cell growth, generally, but not always, without the presence of one or more amino acids (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more amino acids). Minimal medium typically contains: (1) a carbon source for microorganism (*e.g.*, bacterial cell) growth; (2) various salts, which can vary among microorganism (*e.g.*, bacterial) species and growing conditions; and (3) water. The carbon source can vary significantly, from simple sugars like glucose to more complex hydrolysates of other biomass, such as yeast extract, as discussed in more detail below. The salts generally provide essential elements such as magnesium, nitrogen, phosphorus, and sulfur to allow the cells to synthesize proteins and nucleic acids. Minimal medium can also be supplemented with selective agents, such as antibiotics, to select for the maintenance of certain plasmids and the like. For example, if a microorganism is resistant to a certain antibiotic, such as ampicillin or tetracycline, then that antibiotic can be added to the medium in order to prevent cells lacking the resistance from growing. Medium can be supplemented with other compounds as necessary to select for desired physiological or biochemical characteristics, such as particular amino acids and the like.

[0203] Any minimal medium formulation can be used to cultivate the host cells. Exemplary minimal medium formulations include, for example, M9 minimal medium and TM3 minimal medium. Each liter of M9 minimal medium contains (1) 200 ml sterile M9 salts (64 g Na_2HPO_4-$7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5.0 g NH_4Cl per liter); (2) 2 ml of 1 M $MgSO_4$ (sterile); (3) 20 ml of 20% (w/v) glucose (or other carbon source); and (4) 100 μl of 1 M $CaCl_2$ (sterile). Each liter of TM3 minimal medium contains (1) 13.6 g K_2HPO_4; (2) 13.6 g KH_2PO_4; (3) 2 g $MgSO_4*7H_2O$; (4) 2 g Citric Acid Monohydrate; (5) 0.3 g Ferric Ammonium Citrate; (6) 3.2 g $(NH_4)_2SO_4$; (7) 0.2 g yeast extract; and (8) 1 ml of 1000X Trace Elements solution; pH is adjusted to ~6.8 and the solution is filter sterilized. Each liter of 1000X Trace Elements contains: (1) 40 g Citric Acid Monohydrate; (2) 30 g $MnSO_4*H_2O$; (3) 10 g NaCl; (4) 1 g $FeSO_4*7H_2O$; (4)1 g $CoCl_2*6H_2O$; (5) 1 g $ZnSO_4*7H_2O$; (6) 100 mg $CuSO_4*5H_2O$; (7) 100 mg H_3BO_3; and (8) 100 mg $NaMoO_4*2H_2O$; pH is adjusted to ~3.0.

[0204] An additional exemplary minimal media includes (1) potassium phosphate K_2HPO_4, (2) Magnesium Sulfate $MgSO_4 * 7H_2O$, (3) citric acid monohydrate $C_6H_8O_7*H_2O$, (4) ferric ammonium citrate $NH_4FeC_6H_5O_7$, (5) yeast extract (from biospringer), (6) 1000X Modified Trace Metal Solution, (7) sulfuric acid 50% w/v, (8) foamblast 882 (Emerald Performance Materials), and (9) Macro Salts Solution 3.36ml All of the components are added together and dissolved in deionized H_2O and then heat sterilized. Following cooling to room temperature, the pH is adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Vitamin Solution and spectinomycin are added after sterilization and pH adjustment.

[0205] 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 can include any carbon source suitable for maintaining the viability or growing the host cells. In some aspects, the carbon source is a carbohydrate (such as monosaccharide, disaccharide, oligosaccharide, or polysaccharides), or invert sugar (*e.g.*, enzymatically treated sucrose syrup).

[0206] In some aspects, the carbon source includes yeast extract or one or more components of yeast extract. In some aspects, the concentration of yeast extract is 0.1% (w/v), 0.09% (w/v), 0.08% (w/v), 0.07% (w/v), 0.06% (w/v), 0.05% (w/v), 0.04% (w/v), 0.03% (w/v), 0.02% (w/v), or 0.01% (w/v) yeast extract. In some aspects, the carbon source includes both yeast extract (or one or more components thereof) and another carbon source, such as glucose.

[0207] 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).

Exemplary Cell Culture Conditions

[0208] Materials and methods suitable for the maintenance and growth of the recombinant cells of the invention are described *infra*, *e.g.*, in the Examples section. Other materials and methods suitable for the maintenance and growth of cell (*e.g.* bacterial) cultures are well known in the art. Exemplary techniques can be found in International Publication No. WO 2009/076676, U.S. Patent Application No. 12/335,071 (U.S. Publ. No. 2009/0203102), WO 2010/003007, US Publ. No. 2010/0048964, WO 2009/132220, US Publ. No. 2010/0003716,

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. In some aspects, the cells are cultured in a culture medium under conditions permitting the expression of one or more isoprene synthase, DXP pathway (*e.g.*, DXS), IDI, MVA pathway (*e.g.,* but not limited to, mvaE and/or mvaS), or PGL polypeptides encoded by a nucleic acid inserted into the host cells.

[0209] Standard cell culture conditions can be used to culture the cells (*see*, for example, WO 2004/033646 and references cited therein). In some aspects, cells are grown and maintained at an appropriate temperature, gas mixture, and pH (such as at about 20°C to about 37°C, at about 6% to about 84% CO_2, and at a pH between about 5 to about 9). In some aspects, cells are grown at 35°C in an appropriate cell medium. In some aspects, 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). Cells can be grown under aerobic, anoxic, or anaerobic conditions based on the requirements of the host cells. In addition, more specific cell culture conditions can be used to culture the cells. For example, in some embodiments, the cells (*e.g.*, bacterial cells, such as *E. coli* cells) express one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) under the control of a strong promoter in a low to medium copy plasmid and are cultured at 34°C.

[0210] Standard culture conditions and modes of fermentation, such as batch, fed-batch, or continuous fermentation that can be used are described in International Publication No. WO 2009/076676, U.S. Patent Application No. 12/335,071 (U.S. Publ. No. 2009/0203102), WO 2010/003007, US Publ. No. 2010/0048964, WO 2009/132220, US Publ. No. 2010/0003716. Batch and Fed-Batch fermentations are common and well known in the art and examples can be found in Brock, Biotechnology: *A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc.

[0211] In some aspects, 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%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, or 10%) of the amount of glucose that is consumed by the cells. In particular aspects, 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 aspects, 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 aspects, glucose does not accumulate during the time the cells are cultured. In various aspects, 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 aspects, 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 can allow more favorable regulation of the cells.

[0212] In some aspects, the cells (such as bacterial cells) are grown in batch culture. The cells (such as bacterial cells) can also be grown in fed-batch culture or in continuous culture. Additionally, the cells (such as bacterial cells) can be cultured in minimal medium, including, but not limited to, any of the minimal media described above. The minimal medium can be further supplemented with 1.0 % (w/v) glucose, or any other six carbon sugar, or less. Specifically, the minimal medium can be supplemented with 1% (w/v), 0.9% (w/v), 0.8% (w/v), 0.7% (w/v), 0.6% (w/v), 0.5% (w/v), 0.4% (w/v), 0.3% (w/v), 0.2% (w/v), or 0.1% (w/v) glucose. Additionally, the minimal medium can be supplemented 0.1% (w/v) or less yeast extract. Specifically, the minimal medium can be supplemented with 0.1% (w/v), 0.09% (w/v), 0.08% (w/v), 0.07% (w/v), 0.06% (w/v), 0.05% (w/v), 0.04% (w/v), 0.03% (w/v), 0.02% (w/v), or 0.01% (w/v) yeast extract. Alternatively, the minimal medium can be supplemented with 1% (w/v), 0.9% (w/v), 0.8% (w/v), 0.7% (w/v), 0.6% (w/v), 0.5% (w/v), 0.4% (w/v), 0.3% (w/v), 0.2% (w/v), or 0.1% (w/v) glucose and with 0.1% (w/v), 0.09% (w/v), 0.08% (w/v), 0.07% (w/v), 0.06% (w/v), 0.05% (w/v), 0.04% (w/v), 0.03% (w/v), 0.02% (w/v), or 0.01% (w/v) yeast extract.

Recombinant microorganisms capable of increased production of mevalonate

[0213] The recombinant microorganisms (*e.g.*, recombinant bacterial cells) described herein have the ability to produce mevalonate at an amount and/or concentration greater than that of the same cells without any manipulation to the various enzymatic pathways described herein. The recombinant microorganisms (*e.g.*, bacterial cells) that have been engineered for modulation in the various pathways described herein to increase carbon flux to mevalonate can be used to

produce mevalonate. These engineered cells can also contain one or more copies of a heterologous nucleic acid encoding upper MVA pathway polypeptides. In some aspects, the cells can contain one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). In one aspect, the recombinant cells (such as bacterial cells) described herein have the ability to produce mevalonate at a concentration greater than that of the same cells that have not been engineered to increase carbon flux towards mevalonate. In other aspects, the recombinant cells described herein have the ability to produce mevalonate at a concentration greater than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). In one aspect, any of the cells disclosed herein can be cultured in minimal medium. In some cases, the one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide (*e.g.*, an mvaE and/or mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) is a heterologous nucleic acid that is integrated into the host cell's chromosome. The cells (such as bacterial cells) can produce greater than about 85 mg/L/hr/OD of mevalonate. Alternatively, the cells (such as bacterial cells) can produce greater than about 30 mg/L/hr/OD, 40 mg/L/hr/OD, 50 mg/L/hr/OD, 60 mg/L/hr/OD, 70 mg/L/hr/OD, 80 mg/L/hr/OD, 90 mg/L/hr/OD, 100 mg/L/hr/OD, 110 mg/L/hr/OD, 120 mg/L/hr/OD, 130 mg/L/hr/OD, 140 mg/L/hr/OD, 150 mg/L/hr/OD, 160 mg/L/hr/OD, 170 mg/L/hr/OD, 180 mg/L/hr/OD, 190 mg/L/hr/OD, or 200 mg/L/hr/OD of mevalonate, inclusive, as well as any numerical value in between these numbers.

[0214] The host cells (such as bacterial cells) described herein are engineered to have one or more mutations which increase carbon flux towards the MVA pathway and can produce higher peak titers of mevalonate in comparison to cells which have not been similarly engineered. Additionally, the cells described herein produce mevalonate at a higher peak titer than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide. In another aspect, the cells (such as bacterial cells) described herein produce mevalonate at a higher peak titer than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,*

and/or *E. faecalis*) when cultured in minimal medium. The cells (such as bacterial cells) can produce greater than about 105 g/L peak titer of mevalonate after 48 hours of fermentation. Alternatively, the cells (such as bacterial cells) can produce greater than about 50 g/L, 60 g/L, 70 g/L, 80 g/L, 90 g/L, 100 g/L, 110 g/L, 120 g/L, 130 g/L, 140 g/L, 150 g/L, 160 g/L, 170 g/L, 180 g/L, 190 g/L, 200 g/L, 210 g/L, 220 g/L, 230 g/L, 240 g/L, 250 g/L, 260 g/L, 270 g/L, 280 g/L, 290 g/L, 300 g/L peak titer of mevalonate after 48 hours of fermentation, inclusive, as well as any numerical value in between these numbers.

[0215] The host cells (such as bacterial cells) described herein are engineered to have one or more mutations which increase carbon flux towards the MVA pathway which results in a higher cell productivity index (CPI) for mevalonate in comparison to cells which have not been similarly engineered. Additionally, the cells described herein have a higher CPI than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide. In some aspects, the cells (such as bacterial cells) described herein have a higher CPI than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding an mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). In one aspect, the cells can be cultured in minimal medium. The cells (such as bacterial cells) can have a CPI for mevalonate of at least about 4.5 (g/g). Alternatively, the cells (such as bacterial cells) can have a CPI for mevalonate of at least about 1 (g/g), 2 (g/g), 3 (g/g), 4 (g/g), 5 (g/g), 6 (g/g), 7 (g/g), 8 (g/g), 9 (g/g), 10 (g/g), 11 (g/g), 12 (g/g), 13 (g/g), 14 (g/g), 15 (g/g), 20 (g/g), 25 (g/g), or 30 (g/g) inclusive, as well as any numerical value in between these numbers.

[0216] The host cells (such as bacterial cells) described herein are engineered to have one or more mutations which increase carbon flux towards the MVA pathway which results in a higher mass yield of mevalonate in comparison to cells which have not been similarly engineered. Additionally, the cells described herein have a higher mass yield of mevalonate from glucose than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide. In some aspects, the cells (such as bacterial cells) described herein have a higher mass yield of mevalonate from glucose than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). In one aspect, the cells can be cultured in minimal medium.

The cells (such as bacterial cells) can produce a mass yield of mevalonate from glucose of at least about 38%. Alternatively, the cells (such as bacterial cells) can produce a mass yield of mevalonate from glucose of at least about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, or 55%, inclusive, as well as any numerical value in between these numbers.

*Methods of using recombinant cells (*e.g. *recombinant bacterial cells) to produce high amounts of mevalonate*

[0217] Also provided herein are methods for the production of mevalonate. In some aspects, the method for producing mevalonate comprises: (a) culturing a composition comprising recombinant cells (such as bacterial cells) which have been engineered to increase carbon flux to mevalonate as described herein (including any of the cells, such as the bacterial cells described above), or progeny thereof, capable of producing mevalonate; and (b) producing mevalonate. In some aspects, the method of producing mevalonate comprises the steps of culturing any of the recombinant cells described herein under conditions suitable for the production of mevalonate and allowing the recombinant cells to produce mevalonate. In some aspects, the method of producing mevalonate further comprises a step of recovering the mevalonate.

[0218] The method of producing mevalonate can also comprise the steps of: (a) culturing cells heterologously expressing one or more copies of a gene encoding an upper MVA pathway polypeptide; and (b) producing mevalonate. In other aspects, the method of producing mevalonate can comprise the steps of: (a) culturing cells (such as bacterial cells, including, but not limited to, *E. coli* cells) that do not endogenously have an *mvaE* gene and an *mvaS* gene (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) in minimal medium, wherein the cells heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*); and (b) producing mevalonate. Additionally, the cells can produce mevalonate in concentrations greater than that of the same cells lacking one or more heterologous copies of a gene encoding an upper MVA pathway polypeptide and which have not been engineered for greater carbon flux towards mevalonate. In other aspects, the cells (such as bacterial cells) can produce mevalonate in concentrations greater than that of the same cells lacking one or more heterologous copies of a gene encoding an mvaE polypeptide and an mvaS

polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), when the cells are cultured in minimal medium. In some cases, the one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide is a heterologous nucleic acid that is integrated into the host cell's chromosome. In one aspect, the one or more copies of a heterologous nucleic acid encoding an mvaE and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) is a heterologous nucleic acid that is integrated into the host cell's chromosome

[0219] The instant methods for the production of mevalonate can produce greater than about 85 mg/L/hr/OD of mevalonate. Alternatively, mevalonate can be produced in amounts greater than about 30 mg/L/hr/OD, 40 mg/L/hr/OD, 50 mg/L/hr/OD, 60 mg/L/hr/OD, 70 mg/L/hr/OD, 80 mg/L/hr/OD, 90 mg/L/hr/OD, 100 mg/L/hr/OD, 110 mg/L/hr/OD, 120 mg/L/hr/OD, 130 mg/L/hr/OD, 140 mg/L/hr/OD, 150 mg/L/hr/OD, 160 mg/L/hr/OD, 170 mg/L/hr/OD, 180 mg/L/hr/OD, 190 mg/L/hr/OD, or 200 mg/L/hr/OD of mevalonate, inclusive, as well as any numerical value in between these numbers. In some aspects, the method of producing mevalonate further comprises a step of recovering the mevalonate.

[0220] The method of producing mevalonate can similarly comprise the steps of: (a) culturing cells which have been engineered for increased carbon flux to mevalonate as described herein, wherein the cells heterologously express one or more copies of an upper MVA pathway gene encoding one or more upper MVA pathway polypeptides; and (b) producing mevalonate, wherein the cells produce mevalonate with a higher peak titer after 48 hours of fermentation than that of the same cells lacking one or more copies of an upper MVA pathway gene encoding one or more upper MVA pathway polypeptides, and which have not been engineered for increased carbon flux to mevalonate production. In other aspects, the method of producing mevalonate can similarly comprise the steps of: (a) culturing cells (such as bacterial cells) which have been engineered for increased carbon flux to mevalonate as described herein (including, but not limited to, *E. coli* cells) that do not endogenously have an *mvaE* gene and an *mvaS* gene from (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) in minimal medium, wherein the cells (such as bacterial cells) heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium,*

E. gallinarum, E. casseliflavus, and/or *E. faecalis*); and (b) producing mevalonate, wherein the cells (such as bacterial cells) produce mevalonate with a higher peak titer after 48 hours of fermentation than that of the same cells lacking one or more heterologous copies of a gene encoding an mvaE polypeptide and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), and which have not been engineered for increased carbon flux to mevalonate production when the cells are cultured in minimal medium.

[0221] The instant methods for the production of mevalonate can produce greater than about 105 g/L peak titer of mevalonate after 48 hours of fermentation. Alternatively, the cells (such as bacterial cells) can produce greater than about 50 g/L, 60 g/L, 70 g/L, 80 g/L, 90 g/L, 100 g/L, 110 g/L, 120 g/L, 130 g/L, 140 g/L, 150 g/L, 160 g/L, 170 g/L, 180 g/L, 190 g/L, or 200 g/L peak titer of mevalonate after 48 hours of fermentation, inclusive, as well as any numerical value in between these numbers. In some aspects, the method of producing mevalonate further comprises a step of recovering the mevalonate.

[0222] The method of producing mevalonate can similarly comprise the steps of: (a) culturing cells which have been engineered for increased carbon flux to mevalonate as described herein, wherein the cells heterologously express one or more copies of an upper MVA pathway gene encoding one or more upper MVA pathway polypeptides; and (b) producing mevalonate, wherein the cells have a CPI for mevalonate higher than that of the same cells lacking one or more copies of an upper MVA pathway gene encoding one or more upper MVA pathway polypeptides, and which have not been engineered for increased carbon flux to mevalonate production. In other aspects, the method of producing mevalonate can similarly comprise the steps of: (a) culturing cells (such as bacterial cells) which have been engineered for increased carbon flux as described herein (including, but not limited to, *E. coli* cells) that do not endogenously have an *mvaE* gene and an *mvaS* gene (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) in minimal medium, wherein the cells (such as bacterial cells) heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*); and (b) producing mevalonate, wherein the cells (such as bacterial cells) have a CPI for mevalonate higher than that of the same cells lacking one or more heterologous copies of a

gene encoding an mvaE polypeptide and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), and which have not been engineered for increased carbon flux to mevalonate production when the cells are cultured in minimal medium.

[0223] The instant methods for the production of mevalonate can produce mevalonate using cells with a CPI for mevalonate of at least 4.5 (g/g). Alternatively, the cells (such as bacterial cells) can have a CPI of at least 1 (g/g), 2 (g/g), 3 (g/g), 4 (g/g), 5 (g/g), 6 (g/g), 7 (g/g), 8 (g/g), 9 (g/g), 10 (g/g), 11 (g/g), 12 (g/g), 13 (g/g), 14 (g/g), 15 (g/g), 20 (g/g), 25 (g/g), or 30 (g/g) inclusive, as well as any numerical value in between these numbers. In some aspects, the method of producing mevalonate further comprises a step of recovering the mevalonate.

[0224] Provided herein are methods of using any of the cells described above for enhanced mevalonate production. The production of mevalonate by the cells can be enhanced by the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* mvaE and mvaS polypeptides, such as, but not limited to, mvaE and/or mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). The production of mevalonate can be enhanced by about 1,000,000 folds (*e.g.*, about 1 to about 500,000 folds, about 1 to about 50,000 folds, about 1 to about 5,000 folds, about 1 to about 1,000 folds, about 1 to about 500 folds, about 1 to about 100 folds, about 1 to about 50 folds, about 5 to about 100,000 folds, about 5 to about 10,000 folds, about 5 to about 1,000 folds, about 5 to about 500 folds, about 5 to about 100 folds, about 10 to about 50,000 folds, about 50 to about 10,000 folds, about 100 to about 5,000 folds, about 200 to about 1,000 folds, about 50 to about 500 folds, or about 50 to about 200 folds) compared to the production of mevalonate by cells without the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or mvaS polypeptide, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) which have not been engineered for increased carbon flux to MVA production.

[0225] The production of mevalonate by cells according to any of the methods described herein can be enhanced (*e.g.*, enhanced by the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide. In some aspects, the production of mevalonate by the cells according to any of the methods described herein can be enhanced (*e.g.*, enhanced by the expression of one or more heterologous nucleic acids encoding the mvaE and

mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). The production of mevalonate can be enhanced by about 1,000,000 folds (*e.g.*, about 1 to about 500,000 folds, about 1 to about 50,000 folds, about 1 to about 5,000 folds, about 1 to about 1,000 folds, about 1 to about 500 folds, about 1 to about 100 folds, about 1 to about 50 folds, about 5 to about 100,000 folds, about 5 to about 10,000 folds, about 5 to about 1,000 folds, about 5 to about 500 folds, about 5 to about 100 folds, about 10 to about 50,000 folds, about 50 to about 10,000 folds, about 100 to about 5,000 folds, about 200 to about 1,000 folds, about 50 to about 500 folds, or about 50 to about 200 folds) compared to the production of mevalonate by naturally-occurring cells (*e.g.*, cells not expressing one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide, for example, an mvaE and/or mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*)).

[0226] The production of mevalonate can also be enhanced by at least about any of 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, 200 folds, 500 folds, 1000 folds, 2000 folds, 5000 folds, 10,000 folds, 20,000 folds, 50,000 folds, 100,000 folds, 200,000 folds, 500,000 folds, or 1,000,000 folds compared to the production of mevalonate by naturally-occurring cells or by cells without the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* mvaE and mvaS polypeptides, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) which have not been engineered for increased carbon flux to MVA production.

[0227] In addition, more specific cell culture conditions can be used to culture the cells in the methods described herein. For example, the method for the production of mevalonate can comprise the steps of (a) culturing cells (such as any cell engineered for increased carbon flux to mevalonate as described herein) in minimal medium at 34°C, wherein the cells heterologously express one or more copies of a gene encoding an upper MVA pathway polypeptide on a low to medium copy plasmid under the control of a strong promoter; and (b) producing mevalonate. In some aspects, the method for the production of mevalonate comprises the steps of (a) culturing cells (such as bacterial cells, including, but not limited to, *E. coli* cells) that do not endogenously have an *mvaE* gene and an *mvaS* gene (such as, but not limited to, *mvaE* and *mvaS* genes from *L.*

grayi, E. faecium, E. gallinarum, E. casseliflavus, and/or *E. faecalis*) in minimal medium at 34°C, wherein the cells (such as bacterial cells) heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) on a low to medium copy plasmid and under the control of a strong promoter; and (b) producing mevalonate. In some aspects, the method of producing mevalonate further comprises a step of recovering the mevalonate.

Recombinant Cells (such as bacterial cells) Capable of Increased Production of Isoprene

[0228] Isoprene (2-methyl-1,3-butadiene) is an important organic compound used in a wide array of applications. For instance, isoprene is employed as an intermediate or a starting material in the synthesis of numerous chemical compositions and polymers, including in the production of synthetic rubber. Isoprene is also an important biological material that is synthesized naturally by many plants and animals.

[0229] Isoprene is produced from DMAPP by the enzymatic action of isoprene synthase. Therefore, without being bound to theory, it is thought that increasing the cellular production of mevalonate in cells (such as bacterial cells) by any of the compositions and methods described above will similarly result in the production of higher amounts of isoprene. Increasing the molar yield of mevalonate production from glucose translates into higher molar yields of isoprenoid precursors and isoprenoids, including isoprene, produced from glucose when combined with appropriate enzymatic activity levels of mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase and other appropriate enzymes for isoprene and isoprenoid production.

[0230] Production of isoprene can be made by using any of the recombinant host cells described here where one or more of the enzymatic pathways have been manipulated such that enzyme activity is modulated to increase carbon flow towards isoprene production. The recombinant microorganisms described herein that have various enzymatic pathways manipulated for increased carbon flow to mevalonate production can be used to produce isoprene. Any of the recombinant host cells expressing one or more copies of a heterologous nucleic acid encoding upper MVA pathway polypeptides including, but not limited to, an mvaE and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L.*

grayi, E. faecium, E. gallinarum, E. casseliflavus, and/or *E. faecalis*) capable of increased production of mevalonate described above can also be capable of increased production of isoprene. In some aspects, these cells further comprise one or more heterologous nucleic acids encoding polypeptides of the lower MVA pathway and a heterologous nucleic acid encoding an isoprene synthase polypeptide. As an alternative to using *mvaE* and *mvaS* genes (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) any known MVA pathway polypeptides (upper and lower MVA pathway) can be used as well. MVA pathway polypeptides are well known to one of skill in the art.

Nucleic acids encoding polypeptides of the lower MVA pathway

[0231] In some aspects of the invention, the cells described in any of the compositions or methods described herein further comprise one or more nucleic acids encoding a lower mevalonate (MVA) pathway polypeptide(s). In some aspects, the lower MVA pathway polypeptide is an endogenous polypeptide. In some aspects, the endogenous nucleic acid encoding a lower MVA pathway polypeptide is operably linked to a constitutive promoter. In some aspects, the endogenous nucleic acid encoding a lower MVA pathway polypeptide is operably linked to an inducible promoter. In some aspects, the endogenous nucleic acid encoding a lower MVA pathway polypeptide is operably linked to a strong promoter. In a particular aspect, the cells are engineered to over-express the endogenous lower MVA pathway polypeptide relative to wild-type cells. In some aspects, the endogenous nucleic acid encoding a lower MVA pathway polypeptide is operably linked to a weak promoter.

[0232] The lower mevalonate biosynthetic pathway comprises mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and diphosphomevalonte decarboxylase (MVD). In some aspects, the lower MVA pathway can further comprise isopentenyl diphosphate isomerase (IDI). Cells provided herein can comprise at least one nucleic acid encoding isoprene synthase, one or more upper MVA pathway polypeptides, and/or one or more lower MVA pathway polypeptides. Polypeptides of the lower MVA pathway can be any enzyme (a) that phosphorylates mevalonate to mevalonate 5-phosphate; (b) that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (c) that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. More particularly, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate can be from the group consisting of *M. mazei* mevalonate kinase polypeptide, *Lactobacillus* mevalonate kinase polypeptide, *M. burtonii* 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, *Streptomyces* mevalonate kinase polypeptide, and *Streptomyces CL190* mevalonate kinase polypeptide. In another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase.

[0233] In some aspects, the lower MVA pathway polypeptide is a heterologous polypeptide. In some aspects, the cells comprise more than one copy of a heterologous nucleic acid encoding a lower MVA pathway polypeptide. In some aspects, the heterologous nucleic acid encoding a lower MVA pathway polypeptide is operably linked to a constitutive promoter. In some aspects, the heterologous nucleic acid encoding a lower MVA pathway polypeptide is operably linked to an inducible promoter. In some aspects, the heterologous nucleic acid encoding a lower MVA pathway polypeptide is operably linked to a strong promoter. In some aspects, the heterologous nucleic acid encoding a lower MVA pathway polypeptide is operably linked to a weak promoter. In some aspects, the heterologous lower MVA pathway polypeptide is a polypeptide from *Saccharomyces cerevisiae, Enterococcus faecalis,* or *Methanosarcina mazei.*

[0234] The nucleic acids encoding a lower MVA pathway polypeptide(s) can be integrated into a genome of the cells or can be stably expressed in the cells. The nucleic acids encoding a lower MVA pathway polypeptide(s) can additionally be on a vector.

[0235] Exemplary lower MVA pathway polypeptides are also provided below: (i) mevalonate kinase (MVK); (ii) phosphomevalonate kinase (PMK); (iii) diphosphomevalonate decarboxylase (MVD); and (iv) isopentenyl diphosphate isomerase (IDI). In particular, the lower MVK polypeptide can be from the genus *Methanosarcina* and, more specifically, the lower MVK polypeptide can be from *Methanosarcina mazei.* In other aspects, the lower MVK polypeptide can be from *M. burtonii.* Additional examples of lower MVA pathway polypeptides can be found in U.S. Patent Application Publication 2010/0086978 the contents of which are expressly incorporated herein by reference in their entirety with respect to lower MVK pathway polypeptides and lower MVK pathway polypeptide variants.

[0236] Any one of the cells described herein can comprise IDI nucleic acid(s) (*e.g.*, endogenous or heterologous nucleic acid(s) encoding IDI). Isopentenyl diphosphate isomerase polypeptides (isopentenyl-diphosphate delta-isomerase or IDI) catalyzes 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.

[0237] Lower MVA pathway polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of a lower MVA pathway polypeptide. Exemplary lower 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 a lower MVA pathway polypeptide. Exemplary lower MVA pathway polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein. In addition, variants of lower MVA pathway polypeptides that confer the result of better isoprene production can also be used as well.

[0238] In some aspects, the lower MVA pathway polypeptide is a polypeptide from *Saccharomyces cerevisiae, Enterococcus faecalis,* or *Methanosarcina mazei*. In some aspects, the MVK 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, *Streptomyces* mevalonate kinase polypeptide, *Streptomyces* CL190 mevalonate kinase polypeptide, *M. burtonii* mevalonate kinase polypeptide, and *Methanosarcina mazei* mevalonate kinase polypeptide. Any one of the promoters described herein (*e.g.*, promoters described herein and identified in the Examples of the present disclosure including inducible promoters and constitutive promoters) can be used to drive expression of any of the MVA polypeptides described herein.

Nucleic acids encoding isoprene synthase polypeptides

[0239] In some aspects of the invention, the cells described in any of the compositions or methods described herein (including host cells that have been engineered for increased carbon flux as described herein) further comprise one or more nucleic acids encoding an isoprene synthase polypeptide or a polypeptide having isoprene synthase activity. In some aspects, the isoprene synthase polypeptide is an endogenous polypeptide. In some aspects, the endogenous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a constitutive promoter. In some aspects, the endogenous nucleic acid encoding an isoprene synthase polypeptide is operably linked to an inducible promoter. In some aspects, the endogenous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a strong promoter. In a particular aspect, the cells are engineered to over-express the endogenous isoprene synthase pathway polypeptide relative to wild-type cells. In some aspects, the endogenous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a weak promoter. In some aspects, the isoprene synthase polypeptide is a polypeptide from *Pueraria* or *Populus* or a hybrid such as *Populus alba x Populus tremula.*

[0240] In some aspects, the isoprene synthase polypeptide is a heterologous polypeptide. In some aspects, the cells comprise more than one copy of a heterologous nucleic acid encoding an isoprene synthase polypeptide. In some aspects, the heterologous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a constitutive promoter. In some aspects, the heterologous nucleic acid encoding an isoprene synthase polypeptide is operably linked to an inducible promoter. In some aspects, the heterologous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a strong promoter. In some aspects, the heterologous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a weak promoter.

[0241] The nucleic acids encoding an isoprene synthase polypeptide(s) can be integrated into a genome of the host cells or can be stably expressed in the cells. The nucleic acids encoding an isoprene synthase polypeptide(s) can additionally be on a vector.

[0242] 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. 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. Exemplary isoprene synthase polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein. In addition, variants of isoprene synthase can possess improved activity such as improved enzymatic activity. In some aspects, an isoprene synthase variant has other improved properties, such as improved stability (*e.g.*, thermo-stability), and/or improved solubility.

[0243] 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*. 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. In one exemplary assay, DMAPP (Sigma) can be 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 0C. 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) can be 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 370C for 15 minutes with shaking. The reaction can be quenched by adding 200 μL of 250 mM EDTA and quantified by GC/MS.

[0244] In some aspects, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide or a variant thereof. In some aspects, the isoprene synthase polypeptide is an isoprene synthase from *Pueraria* or a variant thereof. In some aspects, the isoprene synthase polypeptide is an isoprene synthase from Populus or a variant thereof. In some aspects, the isoprene synthase polypeptide is a poplar isoprene synthase polypeptide or a variant thereof. In some aspects, the isoprene synthase polypeptide is a kudzu isoprene synthase polypeptide or a variant thereof. In some aspects, the isoprene synthase polypeptide is a polypeptide from *Pueraria* or *Populus* or a hybrid, *Populus alba x Populus tremula*, or a variant thereof.

[0245] In some aspects, the isoprene synthase polypeptide or nucleic acid is from the family Fabaceae, such as the Faboideae subfamily. In some aspects, 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), English Oak (*Quercus robur*) (Zimmer *et al.*, WO 98/02550), or a variant thereof. In some aspects, the isoprene synthase polypeptide is an isoprene synthase from *Pueraria montana*, *Pueraria lobata, Populus tremuloides*, *Populus alba*, *Populus nigra*, or *Populus trichocarpa or* a variant thereof. In some aspects, the isoprene synthase polypeptide is an isoprene synthase from *Populus alba* or a variant thereof. In some aspects, the nucleic acid encoding the isoprene synthase (*e.g.*, isoprene synthase from *Populus alba* or a variant thereof) is codon optimized.

[0246] In some aspects, the isoprene synthase nucleic acid or polypeptide is a naturally-occurring polypeptide or nucleic acid (*e.g.*, naturally-occurring polypeptide or nucleic acid from *Populus*). In some aspects, the isoprene synthase nucleic acid or polypeptide is not a wild-type or naturally-occurring polypeptide or nucleic acid. In some aspects, the isoprene synthase nucleic acid or polypeptide is a variant of a wild-type or naturally-occurring polypeptide or nucleic acid (*e.g.*, a variant of a wild-type or naturally-occurring polypeptide or nucleic acid from *Populus*).

[0247] In some aspects, the isoprene synthase polypeptide is a variant. In some aspects, the isoprene synthase polypeptide is a variant of a wild-type or naturally occurring isoprene synthase. In some aspects, the variant has improved activity such as improved catalytic activity compared to the wild-type or naturally occurring isoprene synthase. The increase in activity (*e.g.*, catalytic activity) can be at least about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95%. In some aspects, the increase in activity such as catalytic activity is at least about any of 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 30 folds, 40 folds, 50 folds, 75 folds, or 100 folds. In some aspects, the increase in activity such as catalytic activity is about 10% to about 100 folds (*e.g.*, about 20% to about 100 folds, about 50% to about 50 folds, about 1 fold to about 25 folds, about 2 folds to about 20 folds, or about 5 folds to about 20 folds). In some aspects, the variant has improved solubility compared to the wild-type or naturally occurring isoprene synthase. The increase in solubility can be at least about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95%. The increase in solubility can be at least about any of 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 30 folds, 40 folds, 50 folds, 75 folds, or 100 folds. In some aspects, the increase in solubility is about 10% to about 100 folds (*e.g.*, about 20% to about

100 folds, about 50% to about 50 folds, about 1 fold to about 25 folds, about 2 folds to about 20 folds, or about 5 folds to about 20 folds). In some aspects, the isoprene synthase polypeptide is a variant of naturally occurring isoprene synthase and has improved stability (such as thermo-stability) compared to the naturally occurring isoprene synthase.

[0248] In some aspects, the variant has at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 110%, at least about 120%, at least about 130%, at least about 140%, at least about 150%, at least about 160%, at least about 170%, at least about 180%, at least about 190%, at least about 200% of the activity of a wild-type or naturally occurring isoprene synthase. The variant can share sequence similarity with a wild-type or naturally occurring isoprene synthase. In some aspects, a variant of a wild-type or naturally occurring isoprene synthase can have at least about any of 40%, 50%, 60%, 70%, 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 99.9% amino acid sequence identity as that of the wild-type or naturally occurring isoprene synthase. In some aspects, a variant of a wild-type or naturally occurring isoprene synthase has any of about 70% to about 99.9%, about 75% to about 99%, about 80% to about 98%, about 85% to about 97%, or about 90% to about 95% amino acid sequence identity as that of the wild-type or naturally occurring isoprene synthase.

[0249] In some aspects, the variant comprises a mutation in the wild-type or naturally occurring isoprene synthase. In some aspects, the variant has at least one amino acid substitution, at least one amino acid insertion, and/or at least one amino acid deletion. In some aspects, the variant has at least one amino acid substitution. In some aspects, the number of differing amino acid residues between the variant and wild-type or naturally occurring isoprene synthase can be one or more, *e.g.* 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, or more amino acid residues. Naturally occurring isoprene synthases can include any isoprene synthases from plants, for example, kudzu isoprene synthases, poplar isoprene synthases, English oak isoprene synthases, and willow isoprene synthases. In some aspects, the variant is a variant of isoprene synthase from *Populus alba.* In some aspects, the variant of isoprene synthase from *Populus alba* has at least one amino acid substitution, at least one amino acid insertion, and/or at least one amino acid deletion. In some aspects, the variant is a truncated *Populus alba* isoprene synthase. In some aspects, the nucleic acid encoding variant (*e.g.*, variant of isoprene synthase

from *Populus alba*) is codon optimized (for example, codon optimized based on host cells where the heterologous isoprene synthase is expressed). In other aspects, the variant of isoprene synthase from *Populus alba* has at least one amino acid substitution, at least one amino acid insertion, and/or at least one amino acid deletion at the amino acid residue shown in **Table 2.** In another aspect, the variant of isoprene synthase comprises at least one amino acid substitution, at least one amino acid deletion, and at least one amino acid insertion at any of the amino acid residues shown in **Table 2**, wherein the amino acid residue number corresponds to the amino acid residue number of *P. alba* isoprene synthase. In one aspect, the *P. alba* isoprene synthase is a truncated isoprene synthase, for example, MEA isoprene synthase which is 16 amino acid shorter than full-length isoprene synthase.

Table 2: Isoprene Synthase Variants of *P. Alba* (MEA)

A118E	E472R	S510C	D323Y	W392S
S22K	K463F	S510V	D323D	W392T
S21R	K463T	I342I	G99D	W392V
S22K	R71K	K348F	K161K	A118P
S22R	R71L	K348Y	W392A	A118Q
E58L	R71M	K348K	W392C	A118A
T481V	R71V	C437L	W392F	E41M
T481Y	R71R	T240C	S288Y	G111S
T502F	K393L	M460M	M228Y	S74Q
T381L	F542L	R461A	A3T	S74S
T381M	P538K	H424P	W392Y	K36D
T381Y	P538R	H424H	W392W	S282H
T383H	P538P	A448L	F89D	S282I
T383L	A503A	A448Q	F89E	S282W
E480I	L436I	A448V	F89F	S282Y
E480R	L436Y	G389D	E41Y	S282S
K393V	L436F	S444E	E41E	K36S
K393I	E488L	S444S	R43E	K36T
E415H	E488M	H511Y	R43L	K36W
E415V	E488T	H511H	K36E	K36Y
E415Y	E488W	R071I	K36H	K36K
R71H	E488E	R071K	K36N	
R71I	I342Y	R071L	K36P	
E58Y	C437M	K374Y	K36Q	
E135G	C437W	K374K	A453I	
A363L	C437Y	L526E	A453V	
K374Y	C437C	L526Q	A453A	

T381I	M460A	L526L	V409I	
L436L	I447T	R242G	V409T	
H254R	I447V	R242R	K161C	
H254C	I447Y	A443G	K161E	
E488C	S444D	A443Q	K161N	
E488F	G389E	A443R	K161Q	
T383Y	L376I	A443S	G99E	
K414I	L376M	S13S	G99G	
K414R	L376L	V268I	S288A	
K414S	I504F	V268V	S288C	
K414W	I504I	K161A	S288T	
E472C	E467H	V409V	W392I	
E472L	E467W	D323F	W392M	

In one embodiment, the MEA *P. alba* isoprene synthase is truncated so that it is 16 amino acids shorter than full length *P. alba* isoprene synthase.

[0250] The isoprene synthase polypeptide provided herein can be any of the isoprene synthases or isoprene synthase variants described in WO 2009/132220, WO 2010/124146, and U.S. Patent Application Publication No.: 2010/0086978, the contents of which are expressly incorporated herein by reference in their entirety with respect to the isoprene synthases and isoprene synthase variants.

[0251] Any one of the promoters described herein (*e.g.*, promoters described herein and identified in the Examples of the present disclosure including inducible promoters and constitutive promoters) can be used to drive expression of any of the isoprene synthases described herein.

[0252] Suitable isoprene synthases include, but are not limited to, those identified by Genbank Accession Nos. AY341431, AY316691, AY279379, AJ457070, and AY182241. Types of isoprene synthases which can be used in any one of the compositions or methods including methods of making microorganisms encoding isoprene synthase described herein are also described in International Patent Application Publication Nos. WO2009/076676, WO2010/003007, WO2009/132220, WO2010/031062, WO2010/031068, WO2010/031076, WO2010/013077, WO2010/031079, WO2010/148150, WO2010/124146, WO2010/078457, and WO2010/148256.

Nucleic acids encoding DXP pathway polypeptides

[0253] In some aspects of the invention, the cells described in any of the compositions or methods described herein (including host cells that have been engineered for increased carbon flux as described herein) further comprise one or more heterologous nucleic acids encoding a DXS polypeptide or other DXP pathway polypeptides. In some aspects, the cells further comprise a chromosomal copy of an endogenous nucleic acid encoding a DXS polypeptide or other DXP pathway polypeptides. In some aspects, the *E. coli* cells further comprise one or more nucleic acids encoding an IDI polypeptide and a DXS polypeptide or other DXP pathway polypeptides. In some aspects, one nucleic acid encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide or other DXP pathway polypeptides. In some aspects, one plasmid encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide or other DXP pathway polypeptides. In some aspects, multiple plasmids encode the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide or other DXP pathway polypeptides.

[0254] 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 polypeptides and nucleic acids and methods of measuring DXS activity are described in more detail in International Publication No. WO 2009/076676, U.S. Patent Application No. 12/335,071 (US Publ. No. 2009/0203102), WO 2010/003007, US Publ. No. 2010/0048964, WO 2009/132220, and US Publ. No. 2010/0003716.

[0255] Exemplary DXP pathways polypeptides include, but are not limited to any of the following polypeptides: DXS polypeptides, DXR polypeptides, MCT polypeptides, CMK polypeptides, MCS polypeptides, HDS polypeptides, HDR polypeptides, and polypeptides (*e.g.*, fusion polypeptides) having an activity of one, two, or more of the DXP pathway polypeptides. In particular, DXP pathway polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of a DXP pathway polypeptide. Exemplary DXP pathway nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of a DXP

pathway polypeptide. Exemplary DXP pathway polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein. Exemplary DXP pathway polypeptides and nucleic acids and methods of measuring DXP pathway polypeptide activity are described in more detail in International Publication No.: WO 2010/148150

[0256] 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 polypeptides and nucleic acids and methods of measuring DXS activity are described in more detail in International Publication No. WO 2009/076676, U.S. Patent Application No. 12/335,071 (US Publ. No. 2009/0203102), WO 2010/003007, US Publ. No. 2010/0048964, WO 2009/132220, and US Publ. No. 2010/0003716.

[0257] In particular, DXS polypeptides convert pyruvate and D-glyceraldehyde 3-phosphate into 1-deoxy-d-xylulose 5-phosphate (DXP). Standard methods can be used to determine whether a polypeptide has DXS polypeptide activity by measuring the ability of the polypeptide to convert pyruvate and D-glyceraldehyde 3-phosphate *in vitro*, in a cell extract, or *in vivo*.

[0258] DXR polypeptides convert 1-deoxy-d-xylulose 5-phosphate (DXP) into 2-C-methyl-D-erythritol 4-phosphate (MEP). Standard methods can be used to determine whether a polypeptide has DXR polypeptides activity by measuring the ability of the polypeptide to convert DXP *in vitro*, in a cell extract, or *in vivo*.

[0259] MCT polypeptides convert 2-C-methyl-D-erythritol 4-phosphate (MEP) into 4-(cytidine 5'-diphospho)-2-methyl-D-erythritol (CDP-ME). Standard methods can be used to determine whether a polypeptide has MCT polypeptides activity by measuring the ability of the polypeptide to convert MEP *in vitro*, in a cell extract, or *in vivo*.

[0260] CMK polypeptides convert 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME) into 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-MEP). Standard

methods can be used to determine whether a polypeptide has CMK polypeptides activity by measuring the ability of the polypeptide to convert CDP-ME *in vitro*, in a cell extract, or *in vivo.*

[0261] MCS polypeptides convert 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-MEP) into 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate (ME-CPP or cMEPP). Standard methods can be used to determine whether a polypeptide has MCS polypeptides activity by measuring the ability of the polypeptide to convert CDP-MEP *in vitro*, in a cell extract, or *in vivo.*

[0262] HDS polypeptides convert 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate into (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP or HDMAPP). Standard methods can be used to determine whether a polypeptide has HDS polypeptides activity by measuring the ability of the polypeptide to convert ME-CPP *in vitro*, in a cell extract, or *in vivo.*

[0263] HDR polypeptides convert (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Standard methods can be used to determine whether a polypeptide has HDR polypeptides activity by measuring the ability of the polypeptide to convert HMBPP *in vitro*, in a cell extract, or *in vivo.*

Source organisms for lower MVA pathway, isoprene synthase, IDI, and DXP pathway polypeptides

[0264] Isoprene synthase, IDI, DXP pathway, and/or lower MVA pathway nucleic acids (and their encoded polypeptides) can be obtained from any organism that naturally contains isoprene synthase, IDI, DXP pathway, and/or lower MVA pathway nucleic acids. Isoprene is formed naturally by a variety of organisms, such as bacteria, yeast, plants, and animals. Some organisms contain the MVA pathway for producing isoprene. Isoprene synthase nucleic acids can be obtained, *e.g.*, from any organism that contains an isoprene synthase. MVA pathway nucleic acids can be obtained, *e.g.*, from any organism that contains the MVA pathway. IDI and DXP pathway nucleic acids can be obtained, *e.g.*, from any organism that contains the IDI and DXP pathway.

[0265] The nucleic acid sequence of the isoprene synthase, DXP pathway, IDI, and/or MVA pathway nucleic acids can be isolated from a bacterium, fungus, plant, algae, or cyanobacterium. Exemplary source organisms include, for example, yeasts, such as species of *Saccharomyces*

(*e.g.*, *S. cerevisiae*), bacteria, such as species of *Escherichia* (*e.g.*, *E. coli*), or species of *Methanosarcina* (*e.g.*, *Methanosarcina mazei*), plants, such as kudzu or poplar (*e.g.*, *Populus alba* or *Populus alba x tremula* CAC35696) or aspen (*e.g.*, *Populus tremuloides*). Exemplary sources for isoprene synthases, IDI, and/or MVA pathway polypeptides which can be used are also described in International Patent Application Publication Nos. WO2009/076676, WO2010/003007, WO2009/132220, WO2010/031062, WO2010/031068, WO2010/031076, WO2010/013077, WO2010/031079, WO2010/148150, WO2010/078457, and WO2010/148256.

[0266] In some aspects, the source organism is a yeast, such as *Saccharomyces sp., Schizosaccharomyces sp.*, *Pichia sp.,* or *Candida sp.*

[0267] In some aspects, the source organism is a bacterium, such as strains of *Bacillus* such as *B. lichenformis* or *B. subtilis,* strains of *Pantoea* such as *P. citrea*, strains of *Pseudomonas* such as *P. alcaligenes,* strains of *Streptomyces* such as *S. lividans* or *S. rubiginosus,* strains of *Escherichia* such as *E. coli,* strains of *Enterobacter*, strains of *Streptococcus*, or strains of *Archaea* such as *Methanosarcina mazei.*

[0268] 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*.

[0269] In some aspects, 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 aspects, the source organism is a gram–negative bacterium, such as *E. coli* or *Pseudomonas sp.*

[0270] In some aspects, the source organism is a plant, such as a plant from the family Fabaceae, such as the Faboideae subfamily. In some aspects, the source organism is kudzu, poplar (such as *Populus alba x tremula* CAC35696), aspen (such as *Populus tremuloides*), or *Quercus robur.*

[0271] In some aspects, the source organism is an algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates.

[0272] In some aspects, 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.*

Nucleic acids encoding phosphoketolase polypeptides

[0273] In some aspects of the invention, the recombinant cells described in any of the compositions or methods described herein can further comprise one or more nucleic acids encoding a phosphoketolase polypeptide or a polypeptide having phosphoketolase activity. In some aspects, the phosphoketolase polypeptide is an endogenous polypeptide. In some aspects, the endogenous nucleic acid encoding a phosphoketolase polypeptide is operably linked to a constitutive promoter. In some aspects, the endogenous nucleic acid encoding a phosphoketolase polypeptide is operably linked to an inducible promoter. In some aspects, the endogenous nucleic acid encoding a phosphoketolase polypeptide is operably linked to a strong promoter. In some aspects, more than one endogenous nucleic acid encoding a phosphoketolase polypeptide is used (*e.g,* 2, 3, 4, or more copies of an endogenous nucleic acid encoding a phosphoketolase polypeptide). In a particular aspect, the cells are engineered to overexpress the endogenous phosphoketolase polypeptide relative to wild-type cells. In some aspects, the endogenous nucleic acid encoding a phosphoketolase polypeptide is operably linked to a weak promoter.

[0274] Phosphoketolase enzymes catalyze the conversion of xylulose 5-phosphate to glyceraldehyde 3-phosphate and acetyl phosphate and/or the conversion of fructose 6-phosphate to erythrose 4-phosphate and acetyl phosphate. In certain embodiments, the phosphoketolase enzyme is capable of catalyzing the conversion of xylulose 5-phosphate to glyceraldehyde 3-phosphate and acetyl phosphate. In other embodiments, the phosphoketolase enzyme is capable

of catalyzing the conversion of fructose 6-phosphate to erythrose 4-phosphate and acetyl phosphate. Thus, without being bound by theory, the expression of phosphoketolase as set forth herein can result in an increase in the amount of acetyl phosphate produced from a carbohydrate source. This acetyl phosphate can be converted into acetyl-CoA which can then be utilized by the enzymatic activities of the MVA pathway to produces mevalonate, isoprenoid precursor molecules, isoprene and/or isoprenoids. Thus the amount of these compounds produced from a carbohydrate substrate may be increased. Alternatively, production of Acetyl-P and AcCoA can be increased without the increase being reflected in higher intracellular concentration. In certain embodiments, intracellular acetyl-P or acetyl-CoA concentrations will remain unchanged or even decrease, even though the phosphoketolase reaction is taking place.

[0275] Exemplary phosphoketolase 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 phosphoketolase polypeptide. Exemplary phosphoketolase 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.

[0276] Standard methods can be used to determine whether a polypeptide has phosphoketolase peptide activity by measuring the ability of the peptide to convert D-fructose 6-phosphate or D-xylulose 5-phosphate into acetyl-P. Acetyl-P can then be converted into ferryl acetyl hydroxamate, which can be detected spectrophotometrically (Meile *et al.*, J. Bact. 183:2929-2936, 2001). Any polypeptide identified as having phosphoketolase peptide activity as described herein is suitable for use in the present invention.

[0277] In other aspects, exemplary phosphoketolase nucleic acids include, for example, a phosphoketolase isolated from *Lactobacillus reuteri, Bifidobacterium longum, Ferrimonas balearica, Pedobactor saltans, Streptomyces griseus*, and/or *Nocardiopsis dassonvillei.* Additional examples of phosphoketolase enzymes which can be used herein are described in U.S. 7,785,858, which is incorporated by reference herein.

Pathways involving the Entner-Doudoroff pathway

[0278] The Entner-Doudoroff (ED) pathway is an alternative to the Emden-Meyerhoff-Parnass (EMP –glycolysis) pathway. Some organisms, like *E. coli*, harbor both the ED and EMP pathways, while others have only one or the other. *Bacillus* subtilis has only the EMP pathway, while *Zymomonas mobilis* has only the ED pathway (Peekhaus and Conway. 1998. J. Bact. 180:3495-3502; Stulke and Hillen. 2000. Annu. Rev. Microbiol. 54, 849–880; Dawes et al. 1966. *Biochem. J.* 98:795-803).

[0279] Phosphogluconate dehydratase (edd) removes one molecule of H_2O from 6-phospho-D-gluconate to form 2-dehydro-3-deoxy-D-gluconate 6-phosphate, while 2-keto-3-deoxygluconate 6-phosphate aldolase (eda) catalyzes an aldol cleavage (Egan et al. 1992. *J. Bact.* 174:4638-4646). The two genes are in an operon.

[0280] Metabolites that can be directed into the phosphoketolase pathway can also be diverted into the ED pathway. To avoid metabolite loss to the ED-pathway, phosphogluconate dehydratase gene (*e.g.*, the endogenous phosphogluconate dehydratase gene) and/or a 2-keto-3-deoxygluconate 6-phosphate aldolase gene (*e.g.*, the endogenous 2-keto-3-deoxygluconate 6-phosphate aldolase gene) activity is attenuated. One way of achieving attenuation is by deleting phosphogluconate dehydratase (edd) and/or 2-keto-3-deoxygluconate 6-phosphate aldolase (eda). This can be accomplished by replacing one or both genes with a chloramphenicol or kanamycin cassette followed by looping out of the cassette. Without these enzymatic activities, more carbon can flux through the phosphoketolase enzyme, thus increasing the yield of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids.

[0281] The activity of phosphogluconate dehydratase (edd) and/or 2-keto-3-deoxygluconate 6-phosphate aldolase (eda) can also be decreased by other molecular manipulations of the enzymes. The decrease of enzyme activity can be any amount of reduction of specific activity or total activity as compared to when no manipulation has been effectuated. In some instances, the decrease of enzyme activity is decreased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%.

[0282] In some cases, attenuating the activity of the endogenous phosphogluconate dehydratase gene and/or the endogenous 2-keto-3-deoxygluconate 6-phosphate aldolase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison

to cells that do not have attenuated endogenous phosphogluconate dehydratase gene and/or endogenous acetate kinase2-keto-3-deoxygluconate 6-phosphate aldolase gene expression.

Pathways involving the oxidative branch of the pentose phosphate pathway

[0283] *E. coli* uses the pentose phosphate pathway to break down hexoses and pentoses and to provide cells with intermediates for various anabolic pathways. It is also a major producer of NADPH. The pentose phosphate pathway is composed from an oxidative branch (with enzymes like glucose 6-phosphate 1-dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*) or 6-phosphogluconate dehydrogenase (*gnd*)) and a non-oxidative branch (with enzymes such as transketolase (*tktA*), transaldolase (*talA* or talB), ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase) (Sprenger. 1995. *Arch. Microbiol.*164:324-330).

[0284] In order to direct carbon towards the phosphoketolase enzyme, the non-oxidative branch of the pentose phosphate pathway (transketolase, transaldolase, ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase) expression can be modulated (*e.g.*, increase enzyme activity) to allow more carbon to flux towards fructose 6-phosphate and xylulose 5-phosphate, thereby increasing the eventual production of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids. Increase of transketolase, transaldolase, ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase activity can be any amount of increase of specific activity or total activity as compared to when no manipulation has been effectuated. In some instances, the enzyme activity is increased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%. In some aspects, the activity of transketolase, transaldolase, ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase is modulated by increasing the activity of an endogenous transketolase, transaldolase, ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase. This can be accomplished by replacing the endogenous transketolase, transaldolase, ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase gene promoter with a synthetic constitutively high expressing promoter. The genes encoding transketolase, transaldolase, ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase can also be cloned on a plasmid behind an appropriate promoter. The increase of the activity of transketolase, transaldolase, ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase can result in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to cells that do

not have increased expression of transketolase, transaldolase, ribulose-5-phosphate-epimerase and (or) ribose-5-phosphate epimerase.

Pathways involving phosphofructokinase

[0285] Phosphofructokinase is a crucial enzyme of glycolysis which catalyzes the phosphorylation of fructose 6-phosphate. *E. coli* has two isozymes encoded by *pfkA* and *pfkB*. Most of the phosphofructokinase activity in the cell is due to *pfkA* (Kotlarz et al. 1975, *Biochim. Biophys. Acta*, 381:257-268).

[0286] In order to direct carbon towards the phosphoketolase enzyme, phosphofructokinase expression can be modulated (*e.g.*, decrease enzyme activity) to allow more carbon to flux towards fructose 6-phosphate and xylulose 5-phosphate, thereby increasing the eventual production of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids. Decrease of phosphofructokinase activity can be any amount of reduction of specific activity or total activity as compared to when no manipulation has been effectuated. In some instances, the decrease of enzyme activity is decreased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%. In some aspects, the activity of phosphofructokinase is modulated by decreasing the activity of an endogenous phosphofructokinase. This can be accomplished by replacing the endogenous phosphofructokinase gene promoter with a synthetic constitutively low expressing promoter. The gene encoding phosphofructokinase can also be deleted. The decrease of the activity of phosphofructokinase can result in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to cells that do not have decreased expression of phosphofructokinase.

Recombinant cells (such as bacterial cells) capable of increased production of isoprene

[0287] The recombinant cells described herein that have been engineered for increased carbon flux to isoprene have the ability to produce isoprene at a concentration greater than that of the same cells that have not been engineered for increased carbon flux to isoprene. In one aspect, the recombinant cells (such as bacterial cells) described herein (*e.g.,* host cells that have been engineered for increased carbon flux to isoprene as described herein) have the ability to produce isoprene at a concentration greater than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or

mvaS polypeptide, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), one or more copies of a heterologous nucleic acid encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding an isoprene synthase polypeptide when cultured in minimal media. In certain aspects, these cells comprise one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide (*e.g.*, an mvaE and/or mvaS polypeptide, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) one or more copies of a heterologous nucleic acid encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding an isoprene synthase polypeptide are heterologous nucleic acids. In one aspect, the one or more heterologous nucleic acids are integrated into the host cell's chromosome. The cells can produce at least 5% greater amounts of isoprene compared to isoprene-producing cells that have not been engineered to increase carbon flux to isoprene. In other aspects, the cells (such as bacterial cells) can produce at least 5% greater amounts of isoprene compared to isoprene-producing cells (such as bacterial cells) that do not comprise the mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). Alternatively, the cells (such as bacterial cells) can produce greater than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15% of isoprene, inclusive, as well as any numerical value in between these numbers.

[0288] In one aspect of the invention, there are provided cells that have been engineered for increased carbon flux to isoprene which comprise one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide, one or more heterologous nucleic acids encoding a lower mevalonate (MVA) pathway polypeptide(s), one or more heterologous nucleic acids encoding a DXP pathway polypeptide(s), and one or more heterologous nucleic acids encoding an isoprene synthase polypeptide. In another aspect of the invention, there are provided cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.*, mvaE and mvaS polypeptides such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), one or more heterologous nucleic acids encoding a lower mevalonate (MVA) pathway polypeptide(s), one or more heterologous nucleic acids encoding a DXP pathway polypeptide(s), and one or more heterologous nucleic acids encoding an isoprene synthase polypeptide. The cells can further comprise one or more heterologous nucleic acids encoding an IDI polypeptide.

The one or more heterologous nucleic acids can be operably linked to constitutive promoters, can be operably linked to inducible promoters, or can be operably linked to a combination of inducible and constitutive promoters. The one or more heterologous nucleic acids can additionally be operably linked strong promoters, weak promoters, and/or medium promoters. One or more of the heterologous nucleic acids encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), a lower mevalonate (MVA) pathway polypeptide(s), a DXP pathway polypeptide(s), and an isoprene synthase polypeptide can be integrated into a genome of the host cells or can be stably expressed in the cells. The one or more heterologous nucleic acids can additionally be on a vector.

[0289] In some aspects, there are provided cells which comprise one or more heterologous nucleic acids encoding an *M. burtonii* MVK polypeptide and one or more nucleic acids encoding an isoprene synthase polypeptide or variants thereof, wherein the cells are capable of producing isoprene.

[0290] The production of isoprene by cells that have been engineered for increased carbon flux to isoprene according to any of the compositions or methods described herein can be enhanced (*e.g.*, enhanced by the expression of one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a lower MVA pathway polypeptide(s), a DXP pathway polypeptide(s), and/or upper MVA pathway polypeptide). In other aspects, the production of isoprene by the cells according to any of the compositions or methods described herein can be enhanced (*e.g.*, enhanced by the expression of one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a lower MVA pathway polypeptide(s), a DXP pathway polypeptide(s), and/or the mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). As used herein, "enhanced" isoprene production refers to an increased cell productivity index (CPI) for isoprene, an increased titer of isoprene, an increased mass yield of isoprene, and/or an increased specific productivity of isoprene by the cells described by any of the compositions and methods described herein compared to cells which do not have one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a lower MVA pathway polypeptide(s), a DXP pathway polypeptide(s), and/or the mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E.*

casseliflavus, and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprene production. The production of isoprene can be enhanced by about 5% to about 1,000,000 folds. The production of isoprene can be enhanced by about 10% to about 1,000,000 folds (*e.g.*, about 1 to about 500,000 folds, about 1 to about 50,000 folds, about 1 to about 5,000 folds, about 1 to about 1,000 folds, about 1 to about 500 folds, about 1 to about 100 folds, about 1 to about 50 folds, about 5 to about 100,000 folds, about 5 to about 10,000 folds, about 5 to about 1,000 folds, about 5 to about 500 folds, about 5 to about 100 folds, about 10 to about 50,000 folds, about 50 to about 10,000 folds, about 100 to about 5,000 folds, about 200 to about 1,000 folds, about 50 to about 500 folds, or about 50 to about 200 folds) compared to the production of isoprene by cells that do not express one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprene production.

[0291] The production of isoprene can also enhanced by at least about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, 200 folds, 500 folds, 1000 folds, 2000 folds, 5000 folds, 10,000 folds, 20,000 folds, 50,000 folds, 100,000 folds, 200,000 folds, 500,000 folds, or 1,000,000 folds.

Methods of using the recombinant cells to produce isoprene

[0292] Also provided herein are methods of producing isoprene comprising culturing any of the recombinant microorganisms that have been engineered for increased carbon flux to isoprene as described herein. In one aspect, isoprene can be produced by culturing recombinant cells (such as bacterial cells) comprising modulation in any of the enzymatic pathways described herein and one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or an mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), a lower MVA pathway polypeptide, and an isoprene synthase polypeptide. In another aspect, isoprene can be produced by culturing recombinant cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or an mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E.*

faecalis), a lower MVA pathway polypeptide, and an isoprene synthase polypeptide. The isoprene can be produced from any of the cells described herein and according to any of the methods described herein. Any of the cells can be used for the purpose of producing isoprene from carbohydrates, including six carbon sugars such as glucose.

[0293] Thus, also provided herein are methods of producing isoprene comprising culturing cells that have been engineered for increased carbon flux to isoprene and which comprise one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide; and (b) producing isoprene. In other aspects, provided herein are methods of producing isoprene comprising culturing cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding an mvaE and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), in a suitable condition for producing isoprene and (b) producing isoprene. The cells can comprise one or more nucleic acid molecules encoding the lower MVA pathway polypeptide(s) described above (*e.g.*, MVK, PMK, MVD, and/or IDI) and any of the isoprene synthase polypeptide(s) described above (*e.g. Pueraria* isoprene synthase). In some aspects, the cells (such as bacterial cells) can be any of the cells described herein. Any of the isoprene synthases or variants thereof described herein, any of the microorganism (*e.g.,* bacterial) or plant strains described herein, any of the promoters described herein, and/or any of the vectors described herein can also be used to produce isoprene using any of the energy sources (*e.g.* glucose or any other six carbon sugar) described herein. In some aspects, the method of producing isoprene further comprises a step of recovering the isoprene.

[0294] In some aspects, there is provided a method for producing isoprene comprising (a) culturing cells which comprise one or more heterologous nucleic acids encoding an *M. burtonii* MVK and an isoprene synthase; and (b) producing isoprene.

[0295] In some aspects, the amount of isoprene produced is measured at the peak absolute productivity time point. In some aspects, the peak absolute productivity for the cells is about any of the amounts of isoprene disclosed herein. In some aspects, the amount of isoprene produced is measured at the peak specific productivity time point. In some aspects, the peak specific productivity for the cells is about any of the amounts of isoprene per cell disclosed herein. In some aspects, the cumulative, total amount of isoprene produced is measured. In

some aspects, the cumulative total productivity for the cells is about any of the amounts of isoprene disclosed herein.

[0296] In some aspects, any of the cells described herein that have been engineered for increased carbon flux to isoprene (for examples the cells in culture) produce isoprene at greater than about any of or about any of 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 aspects, 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 aspects, 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.

[0297] In some aspects, the cells that have been engineered for increased carbon flux to isoprene 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 aspects, 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 aspects, 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.

[0298] In some aspects, the cells that have been engineered for increased carbon flux to isoprene in culture produce a cumulative titer (total amount) of isoprene at greater than about any of or about any of 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 aspects, 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 aspects, 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}.

[0299] In some aspects, the isoprene produced by the cells that have been engineered for increased carbon flux to isoprene in culture comprises at least about 1, 2, 5, 10, 15, 20, or 25% by volume of the fermentation offgas. In some aspects, the isoprene comprises between about 1 to about 25% by volume of the offgas, such as between about 5 to about 15 %, about 15 to about 25%, about 10 to about 20%, or about 1 to about 10 %.

[0300] Provided herein are cells having enhanced isoprene production. The production of isoprene by the cells can be enhanced by the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* but not limited to, an mvaE and/or mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus*, and/or *E. faecalis*), one or more copies of a heterologous nucleic acid encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding an isoprene synthase polypeptide. As used herein, "enhanced" isoprene production refers to an increased cell productivity index (CPI) for isoprene, an increased titer of isoprene, an increased mass yield of isoprene, and/or an increased specific productivity of isoprene by the cells described by any of the compositions and methods described herein compared to cells which do not have one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a lower MVA pathway polypeptide(s), a DXP pathway polypeptide(s), and/or an upper MVA pathway polypeptide (*e.g.,* mvaE and/or mvaS polypeptides such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprene production. The production of isoprene can be enhanced by about 5% to about 1,000,000 folds. The production of isoprene can be enhanced by about 10% to about 1,000,000 folds (*e.g.*, about 50% to about 1,000,000 folds, about 1 to about 500,000 folds, about 1 to about 50,000 folds, about 1 to about 5,000 folds, about 1 to about 1,000 folds, about 1 to about 500 folds, about 1 to about 100 folds, about 1 to about 50 folds, about 5 to about 100,000 folds, about 5 to about 10,000 folds, about 5 to about 1,000 folds, about 5 to about 500

folds, about 5 to about 100 folds, about 10 to about 50,000 folds, about 50 to about 10,000 folds, about 100 to about 5,000 folds, about 200 to about 1,000 folds, about 50 to about 500 folds, or about 50 to about 200 folds) compared to the production of isoprene by the cells that do not endogenously have one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a lower MVA pathway polypeptide(s), a DXP pathway polypeptide(s), and/or an upper MVA pathway polypeptide (*e.g.,* mvaE and/or mvaS polypeptides such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprene production.

[0301] The production of isoprene by the cells that have been engineered for increased carbon flux to isoprene according to any of the methods described herein can be enhanced (*e.g.*, enhanced by the expression of one or more heterologous nucleic acids encoding the isoprene synthase polypeptide). The production of isoprene can be enhanced by about 5% to about 1,000,000 folds. The production of isoprene can be enhanced by about 10% to about 1,000,000 folds (*e.g.*, about 50% to about 1,000,000 folds, about 1 to about 500,000 folds, about 1 to about 50,000 folds, about 1 to about 5,000 folds, about 1 to about 1,000 folds, about 1 to about 500 folds, about 1 to about 100 folds, about 1 to about 50 folds, about 5 to about 100,000 folds, about 5 to about 10,000 folds, about 5 to about 1,000 folds, about 5 to about 500 folds, about 5 to about 100 folds, about 10 to about 50,000 folds, about 50 to about 10,000 folds, about 100 to about 5,000 folds, about 200 to about 1,000 folds, about 50 to about 500 folds, or about 50 to about 200 folds) compared to the production of isoprene by the naturally-occurring cells (*e.g.*, the cells without the expression of one or more heterologous nucleic acids encoding an isoprene synthase polypeptide). The production of isoprene can also enhanced by at least about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, 200 folds, 500 folds, 1000 folds, 2000 folds, 5000 folds, 10,000 folds, 20,000 folds, 50,000 folds, 100,000 folds, 200,000 folds, 500,000 folds, or 1,000,000 folds compared to the production of isoprene by naturally-occurring cells or by cells without the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus*, and/or *E. faecalis* and which have not been engineered for increased carbon flux to isoprene production.

Recombinant cells (such as bacterial cells) capable of increased production of isoprenoid precursors and/or isoprenoids

[0302] Isoprenoids can be produced in many organisms from the synthesis of the isoprenoid precursor molecules which are the end products of the MVA pathway. As stated above, isoprenoids represent an important class of compounds and include, for example, food and feed supplements, flavor and odor compounds, and anticancer, antimalarial, antifungal, and antibacterial compounds.

[0303] As a class of molecules, isoprenoids are classified based on the number of isoprene units comprised in the compound. Monoterpenes comprise ten carbons or two isoprene units, sesquiterpenes comprise 15 carbons or three isoprene units, diterpenes comprise 20 carbons or four isoprene units, sesterterpenes comprise 25 carbons or five isoprene units, and so forth. Steroids (generally comprising about 27 carbons) are the products of cleaved or rearranged isoprenoids.

[0304] Isoprenoids can be produced from the isoprenoid precursor molecules IPP and DMAPP. These diverse compounds are derived from these rather simple universal precursors and are synthesized by groups of conserved polyprenyl pyrophosphate synthases (Hsieh et al., *Plant Physiol.* 2011 Mar;155(3):1079-90). The various chain lengths of these linear prenyl pyrophosphates, reflecting their distinctive physiological functions, in general are determined by the highly developed active sites of polyprenyl pyrophosphate synthases via condensation reactions of allylic substrates (dimethylallyl diphosphate (C_5-DMAPP), geranyl pyrophosphate (C_{10}-GPP), farnesyl pyrophosphate (C_{15}-FPP), geranylgeranyl pyrophosphate (C_{20}-GGPP)) with corresponding number of isopentenyl pyrophosphates (C_5-IPP) (Hsieh et al., *Plant Physiol.* 2011 Mar;155(3):1079-90).

[0305] Production of isoprenoid precursors and/or isoprenoid can be made by using any of the recombinant host cells described here where one or more of the enzymatic pathways have been manipulated such that enzyme activity is modulated to increase carbon flow towards isoprenoid production. In addition, these cells can express one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide for increased production of mevalonate, isoprene, isoprenoid precursors and/or isoprenoids. In other aspects, these cells can express one or more copies of a heterologous nucleic acid encoding an mvaE and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E.*

casseliflavus, and/or *E. faecalis*) for increased production of mevalonate, isoprene, isoprenoid precursors and/or isoprenoids. Any of the recombinant host cells that have been engineered for increased carbon flux to mevalonate expressing one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or an mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) capable of increased production of mevalonate or isoprene described above can also be capable of increased production of isoprenoid precursors and/or isoprenoids. In some aspects, these cells further comprise one or more heterologous nucleic acids encoding polypeptides of the lower MVA pathway, IDI, and/or the DXP pathway, as described above, and a heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide. Without being bound to theory, it is thought that increasing the cellular production of mevalonate in cells (such as bacterial cells) by any of the compositions and methods described above will similarly result in the production of higher amounts of isoprenoid precursor molecules and/or isoprenoids. Increasing the molar yield of mevalonate production from glucose translates into higher molar yields of isoprenoid precursor molecules and/or isoprenoids, including isoprene, produced from glucose when combined with appropriate enzymatic activity levels of mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase and other appropriate enzymes for isoprene and isoprenoid production.

Types of isoprenoids

[0306] The cells (such as bacterial cells) of the present invention that have been engineered for increased carbon flux to mevalonate are capable of increased production of isoprenoids and the isoprenoid precursor molecules DMAPP and IPP. Examples of isoprenoids include, without limitation, hemiterpenoids, monoterpenoids, sesquiterpenoids, diterpenoids, sesterterpenoids, triterpenoids, tetraterpenoids, and higher polyterpenoids. In some aspects, the hemiterpenoid is prenol (*i.e.*, 3-methyl-2-buten-1-ol), isoprenol (*i.e.*, 3-methyl-3-buten-1-ol), 2-methyl-3-buten-2-ol, or isovaleric acid. In some aspects, the monoterpenoid can be, without limitation, geranyl pyrophosphate, eucalyptol, limonene, or pinene. In some aspects, the sesquiterpenoid is farnesyl pyrophosphate, artemisinin, or bisabolol. In some aspects, the diterpenoid can be, without limitation, geranylgeranyl pyrophosphate, retinol, retinal, phytol, taxol, forskolin, or aphidicolin. In some aspects, the triterpenoid can be, without limitation, squalene or lanosterol. The

isoprenoid can also be selected from the group consisting of abietadiene, amorphadiene, carene, α-famesene, β-farnesene, farnesol, geraniol, geranylgeraniol, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, β-pinene, sabinene, γ-terpinene, terpindene and valencene.

[0307] In some aspects, the tetraterpenoid is lycopene or carotene (a carotenoid). As used herein, the term "carotenoid" refers to a group of naturally-occurring organic pigments produced in the chloroplasts and chromoplasts of plants, of some other photosynthetic organisms, such as algae, in some types of fungus, and in some bacteria. Carotenoids include the oxygen-containing xanthophylls and the non-oxygen-containing carotenes. In some aspects, the carotenoids are selected from the group consisting of xanthophylls and carotenes. In some aspects, the xanthophyll is lutein or zeaxanthin. In some aspects, the carotenoid is α-carotene, β-carotene, γ-carotene, β-cryptoxanthin or lycopene.

Heterologous nucleic acids encoding polyprenyl pyrophosphate synthases polypeptides

[0308] In some aspects of the invention, the cells that have been engineered for increased carbon flux to isoprenoids described in any of the compositions or methods herein further comprise one or more nucleic acids encoding a lower mevalonate (MVA) pathway polypeptide(s), as described above, as well as one or more nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptides(s). The polyprenyl pyrophosphate synthase polypeptide can be an endogenous polypeptide. The endogenous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide can be operably linked to a constitutive promoter or can similarly be operably linked to an inducible promoter. The endogenous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide can additionally be operably linked to a strong promoter. Alternatively, the endogenous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide can be operably linked to a weak promoter. In particular, the cells can be engineered to over-express the endogenous polyprenyl pyrophosphate synthase polypeptide relative to wild-type cells.

[0309] In some aspects, the polyprenyl pyrophosphate synthase polypeptide is a heterologous polypeptide. The cells of the present invention can comprise more than one copy of a heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide. In some aspects, the heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide is operably linked to a constitutive promoter. In some aspects, the heterologous

nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide is operably linked to an inducible promoter. In some aspects, the heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide is operably linked to a strong promoter. In some aspects, the heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide is operably linked to a weak promoter.

[0310] The nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide(s) can be integrated into a genome of the host cells or can be stably expressed in the cells. The nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide(s) can additionally be on a vector.

[0311] Exemplary polyprenyl pyrophosphate 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 a polyprenyl pyrophosphate synthase. Polyprenyl pyrophosphate synthase polypeptides convert isoprenoid precursor molecules into more complex isoprenoid compounds. Exemplary polyprenyl pyrophosphate synthase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of an isoprene synthase polypeptide. Exemplary polyprenyl pyrophosphate synthase polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein. In addition, variants of polyprenyl pyrophosphate synthase can possess improved activity such as improved enzymatic activity. In some aspects, a polyprenyl pyrophosphate synthase variant has other improved properties, such as improved stability (*e.g.*, thermo-stability), and/or improved solubility. Exemplary polyprenyl pyrophosphate synthase nucleic acids can include nucleic acids which encode polyprenyl pyrophosphate synthase polypeptides such as, without limitation, geranyl diphosposphate (GPP) synthase, farnesyl pyrophosphate (FPP) synthase, and geranylgeranyl pyrophosphate (GGPP) synthase, or any other known polyprenyl pyrophosphate synthase polypeptide.

[0312] In some aspects of the invention, the cells that have been engineered for increased carbon flux to isoprenoids described in any of the compositions or methods herein further comprise one or more nucleic acids encoding a farnesyl pyrophosphate (FPP) synthase. The FPP synthase polypeptide can be an endogenous polypeptide encoded by an endogenous gene. In some aspects, the FPP synthase polypeptide is encoded by an endogenous ispA gene in *E. coli*. The endogenous nucleic acid encoding an FPP synthase polypeptide can be operably

linked to a constitutive promoter or can similarly be operably linked to an inducible promoter. The endogenous nucleic acid encoding an FPP synthase polypeptide can additionally be operably linked to a strong promoter. In particular, the cells can be engineered to over-express the endogenous FPP synthase polypeptide relative to wild-type cells.

[0313] In some aspects, the FPP synthase polypeptide is a heterologous polypeptide. The cells of the present invention can comprise more than one copy of a heterologous nucleic acid encoding a FPP synthase polypeptide. In some aspects, the heterologous nucleic acid encoding a FPP synthase polypeptide is operably linked to a constitutive promoter. In some aspects, the heterologous nucleic acid encoding a FPP synthase polypeptide is operably linked to an inducible promoter. In some aspects, the heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide is operably linked to a strong promoter.

[0314] The nucleic acids encoding an FPP synthase polypeptide can be integrated into a genome of the host cells or can be stably expressed in the cells. The nucleic acids encoding an FPP synthase can additionally be on a vector.

[0315] Standard methods can be used to determine whether a polypeptide has polyprenyl pyrophosphate synthase polypeptide activity by measuring the ability of the polypeptide to convert IPP into higher order isoprenoids *in vitro*, in a cell extract, or *in vivo*. These methods are well known in the art and are described, for example, in U.S. Patent No.: 7,915,026; Hsieh et al., *Plant Physiol.* 2011 Mar;155(3):1079-90; Danner et al., *Phytochemistry.* 2011 Apr 12 [Epub ahead of print]; Jones et al., *J Biol Chem.* 2011 Mar 24 [Epub ahead of print]; Keeling et al., *BMC Plant Biol.* 2011 Mar 7;11:43; Martin et al., *BMC Plant Biol.* 2010 Oct 21;10:226; Kumeta & Ito, *Plant Physiol.* 2010 Dec;154(4):1998-2007; and Köllner & Boland, *J Org Chem.* 2010 Aug 20;75(16):5590-600.

Recombinant cells (such as bacterial cells) capable of increased production of isoprenoid precursors and/or isoprenoids

[0316] The recombinant microorganisms (*e.g.*, recombinant bacterial cells) described herein have the ability to produce isoprenoid precursors and/or isoprenoids at a amount and/or concentration greater than that of the same cells without any manipulation to the various enzymatic pathways described herein. In addition, the cells described herein have the ability to produce isoprenoid precursors and/or isoprenoids at an amount and/or concentration greater than

that of the same cells that have not been engineered for increased carbon flux to isoprenoids and which lack one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide, one or more copies of a heterologous nucleic acid encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide. In some aspects, the cells described herein have the ability to produce isoprenoid precursors and/or isoprenoids at an amount and/or concentration greater than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides(such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), one or more copies of a heterologous nucleic acid encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide when cultured in minimal media. In some cases, the one or more copies of a heterologous nucleic acid encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) one or more copies of a heterologous nucleic acid encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide are heterologous nucleic acids that are integrated into the host cell's chromosome. The cells can produce at least 5% greater amounts of isoprenoid precursors and/or isoprenoids when compared to isoprenoids and/or isoprenoid precursor -producing cells (such as bacterial cells) that have not been engineered for increased carbon flux to isoprenoids. In other aspects, the cells (such as bacterial cells) can produce at least 5% greater amounts of isoprenoid precursors and/or isoprenoids when compared to isoprenoids and/or isoprenoid precursor -producing cells (such as bacterial cells) that do not comprise the mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*). Alternatively, the cells (such as bacterial cells) can produce greater than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15% of isoprenoid precursors and/or isoprenoids, inclusive, as well as any numerical value in between these numbers.

[0317] In one aspect of the invention, there are provided cells that have been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursors comprising one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide, one or more heterologous nucleic acids encoding a lower mevalonate (MVA) pathway polypeptide(s), one or

more heterologous nucleic acids encoding a DXP pathway polypeptide(s), and one or more heterologous nucleic acids encoding polyprenyl pyrophosphate synthase. In another aspect of the invention, there are provided cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), one or more heterologous nucleic acids encoding a lower mevalonate (MVA) pathway polypeptide(s), one or more heterologous nucleic acids encoding a DXP pathway polypeptide(s), and one or more heterologous nucleic acids encoding polyprenyl pyrophosphate synthase. The cells can further comprise one or more heterologous nucleic acids encoding an IDI polypeptide. Additionally, the polyprenyl pyrophosphate synthase polypeptide can be an FPP synthase polypeptide. The one or more heterologous nucleic acids can be operably linked to constitutive promoters, can be operably linked to inducible promoters, or can be operably linked to a combination of inducible and constitutive promoters. The one or more heterologous nucleic acids can additionally be operably linked strong promoters, weak promoters, and/or medium promoters. One or more of the heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), a lower mevalonate (MVA) pathway polypeptide(s), and a DXP pathway polypeptide(s), and a polyprenyl pyrophosphate synthase polypeptide can be integrated into a genome of the host cells or can be stably expressed in the cells. The one or more heterologous nucleic acids can additionally be on a vector.

[0318] In some aspects, there are provided cells which comprise one or more heterologous nucleic acids encoding an *M. burtonii* MVK polypeptide and one or more nucleic acids encoding an polyprenyl pyrophosphate synthase, wherein the cells are capable of producing isoprenoids.

[0319] Provided herein are methods of using any of the cells that have been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor described above for enhanced isoprenoid precursor and/or isoprenoid production. The production of isoprenoid precursors and/or isoprenoids by the cells can be enhanced by the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide, one or more heterologous nucleic acids encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide. In other aspects, the production of

isoprenoid precursors and/or isoprenoids by the cells can be enhanced by the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), one or more heterologous nucleic acids encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide. As used herein, "enhanced" isoprenoid precursor and/or isoprenoid production refers to an increased cell productivity index (CPI) for isoprenoid precursor and/or isoprenoid production, an increased titer of isoprenoid precursors and/or isoprenoids, an increased mass yield of isoprenoid precursors and/or isoprenoids, and/or an increased specific productivity of isoprenoid precursors and/or isoprenoids by the cells described by any of the compositions and methods described herein compared to cells which do not have one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide, a lower MVA pathway polypeptide(s), a DXP pathway polypeptide(s), and/or an upper MVA pathway polypeptide(s) (*e.g.,* mvaE and/or mvaS polypeptides such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production. The production of isoprenoid precursors and/or isoprenoids can be enhanced by about 5% to about 1,000,000 folds. The production of isoprenoid precursors and/or isoprenoids can be enhanced by about 10% to about 1,000,000 folds (*e.g.*, about 1 to about 500,000 folds, about 1 to about 50,000 folds, about 1 to about 5,000 folds, about 1 to about 1,000 folds, about 1 to about 500 folds, about 1 to about 100 folds, about 1 to about 50 folds, about 5 to about 100,000 folds, about 5 to about 10,000 folds, about 5 to about 1,000 folds, about 5 to about 500 folds, about 5 to about 100 folds, about 10 to about 50,000 folds, about 50 to about 10,000 folds, about 100 to about 5,000 folds, about 200 to about 1,000 folds, about 50 to about 500 folds, or about 50 to about 200 folds) compared to the production of isoprenoid and/or isoprenoid precursors by cells without the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* mvaE and mvaS polypeptides, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production.

[0320] The production of isoprenoid precursors and/or isoprenoids by the cells according to any of the methods described herein can be enhanced (*e.g.*, enhanced by the expression of one or

more heterologous nucleic acids encoding an upper MVA pathway polypeptide, one or more heterologous nucleic acids encoding a lower MVA pathway polypeptide, and/or one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide). In other aspects, the production of isoprenoid precursors and/or isoprenoids by the cells according to any of the methods described herein can be enhanced (*e.g.*, enhanced by the expression of one or more heterologous nucleic acids encoding the mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), one or more heterologous nucleic acids encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide). The production of isoprenoid precursors and/or isoprenoids can be enhanced by about 5% to about 1,000,000 folds. The production of isoprenoid precursors and/or isoprenoids can be enhanced by about 10% to about 1,000,000 folds (*e.g.*, about 1 to about 500,000 folds, about 1 to about 50,000 folds, about 1 to about 5,000 folds, about 1 to about 1,000 folds, about 1 to about 500 folds, about 1 to about 100 folds, about 1 to about 50 folds, about 5 to about 100,000 folds, about 5 to about 10,000 folds, about 5 to about 1,000 folds, about 5 to about 500 folds, about 5 to about 100 folds, about 10 to about 50,000 folds, about 50 to about 10,000 folds, about 100 to about 5,000 folds, about 200 to about 1,000 folds, about 50 to about 500 folds, or about 50 to about 200 folds) compared to the production of isoprenoid precursors and/or isoprenoids by naturally-occurring cells (*e.g.*, cells without the expression of one or more heterologous nucleic acids encoding upper MVA pathway polypeptides, *e.g.*, mvaE and mvaS polypeptides, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*, and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production).

[0321] The production of isoprenoid precursors and/or isoprenoids can also enhanced by at least about any of 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, 200 folds, 500 folds, 1000 folds, 2000 folds, 5000 folds, 10,000 folds, 20,000 folds, 50,000 folds, 100,000 folds, 200,000 folds, 500,000 folds, or 1,000,000 folds compared to the production of isoprenoid precursors and/or isoprenoids by naturally-occurring cells or by cells without the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.*, mvaE and/or mvaS polypeptides, such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E.*

casseliflavus, and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production.

Methods of using the recombinant cells to produce isoprenoids and/or isoprenoid precursor molecules

[0322] Also provided herein are methods of producing isoprenoid precursor molecules and/or isoprenoids comprising culturing recombinant microorganisms (*e.g.*, recombinant bacterial cells) that have been engineered in various enzymatic pathways described herein and/or comprising one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide including, but not limited to, mvaE and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), a lower MVA pathway polypeptide, and an polyprenyl pyrophosphate synthase polypeptide. The isoprenoid precursor molecules and/or isoprenoids can be produced from any of the cells described herein and according to any of the methods described herein. Any of the cells can be used for the purpose of producing isoprenoid precursor molecules and/or isoprenoids from carbohydrates, including six carbon sugars such as glucose.

[0323] Thus, provided herein are methods of making isoprenoid precursor molecules and/or isoprenoids comprising culturing cells that have been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor; and (b) producing isoprenoid precursor molecules and/or isoprenoids. In other aspects, provided herein are methods of making isoprenoid precursor molecules and/or isoprenoids comprising culturing cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding an mvaE and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), in a suitable condition for producing isoprene and (b) producing isoprenoid precursor molecules and/or isoprenoids. The cells can further comprise one or more nucleic acid molecules encoding the lower MVA pathway polypeptide(s) described above (*e.g.*, MVK, PMK, MVD, and/or IDI) and any of the polyprenyl pyrophosphate synthase polypeptide(s) described above. In some aspects, the cells (such as bacterial cells) can be any of the cells described herein. Any of the polyprenyl pyrophosphate synthase or variants thereof described herein, any of the microorganism (*e.g.,* bacterial) or plant strains described herein, any of the promoters described herein, and/or any of the vectors described herein can also be used to produce isoprenoid precursor molecules and/or isoprenoids using any of the

energy sources (*e.g.* glucose or any other six carbon sugar) described herein. In some aspects, the method of producing isoprenoid precursor molecules and/or isoprenoids further comprises a step of recovering the isoprenoid precursor molecules and/or isoprenoids.

[0324] The method of producing isoprenoid precursor molecules and/or isoprenoids can comprise the steps of: (a) culturing cells that have been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursors wherein the cells heterologously express one or more copies of a gene encoding an upper MVA pathway polypeptide; and (b) producing isoprenoid precursor molecules and/or isoprenoids, wherein the cells produce greater amounts of isoprenoid precursors and/or isoprenoids when compared to isoprenoids and/or isoprenoid precursor-producing cells that do not comprise one or more heterologous copies of a gene encoding an upper MVA pathway polypeptide and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursors. In other aspects, the method of producing isoprenoid precursor molecules and/or isoprenoids can similarly comprise the steps of: (a) culturing cells (such as bacterial cells, including, but not limited to, *E. coli* cells) that do not endogenously have an *mvaE* gene and an *mvaS* gene (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), wherein the cells (such as bacterial cells) heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*); and (b) producing isoprenoid precursor molecules and/or isoprenoids , wherein the cells (such as bacterial cells) produce greater amounts of isoprenoid precursors and/or isoprenoids when compared to isoprenoids and/or isoprenoid precursor -producing cells (such as bacterial cells) that do not comprise the mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*).

[0325] The instant methods for the production of isoprenoid precursor molecules and/or isoprenoids can produce at least 5% greater amounts of isoprenoid precursors and/or isoprenoids when compared to isoprenoids and/or isoprenoid precursor-producing cells that have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursors and that do not comprise one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide. In other aspects, provided herein are methods for the production of isoprenoid precursor

molecules and/or isoprenoids can produce at least 5% greater amounts of isoprenoid precursors and/or isoprenoids when compared to isoprenoids and/or isoprenoid precursor -producing cells (such as bacterial cells) that do not comprise the mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production. Alternatively, the cells (such as bacterial cells) can produce greater than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15% of isoprenoid precursors and/or isoprenoids, inclusive. In some aspects, the method of producing isoprenoid precursor molecules and/or isoprenoids further comprises a step of recovering the isoprenoid precursor molecules and/or isoprenoids.

[0326] Provided herein are methods of using any of the cells that have been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursors described above for enhanced isoprenoid and/or isoprenoid precursor molecule production. The production of isoprenoid precursor molecules and/or isoprenoids by the cells can be enhanced by the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide, one or more heterologous nucleic acids encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide. In other aspects, the production of isoprenoid precursor molecules and/or isoprenoids by the cells can be enhanced by the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), one or more heterologous nucleic acids encoding a lower MVA pathway polypeptide, and one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide. As used herein, “enhanced” isoprenoid precursor and/or isoprenoid production refers to an increased cell productivity index (CPI) for isoprenoid precursor and/or isoprenoid production, an increased titer of isoprenoid precursors and/or isoprenoids, an increased mass yield of isoprenoid precursors and/or isoprenoids, and/or an increased specific productivity of isoprenoid precursors and/or isoprenoids by the cells described by any of the compositions and methods described herein compared to cells which do not have one or more heterologous nucleic acids encoding a polyprenyl pyrophosphate synthase polypeptide, a lower MVA pathway polypeptide(s), a DXP pathway polypeptide(s), and/or upper MVA pathway polypeptides (*e.g.,* mvaE and/or mvaS polypeptides such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium,*

E. gallinarum, E. casseliflavus, and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production. The production of isoprenoid precursor molecules and/or isoprenoids can be enhanced by about 5% to about 1,000,000 folds. The production of isoprenoid precursor molecules and/or isoprenoids can be enhanced by about 10% to about 1,000,000 folds (*e.g.*, about 1 to about 500,000 folds, about 1 to about 50,000 folds, about 1 to about 5,000 folds, about 1 to about 1,000 folds, about 1 to about 500 folds, about 1 to about 100 folds, about 1 to about 50 folds, about 5 to about 100,000 folds, about 5 to about 10,000 folds, about 5 to about 1,000 folds, about 5 to about 500 folds, about 5 to about 100 folds, about 10 to about 50,000 folds, about 50 to about 10,000 folds, about 100 to about 5,000 folds, about 200 to about 1,000 folds, about 50 to about 500 folds, or about 50 to about 200 folds) compared to the production of isoprenoid precursor molecules and/or isoprenoids by cells without the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* mvaE and/or mvaS polypeptides such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production.

[0327] The production of isoprenoid precursor molecules and/or isoprenoids can also enhanced by at least about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, 200 folds, 500 folds, 1000 folds, 2000 folds, 5000 folds, 10,000 folds, 20,000 folds, 50,000 folds, 100,000 folds, 200,000 folds, 500,000 folds, or 1,000,000 folds compared to the production of isoprenoid precursor molecules and/or isoprenoids by cells without the expression of one or more heterologous nucleic acids encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) and which have not been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production.

[0328] In addition, more specific cell culture conditions can be used to culture the cells in the methods described herein. For example, in some aspects, the method for the production of isoprenoid precursor molecules and/or isoprenoids comprises the steps of (a) culturing cells (such as bacterial cells, including, but not limited to, *E. coli* cells) that have been engineered for increased carbon flux to isoprenoids and/or isoprenoid precursor production that do not

endogenously have an *mvaE* gene and an *mvaS* gene (such as, but not limited to, *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) at 34°C, wherein the cells (such as bacterial cells) heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide (such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*) on a low to medium copy plasmid and under the control of a strong promoter; and (b) producing isoprenoids and/or isoprenoid precursors. In some aspects, the method of producing isoprenoids and/or isoprenoid precursors further comprises a step of recovering the isoprenoid precursor molecules and/or isoprenoids.

Vectors

[0329] Suitable vectors can be used for any of the compositions and methods described herein. For example, suitable vectors can be used to optimize the expression of one or more copies of a gene encoding an upper MVA pathway polypeptide (*e.g.,* an mvaE polypeptide and/or an mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), an isoprene synthase, a polyprenyl pyrophosphate synthase, and/or one or more MVA pathway polypeptides in anaerobes. In some aspects, the vector contains a selective marker. Examples of selectable markers include, but are not limited to, antibiotic resistance nucleic acids (*e.g.*, kanamycin, ampicillin, carbenicillin, gentamicin, hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol) and/or nucleic acids that confer a metabolic advantage, such as a nutritional advantage on the host cell. In some aspects, one or more copies of an upper MVA pathway polypeptide (*e.g.,* an mvaE polypeptide and/or an mvaS polypeptide such as, but not limited to, mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), an isoprene synthase, a polyprenyl pyrophosphate synthase, and/or one or more lower MVA pathway polypeptide nucleic acid(s) integrate into the genome of host cells without a selective marker.

[0330] Any one of the vectors characterized or used in the Examples of the present disclosure can be used.

Transformation methods

[0331] Nucleic acids encoding one or more copies of an upper MVA pathway polypeptide (*e.g.,* an mvaE and/or an mvaS nucleic acid such as, but not limited to, mvaE and mvaS nucleic acids from *L. grayi, E. faecium, E. gallinarum, E. casseliflavus,* and/or *E. faecalis*), isoprene synthase, and/or lower MVA pathway polypeptides can be inserted into a microorganism using suitable techniques. Additionally, isoprene synthase, IDI, DXP pathway, and/or polyprenyl pyrophosphate synthase 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 introduction of a DNA construct or vector into a host cell, 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). The introduced nucleic acids can be integrated into chromosomal DNA or maintained as extrachromosomal replicating sequences. Transformants can be selected by any method known in the art. Suitable methods for selecting transformants are described in International Publication No. WO 2009/076676, U.S. Patent Application No. 12/335,071 (US Publ. No. 2009/0203102), WO 2010/003007, US Publ. No. 2010/0048964, WO 2009/132220, and US Publ. No. 2010/0003716, the disclosures of which are incorporated by reference herein.

Exemplary Purification Methods

[0332] In some aspects, any of the methods described herein further include a step of recovering the compounds produced. In some aspects, any of the methods described herein further include a step of recovering the isoprene. In some aspects, the isoprene is recovered by absorption stripping (*See, e.g.,* US Appl. Pub. No. US 2011/0178261 A1, the disclosure of which is incorporated by reference herein). In some aspects, any of the methods described herein further include a step of recovering an isoprenoid. In some aspects, any of the methods described herein further include a step of recovering the terpenoid or carotenoid.

[0333] Suitable purification methods are described in more detail in U.S. Patent Application Publication US2010/0196977 A1, the disclosure of which is incorporated by reference herein.

[0334] The invention can be further understood by reference to the following examples, which are provided by way of illustration and are not meant to be limiting.

EXAMPLES

Example 1: Construction of *E. coli* strain CMP451 (containing BL21 pgl+ PL.2 mKKDyI GI1.2 gltA), CMP452 and CMP453

[0335] The promoter in front of the citrate synthase gene (*gltA*) in BL21 (Novagen) has been replaced by a constitutive low expression promoter, namely GI1.2 (US patent 7,371,558). Two wild-type promoters have been described for gltA (Wilde, R, and J. Guest. 1986. *J. Gen. Microbiol.* 132:3239-3251) and the synthetic promoter was inserted just after the -35 region of the distal promoter. A PCR product was obtained using primers UpgltACm-F (5'-TATTTAATTTTTAATCATCTAATTTGACAATCATTCAACAAAGTTGTTACAATTAACCCTCACTAAAGGGCGG-3') and DngltA1.xgiCm-R (5'-TCAACAGCTGTATCCCCGTTGAGGGTGAGTTTTGCTTTTGTATCAGCCATATATTCCACCAGCTATTTGTTAGTGAATAAAAGTGGTTGAATTATTTGCTCAGGATGTGGCATHGTCAAGGGCTAATACGACTCACTATAGGGCTCG-3'), and plasmid FRT-gb2-Cm-FRT from Gene Bridges (Heidelberg, Germany) as a template. The PCR product was purified and used in a lambda red-mediated recombination as described by the manufacturer (Gene Bridges, Heidelberg, Germany). Several colonies were selected for further characterization. The promoter region was PCR-amplified using primers gltAPromSeqF: 5'-GGCAGTATAGGCTGTTCACAAAATC-3' and gltApromSeqR: 5'-CTTGACCCAGCGTGCCTTTCAGC-3' and, as a template, DNA extracted by resuspending a colony in 30 uL H_2O, heating at 95 C for 4 min, spinning down, and using 2 uL of that material as a template in a 50 uL reaction. After observing the sequencing results of the PCR products obtained, a colony harboring each of the three different promoters GI1.2, GI1.5 and GI1.6 (US patent 7,371,558) was saved for further use ((CMP141, CMP142 and CMP143; **Table 3**)

Table 3: *E. coli* strains

Strain	Description	Parent
CMP141	BL21 Cm-GI1.2 gltA	BL21
CMP142	BL21 Cm-GI1.5 gltA	BL21
CMP143	BL21 Cm-GI1.6 gltA	BL21
CMP258	BL21 pgl+	BL21
CMP374	BL21 pgl+ PL.2-mKKDyI ldhA::Kan	MD09-314
CMP440	BL21 pgl+ PL.2 mKKDyI Cm-GI1.2	MD09-314

	gltA	
CMP441	BL21 pgl+ PL.2 mKKDyI Cm-GI1.5 gltA	MD09-314
CMP442	BL21 pgl+ PL.2 mKKDyI Cm-GI1.6 gltA	MD09-314
CMP451	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA	CMP440
CMP452	BL21 pgl+ PL.2 mKKDyI GI1.5 gltA	CMP441
CMP453	BL21 pgl+ PL.2 mKKDyI GI1.6 gltA	CMP442
CMP604	BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ackA-pta::Cm	CMP451
CMP620	BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta::Cm ldhA::Kan	CMP604
CMP635	BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta ldhA	CMP620
CMP646	BL21 attB:Cm (to restore LowerP) col1	BL21 (Novagen)
CMP676	BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta ldhA attB::Cm	CMP635
CMP680	BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta ldhA attB::Cm, pCHL276	CMP676
MCM521	BL21 neo-PL.2-mKKDyI	(U.S. Patent Application No. 12/978,324)
MD09-313	BL21 pgl+ neo-PL.2-mKKDyI	CMP258
MD09-314	BL21 pgl+ PL.2-mKKDyI	MD09-313
MD491	BL21 pgl+ ackA-pta::Cm	CMP258

[0336] Strain MD09-313 was built by transducing CMP258 (see U.S. Patent Application No. 12/978,324) with a P1 lysate from strain MCM521 (see U.S. Patent Application No. 12/978,324) and selecting for colonies on Luria-Bertani plates containing 20 ug/ml kanamycin. P1 lysates are prepared according to the method described in Ausubel, et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. The kanamycin marker was removed using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strain MD09-314.

[0337] A P1 lysate was made from strains CMP141, CMP142 and CMP143 and was used to transduce strain MD09-314, to form CMP440, CMP441 and CMP442 respectively (**Table 3**). The chloramphenicol marker was removed using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strains CMP451, CMP452 and CMP453 respectively (**Table 3**).

Example 2: Construction of *E. coli* strain CMP604 (containing BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta::Cm)

[0338] A DNA fragment containing the ackA-pta genes interrupted by a chloramphenicol marker was amplified by PCR using strain Triple Triple in which the chloramphenicol marker is still in (US7,745,184 B2) as a template and primers ackACF (5'-GTGCAAATTCACAACTCAGCGG) and ptaCR (CACCAACGTATCGGGCAT TGCC-3'). The PCR product obtained was used in a recombineering reaction as recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the ackA-pta locus in strain CMP258 (*See* U.S. Patent Application No. 12/978,324). Colonies were selected on LB + 5 ug/ml of chloramphenicol. One colony was picked and was named MD491. A P1 lysate of MD491 was made and was used to transduce strain CMP451. Colonies were selected on LB + 5 ug/ml of chloramphenicol. One colony was picked and was named CMP604.

Example 3: Construction of *E. coli* strain CMP620 (containing BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta::Cm ldhA::Kan) and CMP635 (containing BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta ldhA)

[0339] A DNA fragment containing the ldhA gene interrupted by a kanamycin marker was amplified by PCR using strain JW 1375 from the Keio collection (Baba et al. 2006. *Mol. Syst. Biol.* 2: 2006.0008) as a template, and primers ldhAseqR (5'-GGCTTACCGTTTACGCTTTCCAGC-3') and ldhAseqF2 (5'-CTAATGCAATACGTGTCCCGAGC-3'). The PCR product obtained was used in a recombineering reaction as recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the ldhA locus in strain MD09-313. Colonies were selected on LB + 20 ug/ml of kanamycin. One colony was picked and was named CMP374. A P1 lysate of CMP374 was made and was used to transduce CMP604. Colonies were selected on LB + 20 ug/ml of kanamycin. One colony was picked and was named CMP620. The chloramphenicol and kanamycin markers were looped out simultaneously by electroporating pCP20 (Datsenko and Wanner. 2000. *PNAS* 97:6640-5) in the strain, selecting two colonies on LB + 50 ug/ml carbenicillin at 30°C, then restreaking those colonies on an LB plate at 42°C. A Cm^S and Kan^S colony was selected from those plates and named CMP635.

Example 4: Construction of *E. coli* strain CMP676 (containing BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta ldhA attB::Cm)

[0340] A DNA fragment containing a chloramphenicol marker flanked by DNA homologous to the upstream and downstream regions of the λ attachment site attB was amplified by PCR using plasmid pKD3 (Datsenko & Wanner, 2000, *PNAS* 97:6640-5) as a template, and primers CMP171 (5'-AAAATTTTCATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTCACATGGAGTTGGCAGGATGTTTGATTACATGGGAATTAGCCATGGTCC-3') and CMP172 (5'-GACCAGCCGCGTAACCTGGCAAAATCGGTTACGGTTGAGTAATAAATGGATGCCCTGCGTAAGCGGGGCATTTTTCTTGGTGTAGGCTGGAGCTGCTTCG-3'). The PCR product obtained was used in a recombineering reaction in BL21 (Novagen) as recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the λ attachment site attB. Strain CMP646 was thereby generated, selected on LB + 5 ug/ml chlroamphenicol. A P1 lysate of CMP646 was made and was used in a transduction reaction on strain CMP635, thereby removing the lower mevalonate pathway (mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentenyl diphosphate isomerase) from the chromosome of that strain. The transduction reaction was plated on LB + chloramphenicol 5 ug/ml and one colony was picked and named CMP676.

Example 5: Construction of *E. coli* strain CMP680 (BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ML ackA-pta ldhA attB::Cm, pCHL276) and detection of mevalonate

[0341] Plasmid pCHL276 (see Example 6 (iii)) introduced into CMP676 by electroporation. Colonies were selected on LB + 50 ug/mL spectinomycin. One colony was picked and named CMP680.

(i) *Mevalonate Yield Assay*

[0342] Overnight cultures of the above-identified strains were inoculated in shake tubes containing 2 mL LB broth supplemented with 50 μg/mL spectinomycin (Novagen). Cultures were then incubated for 14h at 34°C at 250 rpm. Next, the cultures were diluted into an 5 mL 48-well plate (Axygen Scientific) containing 2 mL TM3 media supplemented with 1% Glucose, yeast extract to a final concentration of 0.1%, and 200 μM IPTG to final OD of 0.2. The plate

was sealed with a Breath Easier membrane (Diversified Biotech) and incubated at 34°C in a Shel Lab shaker/incubator at 600 rpm for 24 hours. 1 mL of each culture was centrifuged at 3,000 x g for 5 min. 250 μl of supernatant was added to 19 μL of 20% sulfuric acid and incubated on ice for 5 min. The mixture was then centrifuged for 5 min at 3000 x g and the supernatant was collected for HPLC analysis. 200 μl of supernatant was transferred to a HPLC compatible 96-well conical bottom polypropylene plate (Nunc). The concentration of mevalonate in samples was determined by comparison to a standard curve of mevalonate (Sigma). The glucose concentration was measured by performing a glucose oxidase assay according to the manufacturer's specifications (Pointe Scientific, Inc.)

(ii) *HPLC Detection of Mevalonate:*

[0343] HPLC analysis was performed on an Agilent 1100 series HPLC system containing a refractive index detector using a 300mm x 7.8mm BioRad - Aminex HPX-87H ion exclusion column (catalog # 125-0140) incubated at 50°C and equipped with a BioRad - Microguard Cation H refill 30mm x 4.6mm (Catalog # 125-0129). Samples were run at a flow rate of 0.6 ml/min in 0.01 N sulfuric acid running buffer. Mevalonate was detected using a refractive index detector.

<u>Example 6: Construction of *E. coli* strains MCM1373-1377 expressing *mvaE* and *mvaS* genes from *Listeria grayi DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2,* and *Enterococcus casseliflavus*</u>

(i) *Gene identification and selection*

[0344] A primary sequence homology search using the *E. faecalis mvaE* gene product as the query was performed using the BLASTp program located at the NCBI website (Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402). Sequences of interest were selected from the search results.

[0345] In general, sequences of interest for the mvaE and mvaS genes displayed from 59-66% nucleotide sequence identity (codon optimized; see **Table 4**) and between 59-71% amino acid

sequence identity (**Table 5**) compared to the wild type *E. faecalis mvaE* and *mvaS* nucleic acid and protein sequences, respectively.

Table 4: Percent identity of mvaE and mvaS nucleotides (codon-optimized) compared to *Enterococcus faecalis* WT

Species	*mvaE* gene (% identity)	*mvaS* gene (% identity)
Listeria grayi	62	64
Enterococcus faecium	60	59
Enterococcus gallinarum EG2	60	65
Enterococcus casseliflavus	60	66

Table 5: Percent identity of mvaE and mvaS amino acid sequences compared to *Enterococcus faecalis* WT

Species	*mvaE* gene (% identity)	*mvaS* gene (% identity)
Listeria grayi,	59	70
Enterococcus faecium	61	60
Enterococcus gallinarum EG2	60	69
Enterococcus casseliflavus	59	71

(ii) *Plasmids pDW83, pMCM1223- pMCM1225*

[0346] The coding sequences of *MvaE* and *MvaS* from *Enterococcus casseliflavus* EC10 were optimized for expression in *Escherichia coli* (GeneOracle), and subcloned into the expression vector MCM82 (U.S. Patent Application Publication No. US2010/0196977, para. [1023]) to yield pDW83. Specifically, the cassette harboring the *mvaES* operon was cut from the cloning vector GcD126 (GeneOracle) using the restriction enzymes *BglI*I and *Pme*I (Roche) using standard molecular biology techniques. This fragment was then ligated (Roche Rapid Ligation) into MCM82 which had previously been subjected to restriction digest using the enzymes *BamH*I and *Pme*I (Roche) followed by agarose gel separation (Invitrogen E-Gel) to remove the expression cassette encoding *mvaES* from *Enterococcus faecalis* using standard molecular biology techniques. The ligation mixture was transformed into chemically competent Top10 cells (Invitrogen) according to the manufacturer's recommended protocol. Spectinomycin resistant positive transformants were grown in liquid LB medium, and plasmids were purified

(Qiagen Miniprep) and verified by sequencing (Quintara Biosciences) using the primers Ec Seq 1F through 4R (**Table 6**).

Table 6: Sequencing Primers

Ec Seq 1F	5'-GGGTATGAAAGCGATTCTGA-3'
Ec Seq 2F	5'-AGCCCAAGGCGCTATTACCG-3'
Ec Seq 3F	5'-GGATTAGTTCAAAATTTGGC-3'
Ec Seq 4F	5'-CGGTTAATGGCACGTTATGA-3'
Ec Seq 1R	5'-TCGTTCGCCTGTAAACTGCT-3'
Ec Seq 2R	5'-TGCTCTATTTCAGTACCTTT-3'
Ec Seq 3R	5'-TGTAAGTTCAGGCCCACGCC-3'
Ec Seq 4R	5'-CCTCAGCCTTGTTGTAATAA-3'

[0347] Plasmids encoding *MvaE* and *MvaS* from *Enterococcus faecium*, *Listeria grayi*, and *Enterococcus gallinarum* were constructed by GeneOracle (Mountain View, CA) using the design in **Table 7**. A synthetic DNA encoding mvaE-RBS-mvaS was created and then cloned into pMCM82 between the NcoI and PstI sites, replacing the existing operon. The vector provided an RBS for *mvaE*.

Table 7: Design for plasmids pMCM1223- pMCM1225 encoding *MvaE* and *MvaS* from *Enterococcus faecium*, *Listeria grayi*, and *Enterococcus gallinarum*

Plasmid Identifier	Plasmid Name	Source Organism	*MvaE*	*MvaS*	Origin and Selection
pMCM1223	pCL-Ptrc-Upper_GcMM_161 (*Listeria grayi DSM 20601*)	*L. grayi*, DSM 20601	gi|229554876|ref|ZP_04442665.1| acetyl-CoA acetyltransferase/hydroxymethylglutaryl-CoA reductase, degradative [*Listeria grayi DSM 20601*]	gi|229554877|ref|ZP_04442666.1| hydroxymethylglutaryl-CoA synthase [*Listeria grayi DSM 20601*]	pSC101, Spectinomycin (50ug/mL)
pMCM1224	pCL-Ptrc-Upper_GcMM_162 (Enterococcus faecium)	*E. faecium*	gi|9937391|gb|AAG02444.1|AF290094_2 acetyl-CoA acetyltransferase/HMG-CoA reductase [*Enterococcus faecium*]	gi|9937390|gb|AAG02443.1|AF290094_1 HMG-CoA synthase [*Enterococcus faecium*]	pSC101, Spectinomycin (50ug/mL)
pMCM1225	pCL-Ptrc-Upper_GcMM_163	*E. gallinarum* EG2	gi|257869528|ref|ZP_05649181.1| acetyl-CoA	gi|257869527|ref|ZP_05649180.1| hydroxymethylglut	pSC101, Spectinomycin (50ug/mL)

	(*Enterococcus gallinarum* EG2)		acetyltransferase/hydroxymethylglutaryl-CoA reductase [*Enterococcus gallinarum EG2*]	aryl-CoA synthase [*Enterococcus gallinarum EG2*]	

(iii) *pCL_pTrc-Upper(E. faecalis)-leaderless construction (pCHL276)*

[0348] Primers (CL483F: 5'-AGGAGGAATAAACCATGAAAACAGTAGTTATTATTGATGCATTAC-3'; CL484R: 5'-ACTACTGTTTTCATGGTTTATTCCTCCTTATTTAATCGATAC-3') were designed to remove an extra RBS on pCL_pTrc-Upper(*E. faecalis*), the MCM82 plasmid. The PCR reaction consisted of template DNA, MCM82 (100 ng), 50 uM of each forward and reverse primer, 1ul of 10 mM dNTPs (Roche), 5ul of 10X PfuII reaction buffer (Agilent), 1ul of Pfu II fusion enzyme (Agilent) and 40 ul of water. Eighteen cycles were performed with a temperature profile of 50 seconds at 95C, and 50 seconds at 60 °C, and 9 min at 68 °C and an additional 10 min extension at 68 °C in a Bio-Rad thermocycler. DpnI (1ul) was added after completion of the PCR reaction and incubated at 37 °C for two hours to remove template DNA. An additional 1ul of DpnI was added and incubated at 37 °C overnight. Two microliters of the reaction was transformed into TOP10 cells (Invitrogen) and plate of LB + 50 µg/mL spectinomycin. The correct clone was confirmed by sequencing.

(iv) *pCL_pTrc-Upper(E. casseliflavus)-leaderless construction (pCHL277)*

[0349] Primers (CL485F: 5'-AGGAGGAATAAACCATGGAAGAAGTTGTCATCATTGACGCAC-3'; CL486R: 5'-ACTTCTTCCATGGTTTATTCCTCCTTATTTAATCG-3') were designed to remove the extra RBS on pCL_pTrc-Upper(*E. casseliflavus*), pDW83 plasmid. The PCR reaction consisted of template DNA, pDW83 (100 ng), 50 uM of each forward (CL483F) and reverse primer (CL484R), 1ul of 10 mM dNTPs(Roche), 5ul of 10X Pfu II reaction buffer(Agilent), 1ul of Pfu II fusion enzyme (Agilent) and 40 ul of water. Eighteen cycles were performed with a temperature profile of 50 seconds at 95C, and 50 seconds at 60 °C, and 9 min at 68 °C and an additional 10 min extension at 68 °C in a Bio-Rad thermocycler. DpnI (1ul) was added after

PCR reaction and incubate at 37 °C for two hours to remove template DNA. An additional 1 ul of DpnI was added and incubate at 37 °C overnight. Two microliters of the reaction was transformed into TOP10 cell (Invitrogen) and plate of LA/spec50. The correct clone was confirmed by sequencing.

(v) *Construction of High Yield MVA Production Strains MCM1373-1377*

[0350] Host CMP676 was grown to mid-log in LB at 37C and prepared for electroporation by washing 3x in one half culture volume iced ddH2O and resuspended in one tenth culture volume of the same. 100uL of cell suspension was combined with 1uL plasmid DNA, moved to a 2mm electroporation cuvette, electroporated at 25uFD, 200ohms, 2.5kV, and immediately quenched with 500uL LB. Cells were recovered shaking at 37C for 1hr and then transformants selected overnight on LB plates with 50ug /mL spectinomycin at 37C. Single colonies were grown in LB+ 50ug /mL spectinomycin at 37C to OD600 ~1. 500uL of broth was mixed with 1mL 50% glycerol and frozen on dry ice. Frozen stocks were stored at -80C.

Example 7: Examination of mevalonate productivity metrics in engineered *E. coli* strains expressing genes from the mevalonate pathway, grown in fed-batch culture at the 15-L scale

(i) *Materials*

Medium Recipe (per liter fermentation medium):

[0351] Potassium phosphate K_2HPO_4 7.5 g, Magnesium Sulfate $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate $C_6H_8O_7*H_2O$ 2 g, ferric ammonium citrate $NH_4FeC_6H_5O_7$ 0.34 g, yeast extract (from biospringer) 0.5 g, 1000X Modified Trace Metal Solution 1.5 ml, sulfuric acid 50% w/v 2.26ml, foamblast 882 (Emerald Performance Materials) 0.83ml, Macro Salts Solution 3.36ml. All of the components were added together and dissolved in deionized H_2O. This solution was heat sterilized (123ºC for 20 minutes). After cooling to run temperature, the pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Feed solution #1 16.7 g, Vitamin Solution 11.9 mL, and spectinomycin solution 5ml, were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution (per liter):

[0352] 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 deionized H_2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

Macro Salt Solution (per liter):

[0353] $MgSO_4$ * $7H_2O$ 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

Vitamin Solution (per liter):

[0354] Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

Spectinomycin Solution (per liter):

[0355] 50g spectinomycin was q.s. to volume with deionized water and filter sterilized with 0.22 micron filter.

Feed solution #1 (per kilogram):

[0356] Glucose 0.590 kg, Di H2O 0.394 kg, K_2HPO_4 7.4 g, and Foamblast882 8.94g. All components were mixed together and autoclaved.

(ii) *Experimental Methods*

[0357] Fermentation was performed in a 15-L bioreactor with *E. coli* BL21 strains described in **Table 8**. Each strain was run twice, in identical conditions, so productivity results could be reported as an average of the two results.

Table 8: List of mevalonate producing strains examined in fed-batch culture at 15L scale

CMP680	HMB GI 1.2 gltA ML ackA-pta ldhA attB::Cm, pCLPtrcUpper(rbs) (pCHL276))
MCM1373	HMB GI 1.2 gltA ML ackA-pta ldhA attB::Cm + pCL-Ptrc-Upper_Ef
MCM1374	HMB GI 1.2 gltA ML ackA-pta ldhA attB::Cm + pCL-Ptrc-Upper_Ec
MCM1375	HMB GI 1.2 gltA ML ackA-pta ldhA attB::Cm + pCL-Ptrc-Upper_Listeria
MCM1376	HMB GI 1.2 gltA ML ackA-pta ldhA attB::Cm + pCL-Ptrc-Upper_Efaecium
MCM1377	HMB GI 1.2 gltA ML ackA-pta ldhA attB::Cm + pCL-Ptrc-Upper_Eg

[0358] A frozen vial of the *E. coli* strain was thawed and inoculated into tryptone-yeast extract medium (LB Miller medium) in a 2.8L Erlynmeyer flask to be used as the inoculums for the bioreactor. After the inoculum grew to optical density 1.0, measured at 550 nm (OD550), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0359] This experiment was carried out to monitor mevalonate formation from glucose at the desired fermentation pH 7.0 and temperature 34°C. Aerobic conditions were maintained for the duration of the run by sparging air at a rate of 8 standard liters per minute, holding back pressure of 0.7bar gauge, and a stirring rate of 850 rotations per minute, with impellers and baffling to transfer the power to the liquid medium.

[0360] The glucose feed solution was fed using a pulse feed program. As soon as the batch glucose was depleted, signaled by a pH rise (pH>=7.05), a pulse of 3 g/min for 20 min was added. Afterwards, a glucose feed pulse was induced by a pH trigger (pH>=7.05). The pulse lasted 30 min and the magnitude (g/min) was equal to the total carbon dioxide evolution rate (mmol/hr) divided by a predetermined factor sufficient to keep the residual glucose in the broth in excess. The total amount of glucose feed delivered to the bioreactor during the 52 hr fermentation varied by strain. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). A shot of IPTG was added to the tank to bring the concentration to 400 μM when the cells were at an OD550 of 4. The oxygen, nitrogen and carbon dioxide levels in the off-gas from the bioreactors were determined using a Hiden mass spectrometer. A time course of broth samples was taken at 4 hour intervals from each bioreactor. Broth concentration of glucose, citrate, and mevalonate were determined by HPLC. Optical density was determined by measuring the absorbance of dilute broth suspensions at 550nm and multiplying by the dilution factor, to report the result (OD550). The OD550 reading was converted to dry cell mass by using previously generated factors that compare OD550 to dry cell

weight over the time course of a fermentation. Productivity metrics of mass yield, specific productivity, titer, and cell productivity index are reported as an average of two results at comparable time points from each run, using the definitions given above (*See* "Definitions").

(iii) *Small Scale Mevalonate Yield Assay*

[0361] Overnight cultures were inoculated in shake tubes containing 2 mL LB broth supplemented with 50 µg/mL spectinomycin (Novagen) and 50 µg/mL carbenicillin (Novagen) from frozen stocks. Cultures were then incubated for 14h at 34°C at 250 rpm. Next, the cultures were diluted into an 5 mL 48-well plate (Axygen Scientific) containing 2 mL TM3 media supplemented with 1% Glucose, yeast extract to a total concentration of 1%, and 200 µM IPTG to final OD of 0.2. The plate was sealed with a Breath Easier membrane (Diversified Biotech) and incubated at 34°C in a Shel Lab shaker/incubator at 600 rpm for 24 hours. 1 mL of each culture was centrifuged at 3,000 x g for 5 min. 250 µl of supernatant was added to 19 µL of 20% sulfuric acid and incubated on ice for 5 min. The mixture was then centrifuged for 5 min at 3000 x g and the supernatant was collected for HPLC analysis. 200 µl of supernatant was transferred to a HPLC compatible 96-well conical bottom polypropylene plate (Nunc). The concentration of mevalonate in samples was determined by comparison to a standard curve of mevalonate (Sigma). The glucose concentration was measured by performing a glucose oxidase assay according to the manufacturer's specifications (Pointe Scientific, Inc.).

(iv) HPLC Detection of Mevalonate:

[0362] HPLC analysis was performed on a Waters 2695 Alliance HPLC system containing a Knauer K2301 refractive index detector using a 300mm x 7.8mm BioRad - Aminex HPX-87H ion exclusion column (catalog # 125-0140) incubated at 50°C and equipped with a BioRad - Microguard Cation H refill 30mm x 4.6mm (Catalog # 125-0129). Samples were run at a flow rate of 0.6 ml/min in 0.01 N sulfuric acid running buffer. Broth levels of mevalonate were able to be quantified by comparing the refractive index response of each sample versus a calibration curve generated by running various mevonate containing solutions of known concentration.

[0363] Production of mevalonate in batch culture at mass yields from glucose ranged from 34.8% to 41.1% from *E. coli* containing the *mvaE* and *mvaS* genes from the organisms *Listeria*

grayi_DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2, Enterococcus casseliflavus (**Figure 1**, **Table 9**).

Table 9: Mass yield of mevalonate from glucose. S.D. represents one standard deviation of two replicates.

Strain	IPTG (µM)	Mass Yield (%)	S.D.
CMP680	100	33.6	0.8
MCM1373	100	31.8	0.8
MCM1374	100	35.8	3.9
MCM1375	100	34.6	0.2
MCM1376	100	35.6	3.2
MCM1377	100	41.0	0.1
CMP680	200	35.3	0.1
MCM1373	200	31.9	0.2
MCM1374	200	39.2	3.0
MCM1375	200	34.8	1.0
MCM1376	200	37.9	3.3
MCM1377	200	41.1	4.9

[0364] The production of mevalonate in fed batch culture in a 15L fermentor at mass yields from glucose cumulatively ranged from 39.1% to 43.4% in *E. coli* containing the *mvaE* and *mvaS* genes from the organisms *Listeria* grayi*_DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2, Enterococcus casseliflavus* (**Table 10**).

Table 10: Cumulative mass yield results (average of the 3 final points of the 2 runs for each strain)

Strain	Upper enzymes	Mass Yield (Mevalonate on glucose) (w/w%)	Standard deviation (w/w%)	C.V.%
CMP680	*E. faecalis*	37.3	0.5	1.34%
MCM1374	*Enterococcus casseliflavus*	41.3	1.7	4.12%
MCM1375	*Listeria grayi DSM 20601*	39.1	2.0	5.12%
MCM1376	*Enterococcus faecium*	39.7	0.7	1.76%
MCM1377	*Enterococcus gallinarum EG2*	43.4	1.1	2.53%

[0365] Mevalonate peak specific productivities ranged from 87.5 to 100.1 g/L/h/OD in fed batch culture in a 15L fermentor in *E. coli* containing the *mvaE* and *mvaS* genes from the organisms *Listeria grayi_DSM 20601,* Enterococcus *faecium, Enterococcus gallinarum EG2, Enterococcus casseliflavus* (**Table 11**).

Table 11: Peak specific productivity observed for each strain (average of the peak observed values observed in the 2 runs for each strain)

Strain	Upper enzymes	Peak Specific productivity (mg/L/hr/OD)	Standard deviation (mg/L/hr/OD)	C.V.%
CMP680	*E. faecalis*	87.4	7.2	8.2%
MCM1374	*Enterococcus casseliflavus*	100.1	11.6	11.6%
MCM1375	*Listeria grayi DSM 20601*	87.5	26.7	30.5%
MCM1376	*Enterococcus faecium*	93.9	14.2	15.1%
MCM1377	*Enterococcus gallinarum EG2*	88.6	13.9	15.7%

[0366] Finally, mevalonate titers ranged from 108.2 to 115.4 g/L (**Table 12**), and CPIs ranged from 4.86 to 5.80 g mevalonate/g glucose (**Table 13**) in *E. coli* containing the *mvaE* and *mvaS* genes from the organisms *Listeria grayi_DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2, Enterococcus casseliflavus.*

Table 12: Peak mevalonate titer observed for each strain (average of the broth titer observed at 48hrs for each set of runs)

Strain	Upper enzymes	Peak Mevalonate Titer @ 48hrs EFT (g/L)	Standard deviation (g/L)	C.V.%
CMP680	*E. faecalis*	122.8	5.8	4.7%
MCM1374	*Enterococcus casseliflavus*	115.4	4.1	3.6%
MCM1375	*Listeria grayi DSM 20601*	108.2	4.8	4.4%
MCM1376	*Enterococcus faecium*	110.1	12.0	10.9%
MCM1377	*Enterococcus gallinarum EG2*	111.2	6.1	5.5%

Table 13: CPI values for each strain (average of the CPI values observed at 44 and 48 hours for each set of runs)

Strain	Upper enzymes	CPI (g/g)	Standard deviation (g/g)	C.V.%
CMP680	*E. faecalis*	4.25	0.25	5.9%
MCM1374	*Enterococcus casseliflavus*	5.70	0.37	6.5%
MCM1375	*Listeria grayi DSM 20601*	4.86	0.73	15.0%
MCM1376	*Enterococcus faecium*	5.29	0.12	2.3%
MCM1377	*Enterococcus gallinarum EG2*	5.80	0.52	8.9%

Example 8: Construction of isoprene-producing strains with host mutations

[0367] A lower mevalonate pathway can be introduced by transduction into CMP676 using a lysate from MCM521 (see **Table 3**). The kanamycin marker is looped out according to the manufacturer (Gene Bridges, Heidelberg, Germany). The lower pathway from MCM521 can be modified by changing the promoter upstream of the operon by modifying the rbs in front of each gene via the use of alternative genes. Plasmids pMCM1223 (*L. grayi*), pMCM1224 (*E. faecium*), pMCM1225 (*E. gallinarum*), pCHL276 (*E. faecalis*) or pCHL277 (*E. casseliflavus*)

are co-electroporated with a variation of plasmid pDW34 (*See* U.S. Patent Application Publication No: 2010/0196977; **Figure 2**). The plasmids, which are variants of pDW34, contain an isoprene synthase variant, which is improved for activity. Colonies can be selected on LB+ spectinomycin 50 ug/mL + carbenicillin 50 ug/mL.

[0368] The resultant strain is further engineered to modulate the activity of pyruvate dehydrogenase. This strain can be further engineered to increase the activity of one or more genes of the pyruvate dehydrogenase complex. This strain may also be further engineered to decrease the activity of the pyruvate decarboxylase repressor protein. Alternatively, the resultant strain may also be engineered to modulate the activity of citrate synthase. The activity of citrate synthase in the resultant stain can be modulated by decreasing the activity of citrate synthase. Alternatively, the resultant strain may also be engineered to modulate the activity of phosphotransacetylase and/or acetate kinase. The activity of phosphotransacetylase and/or acetate kinase can be modulated by decreasing the activity of phosphotransacetylase and/or acetate kinase. Alternatively, the resultant strain may also be engineered to modulate the activity of lactate dehydrogenase. The activity of lactate dehydrogenase can be modulated by decreasing the activity of lactate dehydrogenase. Alternatively, the resultant strain may also be engineered to modulate the activity of malic enzyme. The activity of lactate dehydrogenase can be modulated by increasing the activity of malic enzyme.

Example 9**:** Increased production of isoprene in strains containing the plasmids with host mutations compared Wild Type *E. faecalis*.

(i) *Materials*

TM3 media recipe (per liter fermentation media):

[0369] 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 0.2 g, 1000X Trace Metals Solution 1 ml. All of the components are added together and dissolved in diH_2O. The pH is adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media is filter-sterilized with a 0.22 micron filter. Glucose 10.0 g and antibiotics are added after sterilization and pH adjustment.

1000X Trace Metal Solution (per liter fermentation media)

[0370] Citric Acid*H_2O 40g, $MnSO_4$*H_2O 30g, NaCl 10g, $FeSO_4$*$7H_2O$ 1g, $CoCl_2$*$6H_2O$ 1g, $ZnSO_4$*$7H_2O$ 1g, $CuSO_4$*$5H_2O$ 100mg, H_3BO_3 100mg, $NaMoO_4$*$2H_2O$ 100mg. Each component is dissolved one at a time in diH_2O. The pH is adjusted to 3.0 with HCl/NaOH, and then the solution is brought to volume and filter-sterilized with a 0.22 micron filter.

(ii) *Experimental procedure*

[0371] Cells are grown overnight in Luria-Bertani broth + antibiotics. The day after, they are diluted to an OD600 of 0.05 in 20 mL TM3 medium containing 50 ug/ml of spectinomycin and 50 ug/mL carbenicillin (in a 250-mL baffled Erlenmeyer flask), and incubated at 34°C and 200 rpm. After 2h of growth, OD600 is measured and 200 uM IPTG is added. Samples are taken regularly during the course of the fermentation. At each timepoint, OD600 is measured. Also, off-gas analysis of isoprene is performed using a gas chromatograph-mass spectrometer (GC-MS) (Agilent) headspace assay. One hundred microliters of whole broth are placed in a sealed GC vial and incubated at 34°C and 200 rpm for a fixed time of 30 minutes. Following a heat kill step, consisting of incubation at 70°C for 5 minutes, the sample is loaded on the GC. The reported specific productivity is the amount of isoprene in ug/L read by the GC divided by the incubation time (30 min) and the measured OD600.

(iii) *Results*:

[0372] When the strains containing one or more host mutations are compared to the same background containing pCHL276 (*E. faecalis*), increased specific productivity, yield, CPI and/or titer of isoprene are observed.

Example 10: Construction of amorphadiene- or farnesene-producing strains

[0373] A lower mevalonate pathway is introduced by transduction into CMP676 using a lysate from MCM521 (see **Table 3**). The kanamycin marker is looped out according to the manufacturer (Gene Bridges, Heidelberg, Germany). The lower pathway from MCM521 can be modified by changing the promoter upstream of the operon by modifying the rbs in front of each gene via the use of alternative genes. Farnesyl diphosphate synthase (*ispA*) is overexpressed, either by altering the promoter and/or rbs on the chromosome, or by expressing it from a plasmid. Plasmids pMCM1223 (*L. grayi*), pMCM1224 (*E. faecium*), pMCM1225 (*E. gallinarum*), pCHL276 (*E. faecalis*) or pCHL277 (*E. casseliflavus*) are co-electroporated with a

variation of plasmid pDW34 (*See* U.S. Patent Application Publication No: 2010/0196977; Figure 2). The plasmids which are variants of pDW34 contain the farnesene synthase codon optimized for *E. coli* or amorphadiene synthase codon optimized for *E. coli,* instead of isoprene synthase. Colonies are selected on LB+ spectinomycin 50 ug/mL + carbenicillin 50 ug/mL.

[0374] The resultant strain is further engineered to modulate the activity of pyruvate dehydrogenase. This strain can be further engineered to increase the activity of one or more genes of the pyruvate dehydrogenase complex. This strain may also be further engineered to decrease the activity of the pyruvate decarboxylase repressor protein. Alternatively, the resultant strain may also be engineered to modulate the activity of citrate synthase. The activity of citrate synthase in the resultant stain can be modulated by decreasing the activity of citrate synthase. Alternatively, the resultant strain may also be engineered to modulate the activity of phosphotransacetylase and/or acetate kinase. The activity of phosphotransacetylase and/or acetate kinase can be modulated by decreasing the activity of phosphotransacetylase and/or acetate kinase. Alternatively, the resultant strain may also be engineered to modulate the activity of lactate dehydrogenase. The activity of lactate dehydrogenase can be modulated by decreasing the activity of lactate dehydrogenase. Alternatively, the resultant strain may also be engineered to modulate the activity of malic enzyme. The activity of lactate dehydrogenase can be modulated by increasing the activity of malic enzyme.

Example 11: Increased production of amorphadiene or farnesene in strains containing the plasmids with host mutations compared to a Wild Type *E. faecalis.*

(i) *Materials*

TM3 media recipe (per liter fermentation media):

[0375] 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 0.2 g, 1000X Trace Metals Solution 1 ml. All of the components are added together and dissolved in diH_2O. The pH is adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media is then filter-sterilized with a 0.22 micron filter. Glucose 10.0 g and antibiotics are added after sterilization and pH adjustment.

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

[0376] Citric Acid*H_2O 40g, $MnSO_4$*H_2O 30g, NaCl 10g, $FeSO_4$*$7H_2O$ 1g, $CoCl_2$*$6H_2O$ 1g, $ZnSO_4$*$7H_2O$ 1g, $CuSO_4$*$5H_2O$ 100mg, H_3BO_3 100mg, $NaMoO_4$*$2H_2O$ 100mg. Each component is dissolved one at a time in diH_2O. The pH is adjusted to 3.0 with HCl/NaOH, and then the solution is brought to volume and filter-sterilized with a 0.22 micron filter.

(ii) *Experimental procedure*

[0377] Cells are grown overnight in Luria-Bertani broth + antibiotics. The day after, they are diluted to an OD600 of 0.05 in 20 mL TM3 medium containing 50 ug/ml of spectinomycin and 50 ug/mL carbenicillin (in a 250-mL baffled Erlenmeyer flask), and incubated at 34°C and 200 rpm. Prior to inoculation, an overlay of 20% (v/v) dodecane (Sigma-Aldrich) is added to each culture flask to trap the volatile sesquiterpene product as described previously (Newman et. al., 2006).

[0378] After 2h of growth, OD600 is measured and 0.05-0.40 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) is added. Samples are taken regularly during the course of the fermentation. At each timepoint, OD600 is measured. Also, amorphadiene or farnesene concentration in the organic layer is assayed by diluting the dodecane overlay into ethyl acetate. Dodecane/ethyl acetate extracts are analyzed by GC–MS methods as previously described (Martin et. al., *Nat. Biotechnol.* 2003, 21:96–802) by monitoring the molecular ion (204 m/z) and the 189 m/z fragment ion for amorphadiene or the molecular ion (204 m/z) for farnesene. Amorphadiene or farnesene samples of known concentration are injected to produce standard curves for amorphadiene or farnesene, respectively. The amount of amorphadiene or farnesene in samples is calculated using the amorphadiene or farnesene standard curves, respectively.

(iii) *Results*

[0379] When the strains containing one or more host mutations are compared to the same are compared to the same background containing pCHL276 (*E. faecalis*), increased specific productivity, yield, CPI and/or titer of amorphadiene or farnesene are observed.

(iv) *References*

[0380] Newman, J.D., Marshal, J.L., Chang, M.C.Y., Nowroozi, F., Paradise,E.M., Pitera, D.J., Newman, K.L., Keasling, J.D., 2006. High-level production of amorpha-4,11-diene in a

two-phase partitioning bioreactor of metabolically engineered *E. coli. Biotechnol. Bioeng.*95, 684–691.

[0381] Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D., Keasling, J.D.,2003. Engineering a mevalonate pathway in *E. coli* for production of terpenoids. *Nat. Biotechnol.* 21, 796–802.

Example 12: Identification of MvaE Proteins that are not Degraded when Expressed in *E. coli* BL21 or *E. coli* BL21(DE3)

[0382] Degradation of heterologously expressed protein in a cell can result in loss of ATP due to the futile cycle of protein synthesis and protein degradation, decrease in catalytic activity of the protein being degraded, decrease in the steady state intracellular concentration of the protein of interest, induction of stress responses that can alter the physiology of the cell, and other effects that are potentially deleterious to the commercial production of biologically-derived products (S.-O. Enfors, 2004). Therefore, the expression of full length proteins that are less prone to degrade is beneficial for metabolic engineering. The *mvaE* gene product from *Enterococcus faecalis* is partially degraded when expressed in *E. coli* BL21 as indicated by fragments that can be identified by western blot (Fig. 3). Cleaved fragments of *E. faecalis* MvaE were also identified by Safestain staining of His-tagged purified material run on an SDS-PAGE gel (Fig. 4). Identification and use of degradation resistant *mvaE* gene products are beneficial for the increased production of mevalonate, isoprene and isoprenoids.

[0383] We demonstrate that the gene products of *mvaEs* from the organisms *E. faecium*, *E. gallinarum*, *E. casseliflavus*, and *L. grayi* are not degraded when expressed in *E. coli* BL21 (DE3) as indicated by absence of fragments that can be identified on Safestain stained SDS-PAGE gels following His-tag mediated purification or when expressed in mevalonate, isoprene or isoprenoid producing *E. coli* BL21 using the methods of detection described.

(i) *Methods*

[0384] Plasmids are constructed that contain DNA encoding His-tagged MvaE from *E. gallinarum, E. faecium, E. casseliflavus, and L. grayi*. MvaE is expressed in *E. coli* BL21 (DE3) and is purified by Ni-resin chromatography. Purified samples are analyzed by SDS-PAGE. Samples are further purified by anion exchange chromatography and in some cases gel filtration.

Samples purified to >95% homogeneity are sent for production of polyclonal antibodies. Production strains are analysed by western blot and probed using the polyclonal antibodies developed against the MvaE of interest.

(ii) *References*

Enfors, S.O., Scheper, T. Physiological Stress Responses in Bioprocesses. Springer-Verlag Berlin Heidelberg 2004.

Example 13: Construction of CMP451, (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA), CMP452 and CMP453

[0385] The promoter in front of the citrate synthase gene (*gltA*) in BL21 (Novagen) has been replaced by a constitutive low expression promoter, namely GI1.2 (US patent 7,371,558). Two wild-type promoters have been described for *gltA* (Wilde, R, and J. Guest. 1986. J. Gen. Microbiol. 132:3239-3251) and the synthetic promoter was inserted just after the -35 region of the distal promoter. A PCR product was obtained using primers UpgltACm-F (5'-TATTTAATTTTTAATCATCTAATTTGACAATCATTCAACAAAGTTGTTACAATTAACCCTCACTAAAGGGCGG-3') and DngltA1.xgiCm-R (5'-TCAACAGCTGTATCCCCGTTGAGGGTGAGTTTTGCTTTTGTATCAGCCATATATTCCACCAGCTATTTGTTAGTGAATAAAAGTGGTTGAATTATTTGCTCAGGATGTGGCATHGTCAAGGGCTAATACGACTCACTATAGGGCTCG-3'), and FRT-gb2-Cm-FRT template DNA from Gene Bridges (Heidelberg, Germany) as a template. The PCR product was purified and used in a lambda red-mediated recombination as described by the manufacturer (Gene Bridges, Heidelberg, Germany). Several colonies were selected for further characterization. The promoter region was PCR-amplified using primers gltAPromSeqF (5'-GGCAGTATAGGCTGTTCACAAAATC-3') and gltApromSeqR (5'-CTTGACCCAGCGTGCCTTTCAGC-3') and, as a template, DNA extracted by resuspending a colony in 30 uL H2O, heating at 95 C for 4 min, spinning down, and using 2 uL of that material as a template in a 50 uL reaction. After observing the sequencing results of the PCR products obtained, a colony harboring each of the three different promoters GI1.2, GI1.5 and GI1.6 (US patent 7,371,558) was saved for further use. Those colonies were named CMP141, CMP142 and CMP143 respectively (**Table 14**).

Table 14: *E. coli* strains

Strain	Genotype	Parent	Phenotype
CMP141	BL21 Cm-GI1.2 gltA	BL21	Lowered expression of citrate synthase
CMP142	BL21 Cm-GI1.5 gltA	BL21	Lowered expression of citrate synthase
CMP143	BL21 Cm-GI1.6 gltA	BL21	Increased expression of citrate synthase
CMP258	BL21 pgl+	BL21	BL21 strain restored for the presence of phosphogluconolactonase
CMP374	BL21 pgl+ PL.2 mKKDyI ldhA::Kan	MD09-314	Deletion of lactate dehydrogenase
CMP440	BL21 pgl+ PL.2 mKKDyI Cm-GI1.2 gltA	MD09-314	Lowered expression of citrate synthase
CMP441	BL21 pgl+ PL.2 mKKDyI Cm-GI1.5 gltA	MD09-314	Lowered expression of citrate synthase
CMP442	BL21 pgl+ PL.2 mKKDyI Cm-GI1.6 gltA	MD09-314	Increased expression of citrate synthase
CMP451	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA	CMP440	Lowered expression of citrate synthase
CMP452	BL21 pgl+ PL.2 mKKDyI GI1.5 gltA	CMP441	Lowered expression of citrate synthase
CMP453	BL21 pgl+ PL.2 mKKDyI GI1.6 gltA	CMP442	Increased expression of citrate synthase
CMP596	BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ldhA::Kan	CMP451	Deletion of lactate dehydrogenase
CMP604	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ackA-pta::Cm	CMP451	Deletion of acetate kinase and phosphotransacetylase
CMP620	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ackA-pta::Cm ldhA::Kan	CMP604	Deletion of acetate kinase and phosphotransacetylase and lactate dehydrogenase
CMP635	BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ackA-pta ldhA	CMP620	Deletion of acetate kinase and phosphotransacetylase and lactate dehydrogenase
CMP646	BL21 attB::Cm	BL21 (Novagen)	Chlroamphenicol cassette inserted in the chromosome in the attTn7 region
CMP662	BL21 FRT-Cm-FRT-GI1.2 maeB	BL21 (Novagen)	Constitutive low expression of NADPH-dependent malic enzyme
CMP671	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA Cm-GI1.2 maeB	CMP451	Constitutive low expression of NADPH-dependent malic enzyme
CMP674	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA attB:Cm	CMP451	Downregulated citrate synthase and lower pathway removed from chromosome
CMP676	BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ackA-pta ldhA attB::Cm	CMP635	Downregulated citrate synthase and lower pathway removed from chromosome and deletion of acetate kinase and phosphotransacetylase and lactate dehydrogenase
CMP678	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA attB:Cm, pCHL276	CMP674	Downregulated citrate synthase and lower pathway removed from chromosome

CMP680	BL21 pgl+ PL.2 mKKDyI GI1.2gltA ackA-pta ldhA attB::Cm, pCHL276	CMP676	Downregulated citrate synthase, lower pathway removed from chromosome and deletion of acetate kinase and phosphotransacetylase and lactate dehydrogenase
CMP681	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA GI1.2 maeB	CMP671	Constitutive low expression of NADPH-dependent malic enzyme
CMP694	BL21 pgl+ PL.2 mKKDyI, pCHL276	CMP258	Control. No additional host mutations.
CMP706	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA GI1.2 maeB attB::Cm	CMP681	Lowered expression of citrate synthase, constitutive low expression of NADPH-dependent malic enzyme, and lower pathway removed from chromosome
CMP711	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA GI1.2 maeB attB::Cm, pCHL276	CMP706	lower pathway removed from chromosome
CMP723	BL21 pgl+ PL.2 mKKDyI GI1.2gltA ackA-pta	CMP604	Acetate kinase and phsophotransacetylase
CMP725	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA pdhR attB::Cm	MD10-435	Pyruvate decarboxylase repressor deleted, and lower pathway removed from chromosome
CMP729	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA pdhR attB::Cm, pCHL276	CMP725	Pyruvate decarboxylase repressor deleted, and lower pathway removed from chromosome
CMP735	BL21 pgl+ PL.2 mKKDyI GI1.2gltA ackA-pta attB::Cm	CMP723	Acetate kinase and phsophotransacetylase deleted, and lower pathway removed from chromosome
CMP736	BL21 pgl+ PL.2 mKKDyI GI1.2gltA ackA-pta attB::Cm, pCLPtrcUpper(rbs)	CMP735	Acetate kinase and phsophotransacetylase deleted, and lower pathway removed from chromosome
CMP812	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ldhA::Kan attB::Cm	CMP596	Lactate dehydrogenase deleted, and lower pathway removed from chromosome
CMP828	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ldhA attB	CMP812	Lactate dehydrogenase deleted and lower pathway removed from chromosome
CMP832	BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ldhA attB, pCHL276	CMP828	Lactate dehydrogenase deleted and lower pathway removed from chromosome
MCM521	BL21 neo-PL.2-mKKDyI	U.S. Patent App. No: 12/978,324	BL21 strain containing a “lower mevalonate” pathway
MCM1002	BL21 pgl+, pMCM82, pTrcHis2B	CMP258	Control strain
MD09-273	BL21 pgl+ FRT::PL.6-rbs-yIDI-aspA PL.2 mKKDyI	BL21	Strain used for recombineering of fragments in the chromosome
MD09-313	BL21 pgl+ neo-PL.2-mKKDyI	CMP258	Mevalonate lower pathway on the chromosome, neomycin marker
MD09-314	BL21 pgl+ PL.2-mKKDyI	MD09-313	Mevalonate lower pathway on

			the chromosome, no antibiotic marker
MD10-429	BL21 wt+ pgl+ FRT::PL.6-rbs-yIDI-aspA PL.2 mKKDyI pdhR::Kan	MD09-273	Pyruvate decarboxylase repressor deleted
MD10-432	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA pdhR::Kan	CMP451	Pyruvate decarboxylase repressor deleted
MD10-434	BL21 pgl+ PL.6-rbs-yIDI-aspA PL.2 mKKDyI Cm::PL.6-pdh	MD10-426	Strong constitutive promoter upstream of pyruvate decarboxylase structural enzymes
MD10-435	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA pdhR	MD10-432	Pyruvate decarboxylase repressor deleted
MD10-440	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA Cm::PL.6-pdh	MD09-314	Strong constitutive promoter upstream of pyruvate decarboxylase structural enzymes
MD10-446	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA PL.6-pdh	MD09-314	Strong constitutive promoter upstream of pyruvate decarboxylase structural enzymes
MD10-551	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA+ PL.6-pdh attB::Cm	MD10-446	Pyruvate decarboxylase repressor deleted
MD10-554	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA+ PL.6-pdh attB	MD10-551	Strong constitutive promoter upstream of pyruvate decarboxylase structural enzymes, and lower pathway removed from chromosome
MD10-555	BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA+ PL.6-pdh attB, pCHL276	MD10-554	Strong constitutive promoter upstream of pyruvate decarboxylase structural enzymes, and lower pathway removed from chromosome
MD491	BL21 pgl+ ackA-pta::Cm	CMP258	Deletion of acetate kinase and phosphotransacetylase

[0386] Strain MD09-313 was built by transducing CMP258 (*See* U.S. Patent Application No: 12/978,324) with a P1 lysate from strain MCM521 (*See* U.S. Patent Application No: 12/978,324) and selecting for colonies on Luria-Bertani plates containing 20 ug/ml kanamycin. P1 lysates were prepared according to the method described in Ausubel, et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. The kanamycin marker was removed using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strain MD09-314.

[0387] P1 lysates were made from strains CMP141, CMP142 and CMP143 and were used to transduce strain MD09-314, to form CMP440, CMP441 and CMP442 respectively (**Table 3**). The chloramphenicol marker was removed using the protocol recommended by the manufacturer

(Gene Bridges, Heidelberg, Germany) to form strains CMP451, CMP452 and CMP453 respectively (**Table 14**).

Example 14**:** Citrate synthase assay and results

[0388] Citrate synthase (gltA) activity was measured by a method similar to that described in Sudgen, P. and Newsholm, E. 1975. *Biochem. J.* 150:105-111. In summary, it measures at 412 nm the release of CoASH from acetyl-CoA by reaction with DTNB.

[0389] Strains MD09-313, CMP451, CMP452 and CMP453 were grown overnight in 5 ml LB, at 34 °C and 200 rpm. They were diluted to an OD600 of 0.05 in 20 mL TM3 medium (previously described in U.S. patent No. 7,745,184, col. 32, lines 46-61) containing 10 g/L glucose. The cells were grown at 34 °C and 200 rpm to an OD600 of around 2-3. The broth was centrifuged, and the pellet thus obtained was frozen at -80 C. The day of the assay, the pellet was resuspended in assay buffer and the cells were broken using a French pressure cell. The lysate thus obtained was used in the citrate synthase assay.

[0390] Results of the assay are shown in **Figure 6**. Strain 451 and 452 had around half the activity of the wild-type, while strain CMP453 had around 1.5x the activity of the wild-type.

Example 15**:** Construction of strain MCM1002

[0391] Plasmid pMCM82 (pCLPtrcUpper) was electroporated in strain CMP258 and colonies were selected on LB + spectinomycin 50 ug/mL. One colony was picked and electroporated with pTcHis2B (Invitrogen, Carlsbad, CA) and transformants were selected on LB + spectinomycin 50 ug/ml and carbenicillin 50 ug/mL. One colony was picked and named MCM1002.

Example 16: Construction of strain CMP604 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ackA-pta::Cm), CMP723 (BL21 pgl+ PL.2 mKKDyI GI1.2gltA ackA-pta), and CMP735 (BL21 pgl+ PL.2 mKKDyI GI1.2gltA ackA-pta attB::Cm)

[0392] A DNA fragment containing the *ackA-pta* genes interrupted by a chloramphenicol marker was amplified by PCR using strain Triple Triple in which the Chloramphenicol marker is still in (US7,745,184 B2) as a template and primers ackACF (5'-GTGCAAATTCACAACTCAGCGG-3') and ptaCR (5'-CACCAACGTATCGGGCAT TGCC-3'). The PCR product obtained was used in a recombineering reaction as recommended by the

manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the *ackA-pta* locus in strain CMP258 (*See* U.S. Patent Application No: 12/978,324). Colonies were selected on LB + 5 ug/ml of chloramphenicol. One colony was picked and was named MD491. A P1 lysate of MD491 was made and was used to transduce strain CMP451. Colonies were selected on LB + 5 ug/ml of chloramphenicol. One colony was picked and was named CMP604. The chloramphenicol marker was removed using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strain CMP723. CMP723 was transduced with a P1 lysate made from strain CMP646 (see example 18). Colonies were selected on plates containing LB + 5 ug/mL chloramphenicol. One colony was picked and named CMP735.

Example 17: Construction of strain CMP596 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ldhA::Kan), CMP812 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ldhA::Kan attB::Cm) and CMP828 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ldhA attB)

[0393] Strain CMP451 was transduced with a P1 lysate made on strain CMP374 (see below) containing a kanamycin marker in the lactate dehydrogenase gene (ldhA). Colonies were plated on LB + 20 ug/ml kanamycin. One colony was chosen and named CMP596. CMP596 was transduced with the P1 lysate made from strain CMP646 (see example 18). Transductants were selected on LB + 5 ug/ml chloramphenicol. One colony was selected and named CMP812. The kanamycin and chloramphenicol markers were removed in one step using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strain CMP828.

Example 18: Construction of strain CMP620 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ackA-pta::Cm ldhA::Kan) and CMP635 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ackA-pta ldhA)

[0394] A DNA fragment containing the *ldhA* gene interrupted by a kanamycin marker was amplified by PCR using strain JW 1375 from the Keio collection (Baba et al. 2006. *Mol. Syst. Biol.* 2: 2006.0008) as a template, and primers ldhAseqR (5'-GGCTTACCGTTTACGCTTTCCAGC-3') and ldhAseqF2 (5'-CTAATGCAATACGTGTCCCGAGC-3'). The PCR product obtained was used in a recombineering reaction as recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the *ldhA* locus in strain MD09-313. Colonies were selected on LB + 20 ug/ml of kanamycin. One colony was picked and was named CMP374. A

P1 lysate of CMP374 was made and was used to transduce CMP604. Colonies were selected on LB + 20 ug/ml of kanamycin. One colony was picked and was named CMP620. The chloramphenicol and kanamycin markers were looped out simultaneously by electroporating pCP20 (Datsenko and Wanner. 2000. *PNAS* 97:6640-5) in the strain, selecting two colonies on LB + 50 ug/ml carbenicillin at 30°C, then restreaking those colonies on an LB plate at 42°C. A chloramphenicol- and kanamycin- sensitive colony was selected from those plates and named CMP635.

<u>Example 19: Construction of strains CMP674 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA attB::Cm) and CMP676 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ackA-pta ldhA attB::Cm)</u>

[0395] A DNA fragment containing a chloramphenicol marker flanked by DNA homologous to the upstream and downstream regions of the λ attachment site attB was amplified by PCR using plasmid pKD3 (Datsenko, K., and Wanner, B. 2000. *PNAS* 97:6640-6645) as a template, and primers CMP171 (5'-AAAATTTTCATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTCACATGGAGTTGGCAGGATGTTTGATTACATGGGAATTAGCCATGGTCC-3') and CMP172 (5'-GACCAGCCGCGTAACCTGGCAAAATCGGTTACGGTTGAGTAATAAATGGATGCCCTGCGTAAG CGG GGCATT TTTCTTGGTGTAGGCTGGAGCTGCTTCG-3'). The PCR product obtained was used in a recombineering reaction in BL21 (Novagen) as recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the λ attachment site attB. Strain CMP646 was thereby generated, selected on LB + 5 ug/ml chloramphenicol. A P1 lysate of CMP646 was made and was used in a transduction reaction on strains CMP451 and CMP635, thereby removing the lower mevalonate pathway (mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentenyl diphosphate isomerase)from the chromosome of that strain. The transduction reaction was plated on LB + chloramphenicol 5 ug/ml and one colony for each transduction was picked and named CMP674 and CMP676 respectively.

Example 20: Construction of strains CMP678 (BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA, pCHL276), CMP680 (BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA ackA-pta ldhA attB::Cm), CMP694 (BL21 pgl+ PL.2 mKKDyI, pCHL276), CMP736 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ackA-pta attB::Cm, pCHL276) and CMP832 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA ldhA attB, pCHL276)

[0396] Plasmid pCHL276 was introduced into strains CMP674, CMP676, CMP258, CMP735 and CMP828 by electroporation. Colonies were selected on LB + 50 ug/mL spectinomycin. One colony for each transformation was picked and named CMP678, CMP680, CMP694, CMP736 and CMP832 respectively.

Example 21: Increased production of mevalonate in a strain containing the GI1.2gltA construct in comparison to its parent, and increased mevalonate production in a strain containing ackA-pta ldhA in comparison to its parent or a strain containing one mutation at a time.

[0397] This example shows production of mevalonate in strains CMP678, CMP680, CMP694, CMP736 and CMP832.

(i) *Materials*

TM3 media recipe (per liter fermentation media):

[0398] 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 0.2 g, 1000X Trace Metals Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media was filter-sterilized with a 0.22 micron filter. Glucose 10.0 g and antibiotic were added after sterilization and pH adjustment.

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

[0399] Citric Acid*H_2O 40g, $MnSO_4*H_2O$ 30g, NaCl 10g, $FeSO_4*7H_2O$ 1g, $CoCl_2*6H_2O$ 1g, $ZnSO_4*7H_2O$ 1g, $CuSO_4*5H_2O$ 100mg, H_3BO_3 100mg, $NaMoO_4*2H_2O$ 100mg. Each component is dissolved one at a time in diH_2O. The pH is adjusted to 3.0 with HCl/NaOH, and then the solution is brought to volume and filter-sterilized with a 0.22 micron filter.

(ii) *Experimental procedure*

[0400] Cells were grown overnight in Luria-Bertani broth. The day after, they were diluted to an OD600 of 0.05 in 20 mL TM3 medium containing 50 ug/ml of spectinomycin (in a 250-mL baffled Erlenmeyer flask), and incubated at 34°C and 200 rpm. After 2h of growth, OD600 was measured and 100 uM IPTG was added. Samples were taken regularly during the course of the fermentation. At each timepoint, OD600 was measured and mevalonate concentration was measured by HPLC. HPLC analysis was performed in the following way: 15 uL of 70% (w/v) perchloric acid was added to 500 uL of broth and the mixture was incubated on ice for 5 minutes. Next, the sample was centrifuged at 14,000×g for 5 minutes and the supernatant collected for HPLC analysis run under the following conditions: (1) BioRad - Aminex HPX-87H Ion Exclusion Column (300 mm x 7.8 mm)(Catalog # 125-0140)(BioRad, Hercules, California); (2) column temperature = 50°C; (3) BioRad - Microguard Cation H guard colum refill (30 mm x 4.6 mm)(Catalog # 125-0129)(BioRad); (4) running buffer = 0.01N H_2SO_4; (5) running buffer flow rate = 0.6 ml / min; (6) approximate running pressure = ~950 psi; (7) injection volume = 100 microliters; (8) runtime = 26 minutes.

(iii) *Results*

[0401] Strain CMP678 (GI1.2gltA) was assessed using strain CMP694 (parent) as a control.

[0402] The experiment demonstrated that strain CMP678, having the *gltA* promoter downregulated, showed an increased titer from the same amount of glucose when compared to a strain with a wild-type *gltA* promoter (CMP694) (**Figure 8**). The strains with a downregulated *gltA* promoter grew to a slightly lower OD than the strain with a WT promoter (**Figure 7**).

[0403] Strain CMP680 (*ackA-pta ldhA*), CMP736 (*ackA-pta*), and CMP832 (*ldhA*) were assessed using strain CMP678 (parent) as a control. The *ackA-pta* mutation contributed a slower growth and a lower final OD to the strain, while the *ldhA* mutation did not affect growth (**Figure 7**). The combination of *ackA-pta* and *ldhA* mutation slowed growth and decreased final biomass further (**Figure 7)** further. By themselves, the *ackA-pta* or *ldhA* mutations did not contribute to a higher final concentration of mevalonate, but in combination, they provided a 250% increase in titer (and yield since it is linked to titer in small scale) (**Figure 8**).

<u>Example 22: Construction of CMP662 (BL21 FRT-Cm-FRT-GI1.2 maeB)</u>

[0404] The native promoter in front of the NADP-dependent malic enzyme (*maeB*) has been replaced by a constitutive promoter, namely GI1.2 (U.S. Patent No. 7,371,558). The insertion site of the new promoter has been chosen carefully so as not to alter expression of the gene just upstream (*talA*) which is translated in the opposite direction (**Figure 9**). While the *maeB* promoter has not been described, the *talA* promoter has been experimentally determined (Lacour & Landini, 2004, *J. Bact.* 186:7186-7195).

[0405] A PCR product has been obtained using primers maeB_GI1.xpKD3 (5'-ACT GGA AAT TCA TGG AAA TCA AGT GCA CTT TGT TTT AAC TGG TCA TCC ATT ATA TAC CTC CTG CTA TTT GTT AGT GAA TAA AAG TGG TTG AAT TAT TTG CTC AGG ATG TGG CAT n GT CAA GGG CGT GTA GGC TGG AGC TGC TTC-3') and maeBUp_pKD3 (5'-AGA GTT TGG ACT TGC TCA AAG TCT GTA GAC TCC GGC AGG GTA ATA ATG TGC GCC ACG TTG TGG GCA GGG G ATGGGAATTAGCCATGGTCC-3'), and plasmid pKD3 (Datsenko & Wanner 2000, *PNAS* 97:6640-6645) as a template. The enzyme Herculase II (Agilent, Santa Clara, US) was used according to the manufacturer. The PCR product was purified and used in a lambda red-mediated recombination as described by the manufacturer (Gene Bridges, Heidelberg, Germany). Several colonies were selected for further characterization. The promoter region was PCR-amplified using primers maeBPromseqF: GTGAACTGTTTGATGCCGTC and maeBPromSeqR: ccgtaccgttagagatcacc and, as a template, DNA extracted by resuspending a colony in 30 uL H2O, heating at 95 C for 4 min, spinning down, and using 2 uL of that material as a template in a 50 uL reaction. After observing the sequencing results of the PCR products obtained, a colony harboring the GI1.2 promoter (US patent 7,371,558) was saved for further use. This colony was named CMP662 (**Table 3**). A P1 lysate of strain CMP662 was made and was used to transduce CMP451. Colonies were selected on LB + 5 ug/ml of chloramphenicol. One colony was picked and was named MDCMP671. The chloramphenicol marker was removed using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strain CMP68. CMP681 was transduced with a P1 lysate made from strain CMP646 (see example 18). Colonies were selected on plates containing LB + 5 ug/mL chloramphenicol. One colony was picked and named CMP706.

<u>Example 23: Construction of strain MD10-432(BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA pdhR::Kan), MD10-435 (BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA pdhR) and CMP725 (BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA pdhR attB::Cm) - pdhR mutant cell line</u>

[0406] A DNA fragment containing the *pdhR* gene interrupted by a kanamycin marker was amplified by PCR using strain JW0109 from the Keio collection (Baba et al. 2006 *Mol. Syst. Biol.* 2:2006.0008) using primers MQ10-82F (5'-GGTAAGTGAATCGGTTCAATTCGG-3') and MQ10-82R (5'-CGCTCAACACCTTCTTCACGGATG-3'). The PCR product obtained was purified with Qiagen PCR purification Kit (Qiagen, Germantown, MD) and used in a recombineering reaction as recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the pdhR locus in strain MD09-273. Transformants were selected on LA+ Kan12.5 ug/ml plates incubated at 37°C. One colony was picked and checked by PCR using primers MQ10-83F (5'-CCTGTATGGACATAAGGTGAATAC-3') and MQ10-83R (5'-CCTGTCCCATTGAACTCTCGCCGG-3') which were about ~250bp upstream & downstream of pdhR gene region. The correct mutant was named MD10-429.

[0407] A P1 lysate of strain MD10-429 was made and used to transduce CMP451. Colonies were selected on LB+ Kan12.5 mg/ml. Several transductant colonies were screened by PCR using outside primers MQ10-83F (5'-CCTGTATGGACATAAGGTGAATAC-3') and MQ10-83R (5'-CCTGTCCCATTGAACTCTCGCCGG-3'). The correct mutant in CMP451 background strain is named MD10-432.

[0408] The Kanamycin marker was removed by FRT recombination by electroporating pCP20 (Datsenko and Wanner, 2000, *PNAS* 97:6640-5), selecting a couple colonies on LA+ 50 ug/ml Carbenicillin at 30°C, then re-streaking the colony of interest on an LA plate at 37°C. The resulting markerless strain was named MD10-435. Strain MD10-435 was transduced with the P1 lysate made from strain CMP646 (see example 18). Transductants were selected on LB + 5 ug/ml chloramphenicol. One colony was selected and named CMP725.

Example 24 : Construction of strain MD10-434 (BL21 pgl+ PL.6-rbs-yIDI-aspA PL.2 mKKDyI + Cm::PL.6-pdh), MD10-440 (BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA+ Cm::PL.6-pdh) and MD10-446 (BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA+ PL.6-pdh)- PL.6-pdh mutant cell line

[0409] The promoter in front of the Pdh operon (Pyruvate Dehydrogenase complex) in BL21 (Novagen) has been modified by replacing the native promoter by a constitutive high expression promoter, namely PL.6. A PCR product was obtained using primers MQ10-84F (5'-AGAGTTCAATGGGACAGGTTCCAGAAAACTCAACGTTATTAGATAGATAAGGAATAACCCGTGTAGGCTGGAGCTGCTTC-3') and MQ10-84R (5'-

CGTCATTTGGGAAACGTTCTGACATGTTTTTTTACCTCCTTTGCACCTTCATGGTGGT CAGTGCGTCCTGCTGATGTGCTCAGTATCACCGCCAGTGGTATTTATGTCAACACCG CCAGAGATAATTTATCACCGCAGATGGTTATCTGTATGTTTTTTATATGAATTCATAT GAATATCCTCCTTA-3') to amplify the chloramphenicol gene flanked by FRT sites from pKD3 plasmid (Datsenko and Wanner. 2000, *PNAS* 97:6640-5). A second PCR product was generated using BL21 as atemplate to amplify the whole PDH operon (*aceE*, *aceF* and *lpdA*) using primers MQ10-85F (5'-ATGTCAGAACGTTTCCCAAATGACG-3') and MQ10-85R (5'-GCGGCGTGGTTAGCCGCTTTTTTAATTGCCGGATGTTCCGGCAAACGAAAAATTACT TCTTCTTCGCTTTCGGGTTC-3'). Fusion PCR was used to fuse both pieces of DNA together, with primers MQ10-84F & MQ10-85R. An approximately 1.6 Kbp PCR product was obtained and purified using a Qiagen PCR purification Kit (Qiagen, Germantown, MD). Approximately, 300-400 ng of purified PCR product was used in a lambda red-mediated recombination as described by the manufacturer (Gene Bridges, Heidelberg, Germany) targeting the PDH promoter in strain MD09-273. When this integration failed to integrate, the PCR product was re-amplified using a shorter reverse primer version (MQ10-91R 5'-GAAGTGGTTAAAGCACAC-3'). As a result, ~1.6 kbp PCR product was successfully integrated in MD09-273. Transformants were selected on LA+ chloramphenicol 5ug/ml. Several colonies were selected for further characterization using primers MQ10-84F & MQ10-84R. The promoter region was PCR-amplified using other primers MQ10-84F & MQ10-91R for further verification. The PCR product obtained was sequenced and shown to be correct, and the strain was named MD10-434.

[0410] A P1 lysate of MD10-434 was made and used to transduce CMP451. Transductants were selected on LB+ chloramphenicol 5 μg/ml. Several colonies were verified by PCR using primers MQ10-84F & MQ10-84R. One mutant was picked and named MD10-440.

[0411] The chloramphenicol marker was removed by FRT recombination (Datsenko & Wanner. 2000. *PNAS* 97:6640-5), using plasmid pCP20. Once the transformants were obtained on LA+ 50 ug/ml carbenicillin at 30°C, two colonies were re-streaked on a LB plate and incubated at 37°C. A chloramphenicol-sensitive colony was selected from those plates and named MD10-446.

[0412] MD10-446 was transduced with a P1 lysate obtained from strain CMP646 (see example 18). Transductants were selected on LB + 5 ug/ml chloramphenicol. One colony was

selected and named MD10-551. The chloramphenicol marker was removed by FRT recombination (Datsenko & Wanner. 2000. *PNAS* 97:6640-5), using plasmid pCP20. Once the transformants were obtained on LA+ 50 ug/ml carbenicillin at 30°C, two colonies were re-streaked on a LB plate and incubated at 37°C. A chloramphenicol-sensitive colony was selected from those plates and named MD10-554.

<u>Example 25: Construction of strains MD10-555 (BL21 pgl+ PL.2 mKKDyI GI 1.2 gltA PL.6pdh attB::Cm, pCHL276), CMP711 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA GI1.2 maeB attB::Cm, pCHL276), CMP729 (BL21 pgl+ PL.2 mKKDyI Gi1.2-gltA pdhR attB::Cm, pCHL276)</u>

[0413] Plasmid pCHL276 (see example 6 (iii)) was introduced into strains MD10-446, CMP706 and CMP725 by electroporation. Colonies were selected on LB + 50 ug/mL spectinomycin. One colony for each transformation was picked and named MD10-555, CMP711 and CMP729 respectively.

<u>Example 26: Increased production of mevalonate in strains containing the GI1.2maeB, pdhR, and PL.6 pdh mutations in comparison to their parent</u>

[0414] This example shows production of mevalonate in strains CMP678, MD10-555, CMP711 and CMP729.

(i) *Materials*

TM3 media recipe (per liter fermentation media):

[0415] 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 0.2 g, 1000X Trace Metals Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media was filter-sterilized with a 0.22 micron filter. Glucose 10.0 g and antibiotic were added after sterilization and pH adjustment.

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

[0416] Citric Acid*H_2O 40g, $MnSO_4$*H_2O 30g, NaCl 10g, $FeSO_4$*$7H_2O$ 1g, $CoCl_2$*$6H_2O$ 1g, $ZnSO_4$*$7H_2O$ 1g, $CuSO_4$*$5H_2O$ 100mg, H_3BO_3 100mg, $NaMoO_4$*$2H_2O$ 100mg. Each component is dissolved one at a time in diH_2O. The pH is adjusted to 3.0 with HCl/NaOH, and then the solution is brought to volume and filter-sterilized with a 0.22 micron filter.

(ii) *Experimental procedure*

[0417] Cells were grown overnight in Luria-Bertani broth. The day after, they were diluted to an OD600 of 0.05 in 20 mL TM3 medium containing 50 ug/ml of spectinomycin (in a 250-mL baffled Erlenmeyer flask), and incubated at 34°C and 200 rpm. After 2h of growth, OD600 was measured and 100 uM IPTG was added. Samples were taken regularly during the course of the fermentation. At each timepoint, OD600 was measured and mevalonate concentration was measured by HPLC. HPLC analysis was performed in the following way: 15 uL of 70% (w/v) perchloric acid was added to 500 uL of broth and the mixture was incubated on ice for 5 minutes. Next, the sample was centrifuged at 14,000×g for 5 minutes and the supernatant collected for HPLC analysis run under the following conditions: (1) BioRad - Aminex HPX-87H Ion Exclusion Column (300 mm x 7.8 mm)(Catalog # 125-0140)(BioRad, Hercules, California); (2) column temperature = 50°C; (3) BioRad - Microguard Cation H guard colum refill (30 mm x 4.6 mm)(Catalog # 125-0129)(BioRad); (4) running buffer = 0.01N H2SO4; (5) running buffer flow rate = 0.6 ml / min; (6) approximate running pressure = ~950 psi; (7) injection volume = 100 microliters; (8) runtime = 26 minutes.

(iii) *Results*

[0418] Strains MD10-555 (PL.6pdh), CMP711 (GI1.2maeB) and CMP729 (pdhR) were assessed using strain CMP678 (parent) as a control.

[0419] The experiment demonstrated that strain MD10-555 and CMP729, presumably fluxing more pyruvate towards acetyl-CoA, and CMP711, bringing carbon back from the TCA cycle into pyruvate while generating NADPH, showed an increased titer from the same amount of glucose when compared to a parent strain (CMP678) (**Figure 14**). CMP729, with the *pdhR* mutation, had a slower growth rate than the parent (**Figure 13**). The PL.6pdh and GI1.2maeB mutations did not affect growth (**Figure 13**).

Example 27: Isoprene production from *E. coli* expressing genes from the mevalonate pathway and isoprene synthase, grown in fed-batch culture at the 15-L scale.

(i) *Materials*

Medium Recipe (per liter fermentation medium):

[0420] K2HPO4 7.5 g, MgSO4 * 7H2O 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 50% sulphuric acid 1.6 mL, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in Di H2O. This solution was heat sterilized (123°C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution (per liter):

[0421] Citric Acids * H2O 40 g, MnSO4 * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

Vitamin Solution (per liter):

[0422] Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, D-pantothenic acid 4.8 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

Feed solution (per kilogram):

[0423] Glucose 0.57 kg, Di H2O 0.38 kg, K2HPO4 7.5 g, and 100% Foamblast 10 g. All components were mixed together and autoclaved. Macro Salt Solution 3.4 mL, 1000X Modified Trace Metal Solution 0.8 ml, and Vitamin Solution 6.7 mL were added after the solution had cooled to 25°C.

Macro Salt Solution (per liter):

[0424] MgSO4 * 7H2O 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

(ii) *Methods*

[0425] Fermentation was performed in a 15-L bioreactor with *E. coli* BL21 cells overexpressing the upper MVA pathway (pCLUpper – MCM82), the lower MVA pathway (PL.2-mKKDyI) and truncated isoprene synthase from *P. alba* (pTrcAlba(MEA)) and containing a restored chromosomal *pgl* gene as well as a knocked-down *gltA* gene behind the GI1.2 promoter (strain name CMP457). The parental strain, used in the control fermentation, was a strain without the knocked-down *gltA* gene.

[0426] This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 34°C. A frozen vial of the *E. coli* strain was thawed and inoculated into a flask with tryptone-yeast extract medium and the appropriate antibiotics.. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0427] The feed solution was fed at an exponential rate until a top feed rate of 6 g/min was reached. After this time, the glucose feed was fed to meet metabolic demands at rates less than or equal to 6 g/min. The total amount of glucose delivered to the bioreactor during the 52 hr fermentation was 6.8 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). A shot of IPTG was added to the tank to bring the concentration to 100 uM when the cells were at an OD_{550} of 6 and a second shot was added bring the concentration to 100 uM when the cells were at an OD_{550} of 100.

[0428] The isoprene level in the off-gas from the bioreactor was determined using an iSCAN (*Hamilton* Sundstrand) mass spectrometer.

(iii) *Results*

[0429] The fermentation with the GI1.2 gltA promoter in front of gltA had higher a yield than its parental control (**Figure 15**). Isoprene titer, volumetric productivity and cell productivity index were also higher in the GI1.2gltA strain. Results are summarized in **Table 15** (below).

Table 15: Isoprene Productivity Metrics (GI1.2gltA vs wild type gltA)

Strain description	EFT (hrs)	Titer (g/L)	Volumetric Productivity (g/L/hr)	Overall % Yield of MVA on glucose (g/g)	CPI (gMVA/gDCW)
CMP457 (GI1.2 promoter in front of gltA)	52	113.0	1.60	10.7%	1.65
MD09-317 (wt promoter in front of gltA)	53	77.6	0.92	8.6%	0.79

Example 28: Mevalonate (MVA) production from *E. coli* expressing genes from the mevalonate pathway and grown in fed-batch culture at the 15-L scale.

(i) *Materials*

Medium Recipe (per liter fermentation medium):

[0430] K2HPO4 7.5 g, MgSO4 * 7H2O 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 50% sulphuric acid 1.6 mL, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in Di H2O. This solution was heat sterilized (123°C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution (per liter):

[0431] Citric Acids * H2O 40 g, MnSO4 * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

Vitamin Solution (per liter):

[0432] Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

Feed solution (per kilogram):

[0433] Glucose 0.590 kg, Di H2O 0.393 kg, K2HPO4 7.4 g, and 100% Foamblast882 8.9 g. All components were mixed together and autoclaved.

Macro Salt Solution (per liter):

[0434] MgSO4 * 7H2O 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter. Addition strategy A (GI1.2gltA runs): Add 16.8mls directly the tank media before sterilization, with no further addition. Addition strategy B: (wt gltA run) Add 6.895mls per liter of glucose feed solution.

(ii) *Methods*

[0435] Fermentation was performed in a 15-L bioreactor with *E. coli* BL21 cells expressing the upper MVA pathway (pCLUpper) and containing a restored chromosomal *pgl* gene. Therefore, three mevalonate producing strains were compared.

[0436] MCM1002 (wt promoter in front of *gltA*, *E.faecalis* upper) (See Example 12)

[0437] CMP678 (GI1.2 promoter in front of *gltA*, *E.faecalis* upper) (**See Table 3**).

[0438] CMP680 (ackA-pta-, ldhA-host, GI1.2gltA, E.faecalis upper) (See Example 5)

[0439] This experiment was carried out to monitor mevalonate formation from glucose at the desired fermentation pH 7.0 and temperature 34°C. A frozen vial of the *E. coli* strain was thawed and inoculated into a flask with tryptone-yeast extract medium and the appropriate antibiotics. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0440] The batched media had glucose batched in at 9.9 g/L. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). A shot of IPTG was added to the tank to bring the concentration to 400 uM when the cells were at an OD_{550} of 6. Once the glucose was consumed by the culture, as signaled by a rise in pH, the glucose feed solution was fed to meet metabolic demands at rates less than or equal to 10 g/min. The fermentation was run long enough to determine the maximum mevalonate mass yield on glucose, at least 36 hrs elapsed fermentation time.

(iii) *Conclusions*

[0441] The fermentation with the GI1.2 gltA knockdown (CMP678) had higher a mevalonate yield on glucose than its parental control (MCM1002). Additionally, a higher cell productivity index, volumetric productivity and mevalonate titer were noted (**Figures 20, 21** and **24** respectively. Summarized in **Table 16**).

Table 16: MVA Productivity Metrics (GI1.2gltA vs wild type gltA)

Strain description	EFT (hrs)	Titer (g/L)	Volumetric Productivity (g/L/hr)	Overall % Yield of MVA on glucose (g/g)	CPI (gMVA/gDCW)
MCM1002 (wt gltA, E.faecalis upper)	36.4	70.8	1.95	17.5%	1.80
CMP678 (GI1.2gltA, E.faecalis upper)	36.0	137.1	3.81	35.1%	3.28

[0442] The fermentation with the “triple” host (CMP680) had a higher cell productivity index and a higher overall mevalonate mass yield on glucose than the CMP678 strain (**Figure 23** and **Figure 24**, respectively). Very early in the CMP680 fermentation, the mevalonate mass yield on glucose, and the specific productivity of mevalonate (g/L/hr/OD) was much higher than that of CMP678 or MCM1002. (**Figure 24** and **Figure 25**, respectively). In the CMP680 fermentation, the mevalonate volumetric productivity and mevalonate broth concentration were down versus CMP678 (**Figure 26** and **Figure 27**, respectively). Results summarized in **Table 17**. The CMP680 strain (or, the host with triple mutation or triple host) is useful for determining maximum mevalonate mass yield on glucose and maximum specific productivity in 15L fermentation experiments.

Table 17: Mevalonate (MVA) Productivity Metrics ("Triple" host vs GI1.2gltA vs wild type gltA)

Strain description	EFT (hrs)	Titer (g/L)	Volumetric Productivity (g/L/hr)	Overall % Yield of MVA on glucose (g/g)	CPI (gMVA/gDCW)
MCM1002 (wt gltA, E.faecalis upper)	36.4	70.8	1.95	17.5%	1.80
CMP678 (GI1.2gltA, E.faecalis upper)	36.0	137.1	3.81	35.1%	3.28
CMP680 (ackA-,pta-,ldh- host, GI1.2gltA, E.faecalis upper)	36.2	109.5	3.03	36.1%	3.75

[0443] 36hr is sufficient to get a read on mevalonate mass yield, but the runs can be extended to increase the mevalonate broth concentration. A table shows the peak titers observed in these experiments. Results observed at the peak titers are summarized in **Table 18**, below.

Table 18: Mevalonate (MVA) Productivity Metrics at peak titer ("Triple" host vs GI1.2gltA vs wild type gltA)

	EFT at peak titer (hrs)	Peak Titer (g/L)	Volumetric Productivity at peak titer (g/L/hr)	Overall % Yield of MVA on glucose (g/g) at peak titer	CPI at peak titer (gMVA/gDCW)
MCM1002 (wt gltA, E.faecalis upper)	36.4	70.8	1.95	17.5%	1.80
CMP678 (GI1.2gltA, E.faecalis upper)	52.0	156.0	3.00	37.2%	4.15
CMP680 (ackA-,pta-,ldh- host, GI1.2gltA, E.faecalis upper)	55.2	124.6	2.26	38.0%	4.39

(iv) *Broth analysis*

[0444] The mevalonate concentration in the fermentor broth was determined in broth samples taken at 4 hour intervals by an HPLC analysis. Mevalonate broth concentration in samples was determined by comparison of the refractive index response versus a previously generated

calibration curve obtained by running a prepared solution of high purity mevalonate (Sigma Aldrich).

HPLC Information:

[0445] System: Waters Alliance 2695; Column: BioRad - Aminex HPX-87H Ion Exclusion Column 300mm x 7.8mm Catalog # 125-0140; Column Temperature: 50C; Guard column: BioRad - Microguard Cation H refill 30mm x 4.6mm Catalog # 125-0129; Running buffer: 0.01N H_2SO_4; Running buffer flow rate: 0.6 ml / min; Approximate running pressure: ~1100-1200 psi; Injection volume: 20 microliters; Detector: Refractive Index (Knauer K-2301); Runtime: 26 minutes.

Example 29: Production of *E. coli* strains expressing the mevalonate kinase gene from *Methanococcoides burtonii DSM6242*

[0446] This example details the construction of bacterial strains comprising mevalonate kinase from the psychrotolerant methanogen *M. burtonii* for the production of isoprenoid, isoprenoid precursors, and isoprene through the heterologous mevalonate pathway in *E. coli.*

I. Gene identification and pathway construction

[0447] **Gene Identification/Selection:** A primary sequence homology search using the *M. mazei* mevalonate kinase gene product as the query was performed via the BLASTp program located at the NCBI website (Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Res.* 25:3389-3402). Sequences of interest were selected from the search results. *M. burtonii* mevalonate kinase has 61% sequence identity when compared to the mevalonate kinase from *M. mazei*.

[0448] **Plasmids for the expression of MVK:** Plasmids for the expression of N-terminally HIS-tagged mMVK and bMVK were synthesized by GeneOracle. Tagged MVK genes were cloned into pET24b under the control of a T7 promoter. Plasmids were introduced into Invitrogen OneShot BL21(DE3) cells (#C6000-03) following the manufacturer's protocol and transformants selected on LA kan50 plates at 37C overnight.

[0449] Lower Isoprenoid Pathways Expressing mMVK and bMVK at Different Levels: An existing construct (PL.2-mKKDyI, US2011/0159557) expressing the lower MVA pathway (MVK, PMK, MVD, IDI) was modified to express mMVK at different levels and to express bMVK in place of mMVK. The new construct consists of an FRT-flanked chloramphenicol resistance marker, the PL.2 promoter, a bi-cistronic translation initiation sequence, one of two MVK genes, and the PMK, MVD and IDI genes. A PCR product was constructed in two parts to replace the existing promoter, translation initiation sequence and MVK gene with an excisable cmpR marker, promoter, translation initiation sequence and MVK gene. This PCR product was introduced onto the chromosome by double crossover followed by selection of cmpR cells.

[0450] The bi-cistronic translation initiation sequence is used to increase expression of MVK by initiating translation of the mRNA independently of the MVK sequence. See Schoner et al., Translation of a synthetic b-cistron mRNA in Escherichia coli (*Proc. Natl. Acad. Sci. USA,* Vol. 83, pp. 8506-8510, November 1986). Translation of the MVK ORF is controlled by modulating the strength of the upstream RBS in the bi-cistronic construct. RBSs of varied strength were designed using the RBS Calculator (Salis, et al., *Nat Biotechnol.* 2009 October; 27(10): 946–950.) RBS Calculator optimization software was used with RNA thermodynamic parameters calculated using the Vienna RNA Package v.1.8.4 (http://www.tbi.univie.ac.at/~ivo/RNA/, Andreas R. Gruber, Ronny Lorenz, Stephan H. Bernhart, Richard Neubuck, and Ivo L. Hofacker (NAR, 2008) and the ViennaRNA module for the RBS Calculator. RBSs were calculated on a Linux server running Python v. 2.4.3. RBSs were designed using the 5' UTR upstream of the RBS (up to 50nt) and 50nt of ORF sequence. Multiple RBSs of a given target strength were calculated; one or more was synthesized and tested.

[0451] The GeneBridges chloramphenicol template (#A105) was used as template to amplify the upstream half of the construct using primers MCM545 and MCM770. 140µL ddH$_2$O, 4µL plasmid DNA (~100ng/µL), 5µL 10µM each primer, 2µL dNTPs, 40uL buffer and 4µL polymerase were combined and aliquoted to 4x50µL. These reactions were cycled according to manufacturer's protocol (Agilent Herculase II Fusion kit, #600677) in a PCR program consisting of the following cycle: 95 °C 2:00, (95 °C 0:20, 55 °C 0:20, 72 °C 1:15) x30, 72 °C 3:00, 4 °C until cold. Reactions were purified on Qiagen PCR cleanup columns (#28106), eluted in 50µL and pooled

[0452] The downstream half of the construct was amplified using the MVK gene as template and primers indicated in **Table 22,** below. 35µL ddH_20, 1µL plasmid DNA (~100ng/µL), 1.25µL 10µM each primer, 0.5µL dNTPs, 10uL buffer and 1µL polymerase were combined and cycled as follows: 95 °C 2:00, (95 °C 0:20, 55 °C 0:20, 72 °C 0:30 (mMVK) or 72 °C 1:00 (bMVK)) x30, 72 °C 3:00, 4 °C until cold. Reactions were purified on Qiagen PCR cleanup columns and eluted in 50µL.

[0453] 5µL of upstream and downstream fragments were mixed with 10µL buffer, 0.5µL dNTPs and 27µL ddH_2O and PCR cycled: 95 °C 2:00, (95 °C 0:20, 55 °C 0:20, 72 °C 2:00)x5, rt. 1.25µL of primer MCM120 and either primer MCM162 (mMVK) or MCM790 (bMVK) were added and 25 additional cycles were run. The PCR reaction was brought to 4 °C before purification on Qiagen PCR column and elution in 30µL EB.

[0454] Purified, fused PCR products were electroporated into MCM1332 (for mMVK) or MCM1859 cells (for bMVK). MCM1332 is a subculture of CMP522 (CMP451 (IDN 31588) carrying pRed/ET-carb, GeneBridges) and MCM1859 is a subculture of CMP1103 (CMP1075 (IDN 31308) carrying pRed/ET-carb, GeneBridges). Cultures were grown overnight in LB carb50 at 30 °C. 100µL culture was used to inoculate 5mL of LB carb50 and cultures were grown at 30 °C until visibly turbid (~2hr). 150µL 1M arabinose was added and cultures grown at 37 °C for ~2hrs. Cells were pelleted, washed three times in sterile, iced ddH_2O, and resuspended at 1/10th culture volume in sterile, iced ddH_2O. 100µL cells were mixed with 3µL PCR product and electroporated in a 2mm electroporation cuvette, at 25µFD, 200ohms, 2.5kV, and immediately quenched with 500µL LB. Transformed cells were recovered shaking at 37 °C for ~3hrs and selected overnight on LB cmp5 plates at 37 °C.

[0455] Multiple cmpR colonies were streaked to single colonies and screened for the expected integration by PCR and sequencing. Colony purified strains were grown in LA cmp5, brought to 33% glycerol and stored at -80 °C.

Table 19: *E. coli* strains expressing alternative MVK genes

Strain with modified pathway	MVK	5' RBS Target Strength	PCR Template	Upstream Up Primer	Upstream Down Primer	Downstream Up Primer	Downstream Down Primer

MCM1742	*mazei*	1000	pMCM376 (12/818,090)	MCM545	MCM770	MCM773	MCM162
MCM1743	*mazei*	100000	pMCM376	MCM545	MCM770	MCM778	MCM162
MCM1745	*mazei*	1000000	pMCM376	MCM545	MCM770	MCM781	MCM162
MCM1747	*mazei*	10000	pMCM376	MCM545	MCM770	MCM775	MCM162
MCM1861	*burtonii*	10000	pMCM1666	MCM545	MCM770	MCM789	MCM790

II. Hosts for Producing Isoprene using mMVK and bMVK at Different Levels

[0456] The modified lower MVA pathways were transduced into MCM2029 (a subculture of CMP1133, IDN 31308) to create isoprene production hosts.

[0457] P1 lysates of strains MCM1742, 1753, 1745, 1747 and 1861 (Transduced Pathways) were prepared as follows. 100µL of overnight culture in LB cmp5 was diluted into 10mL LB + 5mM $CaCl_2$, 0.2% glucose, cmp5 and shaken at 250rpm, 37°C for 30 min. 100µL of P1 lysate prepared on MG1655 cells was added and culture shaken for at least 3 hours until the media was clear with precipitated lysed cell material. Lysates were vortexed with 100µL chloroform, spun 5min at 3000rpm, and supernatant stored over 100µL chloroform at 4 °C.

[0458] Overnight culture of MCM2029 (Recipient for Transduction) was washed into ½ volume sterile 10mM $MgSO_4$, 5mM $CaCl_2$. 200µL cell suspension was mixed with 100µL lysate and incubated, still, at 30 °C for 30min. 150µL 1M NaCitrate and 500µL LB were added and the reaction recovered, with shaking, at 37 °C for 1-2 hours. Dilutions were plated on LA cmp5 and incubated at 37 °C overnight. Negative controls (no cells or no transducing lysate) were performed in parallel. Chloramphenicol-resistant colonies were picked, restreaked, and single colonies tested by PCR for the presence of the new pathway. The resulting strains, Hosts with Marker, were grown to mid-log in LA and stored at -80 °C in 33% glycerol.

[0459] The chloramphenicol resistance marker in the Hosts with Marker was removed using the Flp recombinase expressed from plasmid pCP20 (GeneBridges). Cells were grown to midlog in LB cmp5 at 37 °C, electroporated with 1µL pCP20 (~100ng/µl), recovered in LA at 30

°C for 1-3hrs, and transformants selected on LA carb50 at 30C overnight or room temperature for 3 days. A single colony was picked, grown at in LA carb50 30C until visibly turbid, and then shifted to 37 °C for several hours. This culture was streaked out and grown at 37 °C overnight. Single colonies were picked and patched to LA, LA carb50 and LA cmp5. A clone sensitive to carb and cmp was selected for each parent strain. The resulting Isoprene Production Hosts (**Table 20**) were grown to midlog in LB and stored at -80 °C in 33% glycerol.

Table 20: Isoprene production hosts

Isoprene Production Hosts	Hosts with Marker	Recipient for Transduction	Transduced Pathways
MCM2065	MCM2050	MCM2029	MCM1861
MCM2066	MCM2053	MCM2029	MCM1742
MCM2067	MCM2054	MCM2029	MCM1743
MCM2069	MCM2057	MCM2029	MCM1745
MCM2070	MCM2059	MCM2029	MCM1747

III. Plasmids for Expression of Isoprene Synthase without mMVK

[0460] Plasmid pDW166 (Trc P. alba IspS variants MEA (Carb50)) was modified to express bMVK rather than mMVK. The bMVK gene was amplified from pMCM1666 with primers MCM810 and MCM811 and the pDW166 vector, minus the mMVK gene, was amplified with primers MCM808 and MCM809 (Herculase II Fusion kit, 35µL ddH$_2$O, 1µL template (~100ng/µL), 0.5µL dNTPs, 1.25µL each primer at 10µM, 1µL polymerase). Reactions were cycled as follows: 95°C 2:00, 30x(95 °C 0:20, 55 °C 0:20, 72 °C 0:45 (insert) or 3:00 (vector)), 72 °C 3:00, 4 °C until cold. PCR products were purified on a Qiagen PCR column and eluted in 30µL EB. The insert was cloned into the vector using the GeneArt Seamless Cloning and Assembly kit (Invitrogen #A13288) in a reaction consisting of 2µL each PCR product, 4µL buffer, 10µL ddH$_2$O and 2µL enzyme. Reaction was incubated at room temperature for 30min then 4µL was transformed into Invitrogen TOP10 chemically competent cells. Following recovery in 250µL LB at 37 °C for 1hr, transformants were selected on LA carb50 plates at 37 °C overnight. Miniprepped plasmid was sequenced.

[0461] Plasmid pDW166 (pTrcAlba(MEA, IspS variants)-mMVK was modified to delete the majority of the mMVK gene. Plasmid was amplified in a PCR reaction using primers MCM814 and 815 (Herculase II Fusion kit, 35µL ddH_2O, 1µL template (~100ng/µL), 0.5µL dNTPs, 1.25µL each primer at 10µM, 1µL polymerase). Reactions were cycled as follows: 95 °C 2:00, 30x(95 °C 0:20, 55 °C 0:20, 72 °C 3:30 (vector)), 72 °C 3:00, 4 °C until cold. PCR product was desalted on a Millipore VSWP02500 disk floated on ~10mL ddH2) for ~15 minutes and then 5µL was electroporated into MCM2065 cells. Following recovery in 500µL LB at 37 °C for 1hr, transformants were selected on LA carb50 plates at 37 °C overnight. Miniprepped DNA was sequenced.

IV. Strains for the Production of Isoprene via mMVK and bMVK at Different Levels

[0462] Host strains (**Table 20**) were grown in 5mL LB at 37 °C, 250 rpm to midlog. Iced culture was washed in sterile, iced ddH_2O three times and resuspended in 1/10th culture volume. 100µL cell suspension was mixed with 1µL plasmid DNA (~100ng/uL) in eppendorf tube. All strains were transformed with pMCM2095. MCM2065 was also transformed with 1µL each pMCM2020 and pMCM1225 (IDN 31552). Reactions were moved to a 2mm electroporation cuvette, electroporated at 25uFD, 200ohms, 2.5kV, and immediately quenched with 500µL LB. Reactions were recovered shaking at 37 °C for at least one hour and transformants selected overnight at 37 °C on LA plates with carb50 (and also spec50 in the case of MCM2065 cotransformed with pMCM2020 and pMCM1225). A colony for each transformation was grown to midlog in LB with antibiotics, brought to 33% glycerol and stored at -80 °C (Host with IspS). Strain MCM2131 was stored in the same manner. The Host with IspS strains were grown in LB carb50 and electroporated with pMCM1225, with transformants selected on LA carb50 spec50. Again, single colonies were grown in LB carb50 spec50, brought to 33% glycerol, and stored at -80 °C (Production Strain, **Table 21**).

Table 21 Production strains

Production Strain	Upper Pathway Plasmid	Host with IspS	IspS Plasmid	Host
MCM2125	pMCM1225	MCM2099	pMCM2095	MCM2065
MCM2126	pMCM1225	MCM2100	pMCM2095	MCM2066
MCM2127	pMCM1225	MCM2101	pMCM2095	MCM2067
MCM2129	pMCM1225	MCM2103	pMCM2095	MCM2069

MCM2130	pMCM1225	MCM2104	pMCM2095	MCM2070
MCM2131	pMCM1225	N/A	pMCM2020	MCM2065

Table 22: Primers used for production of host strains

MCM162	TTAATCTACTTTCAGACCTTGC
MCM545	aaagtagccgaagatgacggtttgtcacatggagttggcaggatgtttgattaaaagcAATTAACCCTCACTAAAGGGCGG
MCM770	TCCTGCTGATGTGCTCAGTATCACCGCCAGTGGTATTTAAGTCAACACCGCCAGAGATAATTTATCACCGCAGATGGTTATCTTAATACGACTCACTATAGGGCTCG
MCM773	ggcggtgatactgagcacatcagcaggacgcactgcTCTAAGGATTAAAGAGGAGAAGATTTCCTGatgtatcgatttaaataaggaggaataacatatggtatcctgttctgcgccg
MCM775	ggcggtgatactgagcacatcagcaggacgcactgcTCCTAGGGGCGATTAGGGGACCTACTACatgtatcgatttaaataaggaggaataacatatggtatcctgttctgcgccg
MCM778	ggcggtgatactgagcacatcagcaggacgcactgcTCTAGAGCGCACTAAGGAGGCAACTGGatgtatcgatttaaataaggaggaataacatatggtatcctgttctgcgccg
MCM781	ggcggtgatactgagcacatcagcaggacgcactgcTGCAGCGAGGAGGTAAGGGatgtatcgatttaaataaggaggaataacatatggtatcctgttctgcgccg
MCM789	ggcggtgatactgagcacatcagcaggacgcactgcTCCTAGGGGCGATTAGGGGACCTACTACatgtatcgatttaaataaggaggaataacatATGATAACGTGCTCTGCGCC
MCM790	GGCTCTCAACTCTGACATGTTTTTTTCCTCCTTAAGGGTGCAGGCCTATCGCAAATTAGCttactgacattctacgcgaaca
MCM810	GTTTAAACTTTAACTAGACTTTACTGACATTCTACGCGAACACCG
MCM811	cataaaggaggtaaaaaaacATGATAACGTGCTCTGCGCCG
MCM814	gggtaagattagtctagttaaagtttaaac
MCM815	TAACTAGACTAATCTTACCCGGCGCAGAAC

V. Protein Purification

[0463] Protein expression and purification: *E. coli* strain MCM1666 harboring *M. burtonii* mevalonate kinase gene was grown at 34°C in 1-L of Terrific broth containing 50 mg/L kanamycin. Cells were induced with 50 µM IPTG at OD_{600} = 0.6 and harvested by centrifugation 5 hours after induction. Cell pellets were resuspended in 0.05 M sodium phosphate, 0.3 M sodium chloride, 0.02 M imidazole (pH 8.0) buffer containing 0.2 mg/ml DNaseI and 0.5 mM of 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) following a freeze-thaw cycle. Lysis was achieved by repeated passes through a French Pressure cell at 20,000 psi. Cell lysates were clarified by ultracentrifugation at 229,000×g for one hour. The supernatants were filtered through a 0.2 µ filter and loaded onto a HiTrap IMAC HP column

charged with nickel sulfate and equilibrated with 0.05 M sodium phosphate, 0.3 M sodium chloride, 0.02 M imidazole (pH 8.0). Mevalonate kinase was isolated using a linear gradient from 0.02 to 0.5 M of imidazole. Fractions containing the mevalonate kinase were identified using SDS-PAGE (Invitrogen) and desalted into 0.05 M Tris, 0.05 M sodium chloride (pH 7.4) using a Hi Prep 26/10 desalting column. Final mevalonate kinase fraction was 95% when assessed by SDS-PAGE and coomassie staining. Quantitation was performed using spectrophotometer readings at 280 nm (conversion factor equal to 0.3 OD/mg/ml, VectorNTI.

[0464] Isolation, purification and kinetic analysis of *M. mazei* MVK was previously described in U.S. Patent Application Publication No. 2010/0086978**.**

Example 30: Solubility of *M. burtonii* and *M. mazei* mevalonate kinases from pET24b plasmid in MCM1666 and MCM1669 strains

[0465] This example examines the respective solubility of mevalonate kinases derived from *M. burtonii* and *M. mazei.*

(i) Materials and Methods

[0466] Strains MCM1666 and MCM1669 were grown at 34°C in 5 ml of LB medium containing 50 mg/L kanamycin. Cells were induced with 50 µM IPTG at OD(600)=0.6 and harvested by centrifugation 4 hours after induction. Cell lysates were prepared in 50 mM Tris, 50 mM NaCl (pH 7.4) with 0.2 mg/ml DNaseI and 0.5 mM AEBSF and passed twice through the French Pressure Cell set at 700 psi to achieve lysis. Protein solubility was analyzed by SDS-PAGE gel.

(ii) Results

[0467] When expressed of pET24b vector *M. burtonii* mevalonate kinase was 70% soluble, where as mevalonate kinase from *M. Mazei* had a solubility of about 5% and the rest of the protein was located in the pellet fraction (**Figure 28**).

Example 31: Kinetic analysis and inhibition of mevalonate kinases from *M. burtonii* and *M. mazei*

[0468] This example details the respective kinetics of catalytic activity and inhibition of mevalonate kinases derived from *M. burtonii* and *M. mazei.*

(i) Materials and Methods

[0469] Kinetics were performed using SpectraMax 190 platereader (Molecular Devices). All kinetic data were analyzed using Kaleidagraph 4.0 graphing program from Synergy software. Adenosine triphosphate (ATP), phosphoenolpyruvate (PEP), NADH, magnesium chloride, sodium chloride, Tris, HEPE, DNase I, as well as MVP and MVPP were purchased from Sigma. Dithiothreitol was purchased from Fluka. Lactate dehydrogenase was purchased from Calbiochem and pyruvate kinase was purchased from MD biomedicals. DMAPP, IPP and GPP were synthesized in-house.

[0470] The catalytic activities of both *M. burtonii* and *M. mazei* mevalonate kinase were measured using a modified spectrophotometric assay that couples ADP formation to pyruvate synthesis and reduction to lactate. The initial rate of disappearance of NADH serves as a measure of phosphorylation of mevalonate by MVK. The assays were performed in triplicate in a 96-well non-binding plate (Costar catalog #9017) format, at 30ºC. Each 100 µl reaction contained 0.4 mM PEP, 0.05 mM DTT, 0.32 mM NADH, 10 mM $MgCl_2$, 2.5 units of LDH and 2.5 units of PK in 50 mM HEPES, 50 mM NaCl (pH 7.4).

[0471] The kinetic constants for the mevalonate kinases from *M. burtonii* and *M. mazei* were determined by applying random bireactant system kinetics model using varying amounts of both mevalonate and ATP substrates. The following concentrations were tested: 0.4 mM, 0.2 mM, 0.1 mM and 0.05 mM of mevalonate and 2 mM, 1 mM, 0.5 mM and 0.25 mM of ATP. The reaction was initiated with the addition of 40 nM of purified M. burtonii mevalonate kinase or 80 nM (0.25 µg) of purified M. mazei mevalonate kinase. Absorbance changes associated with the amount of NADH oxidized to NAD+ were monitored continuously at 340 nm and plotted against time to determine the rate of the mevalonate kinase coupled reactions. Protein inhibition studies were performed by adding terpenyl diphosphates (DMAPP, IPP, GPP, MVPP) as well as terpenyl monophosphate MVP at various concentrations to the reaction mix. Previously purified *S. cerevisiae* mevalonate kinase was used as a positive control for DMAPP, IPP and GPP inhibition where as *S. pneumoniae* mevalonate kinase was used as a positive control for MVP and MVPP inhibition.

(ii) Results

[0472] Kinetic constants were evaluated for *M. burtonii* and *M. mazei* mevalonate kinases with respect to both mevalonate and ATP using random bireactant system model (**Table 23**). *M. burtonii* mevalonate kinase has a k_{cat} of 44.4 s^{-1} for mevalonate and a k_{cat} of 74.3 s^{-1} for ATP, whereas *M. mazei* mevalonate kinase has k_{cat} of 18.3 s^{-1} for mevalonate and a k_{cat} of 26.8 s^{-1} for ATP at 34°C. $K_{a(Mev)}$ values for *M. burtonii* and *M. mazei* mevalonate kinases were very similar, 391 μM and 397 μM respectively. $K_{b(ATP)}$ were 212 μM for *M. burtonii* mevalonate kinase and 460 μM for *M. mazei.*

Table 23: Kinetic and inhibition data for mevalonate kinases derived from *M. burtonii* and *M. mazei*

Mevalonate kinase	Substrate	k_{cat} (s^{-1})	$K_{a/b}$ (μM)	V_{max} (μM/sec)	α	K_i DMAPP (μM)	K_i IPP (μM)	K_i GPP (μM)	K_i MVP (μM)	K_i MVPP (μM)
M. burtonii	Mevalonate	44.4	391	1.78	0.52	> 5000	> 5000	> 5000	> 3000	> 5000
	ATP	74.3	212	2.97	3.74					
M. mazei	Mevalonate	18.3	397	1.46	0.23	> 5000	> 5000	> 5000	> 3000	> 1000
	ATP	26.8	460	2.14	1.62					

<u>Example 32: Growth and isoprene productivity of *E. coli* strains expressing *M. burtonii* or *M. mazei* mevalonate kinase on the *E. coli* chromosome</u>

[0473] This example details an examination of the growth and isoprene productivity in engineered *E. coli* strains expressing *M. burtonii* mevalonate kinase or *M. mazei* mevalonate kinase on the *E. coli* chromosome at small scale.

Materials and Methods

[0474] **Growth assays:** Overnight cultures were inoculated in shake tubes containing 2 mL of LB broth supplemented with 50 μg/mL carbenicillin (Novagen) and 50 μg/mL spectinomycin (Novagen) from frozen stocks. Cultures were then incubated for 14h at 34°C at 240 rpm. Next, the cultures were diluted into a 5 mL 48-well plate (Axygen Scientific) containing 2 mL TM3 media supplemented with 1% glucose, 0.02% yeast extract, 50 μg/mL carbenicillin and 50 μg/mL spectinomycin to a final OD of 0.2. The plate was sealed with Breath Easier membrane (Diversified Biotech) and incubated at 34°C in a Shel Lab shaker/incubator at 600 rpm. The cultures were induced with 200 μM IPTG at OD of 0.4. One hour after induction mevalonate

was added to the cultures to a final concentration of 0, 2, 4, 8, 16, 32 mM. OD measurements were taken at 0, 1, 2, 3, 4, and 5 hrs after induction with IPTG.

Table 24: List of the engineered *E. coli* strains examined at small scale

Strain Name	Abbreviated Genotype
CMP1136	pgl- + pTrcAlba(MEA, G491S)-mMVK + pCL-Ptrc-Upper_Ef
DW708	pgl- + pTrcAlba(MEA, IspS variants)-mMVK + pCL-Ptrc-Upper_gallinarum
MCM2131	pgl- FRT-PL.2-2cis-RBS10000-MVK(burtonii) + pTrcAlba(MEA, IspS variants)-bMVK + pCL-Ptrc-Upper_gallinarum
MCM2125	pgl- FRT-PL.2-2cis-RBS10000-MVK(burtonii) + pTrcAlba(MEA, IspS variants)-mMVK(del) + pCL-Ptrc-Upper_gallinarum
MCM2126	pgl- FRT-PL.2-2cis-RBS1000-mMVK + pTrcAlba(MEA, IspS variants)-mMVK(del) + pCL-Ptrc-Upper_gallinarum
MCM2127	pgl- FRT-PL.2-2cis-RBS100000-mMVK + pTrcAlba(MEA, IspS variants)-mMVK(del) + pCL-Ptrc-Upper_gallinarum
MCM2129	pgl- FRT-PL.2-2cis-RBS1000000-mMVK + pTrcAlba(MEA, IspS variants)-mMVK(del) + pCL-Ptrc-Upper_gallinarum
MCM2130	pgl- FRT-PL.2-2cis-RBS10000-mMVK + pTrcAlba(MEA, IspS variants)-mMVK(del) + pCL-Ptrc-Upper_gallinarum

[0475] Isoprene productivity: Samples for analysis of isoprene productivity by GC/MS from the engineered *E. coli* strains were taken at 1, 2, 3, 4, and 5 hrs after induction. 100 μL of culture broth was pippeted into deep-98-well glass block and sealed with aluminum sealer (Beckman Coulter). The glass block was incubated for 30 min at 34°C water bath, after which it was transferred to 80°C water bath for a 2 min heat-kill incubation. The glass block was cooled and transferred to the GC/MS for isoprene measurements.

[0476] Isoprene Detection by GC/MS: GC/MS 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 GC/MS method utilized helium as the carrier gas at a flow of 1 ml/min. The injection port was held at 250ºC with a split ratio of 50:1. The oven temperature was held at 37ºC 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.

(ii) Results

[0477] Growth of MCM2131 is not inhibited by mevalonate concentrations ranging between 0 and 16 mM. MCM2131 has the highest specific productivity ranging between 30-42 mg/L/h/OD with 32 mM mevalonate added, therefore is able to support very high flux from the upper pathways.

[0478] Engineered strains MCM2125, MCM2127 and MCM2130 with one copy of chromosomal mevalonate kinase are able to achieve specific productivities of 40 mg/L/h/OD with 16 mM mevalonate feed. Their growth is also not inhibited by mevalonate concentrations between 0-16 mM (**Figure 29**).

Example 33: Plasmid and chormosomal expression of *M. mazei* and *M. burtonii* mevalonate kinases in *E. coli.*

[0479] Strains MCM2126 and MCM2127 were run to determine if any benefit can be obtained by expressing the *Mazei* MVK off of the chromosome only.

Materials and Methods

(i) Solutions

[0480] **Medium Recipe (per liter fermentation medium)**: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 50% sulphuric acid 1.6 mL, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in Di H_2O. This solution was heat sterilized (123°C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

[0481] **1000X Modified Trace Metal Solution (per liter):** Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$

100 mg, H_3BO_3 100 mg, $NaMoO^4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

[0482] Macro Salt Solution (per liter): MgSO4 * $7H_2O$ 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

[0483] Vitamin Solution (per liter): Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H_2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

[0484] Feed solution #1 (per kilogram): Glucose 0.590 kg, Di H_2O 0.393 kg, K_2HPO_4 7.4 g, and 100% Foamblast882 8.9 g. All components were mixed together and autoclaved. After autoclaving the feed solution, nutrient supplements are added to the feed bottle in a sterile hood. Post sterilization additions to the feed are (per kilogram of feed solution), Macro Salt Solution 5.54ml, Vitamin Solution 6.55ml, 1000X Modified Trace Metal Solution 0.82ml.

(ii) Methods

[0485] Samples were thawed and normalized to OD=20 in 100 mM Tris, 100 mM NaCl, pH 7.6, 0.1 mg/ml DNaseI, 1 mg/ml lysozyme, and 0.5 mM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride). OD normalized cell suspensions were lysed by repeated pass through the French pressure cell at 700 psi. Lysates were clarified by centrifugation at 14,000 rpm for 10 minutes. Clarified lysates were evaluated for total protein content using Bradford assay (BioRad, 500-0006). Samples were then protein normalized and ran on 4-12% SDS-PAGE gels (Life Technologies). Proteins were transferred onto Nitrocellulose membrane using iBlot transfer apparatus (Life Technologies). Nitrocellulose was developed using BenchPro™ 4100 Western Card Processing Station (Life Technologies), probing for either *M. mazei* and *M. burtonii* MVKs with primary polyclonal antibodies produced in rabbits by ProSci incorporated against purified enzymes and a secondary fluorescent antibody Alexa Fluor 488 goat anti—rabbit IgG (Life Technologies, A-11008). Specific protein

quantitation was achieved using Storm imager and ImageQuant TL software from GE Healthcare.

(iii) Results

[0486] Expression of *M. burtonii* mevalonate kinase in MCM2125 is at least 15 fold lower then expression of *M. mazei* mevalonate kinase in DW708 strain, based on protein quantitation by western blot analysis (**Figure 30**).

Example 34: Isoprene production in *E. coli*

[0487] This example evaluates isoprene production in *E. coli (BL21)* expressing introduced genes from the mevalonate pathway and grown in fed-batch culture at the 15-L scale. Isoprene producing strains were run in a standard isoprene production process, described below. The performance metrics of strain DW708 (cumulative isoprene yield on glucose, instantaneous isoprene yield on glucose, volumetric productivity of isoprene, specific productivity and cell productivity index) are compared here to experimental strains, MCM2125, MCM2126 and MCM2127. These strains were run in the same conditions to determine if any yield improvement can be attributed to the use of the burtonii mevalonate kinase (MVK) in strain MCM2125, compared to a strain that used only Mazei MVK (DW708). Additionally, strains MCM2126 and MCM2127 were run in the same conditions to determine if any benefit can be obtained by expressing the *Mazei* MVK off of the chromosome only.

Table 25: List of strains

Strain Name	Host	MVK in host chromosome	Upper plasmid	Lower plasmid	MVK on lower plasmid	Run numbers
DW708	pgl-	*Mazei*	pCL Ptrc E. gallinarum Upper (pMCM1225)	pTrc P. alba IspS (MEA IspS variants)-mMVK (pDW166)	Yes (*Mazei*)	20120187 20120260
MCM2125	pgl- FRT-PL.2-2cis-RBS10000-MVK(burtonii)	*Burtonii*	pCL-Ptrc-Upper_gallinarum	pTrcAlba(MEA, IspS variants)-mMVK(del)	No	20120188 20120261
MCM2126	pgl- FRT-PL.2-2cistron-RBS1000-mMVK	*Mazei* (lower strength chromosomal promoter)	pCL-Ptrc-Upper_gallinarum	pTrcAlba(MEA, IspS variants)-mMVK(del)	No	20120262
MCM2127	pgl- FRT-PL.2-2cistron-RBS100000-mMVK +	*Mazei* (higher strength chromosomal promoter)	+ pCL-Ptrc-Upper_gallinarum	pTrcAlba(MEA, IspS variants)-mMVK(del)	No	20120263

(ii) *Solutions*

[0488] Medium Recipe (per liter fermentation medium): K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 50% sulphuric acid 1.6 mL, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in Di H_2O. This solution was heat sterilized (123 C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

[0489] 1000X Modified Trace Metal Solution (per liter): Citric Acids * H_2O 40 g, $MnSO_4$ * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

[0490] Vitamin Solution (per liter): Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

[0491] Macro Salt Solution (per liter): MgSO4 * 7H2O 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

[0492] Feed solution (per kilogram): Glucose 0.590 kg, Di H2O 0.393 kg, K2HPO4 7.4 g, and 100% Foamblast882 8.9 g. All components were mixed together and autoclaved. After autoclaving the feed solution, nutrient supplements are added to the feed bottle in a sterile hood. Post sterilization additions to the feed are (per kilogram of feed solution), Macro Salt Solution 5.54ml, Vitamin Solution 6.55ml, 1000X Modified Trace Metal Solution 0.82ml.

(ii) Method

[0493] A frozen vial of the *E. coli* strain was thawed and inoculated into a flask with tryptone-yeast extract medium and the appropriate antibiotics. After the inoculum grew to optical density 1.0, measured at 550 nm (OD550), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0494] The inlet gas using to maintain bioreactor backpressure at 0.7 bar gauge and to provide the oxygen to the production organisms was supplied by Matheson Tri-Gas, Inc in compressed gas cylinders.

[0495] The batched media had glucose batched in at 9.7 g/L. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). A shot of IPTG was added to the tank to bring the concentration to 200 uM when the cells were at an OD550 of 6. Once the glucose was consumed by the culture, as signaled by a rise in pH, the glucose feed solution was fed to meet metabolic demands at rates less than or equal to 10 g/min. The fermentation was run long enough to determine the maximum isoprene mass yield on glucose, a total of 52 to 69 hrs elapsed fermentation time.

(iii) Analysis

[0496] Isoprene is volatile and can be efficiently swept from the tank by the inlet gas. The isoprene level in the bioreactor off-gas was determined using two mass spectrometers, an iSCAN (Hamilton Sundstrand), and a Hiden HPR20 (Hiden Analytical) mass spectrometer. Oxygen, Nitrogen, and CO2 levels in the offgas were determined by the same mass spec units.

[0497] Dissolved Oxygen in the fermentation broth is measured by sanitary, sterilizable probe with an optical sensor provided Hamilton Company.

[0498] The citrate, glucose, acetate, and mevalonate concentrations in the fermentor broth was determined in broth samples taken at 4 hour intervals by an HPLC analysis. Concentration in broth samples were determined by comparison of the refractive index response versus a previously generated calibration curve using standard of a known concentration

[0499] **HPLC Information:** System: Waters Alliance 2695; Column: BioRad - Aminex HPX-87H Ion Exclusion Column 300mm x 7.8mm Catalog # 125-0140; Column Temperature: 50C; Guard column: BioRad - Microguard Cation H refill 30mm x 4.6mm Catalog # 125-0129;

Running buffer: 0.01N H_2SO_4; Running buffer flow rate: 0.6 ml / min; Approximate running pressure: ~1100-1200 psi; Injection volume: 20 microliters; Detector: Refractive Index (Knauer K-2301); Runtime: 26 minute.

(iv) Results

[0500] The results are summarized in **Table 26**. The strain expressing burtonii MVK (MCM2125) achieved a comparable cumulative % yield of isoprene on glucose (**Figure 31**), a comparable instantaneous % yield of isoprene on glucose (**Figure 32**), a comparable cell performance index (CPI; **Figure 33**), a comparable overall volumetric productivity (**Figure 34**), and a comparable specific productivity **(Figure 35)** versus the strain expressing *mazei* MVK (DW708).

Table 26: Isoprene productivity metrics

Strain description / Run Number	Overall Isoprene Volumetric Productivity (g/L/hr) at time of max overall isoprene yield	Max Cumulative % Yield of Isoprene on glucose (g/g)	Peak instantaneous %yield of isoprene on glucose (g/g%)	CPI (g Isoprene /gDCW) at time of max overall isoprene yield	Peak Specific Productivity (mg isoprene /L/hr/OD)
DW708 20120187	1.96	17.1	19.5	3.19	40.39
DW708 20120260	1.61	16.0	19.0	1.51	28.95
MCM2125 20120188	1.95	17.3	22.4	2.84	38.1
MCM2125 20120261	2.18	16.2	19.5	1.86	35.2
MCM2126 20120262	0.65	7.5	10.08	0.70	19.34
MCM2127 20120263	1.94	15.5	20.23	1.92	28.95

[0501] Comparing strains that expressed MVK off of the chromosome only, strain MCM2125, (expressing burtonii MVK) achieved a better cumulative % yield of isoprene on glucose (**Figure 36**), a comparable instantaneous % yield of isoprene on glucose (**Figure 37**), a higher cell performance index (CPI; **Figure 38**), a slightly better volumetric productivity (**Figure 39**), and a

slightly higher specific productivity versus strain MCM2127 (**Figure 40**), which expressed *mazei* MVK.

Example 35: Construction of strain CMP1136 (- PGL)

[0502] A PCR product containing a Kanamycin cassette flanked by FRT sites and regions homologous to upstream and downstream of pgl (*ybhE*) was obtained, using the PCR method described in example 4, Keio strain JW0750 (Baba et al. 2006. Mol. Syst. Biol. 2:1-11) which contains a kanamycin cassette in the pgl locus, and primers pglAmpF (5'-cagcaaatagcaggtgtatccagc-3') and pglAmpR (5'- GCA ACC GAC TGT TGA TAG AAC AAC-3'). This PCR product was used in a recombineering reaction (see protocol described above) with *E. coli* CMP1075. A colony was selected on LB + kanamycin 10 mg/L and named CMP1125. The kanamycin marker was removed using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strain CMP1133.

[0503] CMP1133 was checked by PCR with primers pglAmpF and pglRecCheck (5'-GGT TAC AAA ATG ATT GGC GTA CGC-3') to demonstrate deletion of the pgl gene. Plasmids MCM82 and pCHL243 were electroporated concomitantly into CMP1133. A colony growing on LB + carbenicilin 50 mg/L and spectinomycin 50 mg/L was selected and named CMP1136.

Example 36: Large scale fermentation of CMP1136

[0504] This experiment was performed to evaluate isoprene production from *E. coli(BL21)* expressing introduced genes from the mevalonate pathway and grown in fed-batch culture at the 15-L scale. An isoprene producing strain CMP1082 (HMB GI1.2gltA, PyddVIspA_GO, truncIspA, pMCM82, pDW72) was run in a standard isoprene production process, described below. The performance metrics (cumulative isoprene yield on glucose, instantaneous isoprene yield on glucose, volumetric productivity of isoprene, specific productivity and cell productivity index) are compared to an experimental strain CMP1136 (HMB GI1.2gltA, PyddVIspA_GO, truncIspA,pgl-, pMCM82, pDW72) that was run in the same conditions to identify yield improvement attributed to the deletion of the *pgl* gene in CMP1136.

[0505] **Medium Recipe** (per liter fermentation medium)**:** K2HPO4 7.5 g, MgSO4 * 7H2O 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 50% sulphuric acid 1.6 mL, 1000X Modified Trace Metal Solution 1 ml. All of the components were

added together and dissolved in Di H2O. This solution was heat sterilized (123°C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

[0506] **1000X Modified Trace Metal Solution** (per liter): Citric Acids * H2O 40 g, MnSO4 * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

[0507] **Vitamin Solution** (per liter): Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

[0508] **Macro Salt Solution** (per liter): MgSO4 * 7H2O 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

[0509] **Feed solution** (per kilogram): Glucose 0.590 kg, Di H2O 0.393 kg, K2HPO4 7.4 g, and 100% Foamblast882 8.9 g. All components were mixed together and autoclaved. After autoclaving the feed solution, nutrient supplements are added to the feed bottle in a sterile hood. Post sterilization additions to the feed are (per kilogram of feed solution), Macro Salt Solution 5.54ml, Vitamin Solution 6.55ml, 1000X Modified Trace Metal Solution 0.82ml.

[0510] This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH (7.0) and temperature (34°C). A frozen vial of the *E. coli* strain was thawed and inoculated into a flask with tryptone-yeast extract medium and the appropriate antibiotics. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0511] The batched media had glucose batched in at 9.7 g/L. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). IPTG was added to the tank to bring the concentration to 200 uM when the cells were at an OD_{550} of 6. Once the glucose was

consumed by the culture, as signaled by a rise in pH, the glucose feed solution was fed to meet metabolic demands at rates less than or equal to 10 g/min. The fermentation was run long enough to determine the maximum isoprene mass yield on glucose, a total of 68 to 72 hrs elapsed fermentation time.

Results

[0512] The pgl- strain (CMP1136) achieved a higher % yield of isoprene on glucose than the pgl+ strain (CMP1082). See **Table 27** and **FIG. 41**. The pgl- strain (CMP1136) achieved a higher instantaneous % yield of isoprene on glucose than the pgl+ strain (CMP1082) and was able to maintain this high productivity for a longer period of time (~24hrs at max for pgl- versus ~12hrs at max for pgl+). See **Table 27** and **FIG. 42**. The pgl- strain (CMP1136) achieved a higher cell productivity index than the pgl+ strain (CMP1082). At the end of fermentation 68 to 72hrs, the pgl- strain had a much higher CPI. Also, at the time of maximum cumulative yield of isoprene on glucose (44hrs for the pgl+ strain and 56hrs for the pgl- strain) the CPI is higher in the pgl- strain. See **Table 27** and **FIG. 43**. The pgl- strain (CMP1136) achieved about the same overall volumetric productivity as the pgl+ strain (CMP1082). See **Table 27** and **FIG. 44.** The pgl- strain (CMP1136) achieved about the same peak specific productivity as the pgl+ strain (CMP1082). However, the pgl- strain (CMP1136) was able to maintain this high productivity for a longer period of time than the pgl+ strain (CMP1082) and was notably better late in the fermentation. See **Table 27** and **FIG. 45**.

Table 27: Isoprene productivity metrics

Strain description / Run Number	Peak instantaneous %yield of isoprene on glucose (g/g%)	Overall Isoprene Volumetric Productivity (g/L/hr) at time of max overall isoprene yield	Max Overall % Yield of Isoprene on glucose (g/g)	CPI (g Isoprene /gDCW) at time of max overall isoprene yield	Peak Specific Productivity (mg isoprene /L/hr/OD)
CMP1082 / 20111110	20.1	1.91	16.3	1.81	30.31
CMP1136 / 20111225	22.3	1.82	17.2	2.73	28.61

Example 37: Construction of strains CMP1237 and CMP1238, having an engineered phosphoenolpyruvate carboxylase (ppc) promoter

[0513] The chloramphenicol cassette FRT-gb2-Cm-FRT from GeneBridges (Heidelberg, Germany) was amplified with primers 1.2ppcR2 (5'-cgggctttgcttttcgtCAGTGGTTGAATTATTTGCTCAGGATGTGGCATCGTCAAGGGCTAATACGACTCACTATAGGGCTCG-3') and ppcF (5'-gttacttggggcgatttttaacatttccataagttacgcttatttaaagcAATTAACCCTCACTAAAGGGCGG-3'). The PCR product thus obtained was purified and used in a recombineering reaction according to the manufacturer's recommendation (GeneBridges) to introduce the construct in strain MG1655 1.6ppc (see U.S. Pat. No. 7,745,184, issued June 29, 2010). A PCR product was amplified from the resulting strain (CMP3_49) using primers CMP79 (5'- GGA AAC ACG GTT TAT CAA GCC CAC C -3') and CMP80 (5'- cgtgaagatttcgacaacttacgg -3') and was and used in a recombineering reaction according to the manufacturer's recommendation (GeneBridges) to introduce the construct in BL21(DE3). A P1 lysate was prepared from the latter strain and was used to transduce CMP1133 (internal docket #40109). P1 lysates were prepared and used according to the method described in Ausubel, et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. A colony was selected on LB + chloramphenicol 5 mg/L and named CMP1230. The chloramphenicol marker was removed using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strain CMP1234. Plasmid pairs pMCM82 (US2011/0159557) and pDW72 (See U.S. Patent Application No.: 13/283,564), and pMCM1225 (See **Table 25**) and pDW240 were introduced by electroporation and selection on LB + 50 mg/L carbenicillin + 50 mg/L spectinomycin. The strains thus obtained were named CMP1237 and CMP1238 respectively.

Example 38: Isoprene production in strains containing an engineered ppc promoter- CMP1237 vs CMP1136

[0514] This example examines isoprene production in strains having an engineered phosphoenolpyruvate carboxylase (ppc) promoter.

(i) Solutions

[0515] TM3 media recipe (per liter fermentation media): K2HPO4 13.6 g, KH2PO4 13.6 g, MgSO4*7H2O 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, (NH4)2SO4 3.2 g, yeast extract 0.2 g, 1000X Trace Metals Solution 1 ml. All of the components are added together and dissolved in diH2O. The pH is adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media is filter-sterilized with a 0.22 micron filter. Glucose 10.0 g and antibiotics are added after pH adjustment and sterilization.

[0516] 1000X Trace Metal Solution (per liter fermentation media): Citric Acid*H2O 40g, MnSO4*H2O 30g, NaCl 10g, FeSO4*7H2O 1g, CoCl2*6H2O 1g, ZnSO4*7H2O 1g, CuSO4*5H2O 100mg, H3BO3 100mg, NaMoO4*2H2O 100mg. Each component is dissolved one at a time in diH2O. The pH is adjusted to 3.0 with HCl/NaOH, and then the solution is brought to volume and filter-sterilized with a 0.22 micron filter.

(ii) Methods

[0517] Cells are grown overnight in Luria-Bertani broth + antibiotics. The day after, they are diluted to an OD600 of 0.1 in 20 mL TM3 medium containing 50 µg/ml of spectinomycin and 50 µg/mL carbenicillin (in a 250-mL baffled Erlenmeyer flask), and incubated at 34°C and 200 rpm. After 2h of growth, OD600 is measured and 200 uM IPTG is added. Samples are taken regularly during the course of the fermentation. At each timepoint, OD600 is measured. Also, off-gas analysis of isoprene is performed using a gas chromatograph-mass spectrometer (GC-MS) (Agilent) headspace assay (*see* U.S. Patent Application Publication No.: US 2005/0287655, the contents of which are incorporated herein by reference in its entirety). One hundred microliters of whole broth are placed in a sealed GC vial and incubated at 34°C and 200 rpm for a fixed time of 30 minutes. Following a heat kill step, consisting of incubation at 70°C for 7 minutes, the sample is loaded on the GC. The reported specific productivity is the amount of isoprene in µg/L read by the GC divided by the incubation time (30 min) and the measured OD600.

(iii) Results

[0518] Strains with engineered ppc promoter (CMP1237) grew slightly slower than the strains with wild-type ppc, CMP1136 (**FIG. 46**). Specific productivity of CMP1237 strains was higher than the specific productivity of CMP1136 (**FIG. 47**).

Example 39: Isoprene production in strains containing an engineered ppc promoter on 15L scale

[0519] This experiment was performed to evaluate isoprene production from *E. coli (BL21)* expressing introduced genes from the mevalonate pathway and grown in fed-batch culture at the 15-L scale. An isoprene producing strain CMP1136 (HMB GI1.2gltA, PyddVIspA_GO, truncIspA, pgl-, pMCM82, pDW72) was run in a standard isoprene production process, described below. The performance metrics (cumulative isoprene yield on glucose, instantaneous isoprene yield on glucose, volumetric productivity of isoprene, specific productivity and cell productivity index) are compared here to an experimental strain that was run in the same conditions to see if any yield improvement can be attributed to the expression of the phosphoenolpyruvate carboxylase gene behind the GI1.2promoter in the experimental strain, CMP1237(HMB GI1.2gltA, GI1.2ppc, PyddVIspA_GO, truncIspA, pgl-, pMCM82, pDW72).

(i) Solutions

[0520] **Medium Recipe (per liter fermentation medium):** K2HPO4 7.5 g, MgSO4 * 7H2O 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 50% sulphuric acid 1.6 mL, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in Di H2O. This solution was heat sterilized (123 C for 20 minutes). The pH was adjusted to 7.0 with ammonium hydroxide (28%) and q.s. to volume. Glucose 10 g, Vitamin Solution 8 mL, and antibiotics were added after sterilization and pH adjustment.

[0521] **1000X Modified Trace Metal Solution (per liter):** Citric Acids * H2O 40 g, MnSO4 * H2O 30 g, NaCl 10 g, FeSO4 * 7H2O 1 g, CoCl2 * 6H2O 1 g, ZnSO * 7H2O 1 g, CuSO4 * 5H2O 100 mg, H3BO3 100 mg, NaMoO4 * 2H2O 100 mg. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with a 0.22 micron filter.

[0522] **Vitamin Solution (per liter):** Thiamine hydrochloride 1.0 g, D-(+)-biotin 1.0 g, nicotinic acid 1.0 g, pyridoxine hydrochloride 4.0 g. Each component was dissolved one at a time in Di H2O, pH was adjusted to 3.0 with HCl/NaOH, and then the solution was q.s. to volume and filter sterilized with 0.22 micron filter.

[0523] Macro Salt Solution (per liter): MgSO4 * 7H2O 296 g, citric acid monohydrate 296 g, ferric ammonium citrate 49.6 g. All components were dissolved in water, q.s. to volume and filter sterilized with 0.22 micron filter.

[0524] Feed solution (per kilogram): Glucose 0.590 kg, Di H2O 0.393 kg, K2HPO4 7.4 g, and 100% Foamblast882 8.9 g. All components were mixed together and autoclaved. After autoclaving the feed solution, nutrient supplements are added to the feed bottle in a sterile hood. Post sterilization additions to the feed are (per kilogram of feed solution), Macro Salt Solution 5.54ml, Vitamin Solution 6.55ml, 1000X Modified Trace Metal Solution 0.82ml.

(ii) Methods

[0525] This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH (7.0) and temperature (34°C). A frozen vial of the *E. coli* strain was thawed and inoculated into a flask with tryptone-yeast extract medium and the appropriate antibiotics. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5 L.

[0526] The inlet gas using to maintain bioreactor backpressure at 0.7 bar gauge and to provide the oxygen to the production organisms oxygen concentration of 8 -10% by volume with the balance of the gas being nitrogen.

[0527] The batched media had glucose batched in at 9.7 g/L. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). A shot of IPTG was added to the tank to bring the concentration to 200 uM when the cells were at an OD_{550} of 6. Once the glucose was consumed by the culture, as signaled by a rise in pH, the glucose feed solution was fed to meet metabolic demands at rates less than or equal to 6 g/min. The fermentation was run long enough to determine the maximum isoprene mass yield on glucose, a total of 68 to 72 hrs elapsed fermentation time.

(iii) Analysis

[0528] Isoprene is volatile and can be efficiently swept from the tank by the inlet gas. The isoprene level in the bioreactor off-gas was determined using two mass spectrometers, an iSCAN

(Hamilton Sundstrand), and a Hiden HPR20 (Hiden Analytical) mass spectrometer. Oxygen, Nitrogen, and CO2 levels in the offgas were determined by the same mass spec units.

[0529] Dissolved Oxygen in the fermentation broth is measured by sanitary, sterilizable probe with an optical sensor provided Hamilton Company.

[0530] The citrate, glucose, acetate, and mevalonate concentrations in the fermentor broth was determined in broth samples taken at 4 hour intervals by an HPLC analysis. Concentration in broth samples were determined by comparison of the refractive index response versus a previously generated calibration curve using standard of a known concentration.

(iii) Results

[0531] The results are summarized in **Table 28**. The GI1.2 ppc strain (CMP1237) achieved a higher % yield of isoprene on glucose **(Figure 48)**, a higher instantaneous % yield of isoprene on glucose **(Figure 49)**, a higher cell productivity index **(Figure 50)**, a higher overall volumetric productivity **(Figure 51)**, and about the same peak specific productivity **(Figure 52)** of isoprene compared to the wildtype ppc promoter strain (CMP1136).

Table 28: Isoprene productivity metrics

Strain description / Run Number	Peak instantaneous %yield of isoprene on glucose (g/g%)	Overall Isoprene Volumetric Productivity (g/L/hr) at time of max overall isoprene yield	Max Overall % Yield of Isoprene on glucose (g/g)	CPI (g Isoprene /gDCW) at time of max overall isoprene yield	Peak Specific Productivity (mg isoprene /L/hr/OD)
CMP1237 / 20120407	21.1	2.21	17.4	2.80	31.86
CMP1136 / 20120135	19.7	2.05	16.3	2.43	31.62

<u>Example 40: GI1.2 ppc construct has less phosphoenolpyruvate carboxylase activity than a wild-type ppc promoter</u>

(i) Materials and methods

[0532] Cells from 15-L fermentations with strains CMP1237 (engineered ppc promoter) and DW719 (wild-type ppc) have been harvested by centrifugation. Supernatant was discarded and the pellet was resuspended in lysis buffer (50 mM Tris HCl, pH 8, 1 mg/ml lysozyme, 1 mM DTT, 10 μg/ml DNAse, 1mM PMSF). Cells were subject one freeze/thaw cycle, before centrifugation to remove cell debris. Supernatant was collected and stored on ice.

[0533] The assay monitors the conversion of phosphenolpyruvate (PEP) to oxaloacetate (OAA) by coupling the conversion of OAA to malate by malate dehydrogenase (MDH) and monitoring the oxidation of NADH to NAD^+.

[0534] The assay was carried out in a microtiter plate with the following reagents:0.1 M Tris pH 8, 0.1 mM NADH, 10 mM $KHCO_3$, 10 mM $MgCL_2$, 5 mM PEP, 0.5 mM Acetyl-CoA, 5 u/ml Malate dehydrogenase, 10-20 μL sample. The decrease in absorbance at 340 nm was observed over 100 sec.

(ii) Results

[0535] At around 48 h in the fermentation, phosphoenolpyruvate carboxylase activity of DW719 was 49 activity/OD_{260} while activity of CMP1237 was 34 activity/OD_{260} .

Example 41: Construction of strains CMP1189 and CMP1191, having a restored FNR protein, and production of isoprene by the latter

[0536] A P1 lysate was prepared from strain JW1326 from the Keio collection (Baba et al. 2006. *Mol. Syst. Biol.* 2: 2006.0008) and was used to transduce CMP1133 (internal docket #40109). P1 lysates were prepared and used according to the method described in Ausubel, et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. A colony was selected on LB + kanamycin 10 mg/L and named CMP1180. A P1 transduction is able to transduce up to 100 kB DNA. As ynaJ is in close proximity of FNR (**Figure 53**), selecting for a mutation in ynaJ will most probably also bring the DNA encoding FNR from the strain the lysate was made. The Keio collection has been built in a K-12 strain, which has a functional FNR. That is unlike *E. coli* BL21in which FNR contains a stop codon in the protein (Studier et al. 2009. *J. Mol. Biol.* 394:653-680). FNR was amplified from CMP1180 with primers CMP51 (5'-CAG GTA ATG CAT TAC GGC CAA CTG -3') and CMP52 (5'- caggctgtacgctggctgatgac -3') and sequenced with CMP53 (5'- ATG ATC CCG GAA AAG CGA ATT ATA CG -3'). The sequence proved

that a functional FNR allele had been restored. The kanamycin marker was removed using the protocol recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to form strain CMP1189. Plasmid pairs pMCM82 (US2011/0159557) and pDW72 (See U.S. Patent Application No.: 13/283,564) were introduced by electroporation and selection on LB + 50 mg/L carbenicillin + 50 mg/L spectinomycin. The strains thus obtained were named CMP1191.

[0537] Isoprene production by strain CMP1191 was compared to CMP1136 using the protocol described in Example 38. As FNR is an oxygen sensor, another set of shake flask was done with the same medium but the flasks were incubated at 50 rpm rather than 200 rpm. That led to an oxygen-limited culture.

Results

[0538] Growth of strain with functional FNR was similar to growth of strain with mutated FNR in well-aerated flask (**Fig. 54**). When oxygen was limited by reducing agitation, growth of strain with functional FNR was a bit slower (**Fig. 55**). Specific productivity of CMP1136 (mutated FNR) and CMP1191 (functional FNR) was similar in well-aerated flask (**Fig. 56**). When oxygen was limited by reducing agitation, the strain with the functional FNR had an improved specific productivity (**Fig. 57**).

<u>Example 42: Construction of MD12-746 (having an ackA-pta mutation)</u>

[0539] A DNA fragment containing the ackA-pta genes interrupted by a chloramphenicol marker was amplified by PCR using strain MG1655 ΔackA-pta::Cm (see U.S. Pat. No. 7,745,184, issued June 29, 2010) as a template, and primers ackACF (5'-gtgcaaattcacaactcagcgg-3') and ptaCR (5'- CAC CAA CGT ATC GGG CAT TGC-3'). The PCR product obtained was used in a recombineering reaction as recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the ackA-pta locus in strain CMP258 (internal docket #31588). Colonies were selected on LB + 5 μg/ml of chloramphenicol. One colony was picked and was named MD10-491. A P1 lysate was made from this strain and used to transduce CMP1133. The chloramphenicol marker was looped out by electroporating pCP20 (Datsenko and Wanner. 2000. *PNAS* 97:6640-5) in the strain, selecting two colonies on LB + 50 ug/ml carbenicillin at 30°C, then restreaking those colonies

on an LB plate at 42°C. A Chloramphenicol sensitive colony was selected from those plates and named MD12-746.

[0540] Isoprene production by strain MD12-746 was compared to DW719 using the protocol described in Example 38..

Results

[0541] Increased specific productivity of isoprene was observed in MD12-746 versus DW719 (**Figures 58-59).**

Example 43: Increased specific productivity of isoprene in a strain overexpressing iraM

[0542] This example details a microarray analyses of 15-L fermentations performed by the method below.

(i) Methods

[0543] Strains used in this genome-wide transcription study are CMP457 and MCM1020. Strain MCM1020 was constructed by electroporating plasmids pTrcHis2B (Invitrogen, Carlsbad, CA) and pCL1920 (*see* U.S. Patent Application Publication No. 2009/0203102, the contents of which is incorporated herein by reference) into strain CMP258 (*see* International Patent Application No. PCT/US2011/058188, the contents of which is incorporated herein by reference) and selecting a colony on LB + 50 mg/L spectinomycin + 50 mg/L carbenicillin.

[0544] Fermentation samples were quickly diluted 1:5 in RNALater (Qiagen, Valencia, CA) and frozen at -20 °C. Cells were harvested and lysed in Trizol (Invitrogen) and incubated at room temperature for 5 minutes. Nucleic acids were isolated by extracting by adding 20% ice cold chloroform. The solution was mixed and incubated for 5 minutes at room temperature followed by centrifugation at 13,000 rpm at 4 °C for 15 minutes. The top water phase was isolated and an equal volume of ice cold ethanol was added. RNA was isolated using the RNEasy mini kit (Qiagen). Following the manufactures instructions, DNA was degraded during the procedure by adding a DNase solution (10 µL DNase I stock in 70 µL RDD buffer) (Qiagen) and incubating at room temperature for 30 minutes. RNA was eluted from the RNeasy column in nuclease-free water. A minimum of 20 µg of RNA was collected from each sample as measured using a Nanodrop instrument. RNA was further purified by precipitation by adding 1/10th

volume if 3M sodium acetate. Glycogen (RNA grade from Fermentas) was added to a final concentration of 1 ug/uL followed by the addition of 2.5 volume of ice cold ethanol. The solution was incubated for 60 minutes at -80 °C and then centrifuged for 15 minutes at 10.000 rpm. The supernatant was discarded and the RNA pellet was washed briefly with ice cold 70% ethanol. The RNA pellet was air dried for 20 minutes and dissolved in nuclease-free water at a concentration of 1 µg/µL. Quality and concentration was measured using a Nanodrop instrument and by gel electrophoresis. Synthesis of cDNA, labeling and transcription analysis was performed by Roche NimbleGen (Iceland) using a 385K 4-plex microarray designed specifically for *E. coli* BL21. The resulting data was analyzed using the GenespringGX Version 11 (Agilent).

(ii) Results

[0545] By analyzing the data, it was found that the proteolysis inhibitor iraM was induced in isoprene producting strains (**Figure 60**).

Example 44: Construction of strains MD11-610 and MD11-612

[0546] **MD11-610: PL.6 promoter in front of iraM.** A PCR product (#1) was generated using primers MQ10-127F (5'-GTT AAC TGG TTG CAG TCA CCT GGA GGC ACC AGG CAC CGC ATC AAC AAA GTT CAT TTG TAA AAA TGG AGA TAA TTG TGT AGG CTG GAG CTG CTT C-3') and MQ10-127R (5'-TCG TGT CAA TTA CTA TCC ACT TCA TGT TTT TTT ACC TCC TTT GCA CCT TCA TGG TGG TCA GTG CGT CCT GCT GAT GTG CTC AGT ATC ACC GCC AGT GGT ATT TAT GTC AAC ACC GCC AGA GAT AAT TTA TCA CCG CAG ATG GTT ATC TGT ATG TTT TTT ATA TGA ATT CAT ATG AAT ATC CTC CTT A-3') with plasmid pKD3 (Datsenko and Wanner. 2000. *PNAS* 97:6640-5) as a template. PCR product #2 was generated using primers MQ10-128F (5'-ATG AAG TGG ATA GTA ATT GAC ACG A-3') and MQ11-128R (5'-GCA TTC TTT CAA TAG CTT TGC TTT CTT CAA CGT CTT TTT TGC AAA GGT GGT AAG CAC ATT TTA TTT TCT TAG TCA TTA CTT GAG CCC ATA TGG GCA TAT T-3'), with chromosomal DNA of BL21 (Novagen) as template. Primers MQ10-127F and MQ11-128R-term (5'-GCATTCTTTCAATAGCTTTGCTTTCTTCAACGTCTTTTTGCAAAGGTGGTAAGCACATTTTATTTTCTTAGTCAAGTCAAAAGCCTCCGGTCGGAGGCTTTTGACTTTACTTGAGCCCATATGGGCATATT-3') were used to amplify a third PCR product (PCR#3) using a

mixture of PCR#1 and PCR#2 fragments as a template. The PCR product thus obtained was used in a recombineering reaction according to the manufacturer's protocol (GeneBridges, Heidelberg, Germany) to integrate it at the kill locus in strain CMP451 (BL21 pgl+ PL.2 mKKDyI GI1.2 gltA) to form strain MD11-597. The chloramphenicol marker was looped out with pCP20 to form strain MD11-604. The latter was transformed by electroporation with plasmids pMCM82 and pDW166. A colony was isolated on LB+ 50 µg/mL carbenicillin + 50 µg/mL spectinomycin and named MD11-610.

[0547] MD11-612: GI1.6 promoter in front of iraM. A PCR product (#4) was generated using primers MQ11-127F (5'-GTT AAC TGG TTG CAG TCA CCT GGA GGC ACC AGG CAC CGC ATC AAC AAA GTT CAT TTG TAA AAA TGG AGA TAA TTG TGT AGG CTG GAG CTG CTT C-3')and MQ11-129R (5'-TCG TGT CAA TTA CTA TCC ACT TCA TTT TAT ATA CCT CCT GCT ATT TGT TAG TGA ATA AAA GTG GTT GAA TTA TTT GCT CAG GAT GTG GCA TTG TCA AGG GCC ATA TGA ATA TCC TCC TTA-3') with plasmid pKD3 (Datsenko and Wanner. 2000. PNAS 97:6640-5) as a template. Primers MQ11-127F and MQ11-128R-term were used to amplify a new PCR product (PCR#5) using a mixture of PCR#4 and PCR#2 fragments as a template. The PCR product thus obtained was used in a recombineering reaction according to the manufacturer's protocol (GeneBridges, Heidelberg, Germany) to integrate it at the kill locus in strain CMP451 to form strain MD11-599. The chloramphenicol marker was looped out with pCP20 to form strain MD11-606. The latter was transformed by electroporation with plasmids pMCM82 and pDW166. A colony was isolated on LB+ 50 mg/mL carbenicillin + 50 mg/mL spectinomycin and named MD11-612.

[0548] Strains MD11-610 and MD11-612 were tested in small scale (shake flasks) in comparison to MD11-608 (CMP451 harboring plasmids pMCM82 and pDW166) as a control, using the protocol described in Example 38 (above). All the strains had a similar growth profile (**Fig. 61**). The strains with the engineered iraM promoter had an increased specific productivity (**Fig. 62**).

Example 45: Increased specific productivity of isoprene in a strain MD11-607, with deleted acrA

[0549] acrA is a component of the multidrug efflux pump acrAB-TolC. This complex has been associated with resistance to solvents, dyes and detergents. Intuitively, overexpression of this pump could increase resistance to stress by chemicals such as butanol or isoprene.

Counterintuitively, mutation of acrA has been credited with increased butanol resistance Atsumi *et al.*, 2010, *Mol Sys Biol.*, 6:449)

[0550] As isoprene might have a toxic effect similar to butanol, we tested the effect of acrA deletion on isoprene specific productivity.

[0551] A DNA fragment containing the acrA gene interrupted by a kanamycin marker was amplified by PCR using strain JW0452 from the Keio collection (Baba et al. 2006. *Mol. Syst. Biol.* 2: 2006.0008) as a template, and primers MQ10-121F (5'-GTT CGT GAA TTT ACA GGT GTT AGA TTT AC-3') and MQ10-121R (5'-GTG CAA TCG TAG GAT ATT GCG CCA CCG GC-3'). The PCR product obtained was used in a recombineering reaction as recommended by the manufacturer (Gene Bridges, Heidelberg, Germany) to integrate the PCR product at the acrA locus in strain CMP451 (See U.S. Patent Application No.: 13/283,564). Colonies were selected on LB + 10 ug/ml of kanamycin. One colony was picked and was named MD11-589. The kanamycin marker was looped out by electroporating pCP20 (Datsenko and Wanner. 2000. *PNAS* 97:6640-5) in the strain, selecting two colonies on LB + 50 ug/ml carbenicillin at 30°C, then restreaking those colonies on an LB plate at 42°C. A KanS colony was selected from those plates and named MD11-602. Plasmids pMCM82 (US2011/0159557) and pDW166 were introduced by electroporation and selection on LB + 50 mg/L carbenicillin + 50 mg/L spectinomycin. The strain thus obtained was named MD11-607.

[0552] Strain MD11-607 was tested in small scale (shake flasks) in comparison to MD11-608 (CMP451 harboring plasmids pMCM82 and pDW166) as a control, using the protocol described in Example 38 (above). Growth was similar early in the fermentation but acrA mutant started to slow down later in the fermentation (**Fig. 68**). MD11-607 had an increased specific productivity compared to MD11-608 (**Fig. 69**).

SEQUENCES

L.grayi mvaE:

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ttctttagcgatttcgataggtgccatcggtgaggagatagagcaagtcgcgaaaaaactgcgtgaagctgaaaaaatgaatcagcaaacggcaatacagattttagaaa
aaattcgcgagaaatga **SEQ ID NO:3**

E. faecium mvaS:

atgaaaatcggtattgaccgtctgtccttcttcatcccgaatttgtatttggacatgactgagctggcagaatcacgcggggatgatccagctaaatatcatattggaatcgga
caagatcagatggcagtgaatcgcgcaaacgaggacatcataacactgggtgcaaacgctgcgagtaagatcgtgacagagaaagaccgcgagttgattgatatggta
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tggctagatatgaggagtctattcgctatagccggagaattggtaacctgtacacaggcagcttgtaccttggtcttacaagcttgttggaaaactctaaaagtttacaaccg
ggagatcggatcggcctcttttcctatggcagtggtgcggtgtccgagttctttaccgggtatttagaagaaaattaccaagagtacctgttcgctcaaagccatcaagaaat

gctggatagccggactcggattacggtcgatgaatacgagaccatcttttcagagactctgccagaacatggtgaatgcgccgaatatacgagcgacgtccccttttctat
aaccaagattgagaacgacattcgttattataaaatctga **SEQ ID NO:4**

E. gallinarum mvaE:

atggaagaagtggtaattatagatgcacgtcggactccgattggtaaatatcacgggtcgttgaagaagttttcagcggtggcgctggggacggccgtggctaaagacat
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gatcgcctggtaggcgagataacactgccgctggccatcgctacagttggaggcgctaccaaagtgttgcccaaagctcaagcggcactggagattagtgatgttcact
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gcccggtctctcgcaatcgcggtcggtgctgaaaaagccgagatcgagcaggtcgccgaaaagttgcggcagaacccgccaatgaatcagcagcaggcgctccgttt
tcttggcgagatccgcgaacaatga **SEQ ID NO:5**

E. gallinarum mvaS:

atgaacgtcggcattgacaaaattaatttttttcgttccaccgtattatctggatatggtcgacctggcccacgcacgcgaagtggacccgaacaaatttacaattggaattgg
acaggatcagatggctgtgagcaaaaagacgcacgatatcgtaacattcgcggctagtgccgcgaaggaaattttagaacctgaggacttgcaagctatagacatggtt
atagttggtaccgaatcgggcattgacgagagcaaagcatccgcggtcgttttacatcgtttgttgggcgtacaacctttcgctcgcagttttgaaattaaagaagcctgtta
cggggcaaccgcaggcattcagtttgccaagactcatatacaagcgaacccggagagcaaggtcctggtaattgcaagcgatatagctcggtatggtcttcggtcaggt
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gcacggtatgaagagtctatcgtatattcacgccggatcggcaacttgtatacgggatcattgtacctggggctgatatccttattggaaaacagttctcacctgtcggcgg
gcgaccggataggattgtttagttatgggagtggcgctgtcagcgaatttttctccggtcgtttagtggcaggctatgaaaatcaattgaacaaagaggcgcatacccagct

cctggatcagcgtcagaagctttccatcgaagagtatgaggcgatttttacagattccttagaaattgatcaggatgcagcgttctcggatgacctgccatattccatccgcg
agataaaaaacacgattcggtactataaggagagctga **SEQ ID NO:6**

E. casseliflavus mvaE:

atggaagaagttgtcatcattgacgcactgcgtactccaataggaaagtaccacggttcgctgaaagattacacagctgttgaactggggacagtagcagcaaaggcgtt
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cgccctggcgctaactggcgttgagacggcgtcggaactggccagcctggcggcgagtgtgggattagttcaaaatttggccgctttacgagcactagtgagcgaggg
cattcagcaagggcacatgagtatgcaagctagatccctggccattagcgtaggtgcgaaaggtactgaaatagagcaactagctgcgaagctgagggcagcgacgc
aaatgaatcaggagcaggctcgtaaatttctgaccgaaataagaaattaa **SEQ ID NO:7**

E. casseliflavus mvaS:

atgaacgttggaattgataaaatcaatttttcgttccgccctatttcattgatatggtggatctcgctcatgcaagagaagttgaccccaacaagttcactataggaataggcc
aagatcagatggcagtaaacaagaaaacgcaagatatcgtaacgttcgcgatgcacgccgcgaaggatattctgactaaggaagatttacaggccatagatatggtaat
agtggggactgagtctgggatcgacgagagcaaggcaagtgctgtcgtattgcatcggcttttaggtattcagccttttgcgcgctcctttgaaattaaggaggcatgctat
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gacgaccggcaaaaactgactatcgcagagtacgaagccatgtttaatgaaaccattgatattgatcaggaccagtcatttgaggatgacttactgtactccatcagagag
atcaaaaacactattcgctactataacgaggagaatgaataa **SEQ ID NO:8**

E. faecalis mvaE:

atgaaaacagtagttattattgatgcattacgaacaccaattggaaaatataaaggcagcttaagtcaagtaagtgccgtagacttaggaacacatgttacaacacaactttt
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aaggacacatggctctacaagcacgttctttagcgatgacggtcggagctactggtaaagaagttgaggcagtcgctcaacaattaaaacgtcaaaaaacgatgaacca
agaccgagccatggctattttaaatgatttaagaaaacaataa **SEQ ID NO:9**

E. faecalis mvaS:

Atgacaattgggattgataaaattagttttttgtgcccccttattatattgatatgacggcactggctgaagccagaaatgtagaccctggaaaatttcatattggtattgggc
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cagaactttctatcgctgaatatgaagccatgtttgcagaaactttagacacagacattgatcaaacgttagaagatgaattaaaatatagtatttctgctattaataatac
cgttcgttcttatcgaaactaa **SEQ ID NO:10**

E. gallinarum EG2 (mvaE):

MEEVVIIDARRTPIGKYHGSLKKFSAVALGTAVAKDMFERNQKIKEEIAQVIIGNVLQAGNGQNPARQVA
LQSGLSVDIPASTINEVCGSGLKAILMGMEQIQLGKAQVVLAGGIESMTNAPSLSHYNKAEDTYSVPVSS
MTLDGLTDAFSSKPMGLTAENVAQRYGISREAQDQFAYQSQMKAAKAQAENKFAKEIVPLAGETKTITAD
EGIRSQTTMEKLASLKPVFKTDGTVTAGNASTINDGAALVLLASKTYCETNDIPYLATIKEIVEVGIDPE
IMGISPIKAIQTLLQNQKVSLEDIGVFEINEAFAASSIVVESELGLDPAKVNRYGGGISLGHAIGATGAR
LATSLVYQMQEIQARYGIASLCVGGGLGLAMLLERPTIEKAKPTDKKFYELSPAERLQELENQQKISSET
KQQLSQMMLAEDTANHLIENQISEIELPMGVGMNLKVDGKAYVVPMATEEPSVIAAMSNGAKMAGEIHTQ
SKERLLRGQIVFSAKNPNEIEQRIAENQALIFERAEQSYPSIVKREGGLRRIALRHFPADSQQESADQST
FLSVDLFVDVKDAMGANIINAILEGVAALFREWFPNEEILFSILSNLATESLVTAVCEVPFSALSKRGGA
TVAQKIVQASLFAKTDPYRAVTHNKGIMNGVEAVMLATGNDTRAVSAACHGYAARTGSYQGLTNWTIESD
RLVGEITLPLAIATVGGATKVLPKAQAALEISDVHSSQELAALAASVGLVQNLAALRALVSEGIQKGHMS
MQARSLAIAVGAEKAEIEQVAEKLRQNPPMNQQQALRFLGEIREQ **SEQ ID NO:11**

E. gallinarum EG2 (mvaS)

MNVGIDKINFFVPPYYLDMVDLAHAREVDPNKFTIGIGQDQMAVSKKTHDIVTFAASAAKEILEPEDLQA
IDMVIVGTESGIDESKASAVVLHRLLGVQPFARSFEIKEACYGATAGIQFAKTHIQANPESKVLVIASDI
ARYGLRSGGEPTQGAGAVAMLLTANPRILTFENDNLMLTQDIYDFWRPLGHAYPMVDGHLSNQVYIDSFK
KVWQAHCERNQASISDYAAISFHIPYTKMGKKALLAVFADEVETEQERVMARYEESIVYSRRIGNLYTGS
LYLGLISLLENSSHLSAGDRIGLFSYGSGAVSEFFSGRLVAGYENQLNKEAHTQLLDQRQKLSIEEYEAI
FTDSLEIDQDAAFSDDLPYSIREIKNTIRYYKES **SEQ ID NO:12**

L. grayi (mvaE):

MVKDIVIIDALRTPIGKYRGQLSKMTAVELGTAVTKALFEKNDQVKDHVEQVIFGNVLQAGNGQNPARQI
ALNSGLSAEIPASTINQVCGSGLKAISMARQQILLGEAEVIVAGGIESMTNAPSITYYNKEEDTLSKPVP
TMTFDGLTDAFSGKIMGLTAENVAEQYGVSREAQDAFAYGSQMKAAKAQEQGIFAAEILPLEIGDEVITQ
DEGVRQETTLEKLSLLRTIFKEDGTVTAGNASTINDGASAVIIASKEFAETNQIPYLAIVHDITEIGIDP
SIMGIAPVSAINKLIDRNQISMEEIDLFEINEAFAASSVVVQKELSIPDEKINIGGSGIALGHPLGATGA
RIVTTLAHQLKRTHGRYGIASLCIGGGLGLAILIEVPQEDQPVKKFYQLAREDRLARLQEQAVISPATKH
VLAEMTLPEDIADNLIENQISEMEIPLGVALNLRVNDKSYTIPLATEEPSVIAACNNGAKMANHLGGFQS
ELKDGFLRGQIVLMNVKEPATIEHTITAEKAAIFRAAAQSHPSIVKRGGGLKEIVVRTFDDDPTFLSIDL
IVDTKDAMGANIINTILEGVAGFLREILTEEILFSILSNYATESIVTASCRIPYEALSKKGDGKRIAEKV
AAASKFAQLDPYRAATHNKGIMNGIEAVVLASGNDTRAVAAAAHAYASRDQHYRGLSQWQVAEGALHGEI
SLPLALGSVGGAIEVLPKAKAAFEIMGITEAKELAEVTAAVGLAQNLAALRALVSEGIQQGHMSLQARSL
ALSVGATGKEVEILAEKLQGSRMNQANAQTILAEIRSQKVEL **SEQ ID NO:13**

L. grayi (mvaS):

MTMNVGIDKMSFFVPPYFVDMTDLAVARDVDPNKFLIGIGQDQMAVNPKTQDIVTFATNAAKNILSAEDL
DKIDMVIVGTESGIDESKASAVVLHRLLGIQKFARSFEIKEACYGGTAALQFAVNHIRNHPESKVLVVAS
DIAKYGLASGGEPTQGAGAVAMLVSTDPKIIAFNDDSLALTQDIYDFWRPVGHDYPMVDGPLSTETYIQS
FQTVWQEYTKRSQHALADFAALSFHIPYTKMGKKALLAILEGESEEAQNRILAKYEKSIAYSRKAGNLYT
GSLYLGLISLLENAEDLKAGDLIGLFSYGSGAVAEFFSGRLVEDYQEQLLKTKHAEQLAHRKQLTIEEYE
TMFSDRLDVDKDAEYEDTLAYSISSVRNTVREYRS **SEQ ID NO:14**

E. faecium (mvaE):

MKEVVMIDAARTPIGKYRGSLSPFTAVELGTLVTKGLLDKTKLKKDKIDQVIFGNVLQAGNGQNVARQIA
LNSGLPVDVPAMTINEVCGSGMKAVILARQLIQLGEAELVIAGGTESMSQAPMLKPYQSETNEYGEPISS
MVNDGLTDAFSNAHMGLTAEKVATQFSVSREEQDRYALSSQLKAAHAVEAGVFSEEIIPVKISDEDVLSE
DEAVRGNSTLEKLGTLRTVFSEEGTVTAGNASPLNDGASVVILASKEYAENNNLPYLATIKEVAEVGIDP

SIMGIAPIKAIQKLTDRSGMNLSTIDLFEINEAFAASSIVVSQELQLDEEKVNIYGGAIALGHPIGASGA
RILTTLAYGLLREQKRYGIASLCIGGGLGLAVLLEANMEQTHKDVQKKKFYQLTPSERRSQLIEKNVLTQ
ETALIFQEQTLSEELSDHMIENQVSEVEIPMGIAQNFQINGKKKWIPMATEEPSVIAAASNGAKICGNIC
AETPQRLMRGQIVLSGKSEYQAVINAVNHRKEELILCANESYPSIVKRGGGVQDISTREFMGSFHAYLSI
DFLVDVKDAMGANMINSILESVANKLREWFPEEEILFSILSNFATESLASACCEIPFERLGRNKEIGEQI
AKKIQQAGEYAKLDPYRAATHNKGIMNGIEAVVAATGNDTRAVSASIHAYAARNGLYQGLTDWQIKGDKL
VGKLTVPLAVATVGGASNILPKAKASLAMLDIDSAKELAQVIAAVGLAQNLAALRALVTEGIQKGHMGLQ
ARSLAISIGAIGEEIEQVAKKLREAEKMNQQTAIQILEKIREK **SEQ ID NO:15**

E. faecium (mvaS)

MKIGIDRLSFFIPNLYLDMTELAESRGDDPAKYHIGIGQDQMAVNRANEDIITLGANAASKIVTEKDREL
IDMVIVGTESGIDHSKASAVIIHHLLKIQSFARSFEVKEACYGGTAALHMAKEYVKNHPERKVLVIASDI
ARYGLASGGEVTQGVGAVAMMITQNPRILSIEDDSVFLTEDIYDFWRPDYSEFPVVDGPLSNSTYIESFQ
KVWNRHKELSGRGLEDYQAIAFHIPYTKMGKKALQSVLDQTDEDNQERLMARYEESIRYSRRIGNLYTGS
LYLGLTSLLENSKSLQPGDRIGLFSYGSGAVSEFFTGYLEENYQEYLFAQSHQEMLDSRTRITVDEYETI
FSETLPEHGECAEYTSDVPFSITKIENDIRYYKI **SEQ ID NO:16**

E. casseliflavus (mvaE):

MEEVVIIDALRTPIGKYHGSLKDYTAVELGTVAAKALLARNQQAKEHIAQVIIGNVLQAGSGQNPGRQVS
LQSGLSSDIPASTINEVCGSGMKAILMGMEQIQLNKASVVLTGGIESMTNAPLFSYYNKAEDQYSAPVST
MMHDGLTDAFSSKPMGLTAETVAERYGITRKEQDEFAYHSQMKAAKAQAAKKFDQEIVPLTEKSGTVLQD
EGIRAATTVEKLAELKTVFKKDGTVTAGNASTINDGAAMVLIASKSYCEEHQIPYLAVIKEIVEVGFAPE
IMGISPIKAIDTLLKNQALTIEDIGIFEINEAFAASSIVVERELGLDPKKVNRYGGGISLGHAIGATGAR
IATTVAYQLKDTQERYGIASLCVGGGLGLAMLLENPSATASQTNFDEESASEKTEKKKFYALAPNERLAF
LEAQGAITAAETLVFQEMTLNKETANHLIENQISEVEIPLGVGLNLQVNGKAYNVPLATEEPSVIAAMSN
GAKMAGPITTTSQERLLRGQIVFMDVQDPEAILAKVESEQATIFAVANETYPSIVKRGGGLRRVIGRNFS
PAESDLATAYVSIDLMVDVKDAMGANIINSILEGVAELFRKWFPEEEILFSILSNLATESLVTATCSVPF
DKLSKTGNGRQVAGKIVHAADFAKIDPYRAATHNKGIMNGVEALILATGNDTRAVSAACHGYAARNGRMQ
GLTSWTIIEDRLIGSITLPLAIATVGGATKILPKAQAALALTGVETASELASLAASVGLVQNLAALRALV
SEGIQQGHMSMQARSLAISVGAKGTEIEQLAAKLRAATQMNQEQARKFLTEIRN **SEQ ID NO:17**

E. casseliflavus (mvaS)

MNVGIDKINFFVPPYFIDMVDLAHAREVDPNKFTIGIGQDQMAVNKKTQDIVTFAMHAAKDILTKEDLQA
IDMVIVGTESGIDESKASAVVLHRLLGIQPFARSFEIKEACYGATAGLQFAKAHVQANPQSKVLVVASDI
ARYGLASGGEPTQGVGAVAMLISADPAILQLENDNLMLTQDIYDFWRPVGHQYPMVDGHLSNAVYIDSFK
QVWQAHCEKNQRTAKDYAALSFHIPYTKMGKKALLAVFAEEDETEQKRLMARYEESIVYSRRTGNLYTGS
LYLGLISLLENSSSLQANDRIGLFSYGSGAVAEFFSGLLVPGYEKQLAQAAHQALLDDRQKLTIAEYEAM
FNETIDIDQDQSFEDDLLYSIREIKNTIRYYNEENE **SEQ ID NO:18**

Acetoactyl-CoA-synthase:

MTDVRFRIIGTGAYVPERIVSNDEVGAPAGVDDDWITRKTGIRQ
RRWAADDQATSDLATAAGRAALKAAGITPEQLTVIAVATSTPDRPQPPTAAYVQHHLG
ATGTAAFDVNAVCSGTVFALSSVAGTLVYRGGYALVIGADLYSRILNPADRKTVVLFG
DGAGAMVLGPTSTGTGPIVRRVALHTFGGLTDLIRVPAGGSRQPLDTDGLDAGLQYFA
MDGREVRRFVTEHLPQLIKGFLHEAGVDAADISHFVPHQANGVMLDEVFGELHLPRAT
MHRTVETYGNTGAASIPITMDAAVRAGSFRPGELVLLAGFGGGMAASFALIEW **SEQ ID NO:19**

Isoprene synthase:

Atggaagctcgtcgttctgcgaactacgaacctaacagctgggactatgattacctgctgtcctccgacacggacgagtccatcgaagtatacaaagacaaagcg
aaaaagctggaagccgaagttcgtcgcgagattaataacgaaaaagcagaatttctgaccctgctggaactgattgacaacgtccagcgcctgggcctgggttacc

gtttcgagtctgatatccgtggtgcgctggatcgcttcgtttcctccggcggcttcgatgcggtaaccaagacttccctgcacggtacggcactgtctttccgtctgctg
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ctgaccaaagcctgggctgacctgtgcaacgctttcctgcaagaagccaagtggctgtacaacaaatctactccgacctttgacgactacttcggcaacgcatgga
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ispA:

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tgcagtatggcgcattattaggtggtaagcgcctgcgacctttcctggtttatgccaccggtcatatgtttggcgttagcacaaacacgctggacgcacccgctgctg
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tcataaaaccggcgcattgattcgcgccgccgttcgccttggtgcattaagcgccggagataaagggcgtcgtgctctgccagtactcgacaagtacgcagagag
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taccctgcacttctgggtcttgagcaagcccggaagaaagcccgggatctgatcgacgatgcccgtcagtcgctgaaacaactggctgaacagtcactcgatacct
cggcactggaagcgctagcggactacatcatccagcgtaataaataa

Amorphadiene synthase codon-optimized for *E. coli:*

ATGAGCCTGACCGAAGAAAAACCGATTCGTCCGATTGCAAATTTTCCGCCTAGCATTTGGGGTGAT
CAGTTTCTGATTTATGAGAAACAGGTTGAACAGGGCGTTGAGCAGATTGTTAATGATCTGAAAAA
AGAAGTTCGCCAGCTGCTGAAAGAAGCACTGGATATTCCGATGAAACATGCCAATCTGCTGAAAC
TGATTGATGAAATTCAGCGTCTGGGTATCCCGTATCATTTTGAACGTGAAATTGATCATGCCCTGC
AGTGCATTTATGAAACCTATGGTGATAATTGGAATGGTGATCGTAGCAGCCTGTGGTTTCGTCTGA
TGCGTAAACAGGGTTATTATGTTACCTGCGACGTGTTTAACAACTATAAAGATAAAAACGGTGCCT
TTAAACAGAGCCTGGCAAATGATGTTGAAGGTCTGCTGGAACTGTATGAAGCAACCAGCATGCGT
GTTCCGGGTGAAATTATTCTGGAAGATGCACTGGGTTTTACCCGTAGCCGTCTGAGCATGATGACC
AAAGATGCATTTAGCACCAATCCGGCACTGTTTACCGAAATCCAGCGTGCACTGAAACAGCCGCT
GTGGAAACGTCTGCCTCGTATTGAAGCAGCACAGTATATTCCGTTTTATCAGCAGCAGGATAGCCA
TAACAAAACCCTGCTGAAACTGGCAAAACTGGAATTTAATCTGCTGCAGAGCCTGCATAAAGAAG
AACTGAGCCACGTTTGTAAATGGTGGAAAGCCTTCGACATCAAAAAAAACGCACCGTGTCTGCGT
GATCGTATTGTTGAATGTTATTTTTGGGGTCTGGGTAGCGGTTTTGAACCGCAGTATAGCCGTGCA
CGTGTGTTTTTTACCAAAGCAGTTGCAGTTATTACCCTGATCGATGATACCTATGACGCATATGGC
ACCTATGAGGAACTGAAAATCTTTACCGAAGCCGTTGAACGTTGGAGCATTACCTGTCTGGATACC
CTGCCGGAATATATGAAACCGATCTATAAACTGTTCATGGACACCTATACCGAGATGGAAGAATTT
CTGGCAAAAGAAGGTCGTACCGACCTGTTTAATTGCGGTAAAGAATTTGTGAAAGAATTCGTGCGT
AACCTGATGGTTGAAGCAAAATGGGCCAATGAAGGTCATATTCCGACCACCGAAGAACATGATCC
GGTTGTGATTATTACCGGTGGTGCAAACCTGCTGACCACCACCTGTTATCTGGGTATGAGCGATAT
TTTCACCAAAGAAAGCGTTGAATGGGCAGTTAGCGCACCGCCTCTGTTTCGTTATAGCGGTATTCT
GGGTCGTCGTCTGAACGATCTGATGACCCATAAAGCAGAACAAGAA

RECTIFIED SHEET (RULE 91) ISA/EP

CGTAAACATAGCAGCAGCAGCCTGGAAAGCTATATGAAAGAATATAACGTGAACGAAGAGTATGCAC
AGACCCTGATTTACAAAGAAGTTGAGGACGTTTGGAAAGATATCAACCGTGAATATCTGACCACGAA
AAACATTCCGCGTCCGCTGCTGATGGCAGTTATTTATCTGTGTCAGTTCCTGGAAGTTCAGTATGCAGG
TAAAGATAACTTTACGCGTATGGGCGACGAATATAAACATCTGATTAAAAGCCTGCTGGTGTATCCGA
TGAGCATTTAA

Farnesene synthase codon-optimized for *E. coli:*

ATGAGCACCCTGCCGATTAGCAGCGTTAGCTTTAGCAGCAGCACCAGTCCGCTGGTTGTTGATGATAA
AGTTAGCACCAAACCGGATGTTATTCGTCACACCATGAACTTTAATGCAAGCATTTGGGGTGATCAGT
TTCTGACCTATGATGAACCGGAAGATCTGGTGATGAAAAAACAGCTGGTTGAAGAACTGAAAGAAGA
AGTTAAAAAAGAGCTGATCACCATCAAAGGTAGCAATGAACCGATGCAGCATGTTAAACTGATTGAA
CTGATCGATGCCGTTCAGCGTCTGGGTATTGCATATCATTTTGAAGAAGAAATCGAAGAAGCCCTGCA
GCATATTCATGTTACCTATGGTGAACAGTGGGTGGATAAAGAAAATCTGCAGAGCATTAGCCTGTGGT
TTCGTCTGCTGCGTCAGCAGGGTTTTAATGTTAGCAGCGGTGTGTTTAAAGATTTTATGGACGAGAAA
GGCAAATTCAAAGAAAGCCTGTGTAATGATGCACAGGGTATTCTGGCACTGTATGAAGCAGCATTTAT
GCGTGTTGAAGATGAAACCATTCTGGATAATGCACTGGAATTTACCAAAGTGCACCTGGATATCATTG
CAAAAGATCCGAGCTGTGATAGCAGCCTGCGTACCCAGATTCATCAGGCACTGAAACAGCCGCTGCG
TCGTCGTCTGGCACGCATTGAAGCACTGCATTATATGCCGATTTATCAGCAAGAAACCAGCCATAATG
AAGATCTGCTGAAACTGGCAAAACTGGATTTTAGCGTTCTGCAGTCCATGCACAAAAAAGAACTGAG
CCATATTTGTAAATGGTGGAAAGATCTGGATCTGCAGAATAAACTGCCGTATGTTCGTGATCGTGTTG
TGGAAGGTTATTTTTGGATTCTGAGCATCTATTATGAACCGCAGCATGCACGTACCCGTATGTTTCTGA
TGAAAACCTGTATGTGGCTGGTTGTGCTGGATGATACGTTTGATAATTATGGCACCTACGAGGAACTG
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AACATTAGCAATAGTCTGTGTCAGGGTCGTTGGCAGAAAGAACTGGGTAGTCAGATTACCCTGGTTGA
AACCAAAATGGCAAAACGTGGTGTTCATGCCCAGCCGCTGGAAGAGTATATGAGCGTTAGCATGGTT
ACCGGCACCTATGGTCTGATGATTGCACGTAGCTATGTTGGTCGTGGTGATATTGTTACCGAAGATAC
CTTTAAATGGGTGAGCAGCTATCCGCCTATTATCAAAGCAAGCTGTGTTATTGTTCGCCTGATGGATG
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GGCACGTATGTGTGAAGTTCTGTATAGCGTTAATGATGGTTTTACCCATGCCGAAGGTGATATGAAAT
CCTATATGAAAAGCTTCTTCGTGCATCCGATGGTTGTTTAA

pMCM1223 - pCL-Ptrc-Upper_GcMM_161 (*Listeria gray*i *DSM 20601*):

cccgtcttactgtcgggaattcgcgttggccgattcattaatgcagattctgaaatgagctgttgacaattaatcatccggctcgtataatgtgtggaattgtgagcggataac
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CLAIMS

What is claimed is:

1. Recombinant cells capable of increased production of isoprene wherein the cells are engineered for increased carbon flux towards isoprene production such that the activity of one or more enzymes from the group consisting of:

(a) citrate synthase,
(b) phosphotransacetylase
(c) acetate kinase,
(d) lactate dehydrogenase,
(e) malate dehydrogenase,
(f) pyruvate dehydrogenase,
(g) phosphogluconolactonase, and
(h) and phosphoenolpyruvate carboxylase

is modulated, and wherein said cells further comprise (i) one or more nucleic acids encoding one or more mevalonate (MVA) pathway polypeptides and (ii) one or more heterologous nucleic acids encoding an isoprene synthase polypeptide; and wherein said cells produce increased amounts of isoprene compared to isoprene-producing cells that have not been engineered for increased carbon flux towards isoprene.

2. The cells of claim 1 wherein the one or more nucleic acids encoding MVA pathway polypeptides are from the upper MVA pathway, wherein the upper MVA pathway nucleic acids are selected from the group consisting of AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase nucleic acids.

3. The cells of claim 2 wherein said one or more nucleic acids encoding an upper mevalonate (MVA) pathway polypeptide is an *mvaE* gene and an *mvaS* gene.

4. The cells of claim 3, wherein the *mvaE* gene and the *mvaS* gene is selected from the group consisting of: (a) an *mvaE* gene and an *mvaS* gene from *L. grayi*; (b) an *mvaE* gene and an *mvaS* gene from *E. faecium*; (c) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*; and (e) an *mvaE* gene and an *mvaS* gene from *E. faecalis*.

5. The cells of claim 1 wherein the one or more nucleic acids encoding MVA pathway polypeptides are from the lower MVA pathway, wherein the lower MVA pathway nucleic acids are selected from the group consisting of MVK, PMK, and MVD nucleic acids.

6. The cells of claim 5, wherein the MVK is selected from the group consisting of *M. mazei* mevalonate kinase, *M. burtonii* mevalonate kinase polypeptide, *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, *Streptomyces* mevalonate kinase polypeptide, and *Streptomyces* CL190 mevalonate kinase polypeptide.

7.The cells of claim 1, wherein the cells further comprise one or more heterologous nucleic acids encoding one or DXP pathway polypeptides.

8. 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* or variant thereof.

9. 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.

10. The cells of claim 1, wherein the activity of citrate synthase is modulated by decreasing the activity of an endogenous citrate synthase gene.

11. The cells of claim 10, wherein the activity of citrate synthase is modulated by chromosomal replacement of an endogenous citrate synthase gene with a transgene encoding an NADH-insensitive citrate synthase.

12. The cells of claim 11, wherein the transgene encoding an NADH-insensitive citrate synthase is derived from *Bacillus subtilis.*

13. The cells of claim 10, wherein the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter.

14. The cells of any one of claims 10-13, wherein decreasing the activity of citrate synthase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have decreased expression of citrate synthase.

15. The cells claim 1, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphotransacetylase and/or acetate kinase.

16. The cells of claim 15, wherein the activity of phosphotransacetylase and/or acetate kinase is modulated by attenuating the activity of an endogenous phosphotransacetylase gene and/or an endogenous acetate kinase gene.

17. The cells of claim 16, wherein endogenous phosphotransacetylase and/or endogenous acetate kinase gene expression is attenuated by deletion of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene.

18. The cells of claim 15 or 16, wherein the cells produces decreased amounts of acetate in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression.

19. The cells of any one of claims 1-9 or 15-18, wherein attenuating the activity of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression

20. The cells of claim 1, wherein carbon flux is directed towards mevalonate production by modulating the activity of lactate dehydrogenase.

21. The cells of claim 20, wherein the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene.

22. The cells of claim 21, wherein endogenous lactate dehydrogenase gene expression is attenuated by deletion of the endogenous lactate dehydrogenase gene.

23. The cells of any one of claims 20-22, wherein the cells produces decreased amounts of lactate in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression.

24. The cells of any one of claims 20-23, wherein attenuating the activity of the endogenous lactate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression.

25. The cells of any one of claims 1, wherein carbon flux is directed towards mevalonate production by modulating the activity of NADP-dependent malate dehydrogenase.

26. The cells of claim 25, wherein the activity of NADP-dependent malate dehydrogenase is modulated by increasing the activity of an NADP-dependent malate dehydrogenase gene.

27. The cells of claim 26, wherein the NADP-dependent malate dehydrogenase gene is an endogenous gene.

28. The cells of claim 27, wherein expression of the endogenous NADP-dependent malate dehydrogenase gene is increased by replacing the endogenous NADP-dependent malate dehydrogenase gene promoter with a synthetic constitutively expressing promoter.

29. The cells of claim 25 or 26, wherein the cells further comprises a heterologous nucleic acid encoding an NADP-dependent malate dehydrogenase polypeptide.

30. The cells of any one of claims 25-29, wherein the cells produces increased amounts of pyruvate in comparison to microorganisms that do not have increased expression of an NADP-dependent malate dehydrogenase gene.

31. The cells of any one of claims 25-30, wherein increasing the activity of an NADP-dependent malate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have increased NADP-dependent malate dehydrogenase gene expression.

32. The cells of any one of claims 1, wherein carbon flux is directed towards mevalonate production by modulating the activity of pyruvate dehydrogenase.

33. The cells of claim 32, wherein the activity of pyruvate dehydrogenase is modulated by increasing the activity of one or more genes of the pyruvate dehydrogenase complex consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase.

34. The cells of claims 32 or 33, wherein the activity of the pyruvate dehydrogenase complex is modulated by attenuating the activity of an endogenous pyruvate dehydrogenase complex repressor gene.

35. The cells of claim 34, wherein the one or more genes of the pyruvate dehydrogenase complex are endogenous genes.

36. The cells of claim 35, wherein expression of the one or more endogenous genes of the pyruvate dehydrogenase complex is increased by replacing one or more endogenous gene promoters with one or more synthetic constitutively expressing promoters.

37. The cells of claim 32 or 33, wherein the cells further comprises one or more heterologous nucleic acids encoding one or more polypeptides from the group consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase.

38. The cells of claim 34, wherein the activity of an endogenous pyruvate dehydrogenase complex repressor is attenuated by deletion of the endogenous pyruvate dehydrogenase complex repressor gene.

39. The cells of any one of claims 32-38, wherein the cells produces increased amounts of acetyl Co-A in comparison to microorganisms wherein the activity of pyruvate dehydrogenase is not modulated.

40. The cells of any one of claims 32-39, wherein modulating the activity of pyruvate dehydrogenase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have modulated pyruvate dehydrogenase expression.

41. The cells of claim 1, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphogluconolactonase (PGL).

42. The cells of claim 41, wherein the activity of PGL is modulated by attenuating the activity of an endogenous PGL gene.

43. The cells of claim 42, wherein the activity of PGL is attenuated by replacing the endogenous PGL gene promoter with a synthetic constitutively low expressing promoter.

44. The cells of claim 43, wherein the activity of an endogenous PGL is attenuated by deletion of the endogenous PGL gene.

45. The cells of claim 1, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphoenolpyruvate carboxylase.

46. The cells of claim 45, wherein the activity of phosphoenolpyruvate carboxylase is modulated by attenuating the activity of an endogenous phosphoenolpyruvate carboxylase gene.

47. The cells of claim 46, wherein the activity of phosphoenolpyruvate carboxylase is attenuated by replacing the endogenous phosphoenolpyruvate carboxylase gene promoter with a synthetic constitutively low expressing promoter.

48. The cells of claim 47, wherein the activity of an endogenous phosphoenolpyruvate carboxylase is attenuated by deletion of the endogenous phosphoenolpyruvate carboxylase gene.

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

50. Recombinant cells capable of increased production of mevalonate wherein the cells are engineered for increased carbon flux towards mevalonate, production such that the activity of one or more enzymes from the group consisting of:

(a) citrate synthase,

(b) phosphotransacetylase

(c) acetate kinase,

(d) lactate dehydrogenase,

(e) malate dehydrogenase,

(f) pyruvate dehydrogenase,

(g) phosphogluconolactonase, and

(h) and phosphoenolpyruvate carboxylase

is modulated and wherein said cells further comprise one or more nucleic acids encoding one or more upper mevalonate (MVA) pathway polypeptides; and wherein the cells produce increased amounts of mevalonate compared to mevalonate-producing cells that have not been engineered for increased carbon flux towards mevalonate.

51. The cells of claim 50, wherein said one or more nucleic acids encoding an upper mevalonate (MVA) pathway polypeptide is an *mvaE* gene and an *mvaS* gene.

52. The cells of claim 50, wherein the *mvaE* gene and the *mvaS* gene is selected from the group consisting of: (a) an *mvaE* gene and an *mvaS* gene from *L. grayi*; (b) an *mvaE* gene and an *mvaS* gene from *E. faecium*; (c) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; (d) an

mvaE gene and an *mvaS* gene from *E. casseliflavus*; and (e) an *mvaE* gene and an *mvaS* gene from *E. faecalis*.

53. The cells of claim 50, wherein the activity of citrate synthase is modulated by decreasing the activity of an endogenous citrate synthase gene.

54. The cells of claim 53 wherein the activity of citrate synthase is modulated by chromosomal replacement of an endogenous citrate synthase gene with a transgene encoding an NADH-insensitive citrate synthase.

55. The cells of claim 54, wherein the transgene encoding an NADH-insensitive citrate synthase is derived from *Bacillus subtilis*.

56. The cells of claim 53, wherein the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter.

57. The cells of any one of claims 53-54, wherein decreasing the activity of citrate synthase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have decreased expression of citrate synthase.

58. The cells of claim 50, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphotransacetylase and/or acetate kinase.

59. The cells of claim 58, wherein the activity of phosphotransacetylase and/or acetate kinase is modulated by attenuating the activity of an endogenous phosphotransacetylase gene and/or an endogenous acetate kinase gene.

60. The cells of claim 59, wherein endogenous phosphotransacetylase and/or endogenous acetate kinase gene expression is attenuated by deletion of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene.

61. The cells of claim 58 or claim 59, wherein the cells produces decreased amounts of acetate in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression.

62. The cells of any one of claims 50-52 or 58-61, wherein attenuating the activity of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression.

63. The cells of claim 50, wherein carbon flux is directed towards mevalonate production by modulating the activity of lactate dehydrogenase.

64. The cells of claim 63, wherein the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene.

65. The cells of claim 64, wherein endogenous lactate dehydrogenase gene expression is attenuated by deletion of the endogenous lactate dehydrogenase gene.

66. The cells of any one of claims 64-65, wherein the cells produces decreased amounts of lactate in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression.

67. The cells of any one of claims 64-66, wherein attenuating the activity of the endogenous lactate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression.

68. The cells of claim 50, wherein carbon flux is directed towards mevalonate production by modulating the activity of NADP-dependent malate dehydrogenase.

69. The cells of claim 68, wherein the activity of NADP-dependent malate dehydrogenase is modulated by increasing the activity of an NADP-dependent malate dehydrogenase gene.

70. The cells of claim 69, wherein the NADP-dependent malate dehydrogenase gene is an endogenous gene.

71. The cells of claim 70, wherein expression of the endogenous NADP-dependent malate dehydrogenase gene is increased by replacing the endogenous NADP-dependent malate dehydrogenase gene promoter with a synthetic constitutively expressing promoter.

72. The cells of claim 68 or 69, wherein the cells further comprises a heterologous nucleic acid encoding an NADP-dependent malate dehydrogenase polypeptide.

73. The cells of any one of claims 68-72, wherein the cells produces increased amounts of pyruvate in comparison to microorganisms that do not have increased expression of an NADP-dependent malate dehydrogenase gene.

74. The cells of any one of claims 68-73, wherein increasing the activity of an NADP-dependent malate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have increased NADP-dependent malate dehydrogenase gene expression.

75. The cells of claim 50, wherein carbon flux is directed towards mevalonate production by modulating the activity of pyruvate dehydrogenase.

76. The cells of claim 75, wherein the activity of pyruvate dehydrogenase is modulated by increasing the activity of one or more genes of the pyruvate dehydrogenase complex consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase.

77. The cells of claims 75 or 76, wherein the activity of the pyruvate dehydrogenase complex is modulated by attenuating the activity of an endogenous pyruvate dehydrogenase complex repressor gene.

78. The cells of claim 77, wherein the one or more genes of the pyruvate dehydrogenase complex are endogenous genes.

79. The cells of claim 78, wherein expression of the one or more endogenous genes of the pyruvate dehydrogenase complex is increased by replacing one or more endogenous gene promoters with one or more synthetic constitutively expressing promoters.

80. The cells of claim 75 or 76, wherein the cells further comprises one or more heterologous nucleic acids encoding one or more polypeptides from the group consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase.

81. The cells of claim 77, wherein the activity of an endogenous pyruvate dehydrogenase complex repressor is attenuated by deletion of the endogenous pyruvate dehydrogenase complex repressor gene.

82. The cells of any one of claims 75-81, wherein the cells produces increased amounts of acetyl Co-A in comparison to microorganisms wherein the activity of pyruvate dehydrogenase is not modulated.

83. The cells of any one of claims 75-82, wherein modulating the activity of pyruvate dehydrogenase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have modulated pyruvate dehydrogenase expression.

84. The cells of claim 50, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphogluconolactonase (PGL).

85. The cells of claim 84, wherein the activity of PGL is modulated by attenuating the activity of an endogenous PGL gene.

86. The cells of claim 85, wherein the activity of PGL is attenuated by replacing the endogenous PGL gene promoter with a synthetic constitutively low expressing promoter.

87. The cells of claim 85, wherein the activity of an endogenous PGL is attenuated by deletion of the endogenous PGL gene.

88. The cells of claim 50, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphoenolpyruvate carboxylase.

89. The cells of claim 88, wherein the activity of phosphoenolpyruvate carboxylase is modulated by attenuating the activity of an endogenous phosphoenolpyruvate carboxylase gene.

90. The cells of claim 89, wherein the activity of phosphoenolpyruvate carboxylase is attenuating by replacing the endogenous phosphoenolpyruvate carboxylase gene promoter with a synthetic constitutively low expressing promoter.

91. The cells of claim 89, wherein the activity of an endogenous phosphoenolpyruvate carboxylase is attenuated by deletion of the endogenous phosphoenolpyruvate carboxylase gene.

92. A method of producing mevalonate, comprising: (a) culturing the cells of claim 50 under suitable culture conditions for production of isoprene; and (b) producing the mevalonate.

93. The cells of any one of claims 50-92, wherein mevalonate production is increased compared to microorganisms that are not grown under conditions of tri-carboxylic acid (TCA) cycle activity, wherein metabolic carbon flux in the cells is directed towards mevalonate production by modulating the activity of one or more enzymes from the group consisting of (a) citrate synthase, (b) phosphotransacetylase and/or acetate kinase, (c) lactate dehydrogenase, (d) malate dehydrogenase, and (e) pyruvate decarboxylase complex.

94. The cells of any one of claims 50-92, wherein the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter, the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene, and the activity of acetate kinase is modulated by attenuating the activity of an endogenous acetate kinase gene.

95. Recombinant cells capable of increased production of isoprenoids wherein the cells are engineered for increased carbon flux towards mevalonate, production such that the activity of one or more enzymes from the group consisting of:

(a) citrate synthase,
(b) phosphotransacetylase
(c) acetate kinase,
(d) lactate dehydrogenase,
(e) malate dehydrogenase,
(f) pyruvate dehydrogenase
(g) phosphogluconolactonase, and
(h) and phosphoenolpyruvate carboxylase

is modulated and wherein said cells further comprise (i) one or more nucleic acids encoding one or more mevalonate (MVA) pathway polypeptides and (ii) one or more nucleic acids encoding polyprenyl pyrophosphate synthases; and wherein the cells produce increased amounts of isoprenoids compared to isoprenoid-producing cells that have not been engineered for increased carbon flux towards mevalonate.

96. The cells of claim 95 wherein the one or more nucleic acids encoding MVA pathway polypeptides are from the upper MVA pathway, wherein the upper MVA pathway nucleic acids are selected from the group consisting of AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase nucleic acids.

97. The cells of claim 96 wherein said one or more nucleic acids encoding an upper mevalonate (MVA) pathway polypeptide is an *mvaE* gene and an *mvaS* gene.

98. The cells of claim 97, wherein the *mvaE* gene and the *mvaS* gene is selected from the group consisting of: (a) an *mvaE* gene and an *mvaS* gene from *L. grayi*; (b) an *mvaE* gene and an

mvaS gene from *E. faecium*; (c) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*; and (e) an *mvaE* gene and an *mvaS* gene from *E. faecalis*.

99. The cells of claim 95 wherein the one or more nucleic acids encoding MVA pathway polypeptides are from the lower MVA pathway, wherein the lower MVA pathway nucleic acids are selected from the group consisting of MVK, PMK, and, MVD nucleic acids.

100. The cells of claim 99, wherein the MVK is selected from the group consisting of *M. mazei* mevalonate kinase, *M. burtonii* mevalonate kinase polypeptide, *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, *Streptomyces* mevalonate kinase polypeptide, and *Streptomyces* CL190 mevalonate kinase polypeptide.

101. The cells of claim 95, wherein the cells further comprise one or more heterologous nucleic acids encoding one or DXP pathway polypeptides.

102. The cells of claim 95, 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.

103. The cells of claim 95, wherein the activity of citrate synthase is modulated by decreasing the activity of an endogenous citrate synthase gene.

104. The cells of claim 103, wherein the activity of citrate synthase is modulated by chromosomal replacement of an endogenous citrate synthase gene with a transgene encoding an NADH-insensitive citrate synthase.

105. The cells of claim 104, wherein the transgene encoding an NADH-insensitive citrate synthase is derived from *Bacillus subtilis*.

106. The cells of claim 103, wherein the activity of citrate synthase is modulated by replacing the endogenous citrate synthase gene promoter with a synthetic constitutively low expressing promoter.

107. The cells of any one of claims 103-106, wherein decreasing the activity of citrate synthase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have decreased expression of citrate synthase.

108. The cells claim 95, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphotransacetylase and/or acetate kinase.

109. The cells of claim 108, wherein the activity of phosphotransacetylase and/or acetate kinase is modulated by attenuating the activity of an endogenous phosphotransacetylase gene and/or an endogenous acetate kinase gene.

110. The cells of claim 109, wherein endogenous phosphotransacetylase and/or endogenous acetate kinase gene expression is attenuated by deletion of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene.

111. The cells of claim 108 or 109, wherein the cells produces decreased amounts of acetate in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression.

112. The cells of any one of claims 95-102 or 108-111, wherein attenuating the activity of the endogenous phosphotransacetylase gene and/or the endogenous acetate kinase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous phosphotransacetylase gene and/or endogenous acetate kinase gene expression

113. The cells of claim 95, wherein carbon flux is directed towards mevalonate production by modulating the activity of lactate dehydrogenase.

114. The cells of claim 113, wherein the activity of lactate dehydrogenase is modulated by attenuating the activity of an endogenous lactate dehydrogenase gene.

115. The cells of claim 114, wherein endogenous lactate dehydrogenase gene expression is attenuated by deletion of the endogenous lactate dehydrogenase gene.

116. The cells of any one of claims 113-115, wherein the cells produces decreased amounts of lactate in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression.

117. The cells of any one of claims 113-116, wherein attenuating the activity of the endogenous lactate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have attenuated endogenous lactate dehydrogenase gene expression.

118. The cells of any one of claims 95, wherein carbon flux is directed towards mevalonate production by modulating the activity of NADP-dependent malate dehydrogenase.

119. The cells of claim 118, wherein the activity of NADP-dependent malate dehydrogenase is modulated by increasing the activity of an NADP-dependent malate dehydrogenase gene.

120. The cells of claim 119, wherein the NADP-dependent malate dehydrogenase gene is an endogenous gene.

121. The cells of claim 119, wherein expression of the endogenous NADP-dependent malate dehydrogenase gene is increased by replacing the endogenous NADP-dependent malate dehydrogenase gene promoter with a synthetic constitutively expressing promoter.

122. The cells of claim 118 or 119, wherein the cells further comprise a heterologous nucleic acid encoding an NADP-dependent malate dehydrogenase polypeptide.

123. The cells of any one of claims 118-122, wherein the cells produces increased amounts of pyruvate in comparison to microorganisms that do not have increased expression of an NADP-dependent malate dehydrogenase gene.

124. The cells of any one of claims 118-123, wherein increasing the activity of an NADP-dependent malate dehydrogenase gene results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have increased NADP-dependent malate dehydrogenase gene expression.

125. The cells of any one of claims 95, wherein carbon flux is directed towards mevalonate production by modulating the activity of pyruvate dehydrogenase.

126. The cells of claim 125, wherein the activity of pyruvate dehydrogenase is modulated by increasing the activity of one or more genes of the pyruvate dehydrogenase complex consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase.

127. The cells of claims 125 or 126, wherein the activity of the pyruvate dehydrogenase complex is modulated by attenuating the activity of an endogenous pyruvate dehydrogenase complex repressor gene.

128. The cells of claim 127, wherein the one or more genes of the pyruvate dehydrogenase complex are endogenous genes.

129. The cells of claim 128, wherein expression of the one or more endogenous genes of the pyruvate dehydrogenase complex is increased by replacing one or more endogenous gene promoters with one or more synthetic constitutively expressing promoters.

130. The cells of claim 125 or 126, wherein the cells further comprises one or more heterologous nucleic acids encoding one or more polypeptides from the group consisting of (a) pyruvate dehydrogenase (E1), (b) dihydrolipoyl transacetylase, and (c) dihydrolipoyl dehydrogenase.

131. The cells of claim 130, wherein the activity of an endogenous pyruvate dehydrogenase complex repressor is attenuated by deletion of the endogenous pyruvate dehydrogenase complex repressor gene.

132. The cells of any one of claims 125-131, wherein the cells produces increased amounts of acetyl Co-A in comparison to microorganisms wherein the activity of pyruvate dehydrogenase is not modulated.

133. The cells of any one of claims 125-132, wherein modulating the activity of pyruvate dehydrogenase results in more carbon flux into the mevalonate dependent biosynthetic pathway in comparison to microorganisms that do not have modulated pyruvate dehydrogenase expression.

134. The cells of claim 95, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphogluconolactonase (PGL).

135. The cells of claim 134, wherein the activity of PGL is modulated by attenuating the activity of an endogenous PGL gene.

136. The cells of claim 135, wherein the activity of PGL is decreased by replacing the endogenous PGL gene promoter with a synthetic constitutively low expressing promoter.

137. The cells of claim 136, wherein the activity of an endogenous PGL is attenuated by deletion of the endogenous PGL gene.

138. The cells of claim 95, wherein carbon flux is directed towards mevalonate production by modulating the activity of phosphoenolpyruvate carboxylase.

139. The cells of claim 138, wherein the activity of phosphoenolpyruvate carboxylase is modulated by attenuating the activity of an endogenous phosphoenolpyruvate carboxylase gene.

140. The cells of claim 139, wherein the activity of phosphoenolpyruvate carboxylase is decreased by replacing the endogenous phosphoenolpyruvate carboxylase gene promoter with a synthetic constitutively low expressing promoter.

141. The cells of claim 140, wherein the activity of an endogenous phosphoenolpyruvate carboxylase is attenuated by deletion of the endogenous phosphoenolpyruvate carboxylase gene.

142. The cells of claims 93-141, wherein the isoprenoid is selected from group consisting of monoterpenes, diterpenes, triterpenes, tetraterpenes, sequiterpenes, and polyterpenes.

143. The cells of claim 142, wherein the isoprenoid is a sesquiterpene.

144. The cells of claim 142, wherein the isoprenoid is selected from the group consisting of abietadiene, amorphadiene, carene, α-farnesene, β-farnesene, farnesol, geraniol, geranylgeraniol, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, β-pinene, sabinene, γ-terpinene, terpindene and valencene.

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

146. The method of claim 49, further comprising (c) recovering the isoprene.

147. The method of claim 92, further comprising (c) recovering the mevalonate.

148. The method of claim 145, further comprising (c) recovering the isoprenoids.

149. The cells of claim 1, further comprising one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide.

150. The cells of claim 95, further comprising one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide.

Figure 1

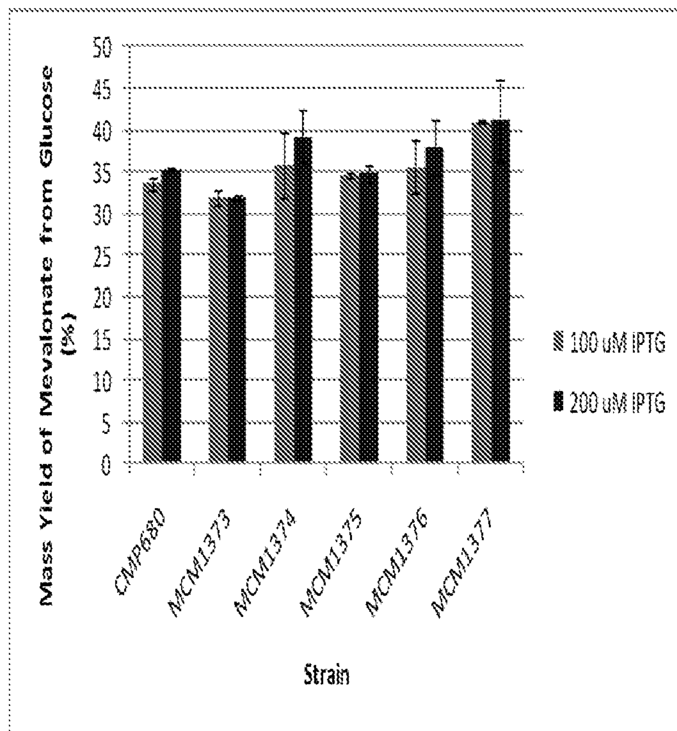


FIGURE 2:

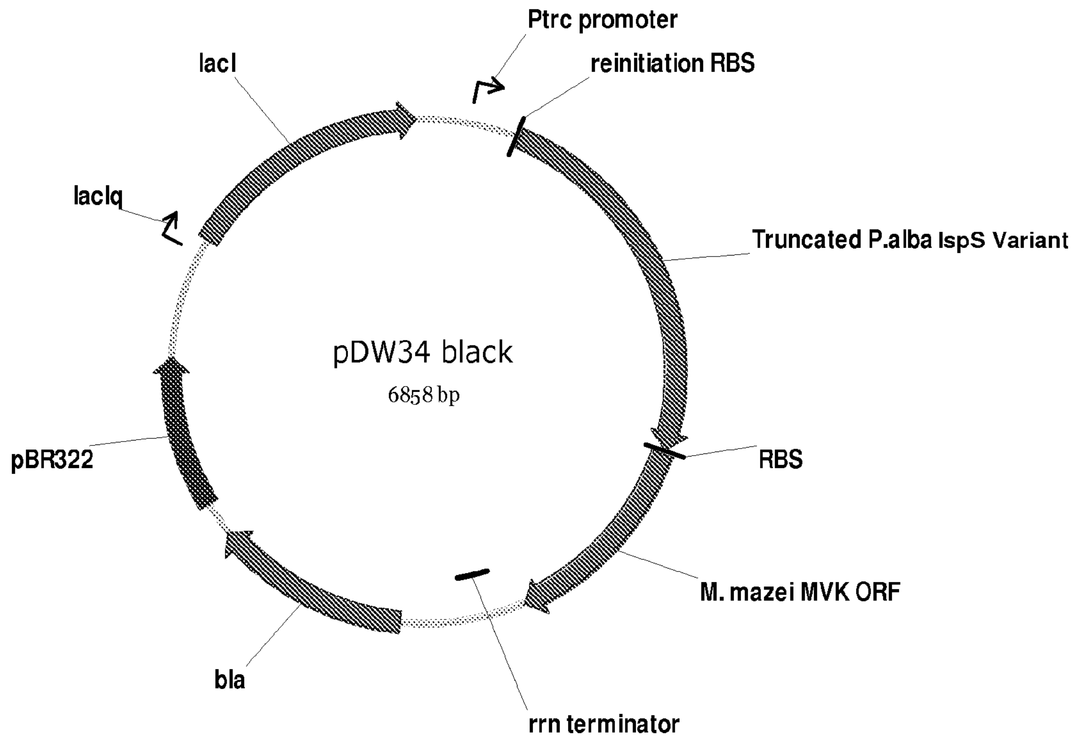


FIGURE 3:

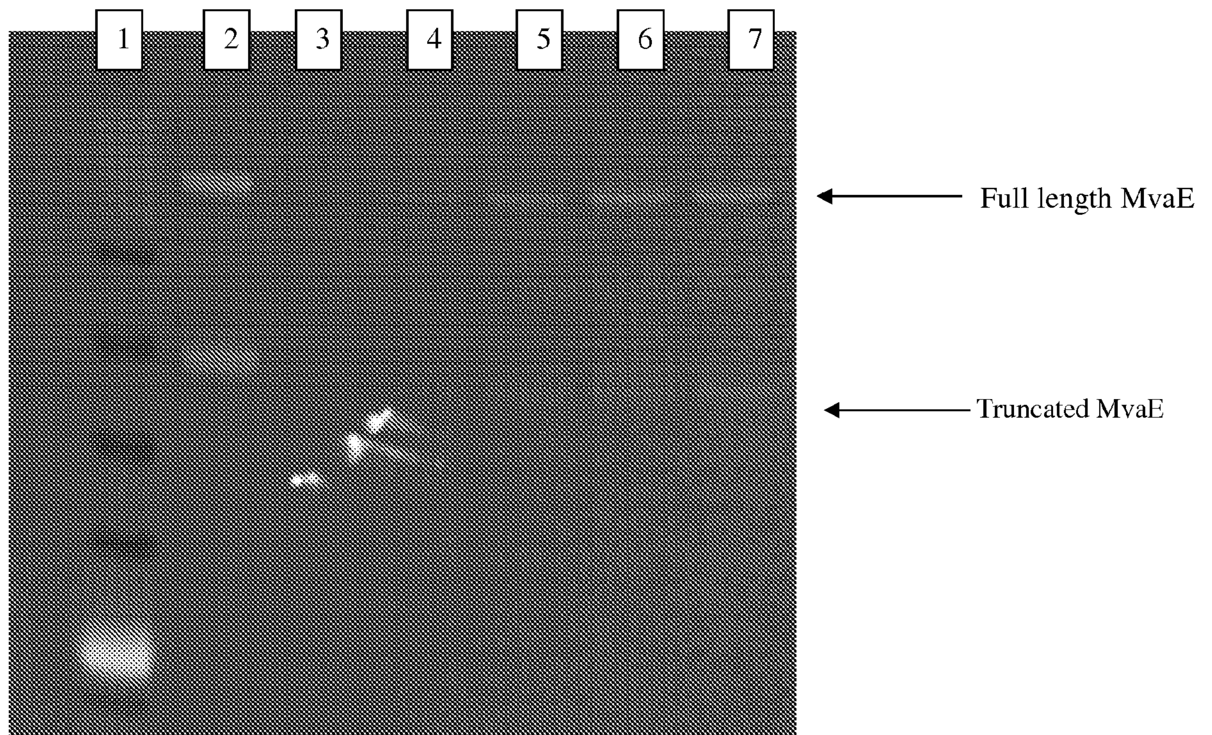


FIGURE 4:

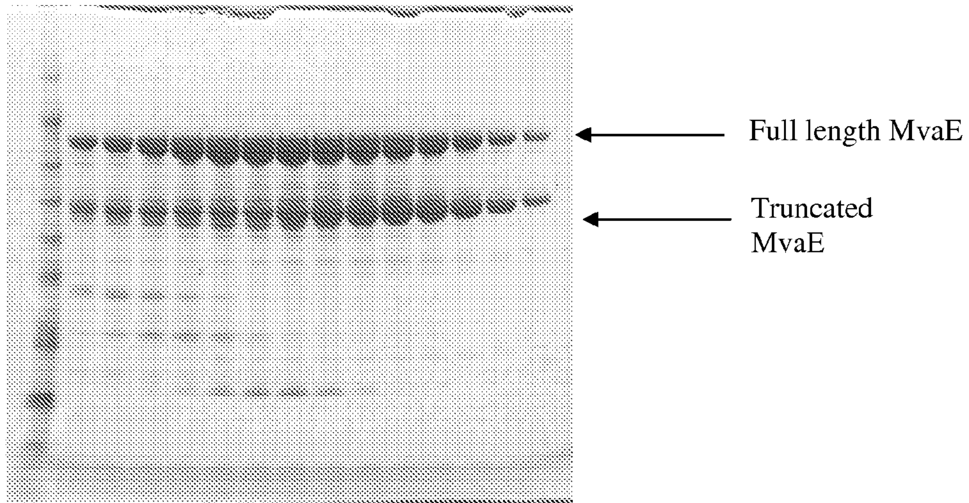


FIGURE 5:

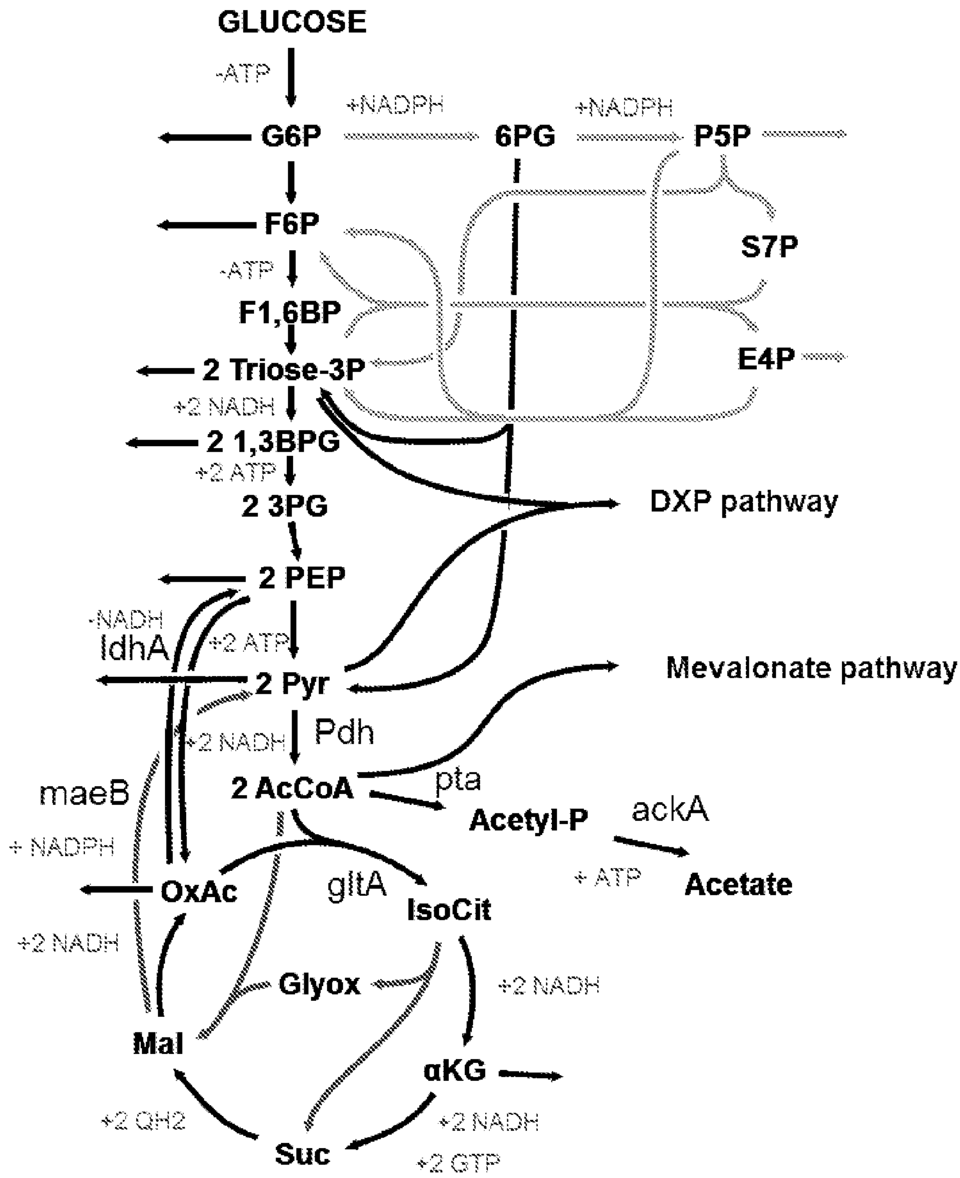


FIGURE 6:

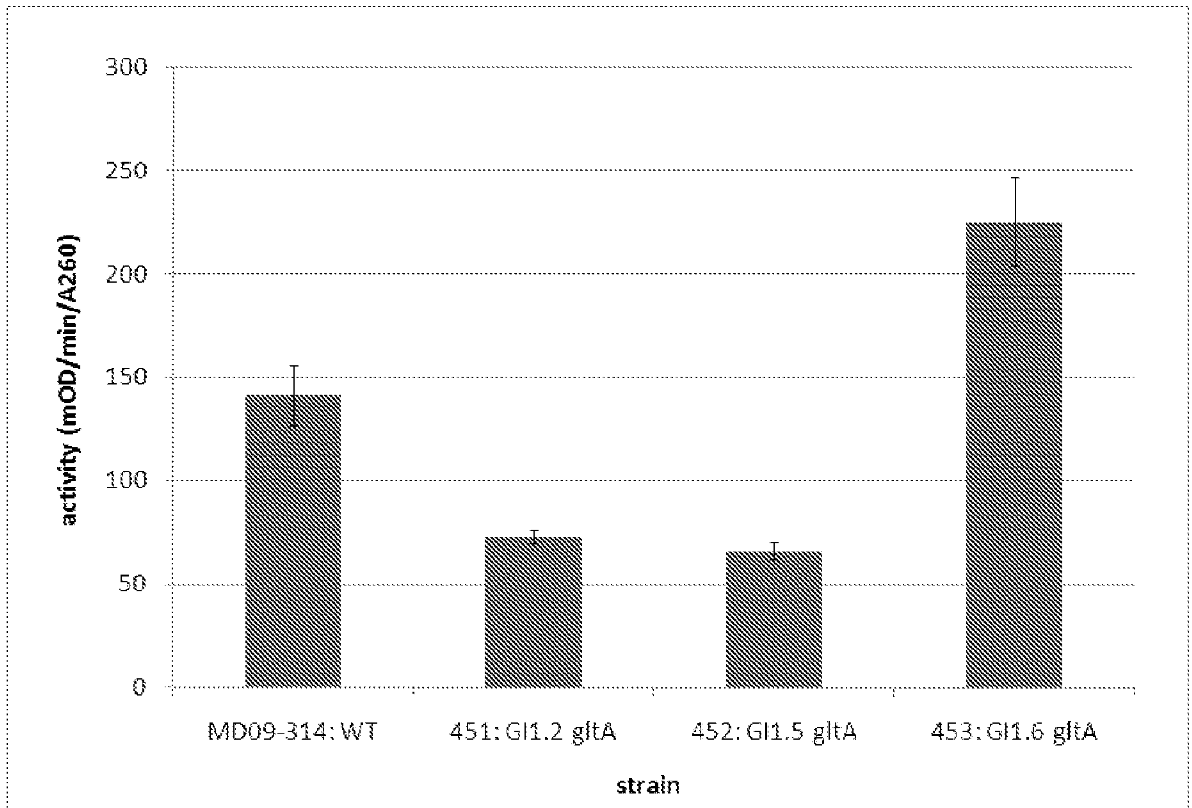


FIGURE 7:

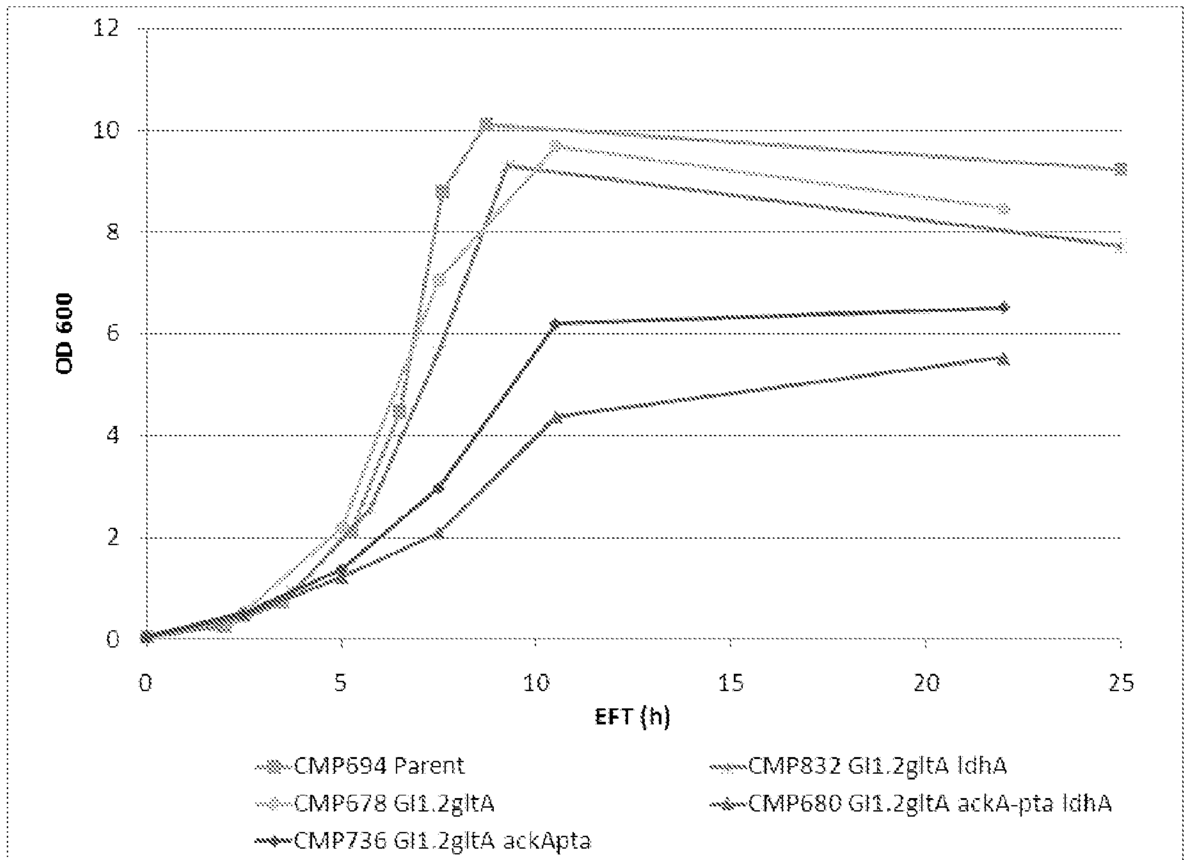


FIGURE 8:

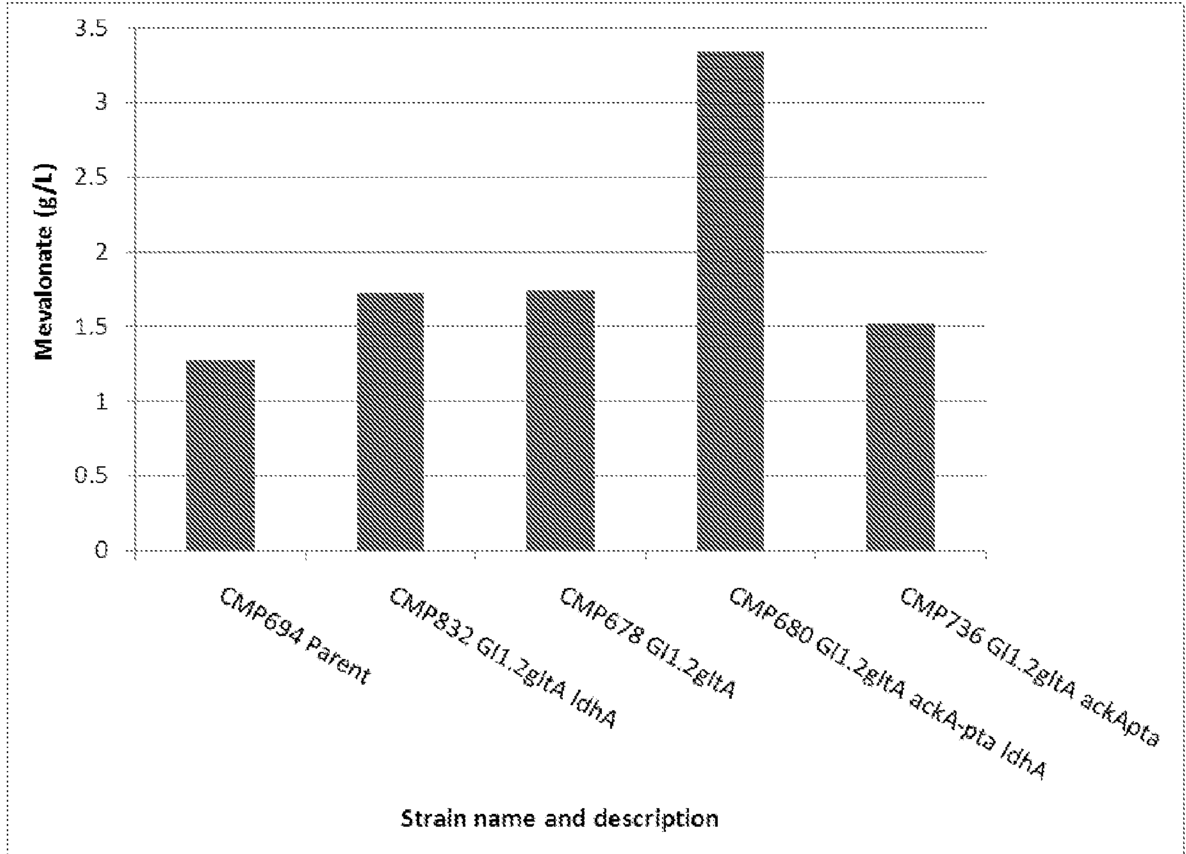


FIGURE 9:

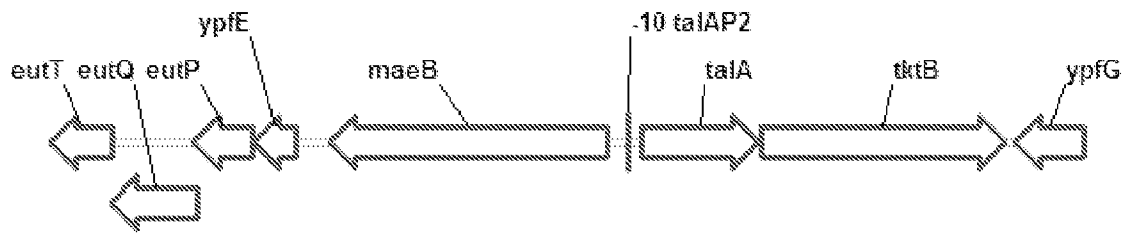


FIGURE 10:

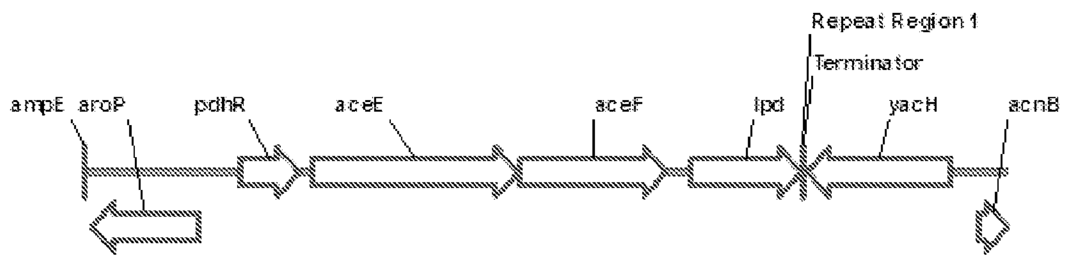


FIGURE 11:

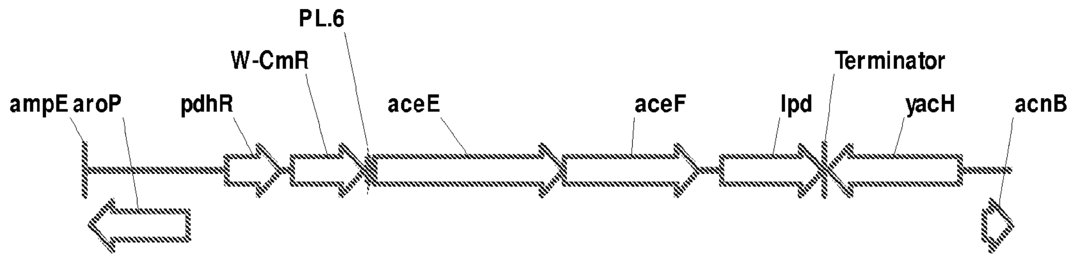


FIGURE 12:

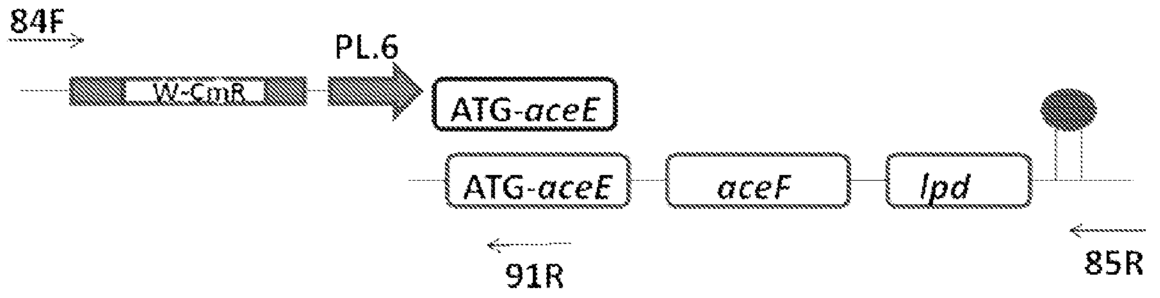


FIGURE 13:

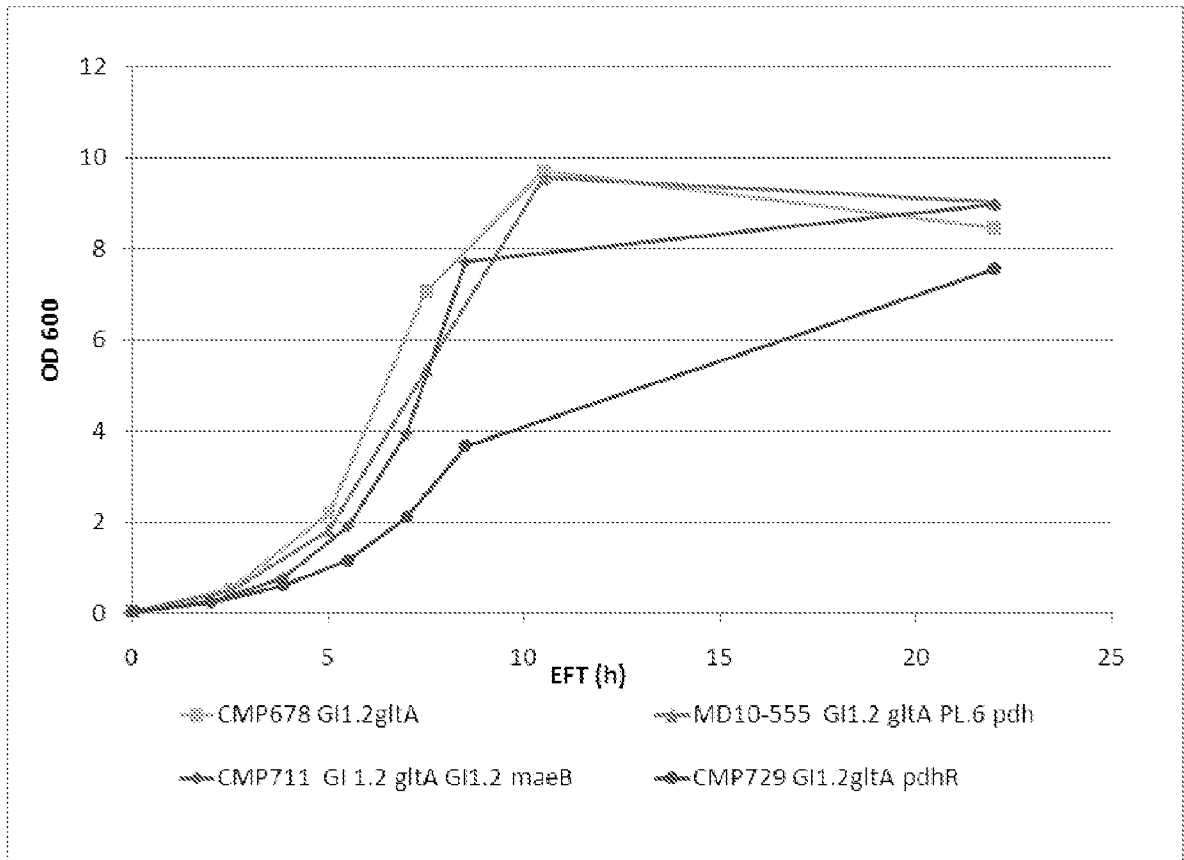
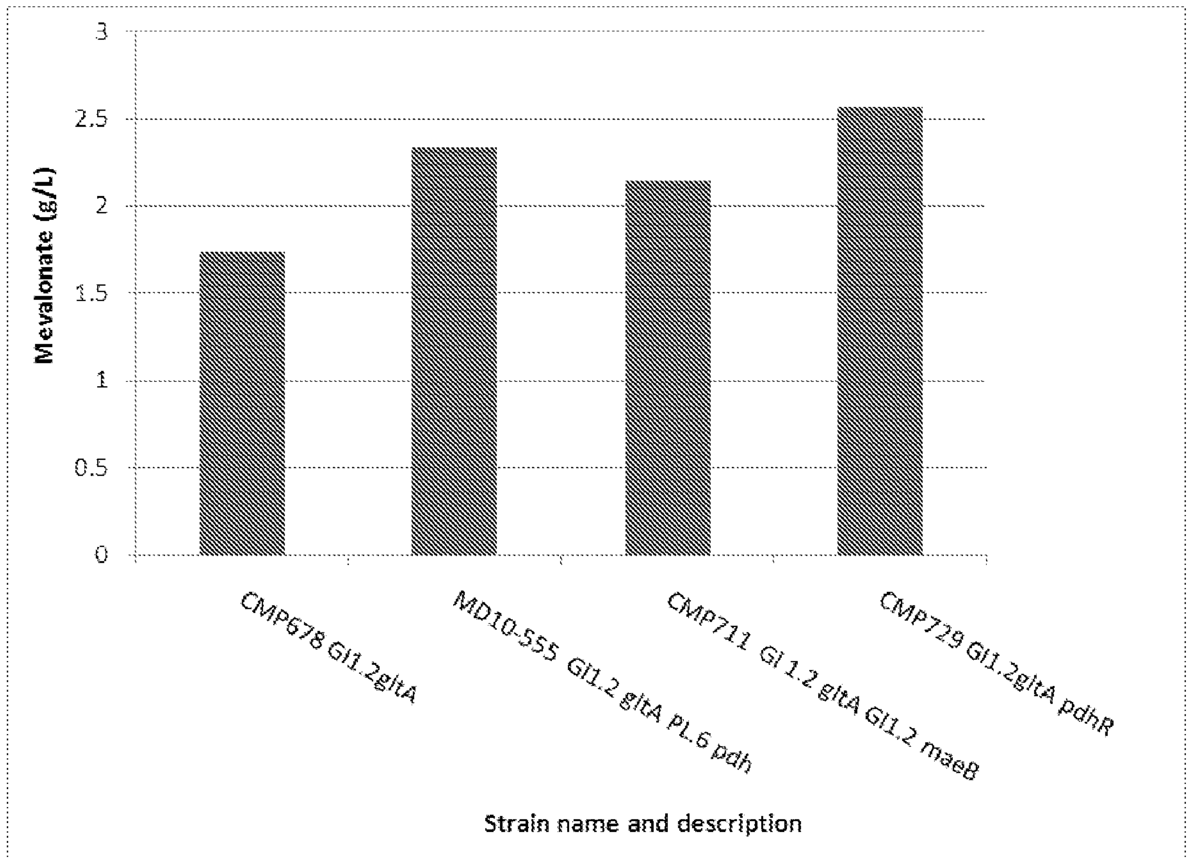


FIGURE 14:



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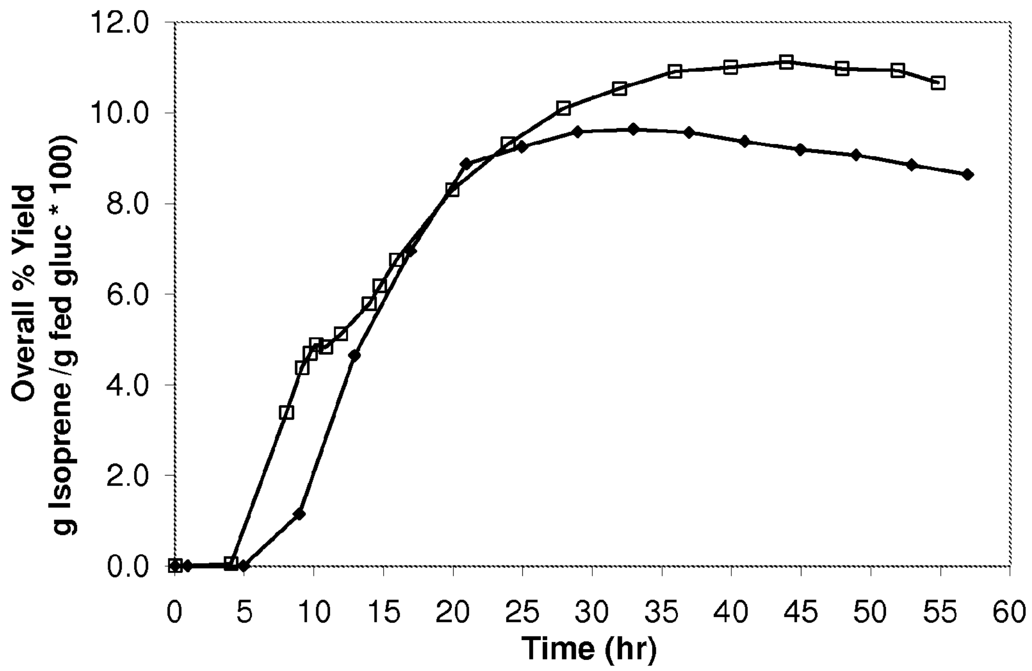

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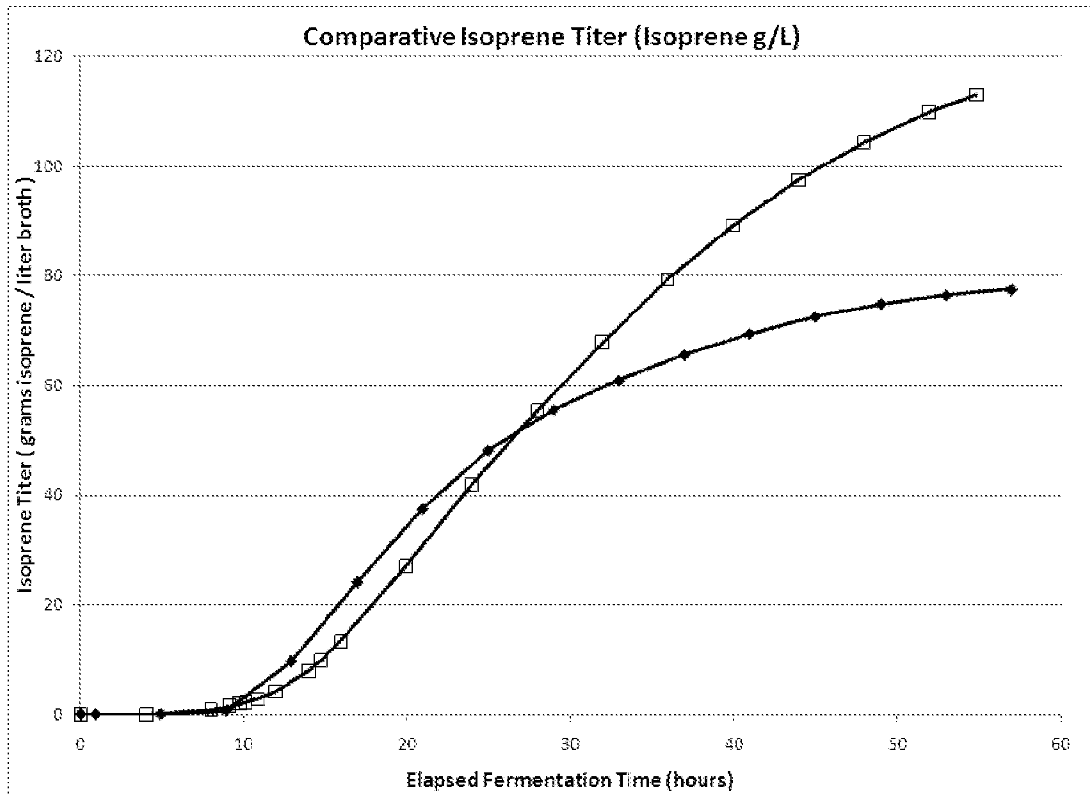

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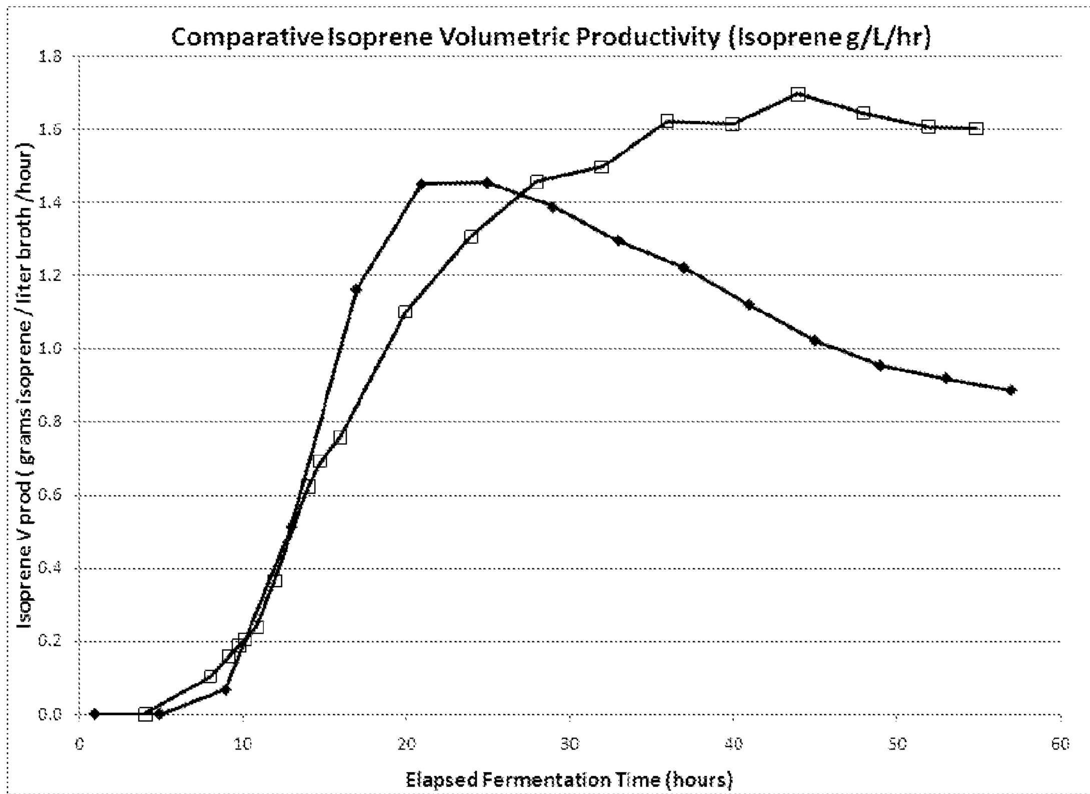

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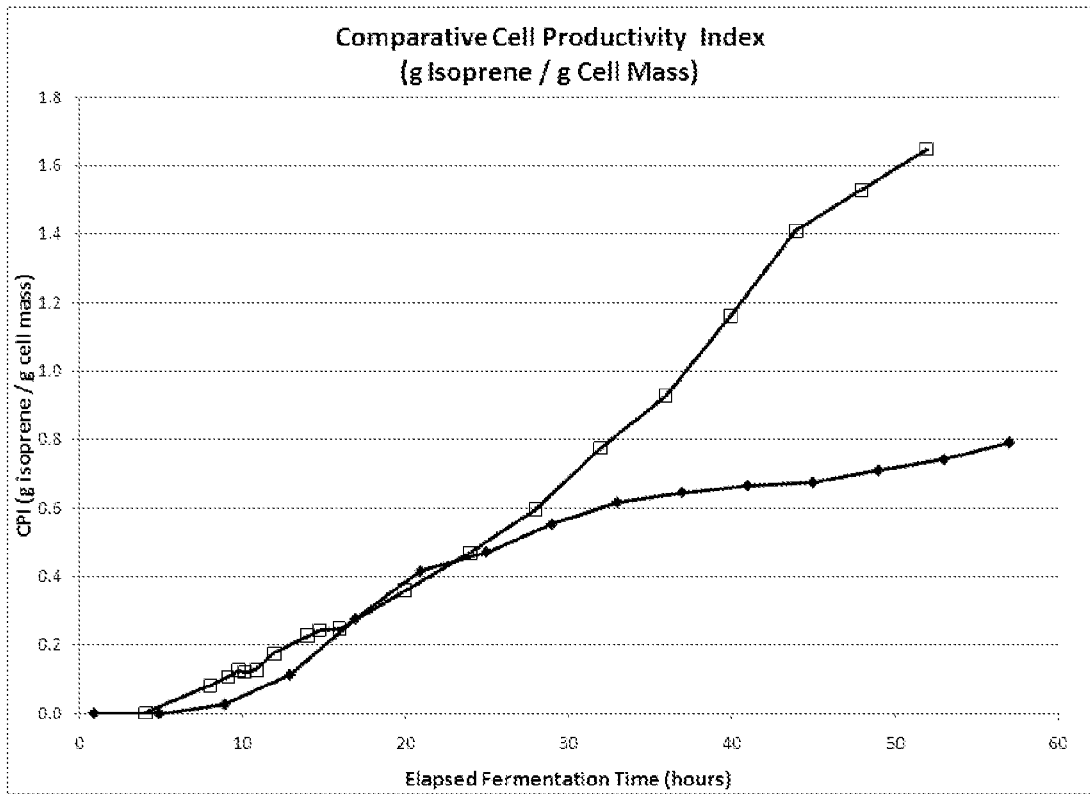

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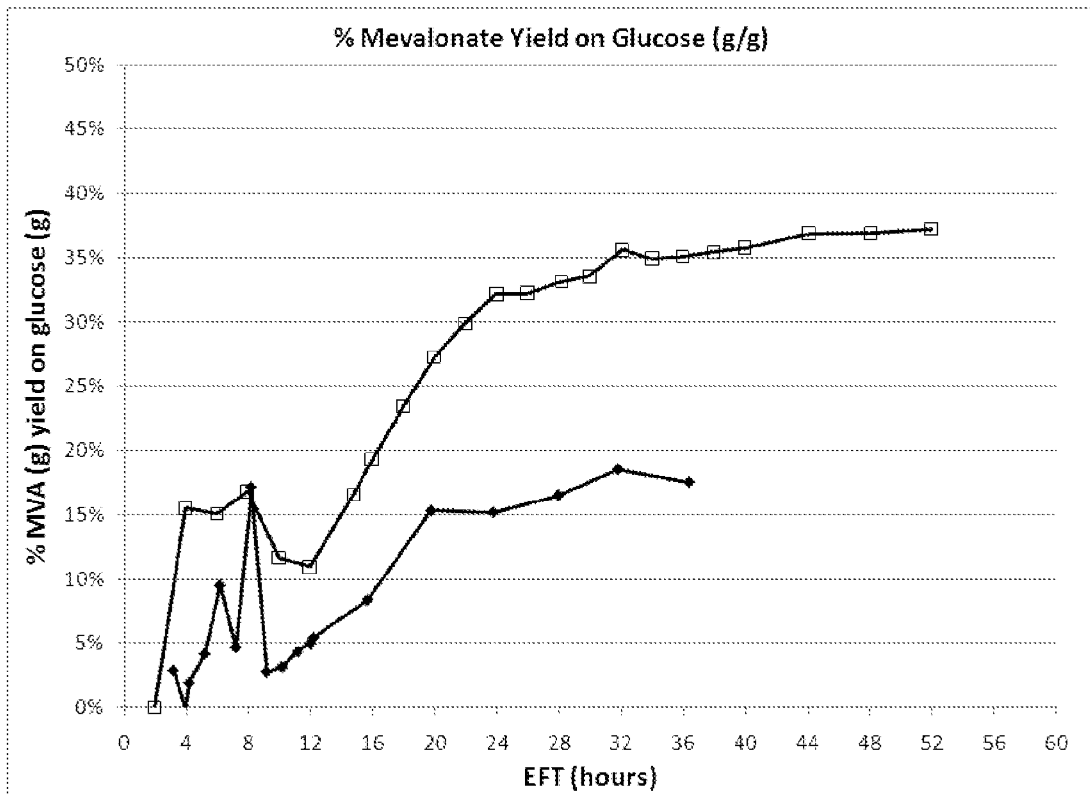

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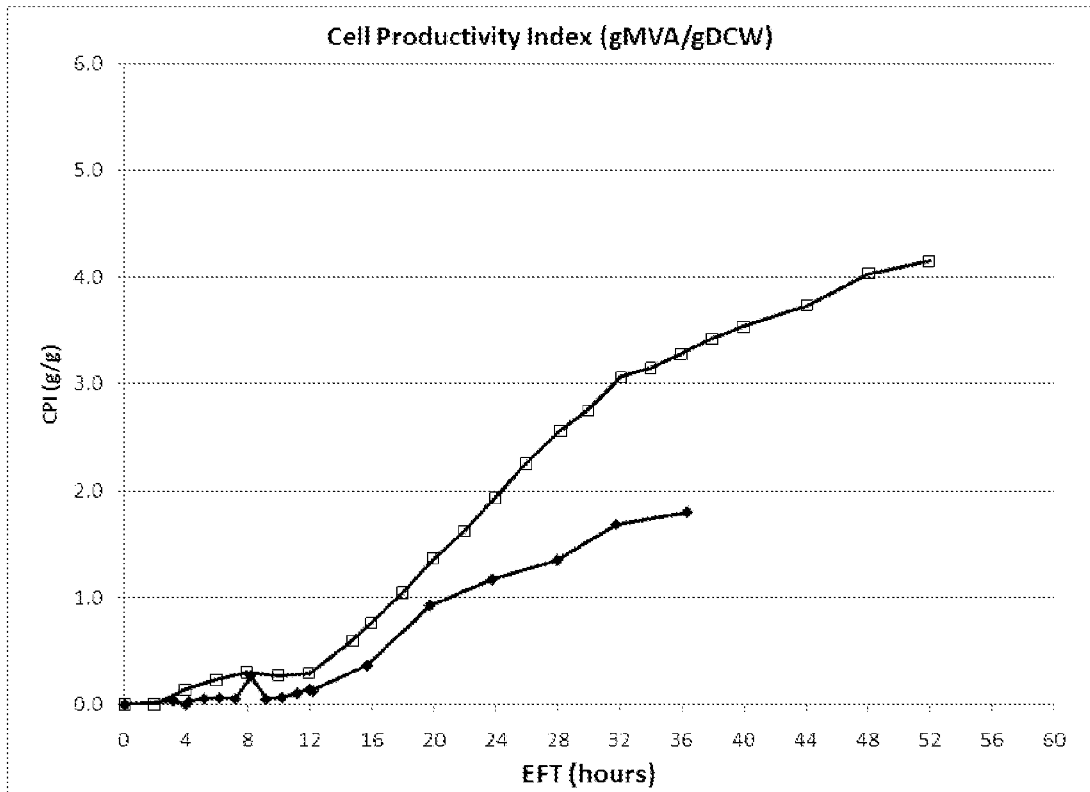

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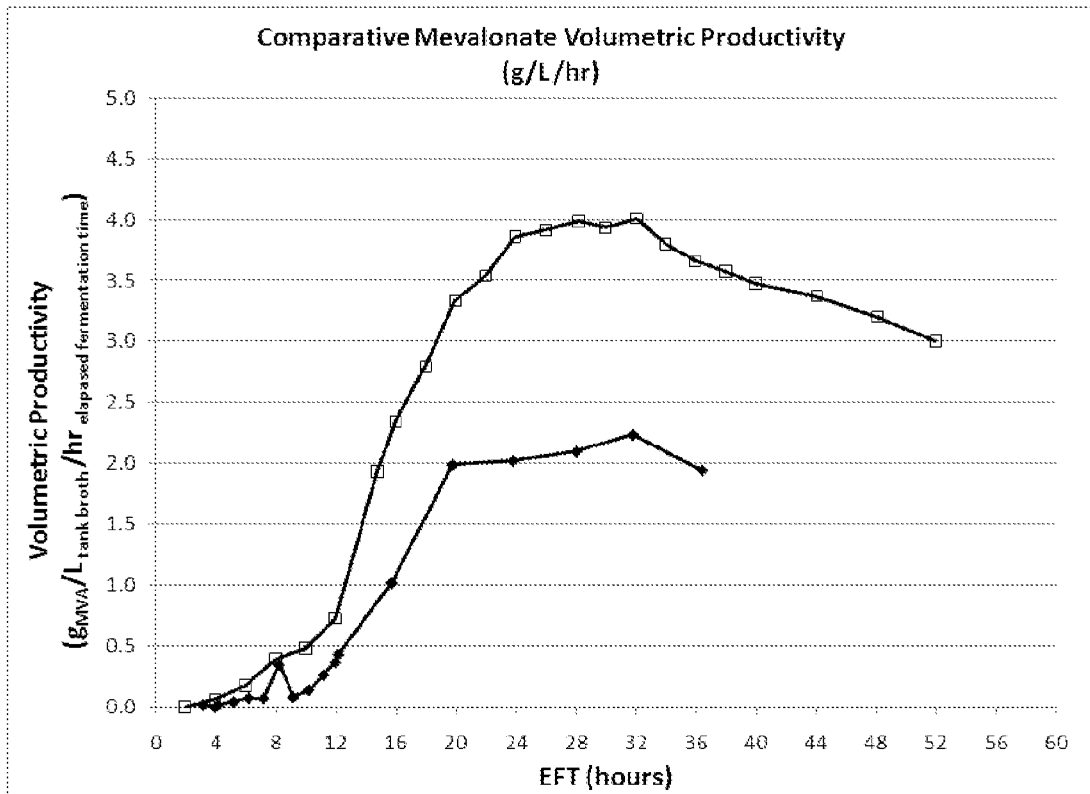

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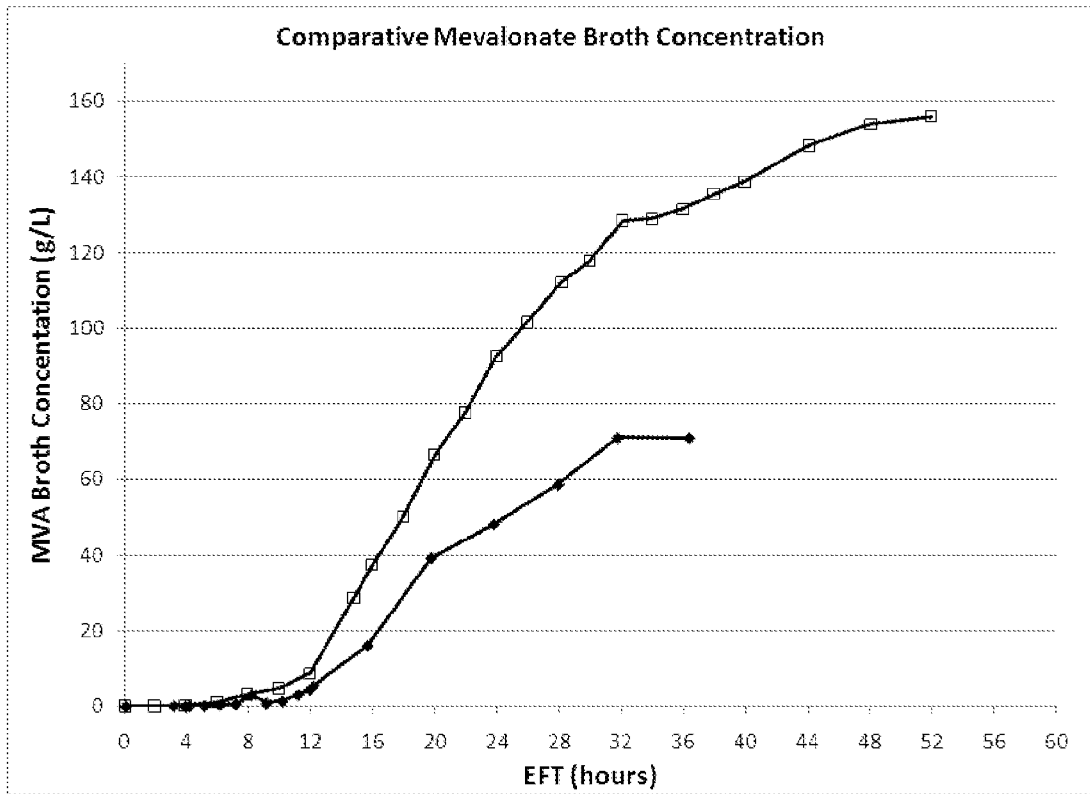

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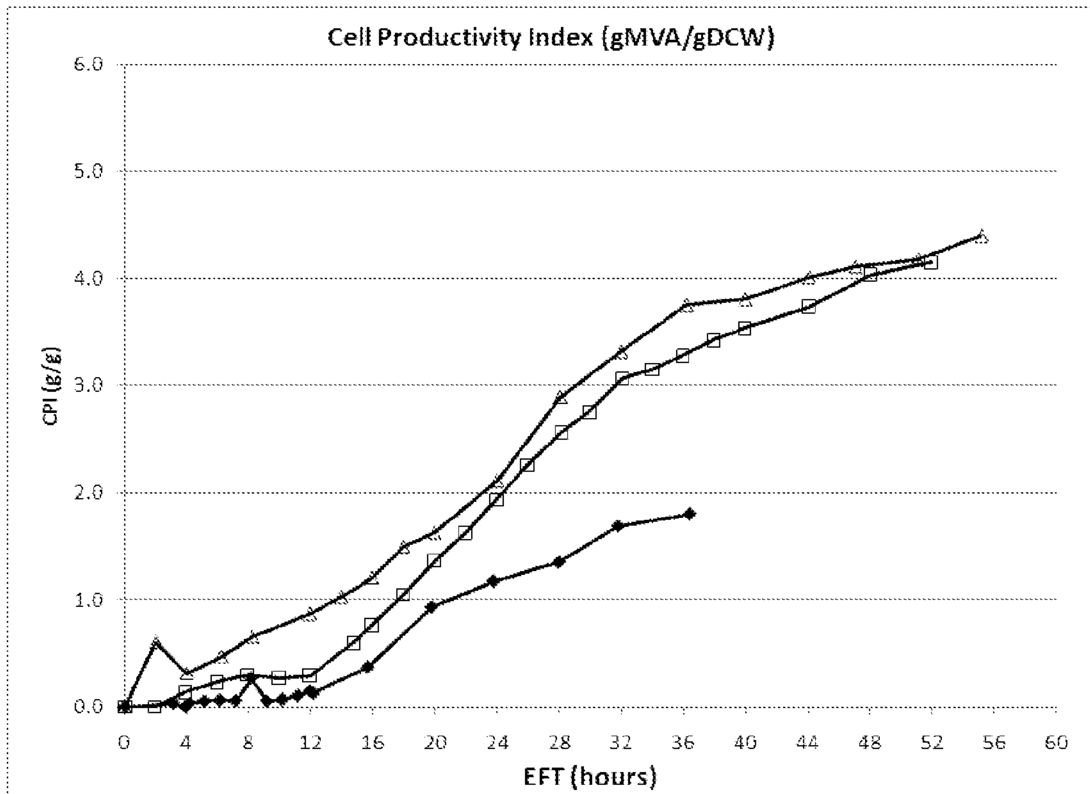

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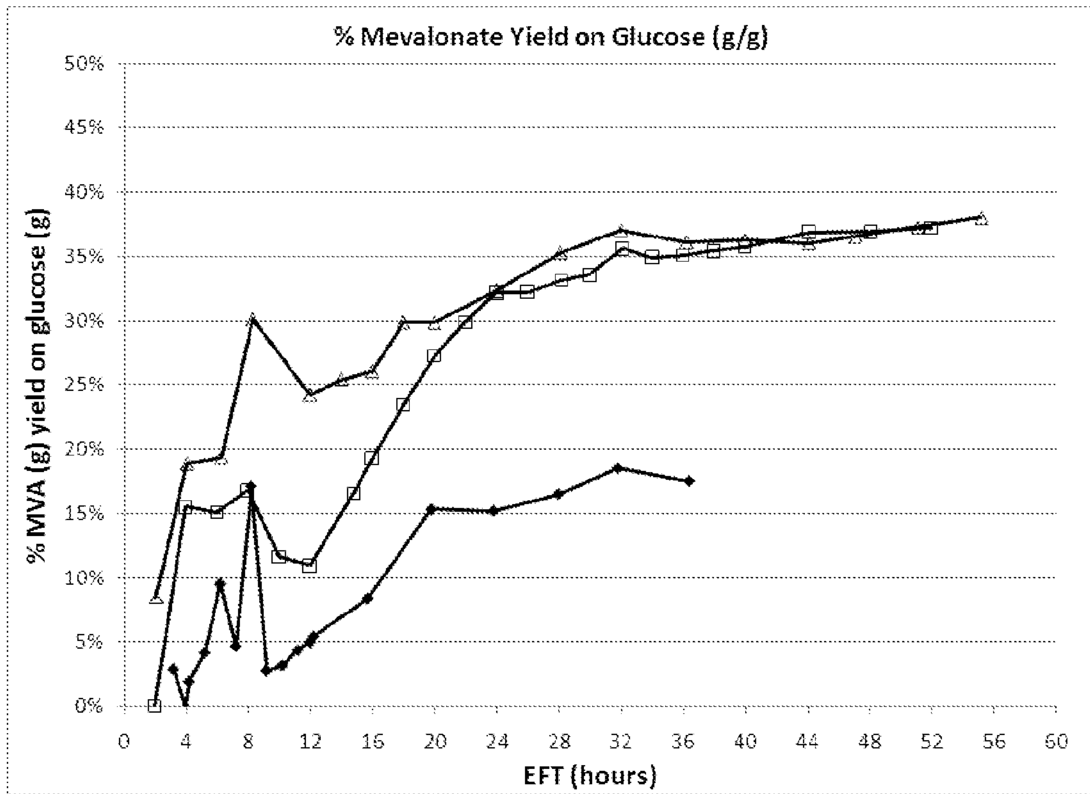

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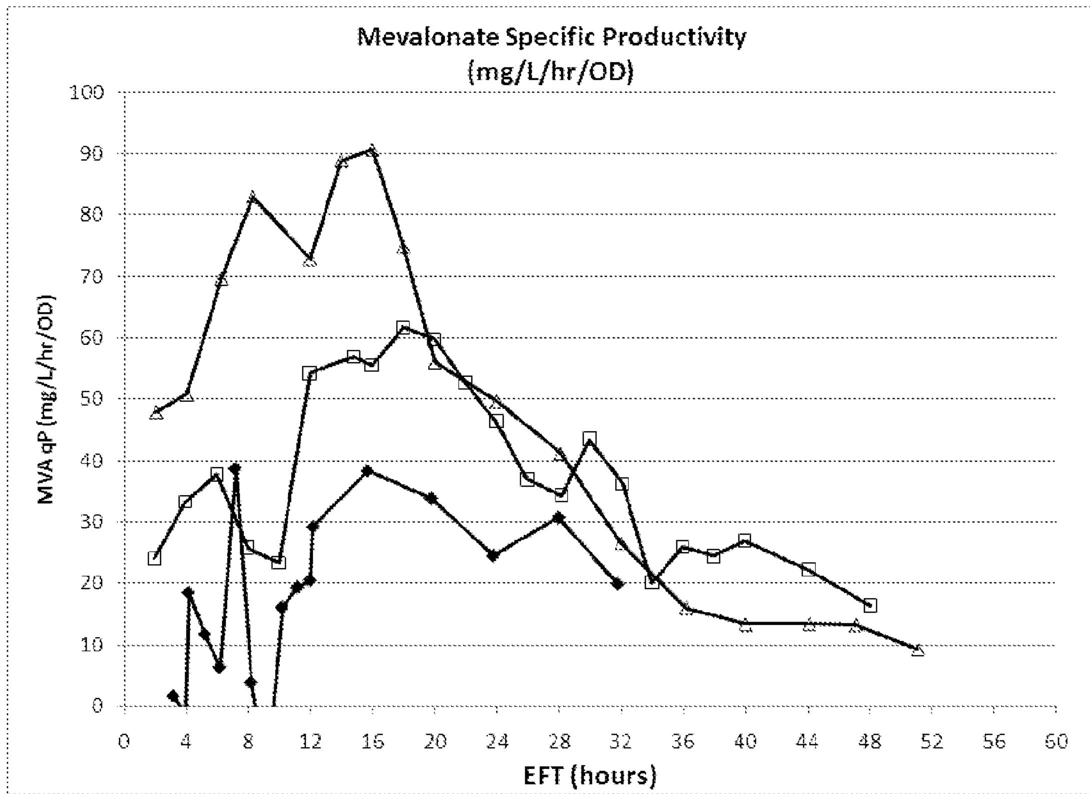

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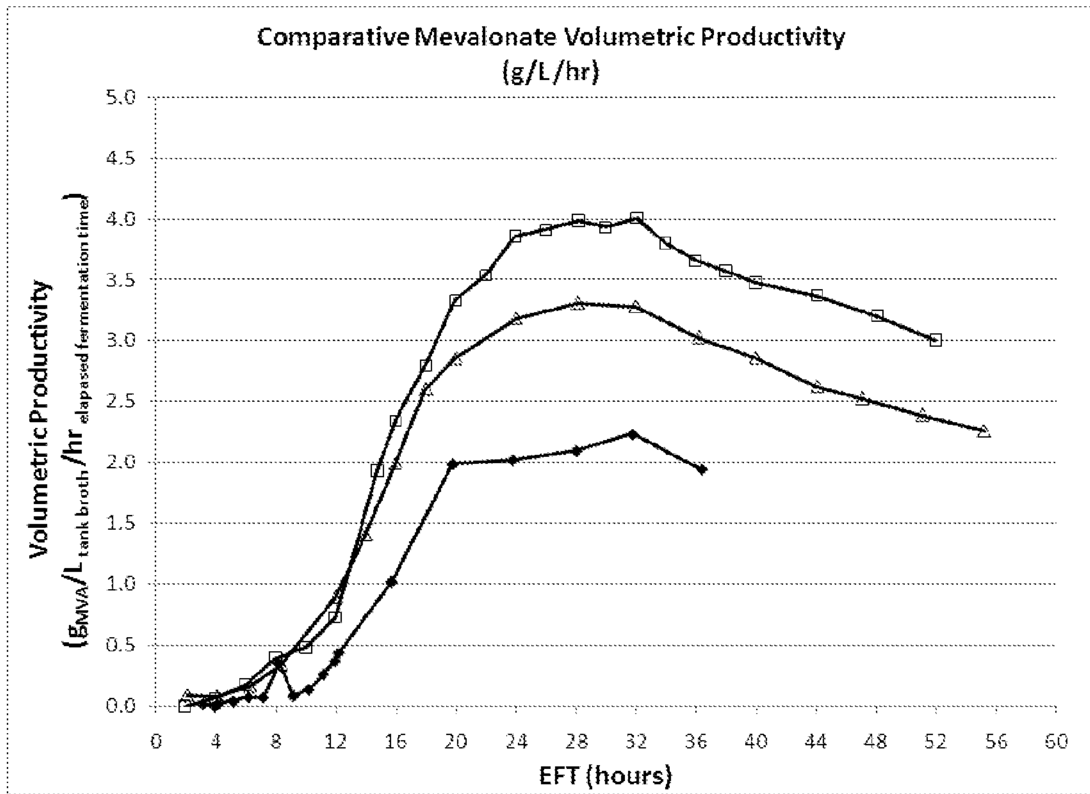

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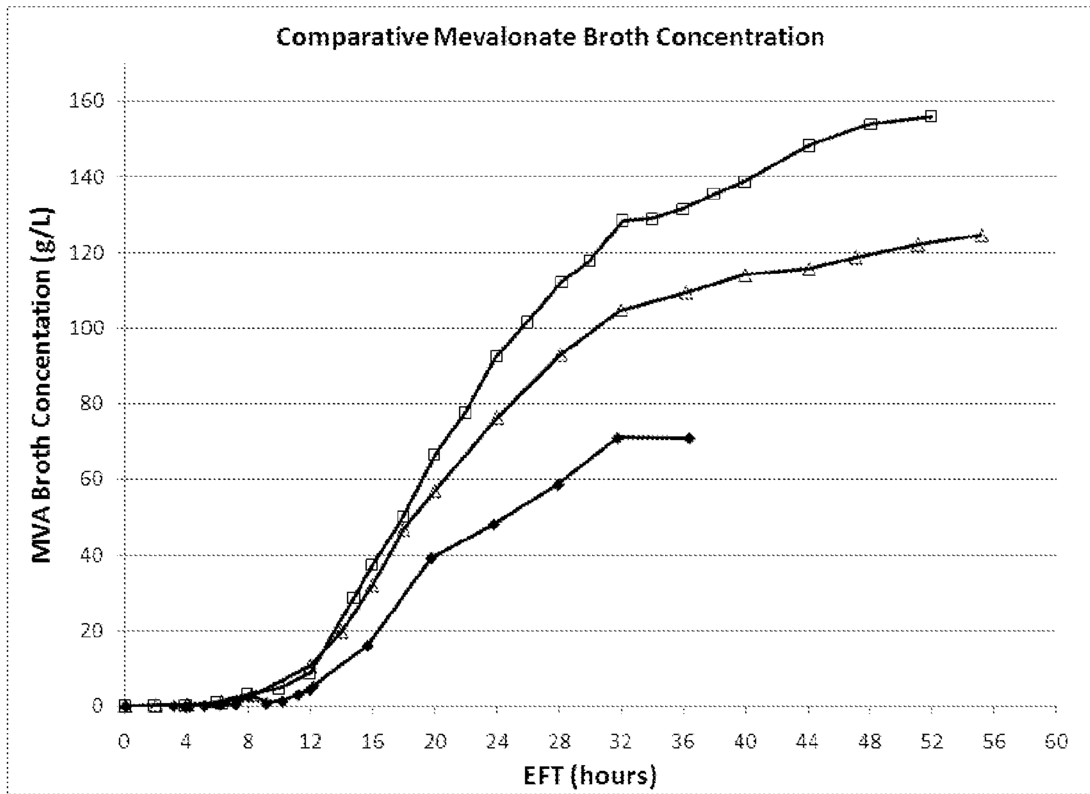

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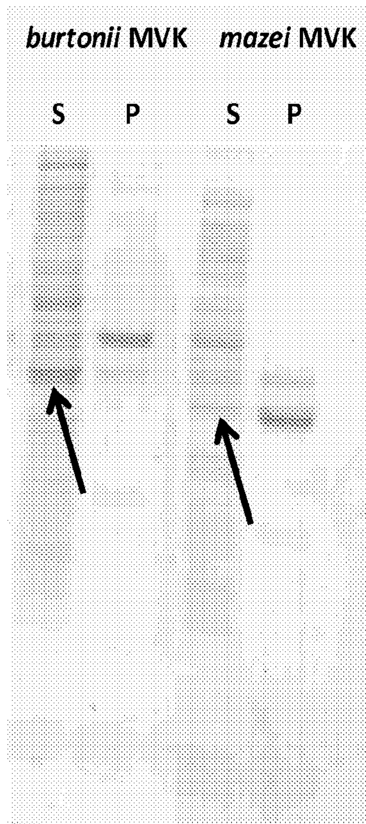

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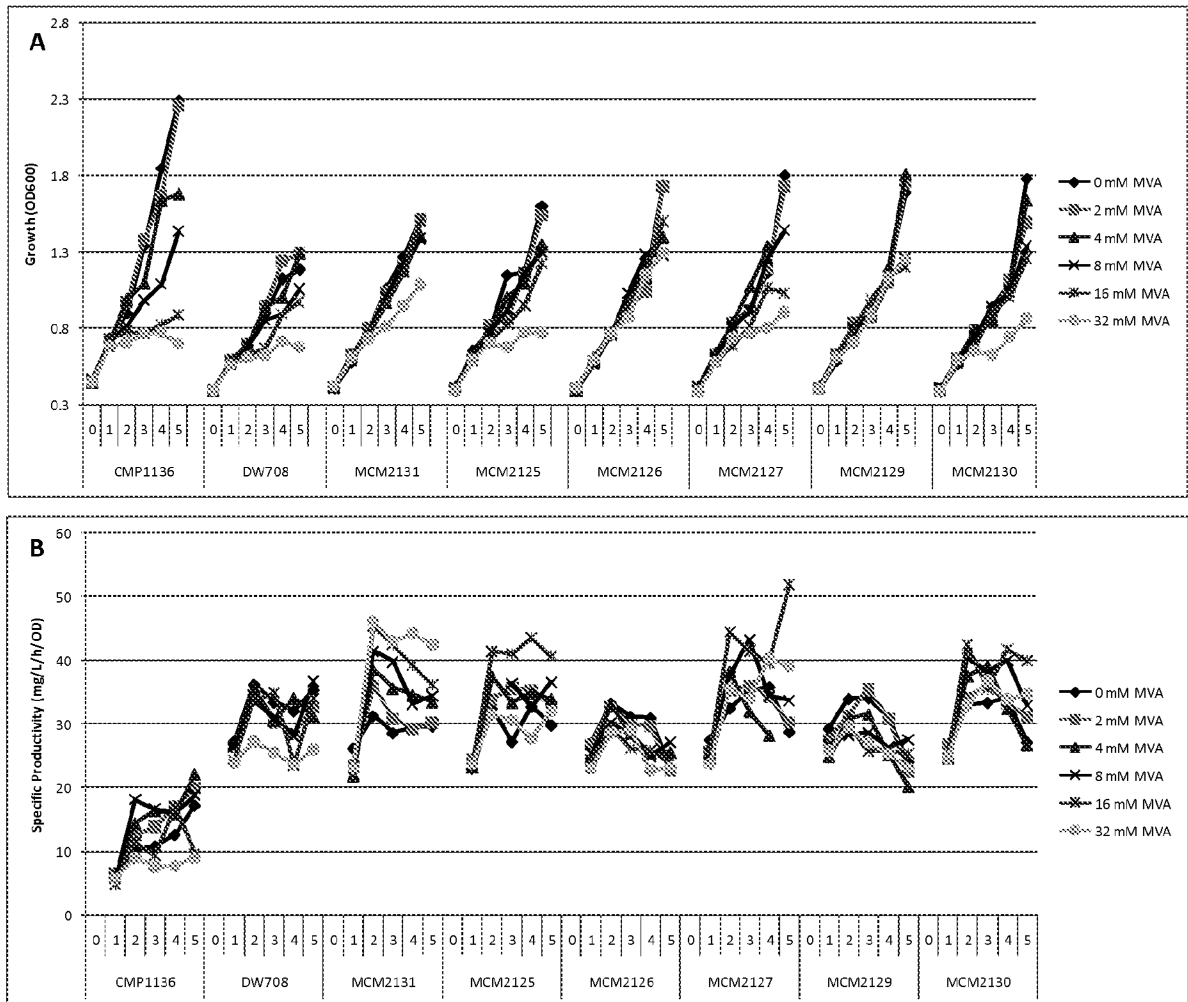

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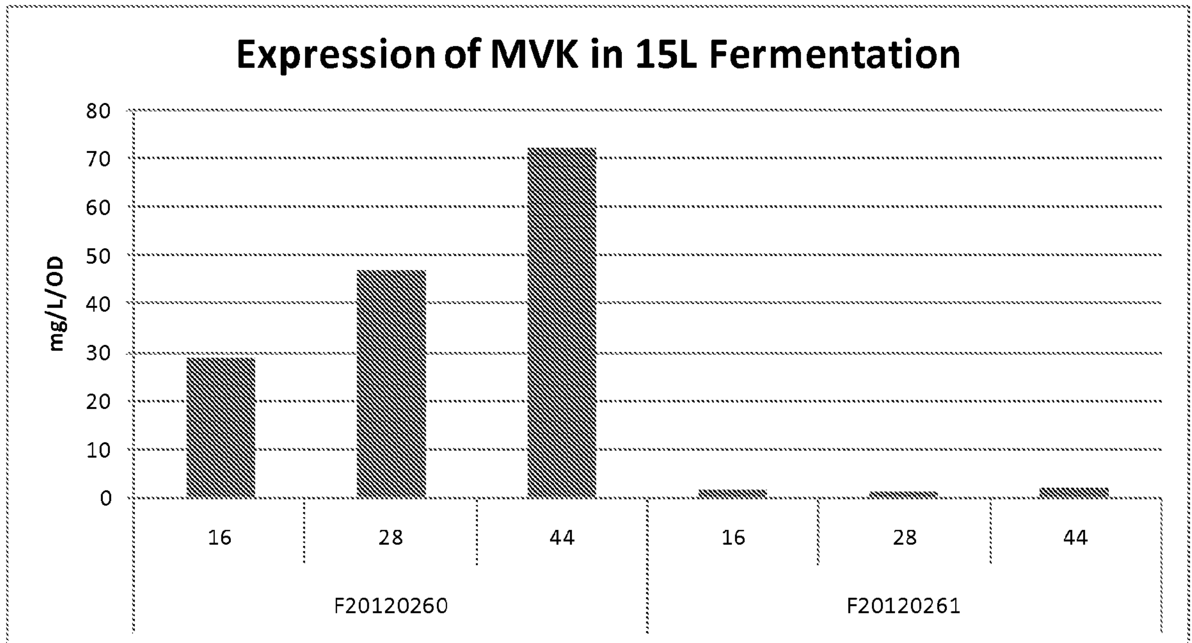

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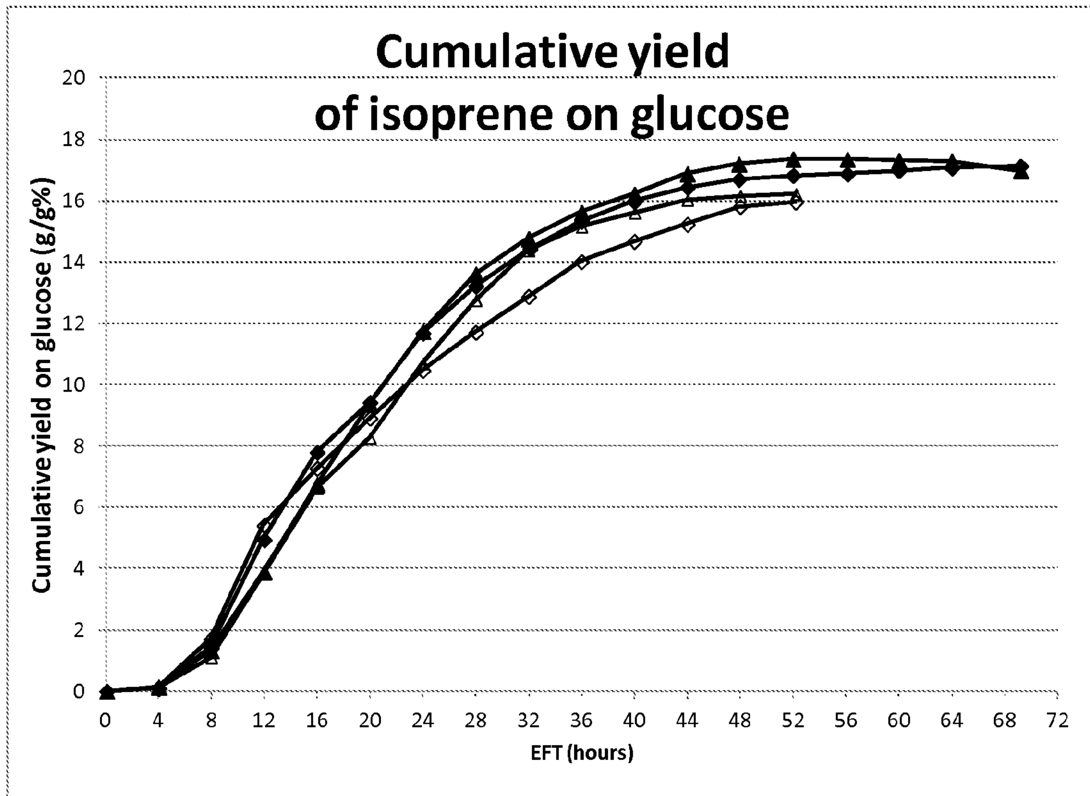

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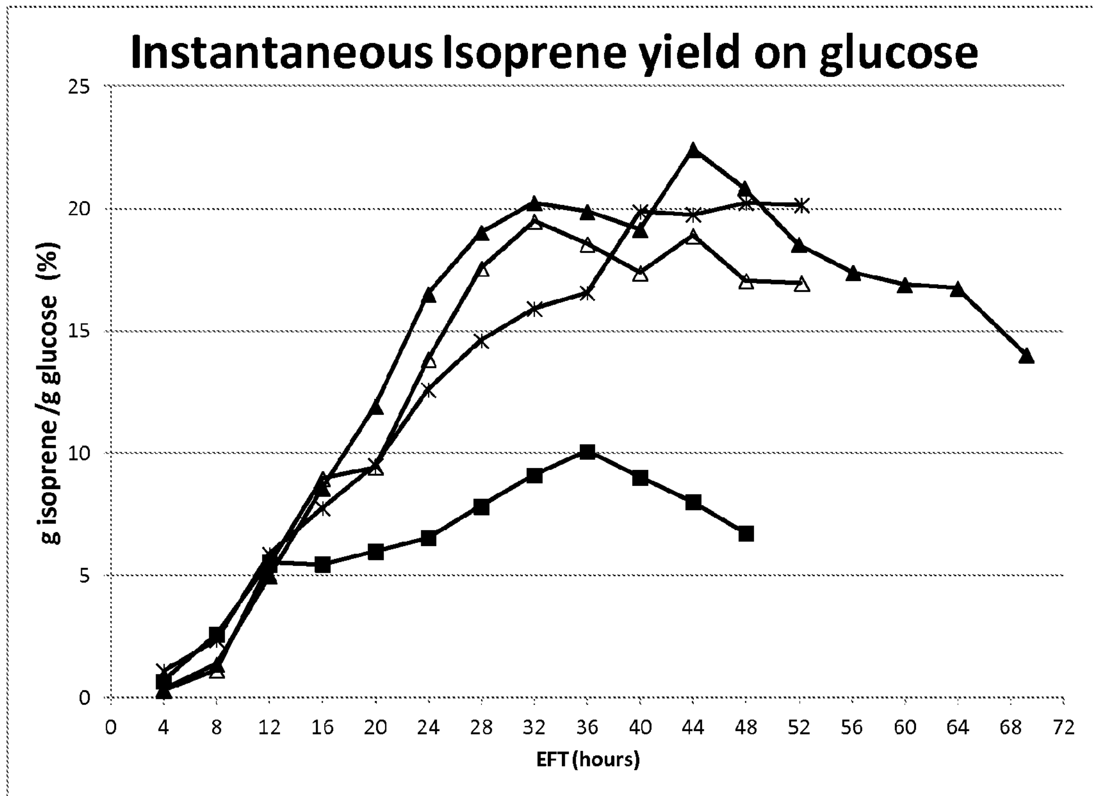

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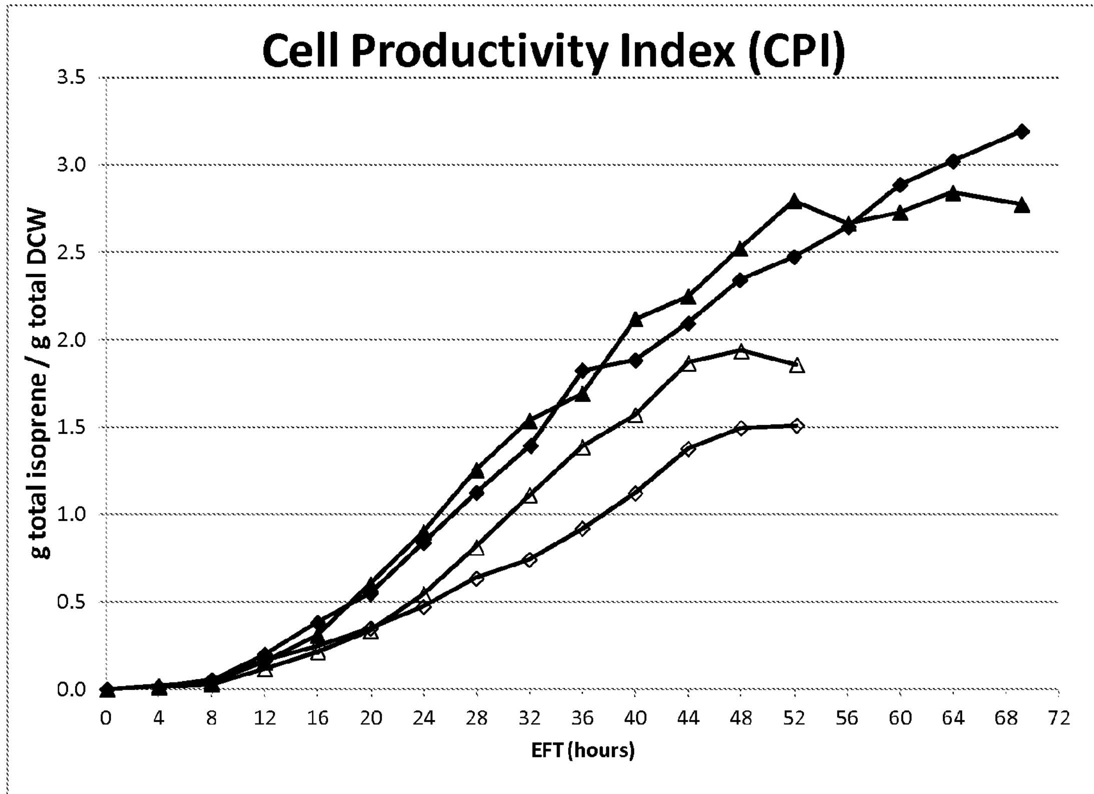

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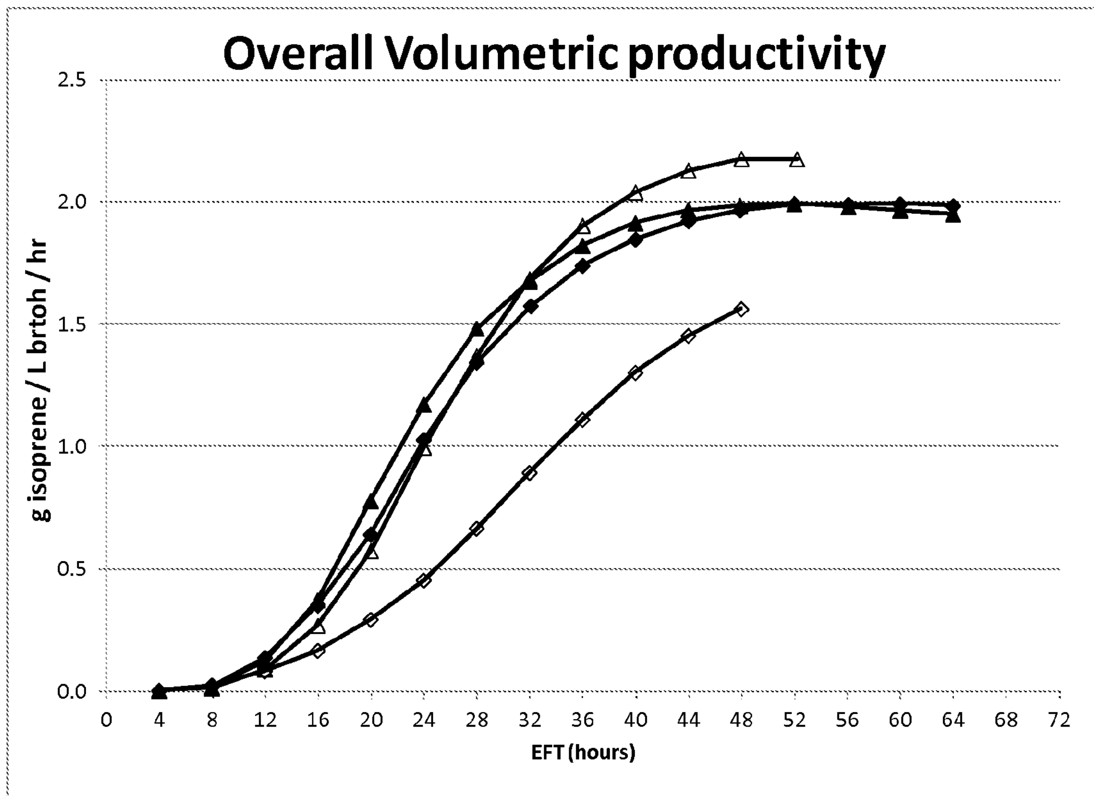

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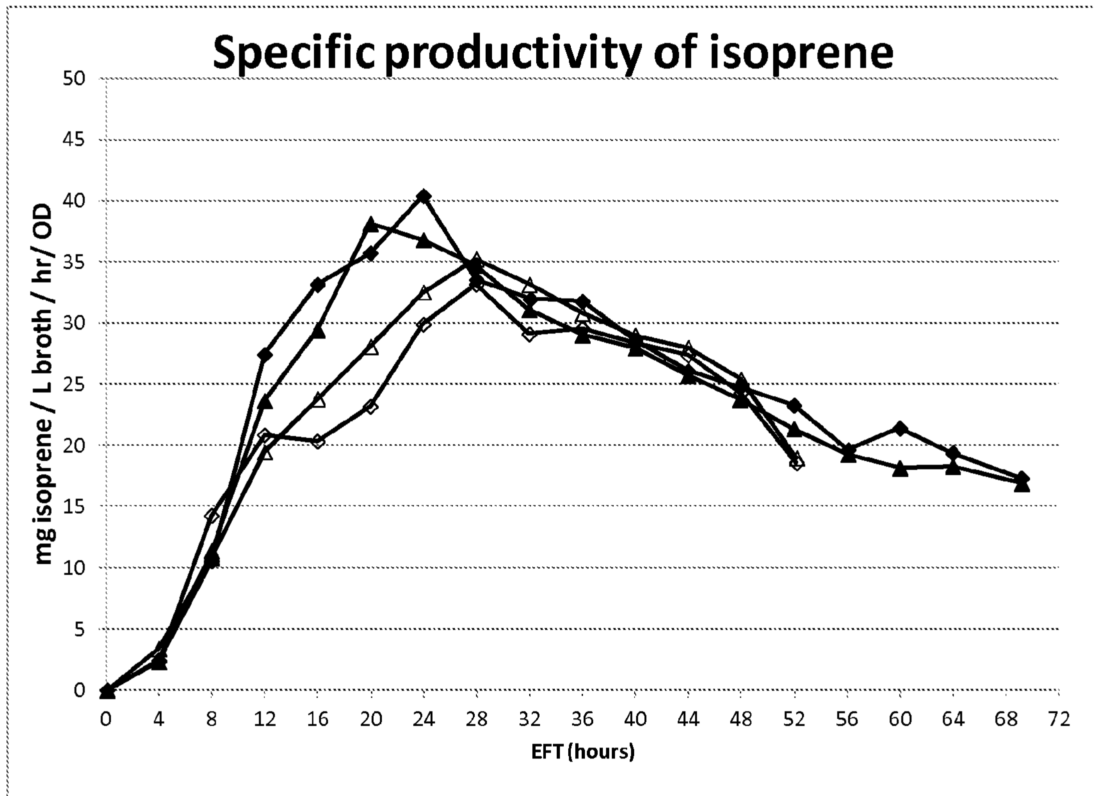

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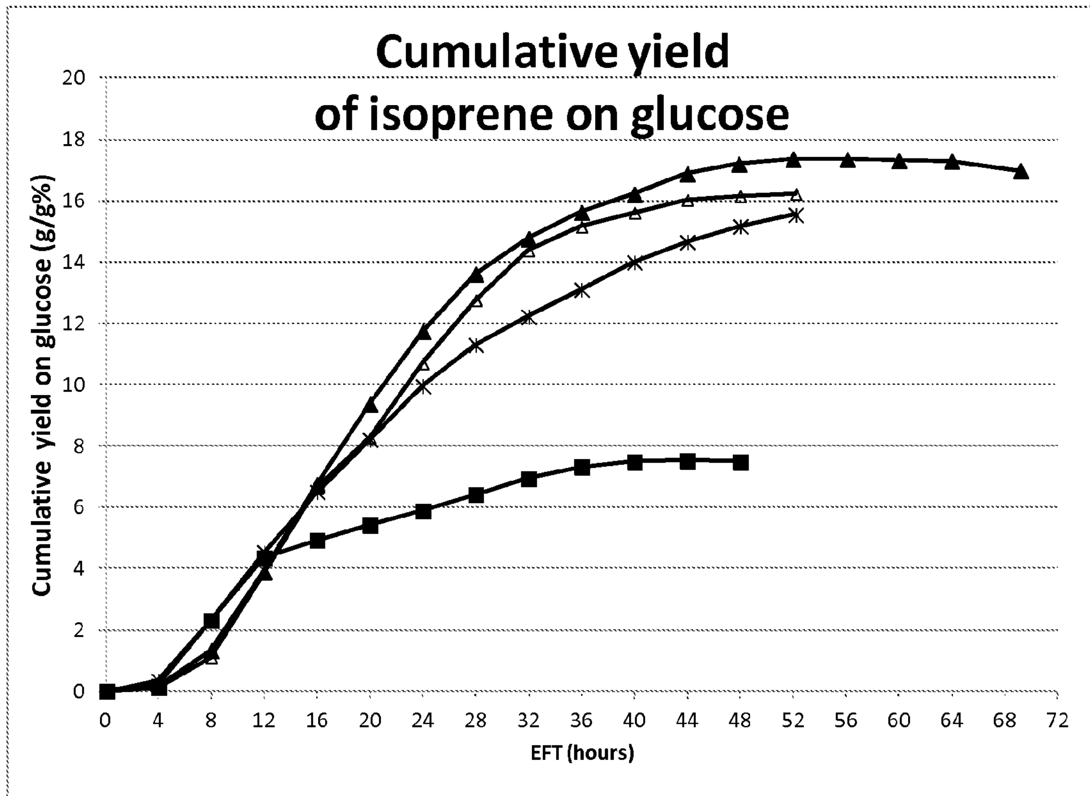

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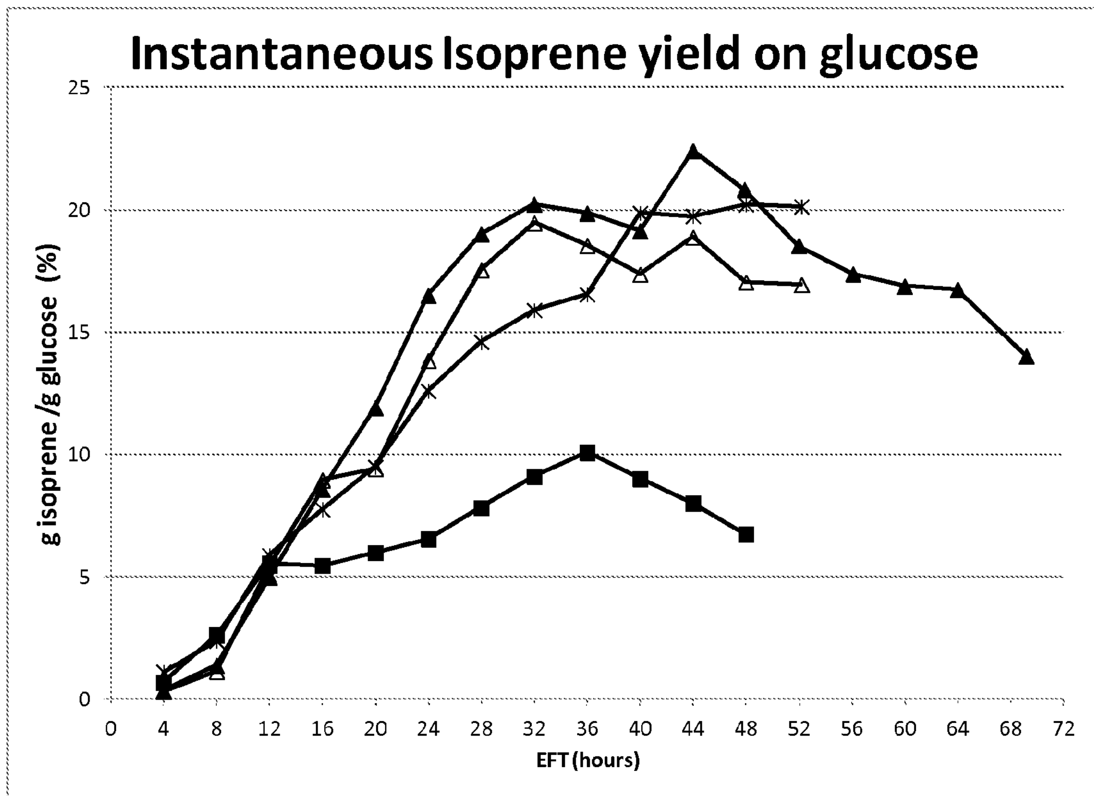

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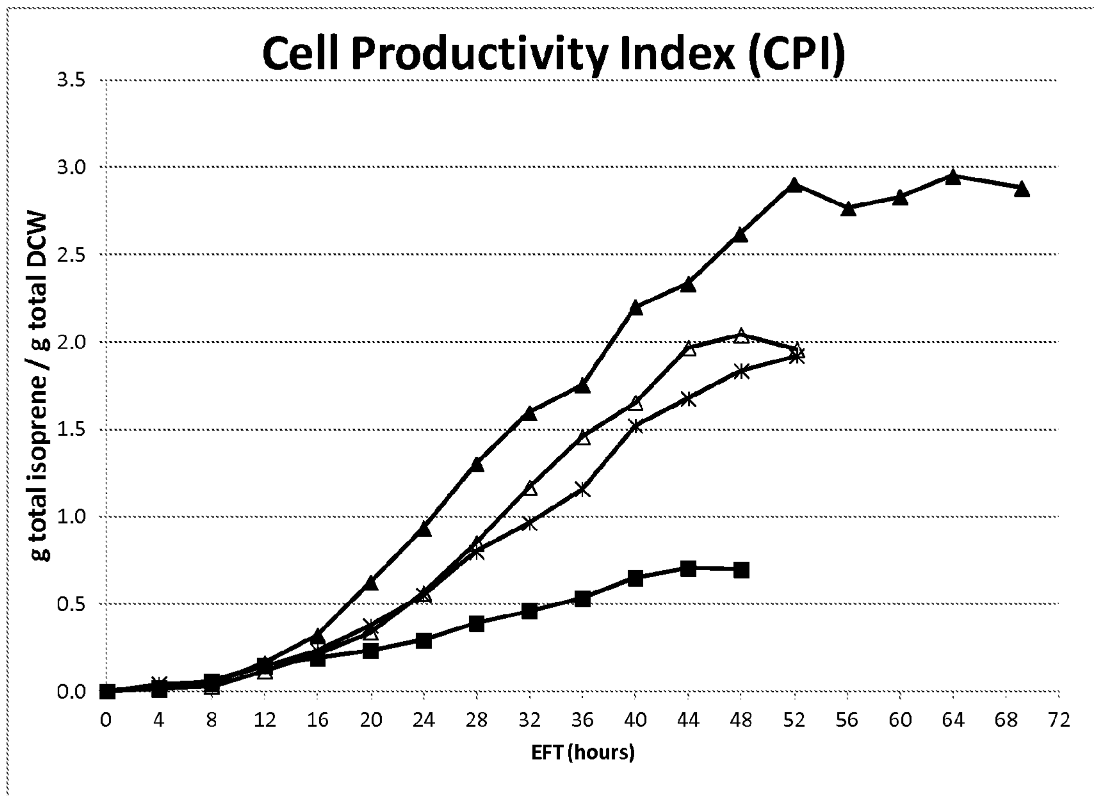

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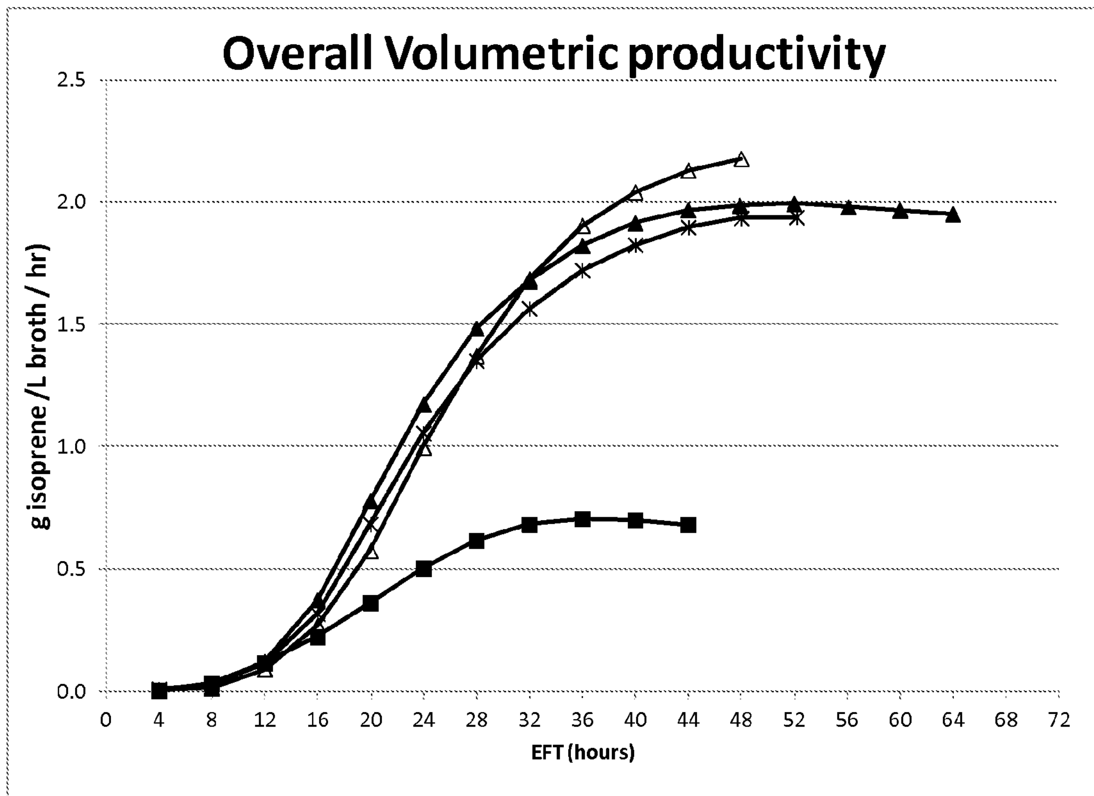

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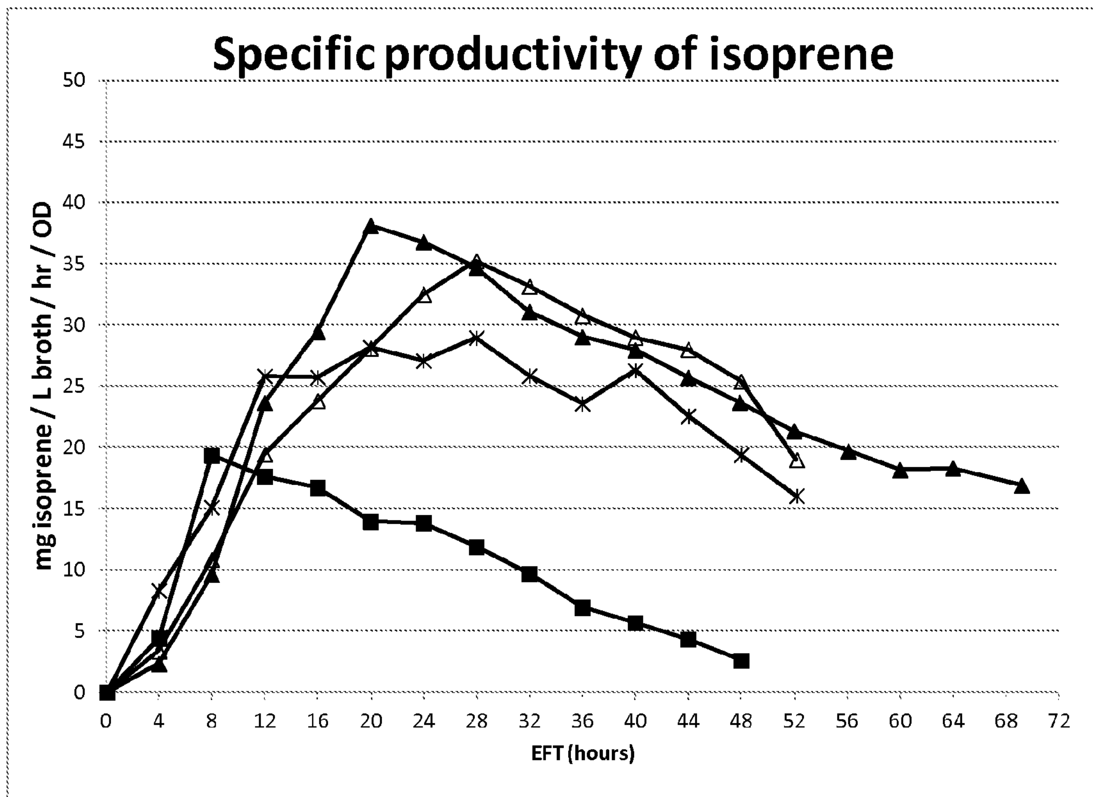

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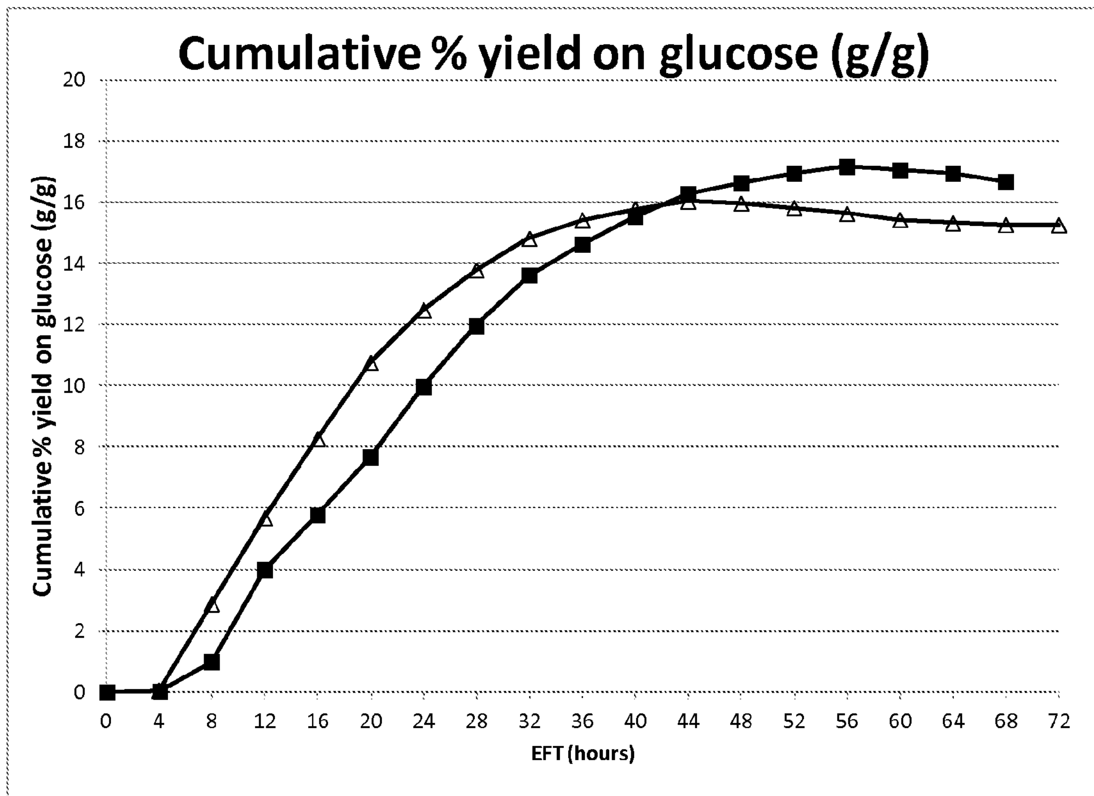

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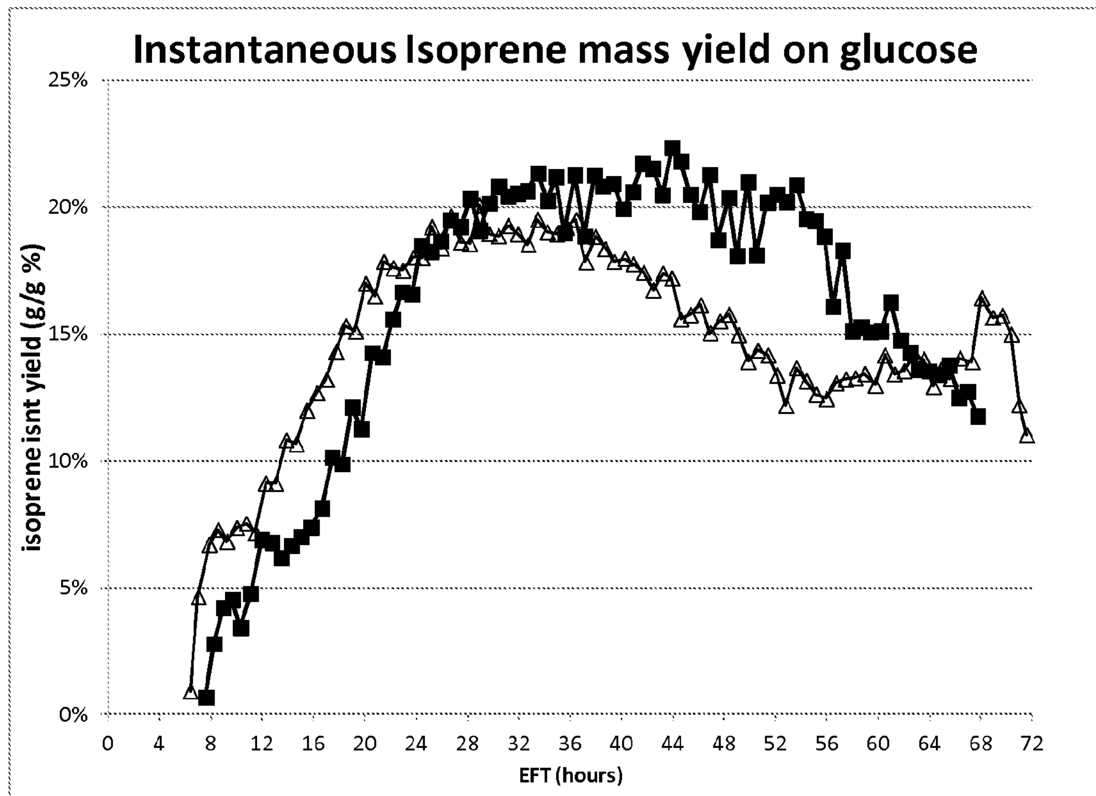

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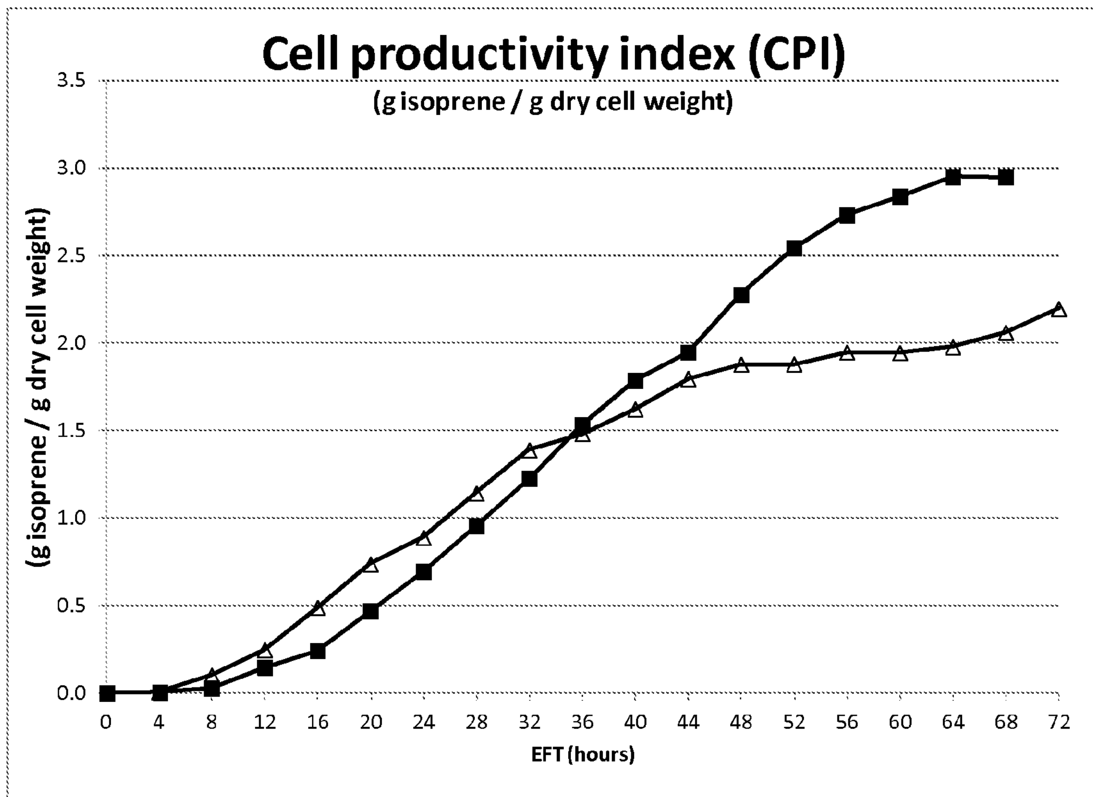

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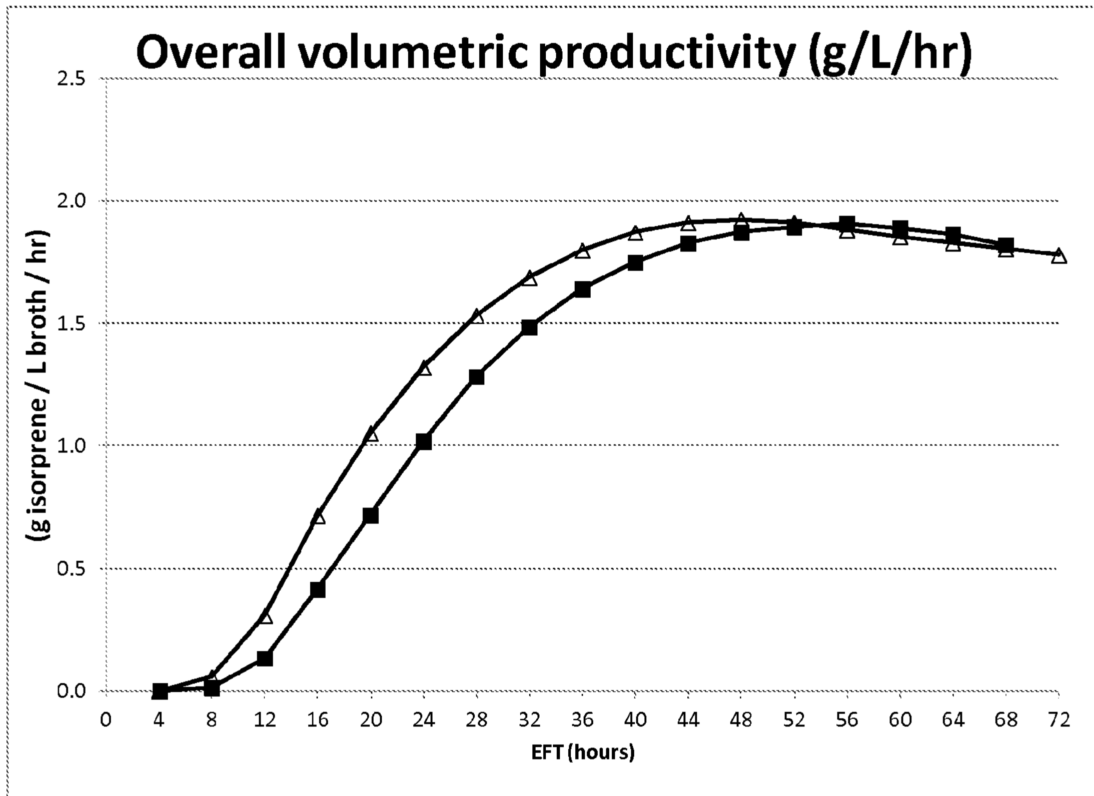

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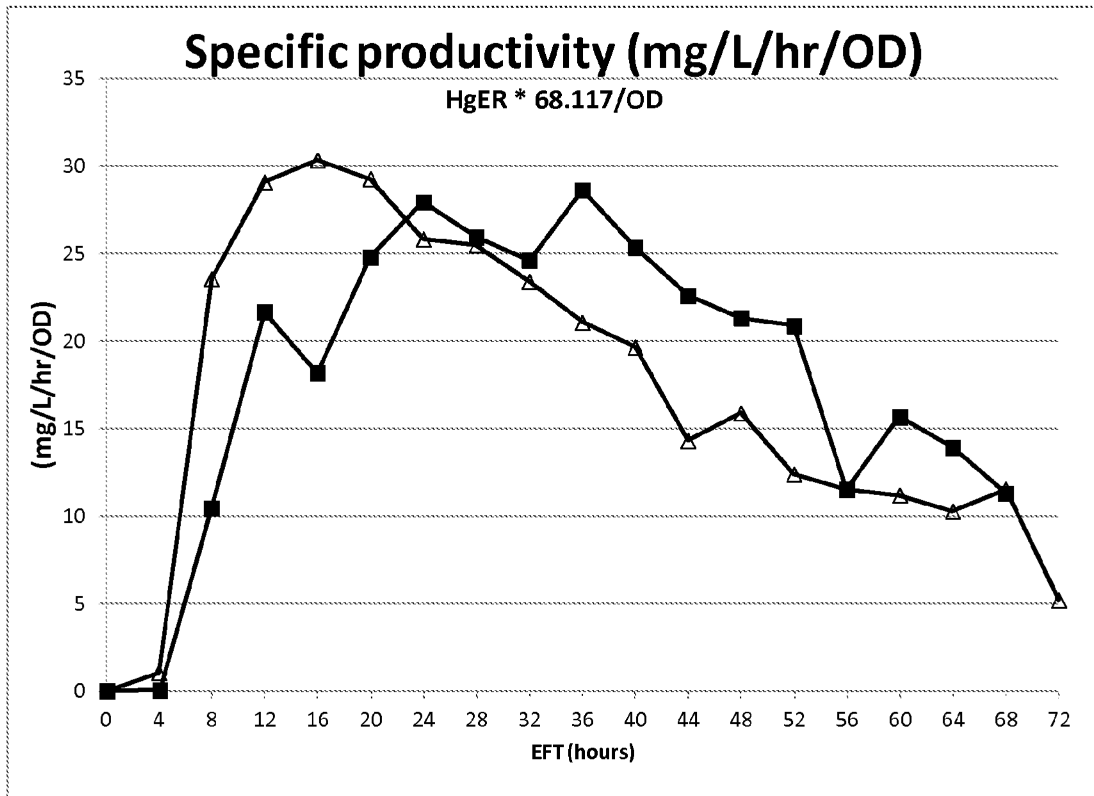

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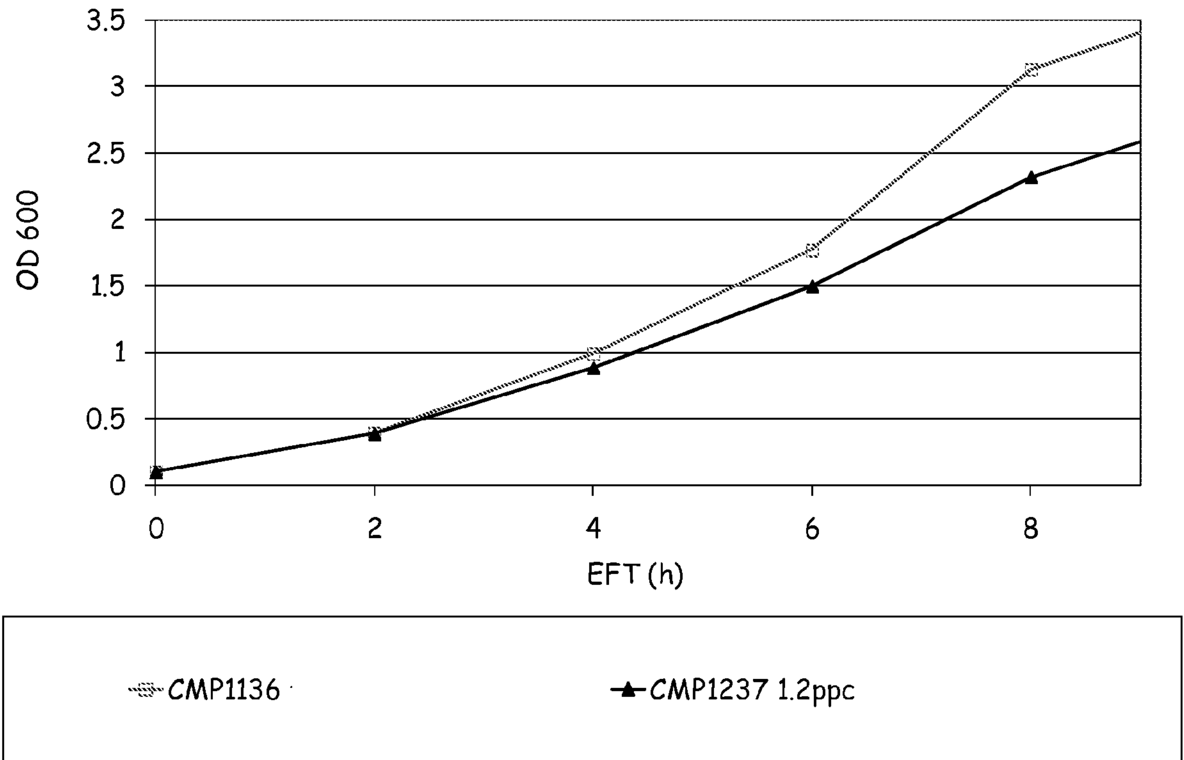

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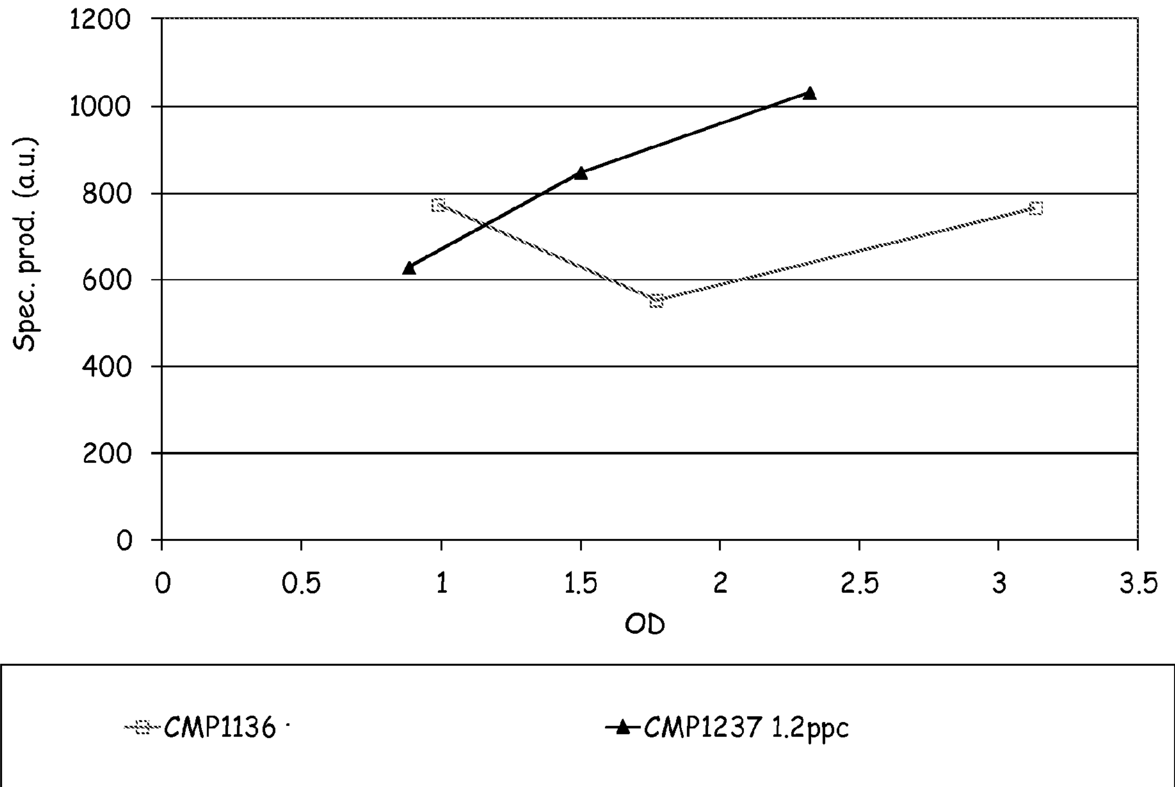

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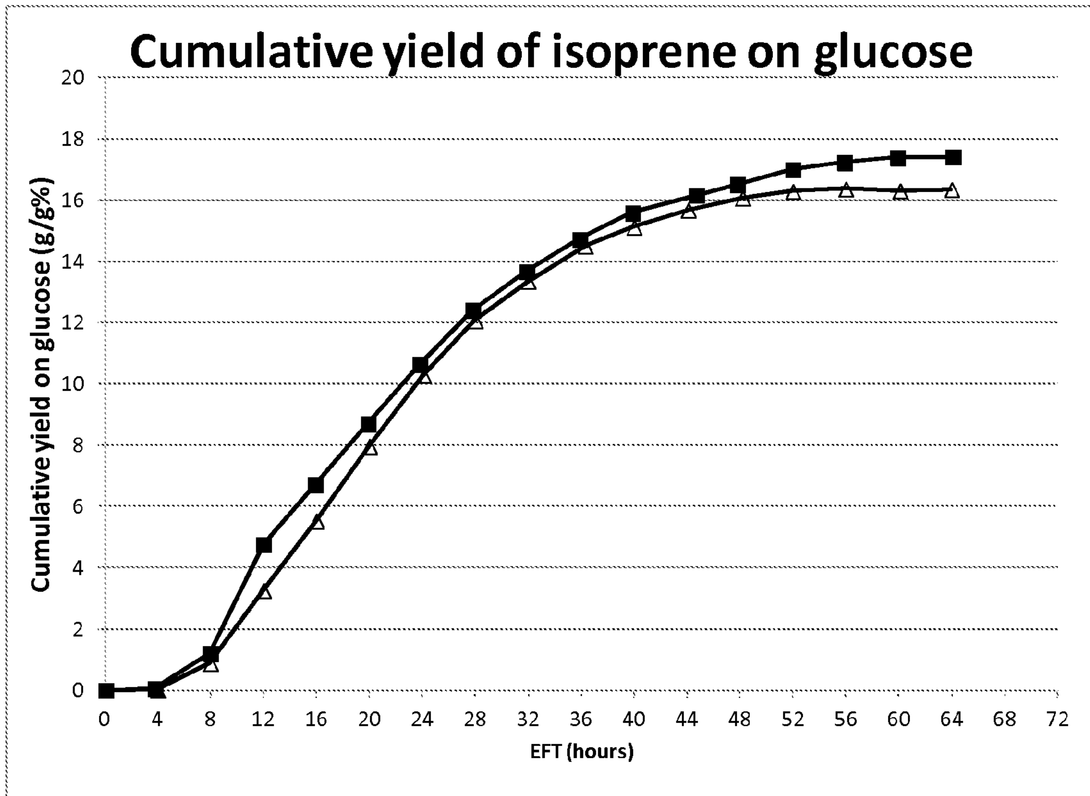

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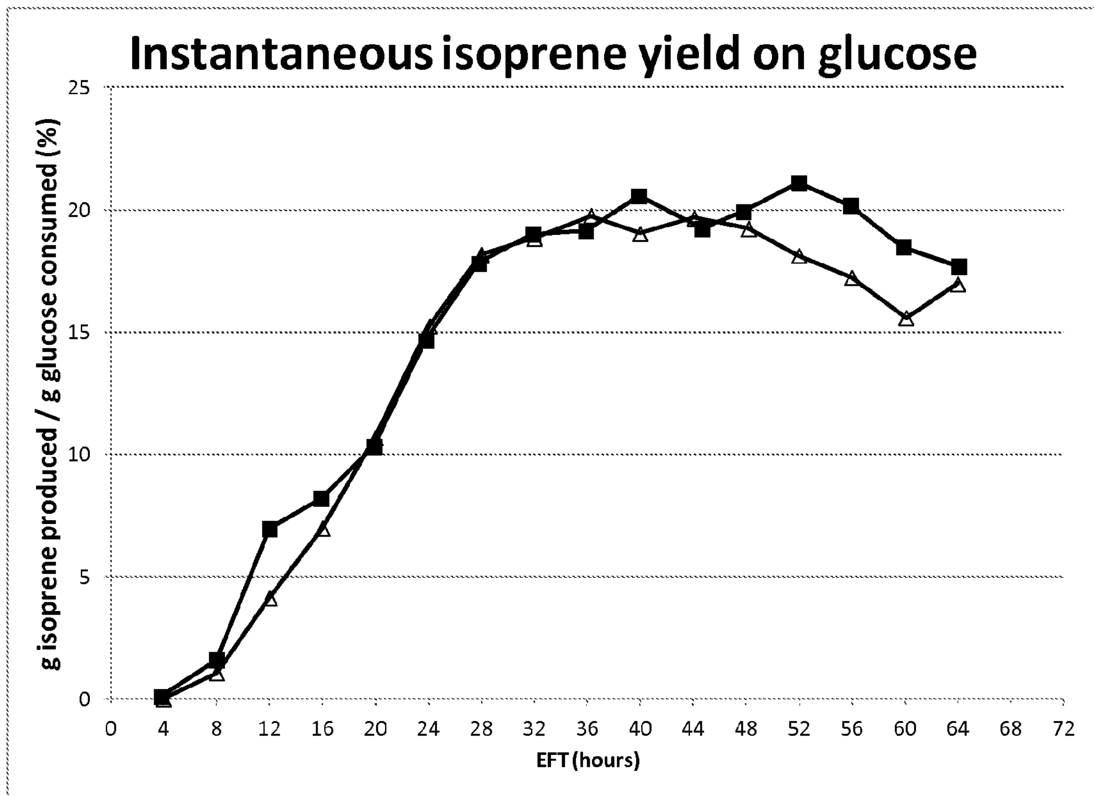

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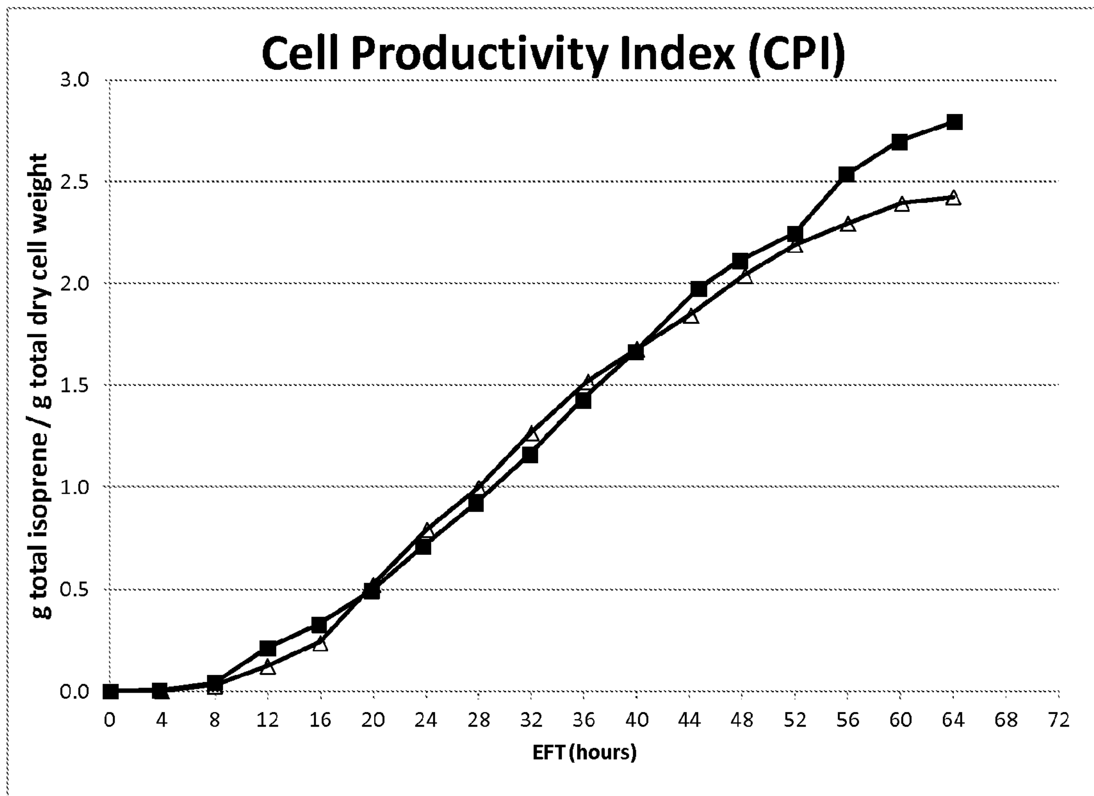

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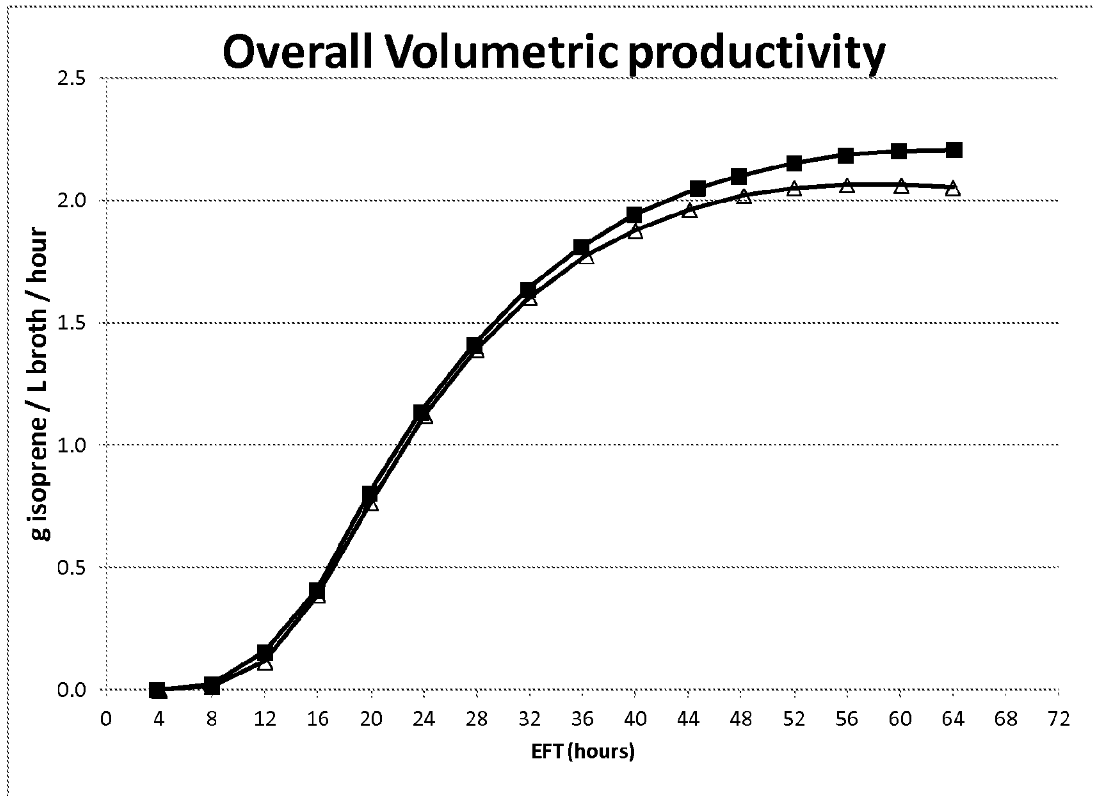

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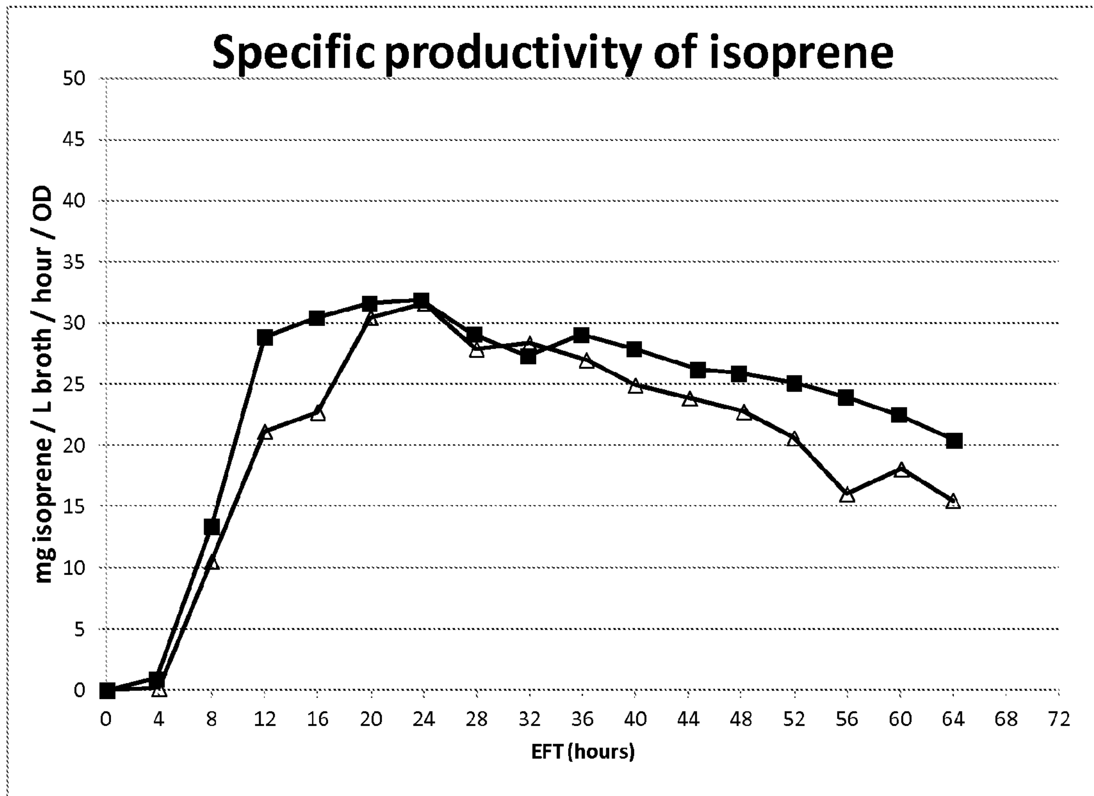

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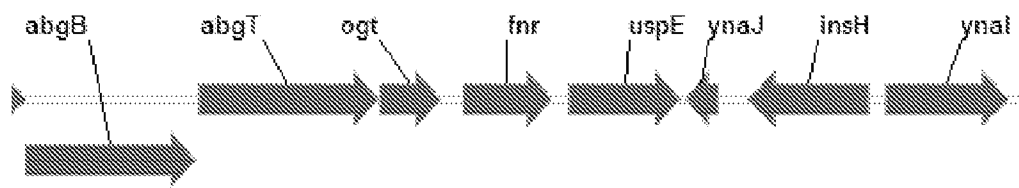

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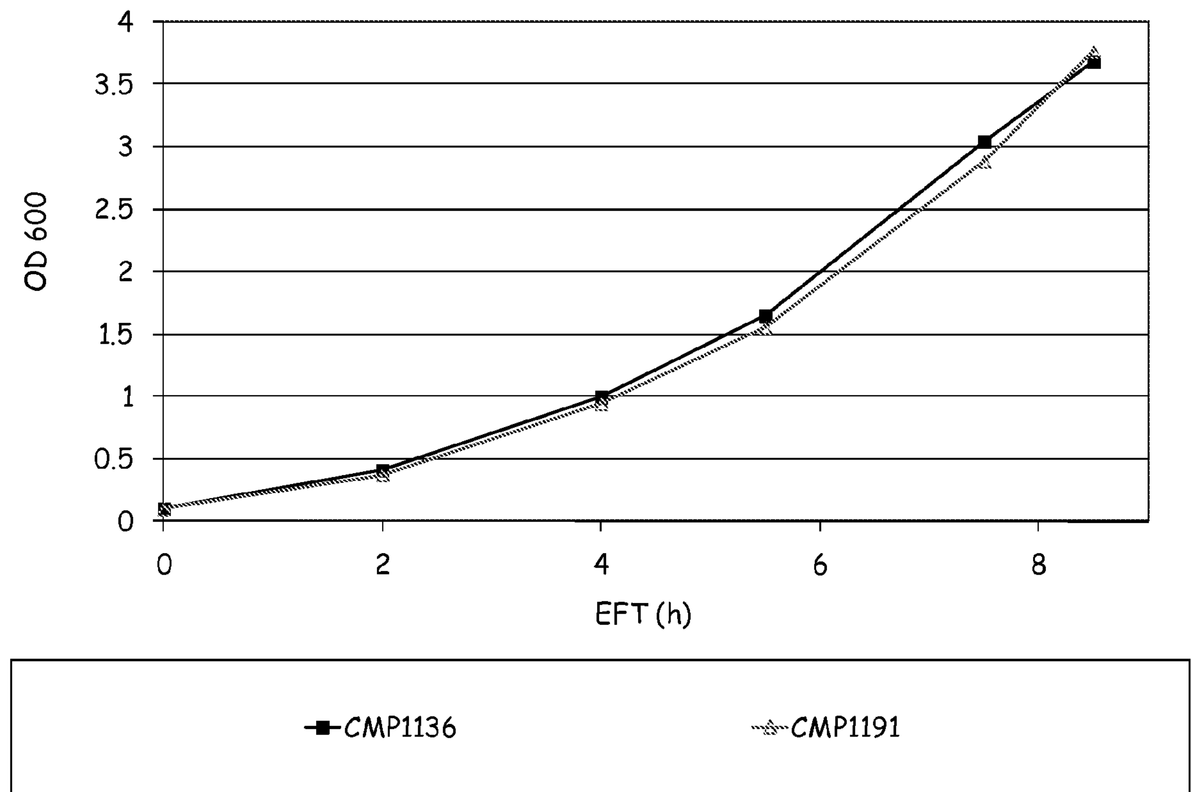

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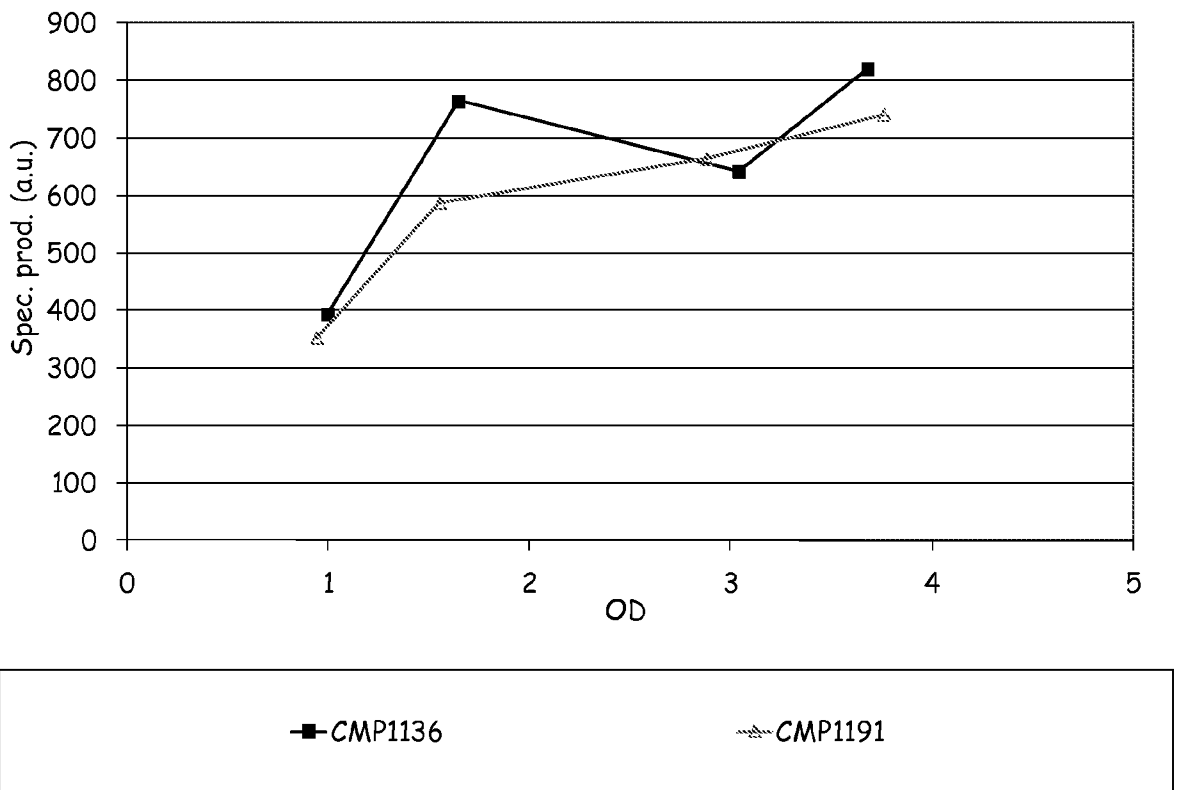

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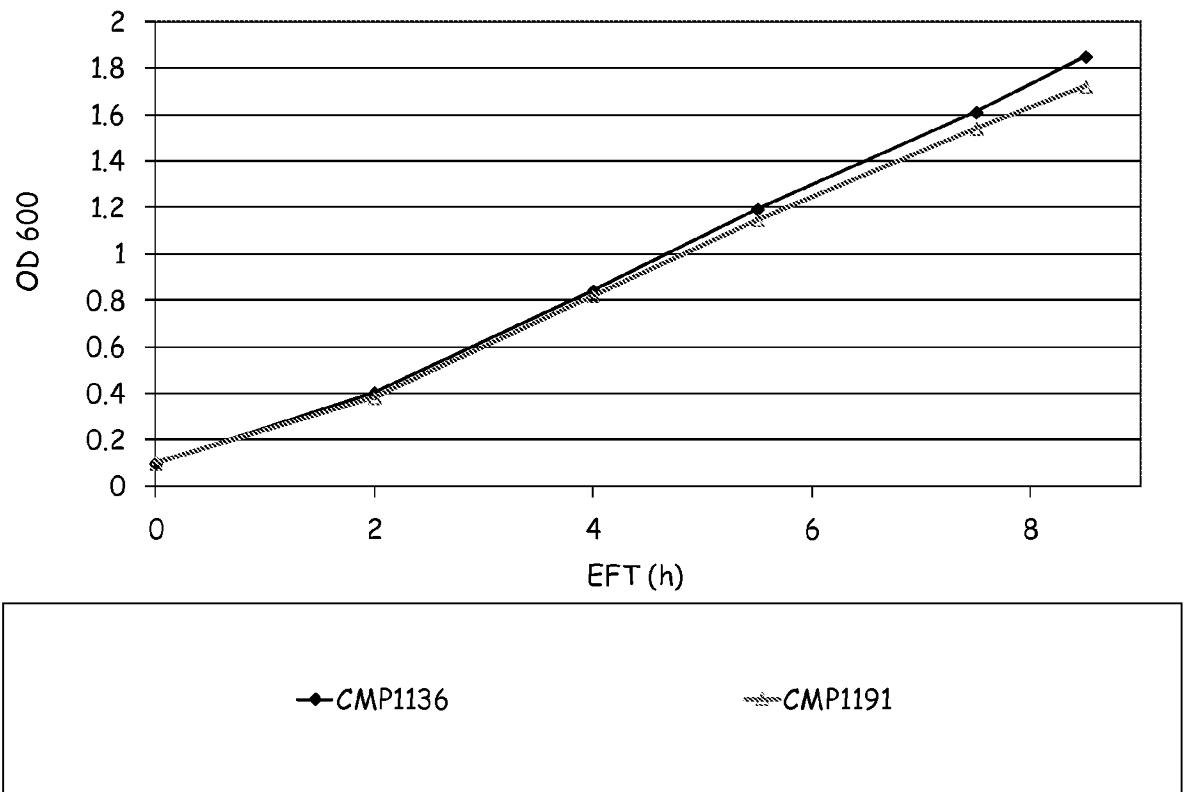

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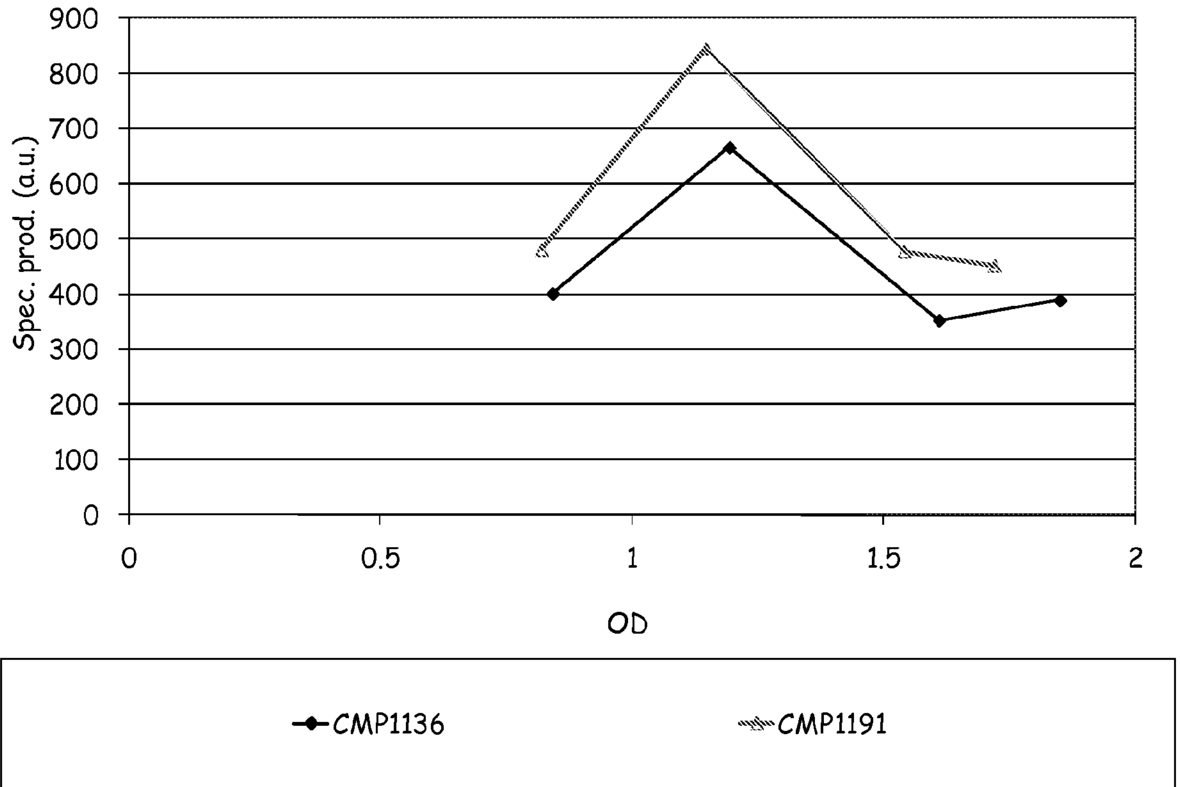

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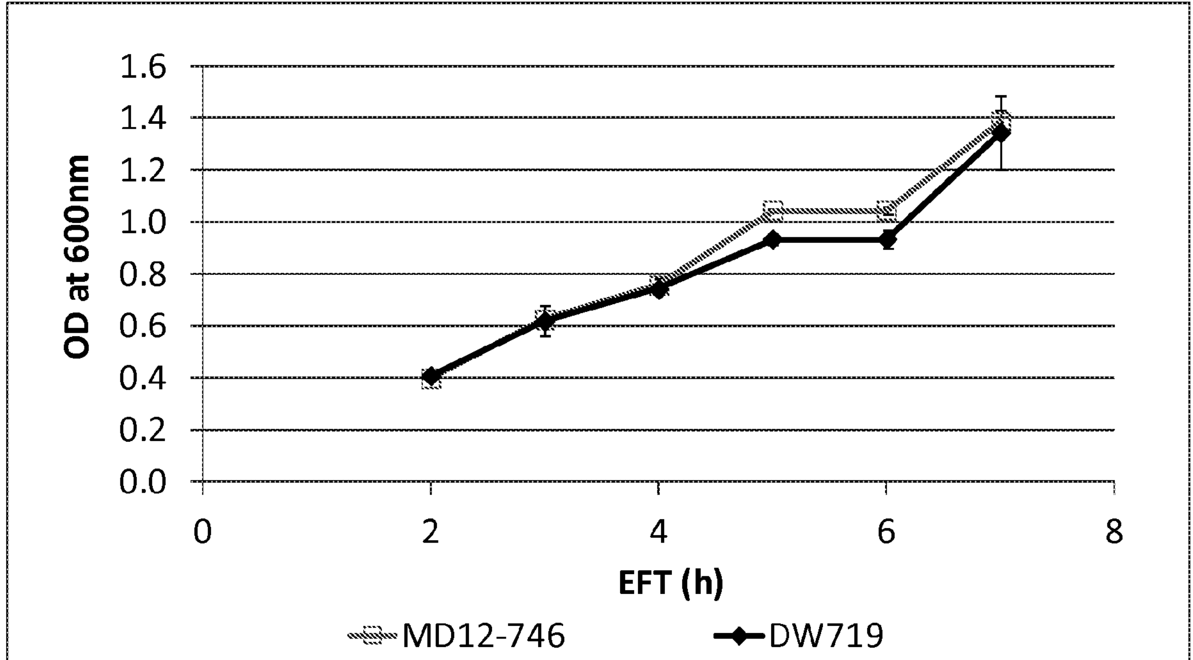

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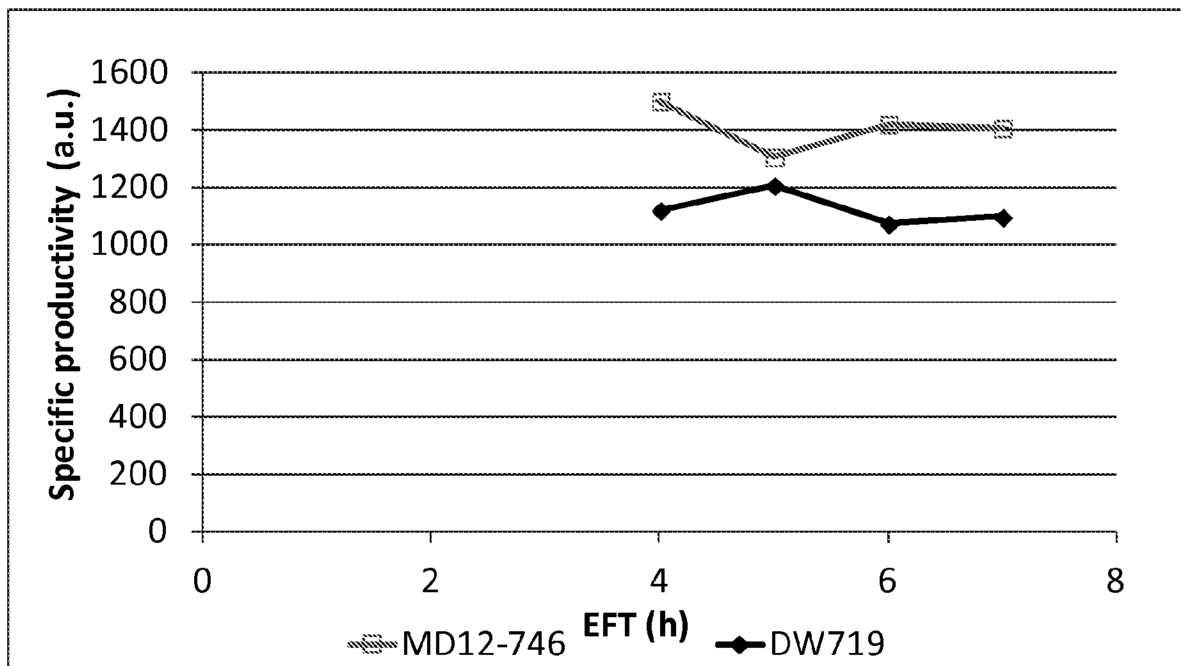

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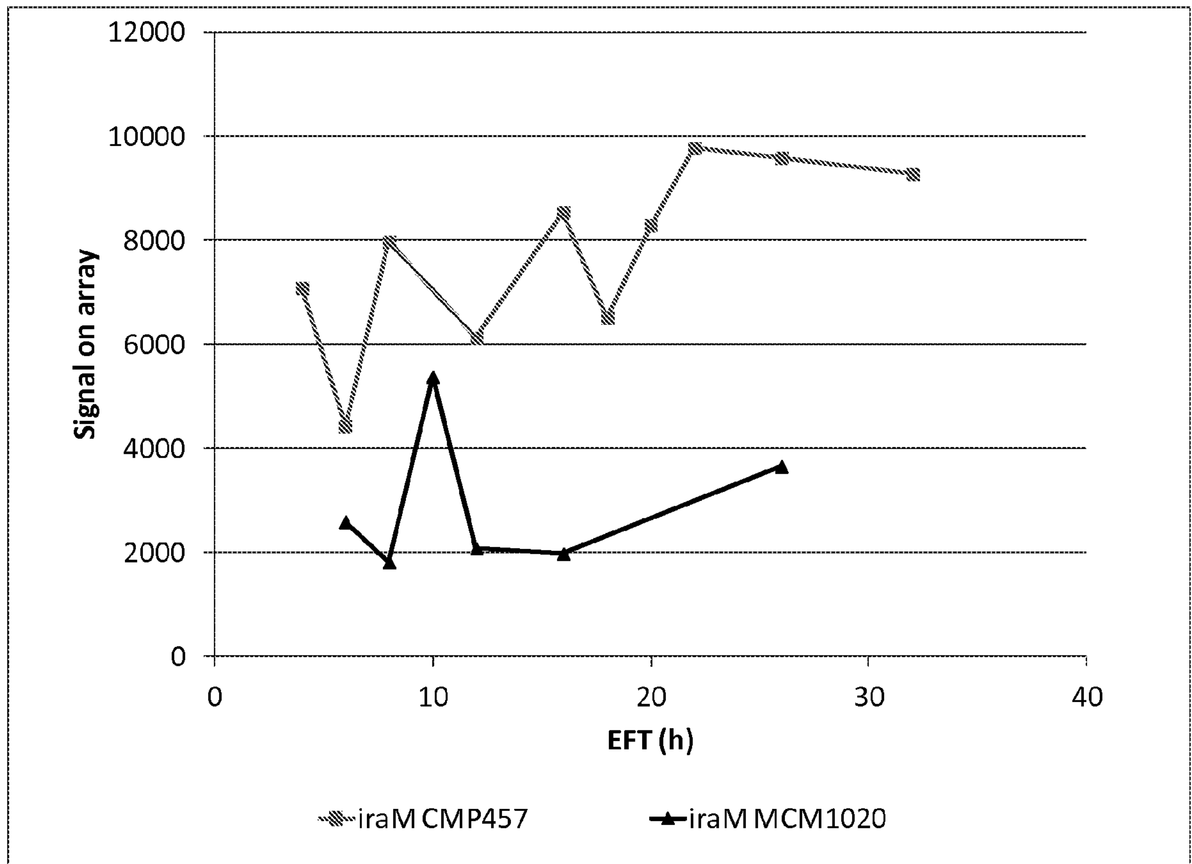

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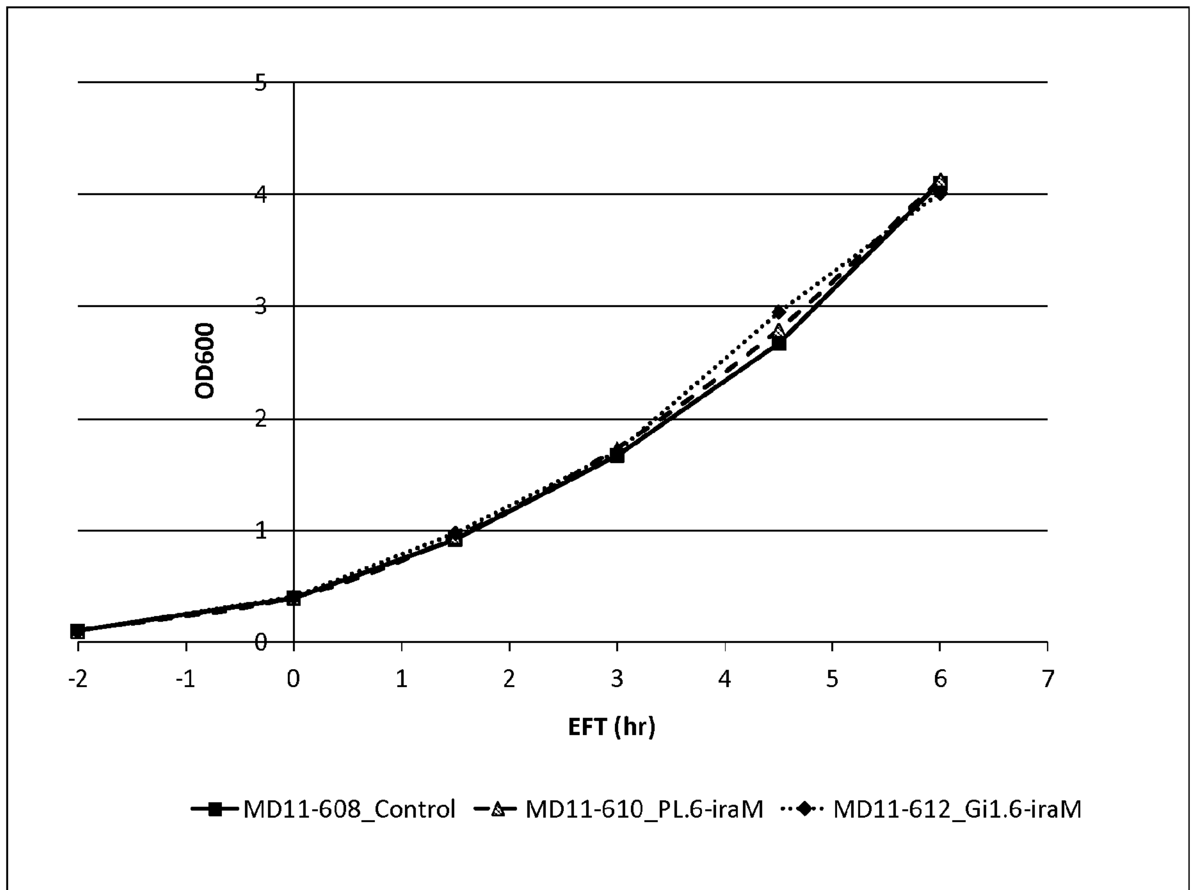

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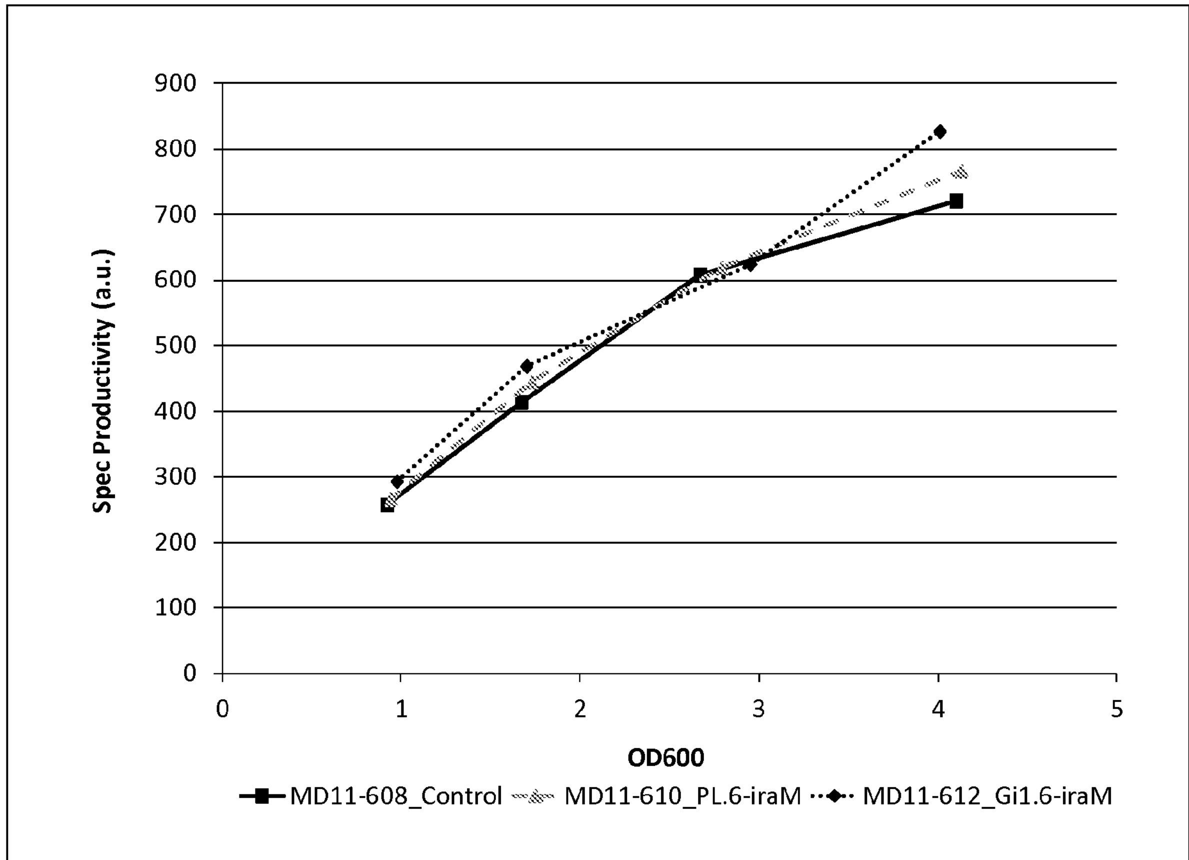

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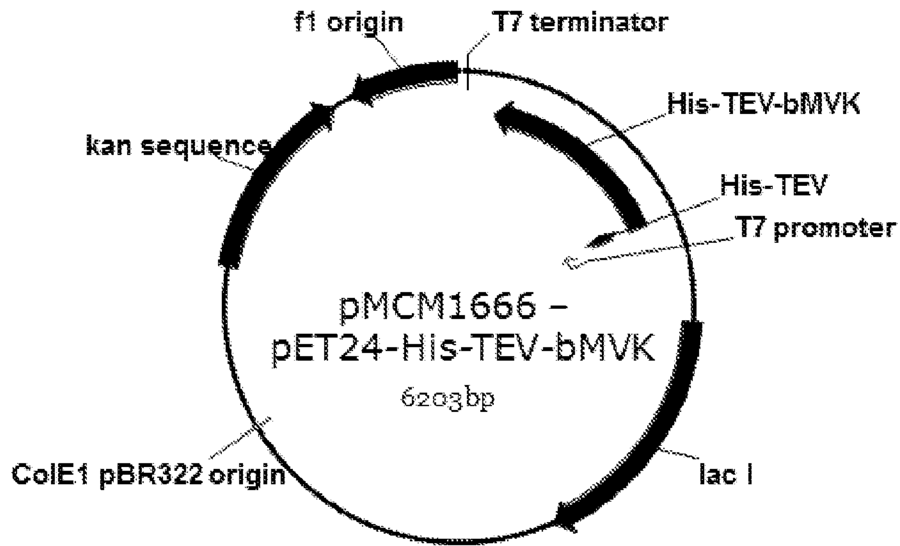

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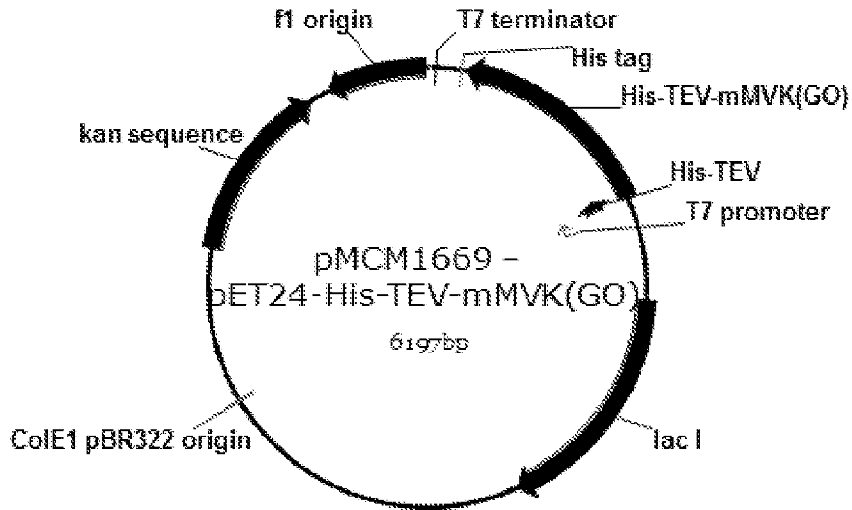

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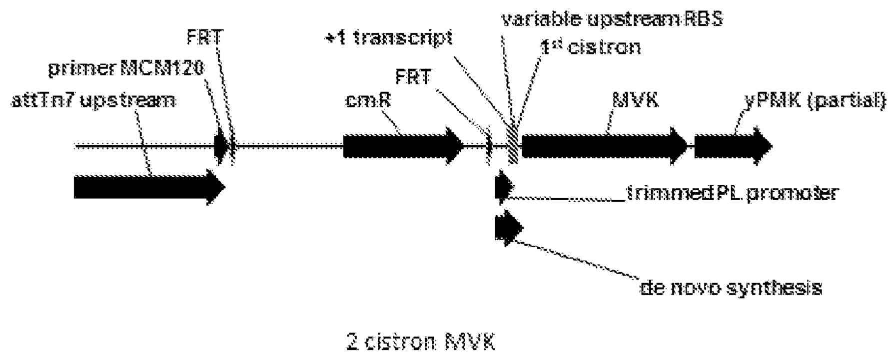

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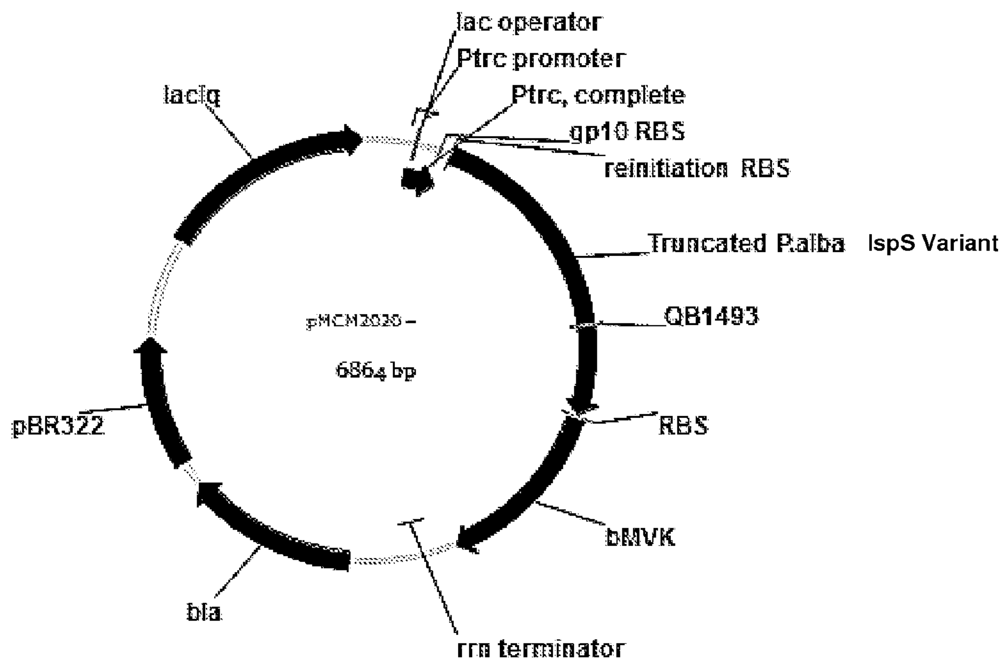

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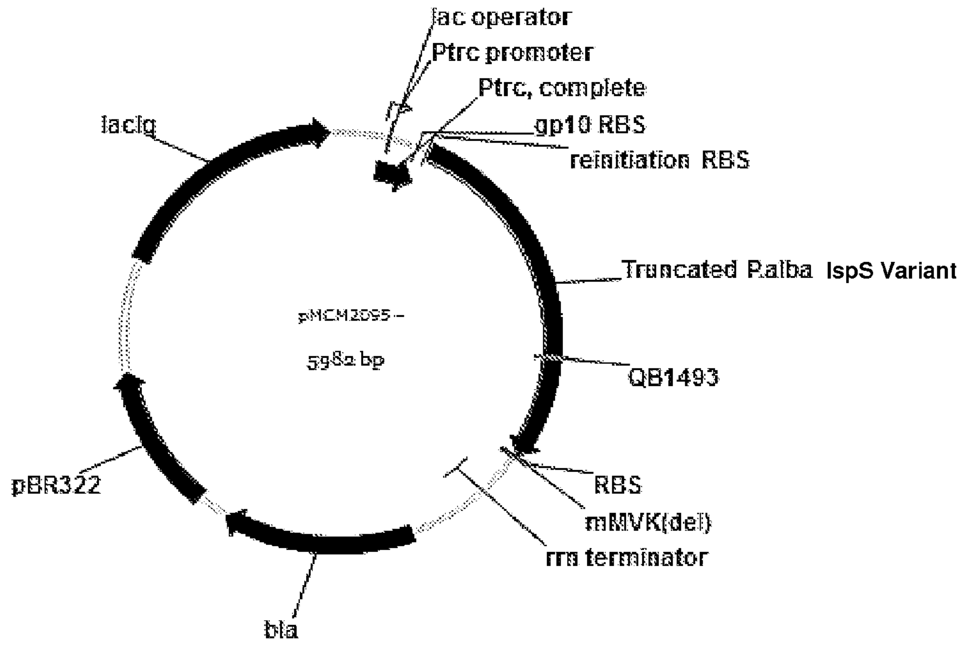

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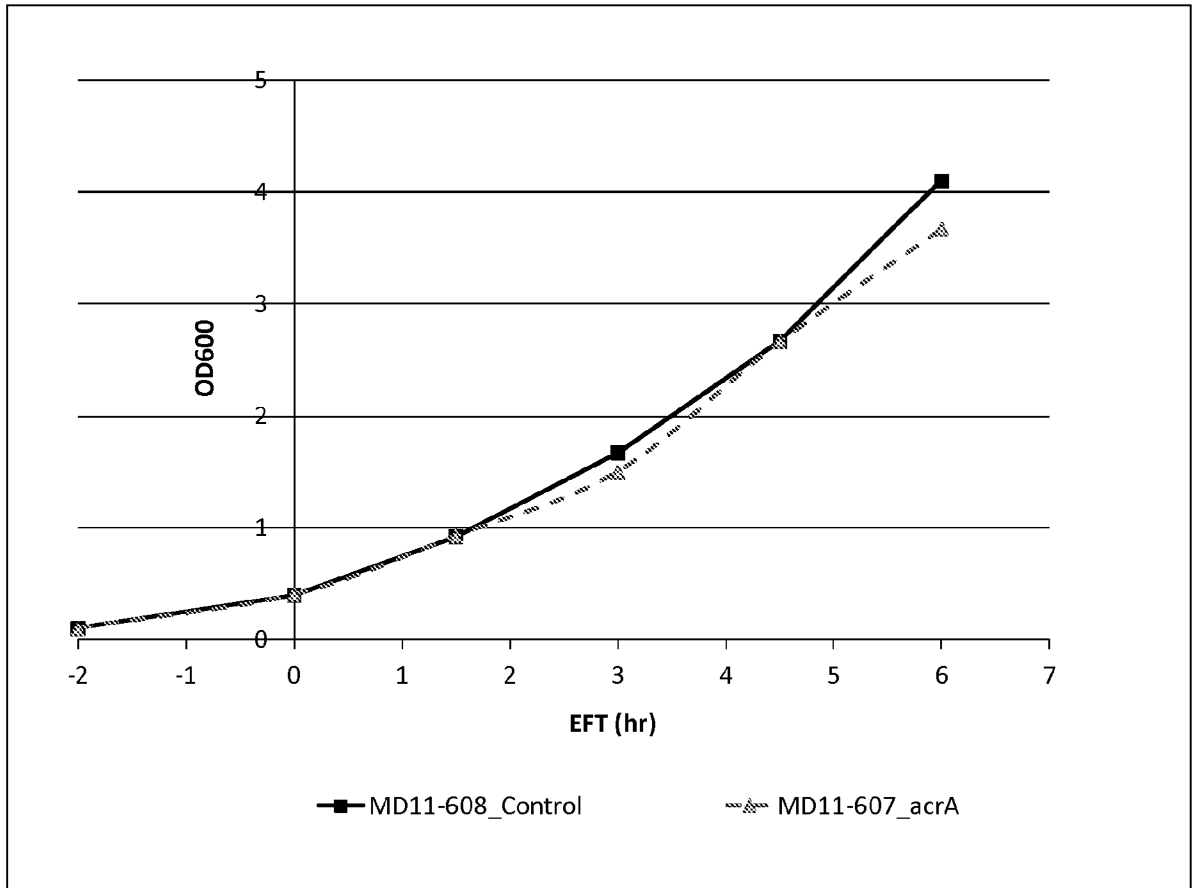

FIGURE 69: