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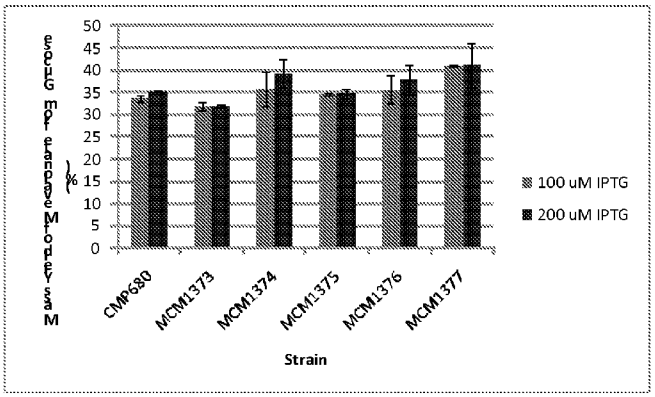
(54) **Title**: PRODUCTION OF MEVALONATE, ISOPRENE, AND ISOPRENOIDS USING GENES ENCODING POLYPEPTIDES HAVING THIOLASE, HMG-COA SYNTHASE AND HMG-COA REDUCTASE ENZYMATIC ACTIVITIES

FIGURE 1:



(57) **Abstract**: The invention features compositions and methods for the increased production of mevalonate, isoprene, isoprenoid precursor molecules, and/or isoprenoids in microorganisms via the heterologous expression of the *mvaE* and *mvaS* genes from the organisms *Listeria grayi DSM* 20601, *Enterococcus faecium*, *Enterococcus gallinarum EG2*, and *Enterococcus casseliflavus*.

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PRODUCTION OF MEVALONATE, ISOPRENE, AND ISOPRENOIDS USING GENES ENCODING POLYPEPTIDES HAVING THIOLASE, HMG-COA SYNTHASE AND HMG-COA REDUCTASE ENZYMATIC ACTIVITIES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/481,098, filed on April 29, 2011, the disclosure of which is hereby incorporated herein by reference 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 microorganisms, as well as methods for producing 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] Methods for the production of isoprene and isoprenoids at rates, titers, and purities have been disclosed (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 isoprene by recombinant cells. The invention also provides compositions ad methods for the increased production of mevalonate, isoprenoid precursor molecules, and/or isoprenoids in microorganisms by the expression (*e.g.*, heterologous expression) of the *mvaE* and *mvaS* genes from the organisms *Listeria grayi_DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2,* and *Enterococcus casseliflavus.*

[0012] Accordingly, provided herein are recombinant cells capable of increased production of isoprene, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences selected from the group consisting of: a *mvaE* gene and a *mvaS* gene from *E. gallinarum*; a *mvaE* gene and a *mvaS* gene from *E. casseliflavus*; a *mvaE* gene and a *mvaS* gene from *E. faecium*; and a *mvaE* gene and a *mvaS* gene from *L. grayi,*wherein said mvaE gene and mvaS gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cells further comprise: one or more nucleic acids encoding polypeptides of the lower MVA pathway; and a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the cells produce increased amounts of isoprene compared to isoprene-producing cells that do not comprise said *mvaE* gene and *mvaS* gene. In some aspects, the nucleic acids encoding polypeptides of the lower MVA pathway comprise enzymes selected from: (a) an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate; (b) an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (c) an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. In some aspects of any of the aspects disclosed herein, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate 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 enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase. In some aspects of any of the aspects disclosed herein, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide or variants thereof. In some aspects, the isoprene synthase polypeptide is a polypeptide from *Pueraria* or *Populus* or a hybrid, *Populus alba x Populus tremula*, or variants thereof. In some aspects, the isoprene synthase polypeptide is selected from the group consisting of *Pueraria montana, Pueraria lobata*, *Populus tremuloides*, *Populus alba*, *Populus nigra*, and *Populus trichocarpa*. In some aspects, the plant isoprene synthase polypeptide is a *Populus alba* isoprene synthase polypeptide. In some aspects of any of the aspects disclosed herein, the cells further comprise one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide. In some aspects, wherein the nucleic acid encoding an IDI polypeptide is a heterologous nucleic acid encoding an IDI polypeptide. In some aspects, the IDI polypeptide is a yeast IDI polypeptide. In some aspects, the nucleic acid encoding an IDI polypeptide is a copy of an endogenous nucleic acid encoding an IDI polypeptide. In some aspects of any of the aspects disclosed herein, the one or more nucleic acids is placed under an inducible promoter or a constitutive promoter. In some aspects of any of the aspects disclosed herein, the one or more nucleic acids is cloned into a multicopy plasmid. In some aspects of any of the aspects disclosed herein, the one or more nucleic acids is integrated into a chromosome of the cells. In some aspects of any of the aspects disclosed herein, the cells are gram-positive bacterial cells or gram-negative bacterial cells, *Escherichia* cells, *Pantoea* cells, fungal cells, filamentous fungal cells, *Trichoderma* cells, *Aspergillus* cells, or yeast cells. In some aspects, the cells are selected from the group consisting of *E. coli, 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 cells are *E. coli*.

[0013] In another aspect, provided herein is a method of producing isoprene, comprising: culturing the host cells disclosed in any of the aspects provided herein under suitable culture conditions for production of isoprene; and producing the isoprene. In one aspect, the method further comprises recovering the isoprene.

[0014] In a further aspect, provided herein are recombinant cells capable of increased production of isoprenoid precursors, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences selected from the group consisting of: an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*; an *mvaE* gene and an *mvaS* gene from *E. faecium*; and an *mvaE* gene and an *mvaS* gene from *L. grayi*, wherein the mvaE gene and mvaS gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cells produce increase amounts of isoprenoid precursors compared to isoprenoid precursor-producing cells that do not comprise said *mvaE* gene and *mvaS* gene. In some aspects, the one or more nucleic acids is placed under an inducible promoter or a constitutive promoter. In some aspects of any of the aspects disclosed herein, the one or more nucleic acids is cloned into a multicopy plasmid. In some aspects of any of the aspects disclosed herein, the one or more nucleic acids is integrated into a chromosome of the cells. In some aspects of any of the aspects disclosed herein, the cells are gram-positive bacterial 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 cells are selected from the group consisting of *E. coli, 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 cells are *E. coli*. In some aspects of any of the aspects disclosed herein, the isoprenoid precursor is mevalonate (MVA).

[0015] In another aspect, provided herein are methods for producing isoprenoid precursors, comprising: culturing the host cells described in any of the aspects disclosed herein under suitable culture conditions for production of isoprenoid precursors; and producing the isoprenoid precursors. In one aspect, the method further comprises recovering the isoprenoid precursors.

[0016] In yet other aspects, provided herein are recombinant cells capable of increased production of isoprenoids, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences selected from the group consisting of: an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*; an *mvaE* gene and an *mvaS* gene from *E. faecium*; and an *mvaE* gene and an *mvaS* gene from *L. grayi*, wherein said *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA

synthase, and HMG-CoA reductase catalytic activities, and wherein the cell further comprise: one or more nucleic acids encoding polypeptides of the lower MVA pathway; and one or more nucleic acids encoding polyprenyl pyrophosphate synthases, wherein the cells produce increased amounts of isoprenoids compared to isoprenoid-producing cells that do not comprise said *mvaE* gene and *mvaS* gene. In some aspects, the nucleic acids encoding polypeptides of the lower MVA pathway comprise enzymes selected from: (a) an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate; (b) an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (c) an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. In some aspects of any of the aspects disclosed herein, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate 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 enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase. In some aspects of any of the aspects disclosed herein, the one or more nucleic acids is placed under an inducible promoter or a constitutive promoter. In some aspects of any of the aspects disclosed herein, the one or more nucleic acids is cloned into a multicopy plasmid. In some aspects of any of the aspects disclosed herein, the one or more nucleic acids is integrated into a chromosome of the cells. In some aspects of any of the aspects disclosed herein, the cells are gram-positive bacterial 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 cells are selected from the group consisting of *E. coli, 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 cells are *E. coli*. In some aspects of any of the aspects disclosed herein, 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 of any of the aspects disclosed herein, 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.

[0017] In another aspect, there is provided a method for producing isoprenoids, comprising: culturing the host cells described in any of the aspects disclosed herein under suitable culture conditions for production of isoprenoids; and producing the isoprenoids. In one aspect, the method further comprises recovering the isoprenoids.

[0018] In one aspect, the invention provides recombinant cells (such as bacterial cells) capable of increased production of mevalonate, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cells produce a higher mass yield of mevalonate compared to cells (such as bacterial cells) that do not comprise the *mvaE* gene and *mvaS* gene from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*. In one aspect, the *mvaE* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:1. In another aspect, the *mvaS* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:2. In another aspect, the *mvaE* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:3. In another aspect, the *mvaS* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:4. In another aspect, the *mvaE* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:5. In another aspect, the *mvaS* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:6. In another aspect, the *mvaE* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:7. In another aspect, the *mvaS* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:8. In one aspect, the one or more heterologous nucleic acids can be placed under the control of an inducible promoter or under the control of a constitutive promoter. In one aspect, the one or more heterologous nucleic acids are codon optimized. In some aspects, the one or more heterologous nucleic acids are cloned into a multicopy plasmid. In another aspect, the one or more heterologous nucleic acids are integrated into a chromosome of the cell (such as a bacterial cell). In one aspect, the cells are bacterial

cells which are either gram-positive cells or gram negative cells. In another aspect, the cells are bacterial cells which are selected from the group consisting of *E. coli, 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 another aspect, the bacterial cells are *E. coli* cells.

[0019] In another aspect, the invention provides recombinant cells (such as bacterial cells) capable of increased production of mevalonate, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cells produce a higher peak titer of mevalonate compared to cells (such as bacterial cells) that do not comprise the *mvaE* gene and *mvaS* gene from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*. In one aspect, the *mvaE* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:1. In another aspect, the *mvaS* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:2. In another aspect, the *mvaE* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:3. In another aspect, the *mvaS* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:4. In another aspect, the *mvaE* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:5. In another aspect, the *mvaS* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:6. In another aspect, the *mvaE* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:7. In another aspect, the *mvaS* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:8. In one aspect, the one or more heterologous nucleic acids can be placed under the control of an inducible promoter or under the control of a constitutive promoter. In one aspect, the one or more heterologous nucleic acids are codon optimized. In some aspects, the one or more heterologous nucleic acids are cloned into a multicopy plasmid. In another aspect, the one or more heterologous nucleic acids are integrated into a chromosome of the cell (such as bacterial cell). In one aspect, the cells are bacterial cells which are either gram-positive cells or gram negative cells. In another aspect, the cells are bacterial cells are selected from the group consisting of *E. coli, 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 another aspect, the bacterial cells are *E. coli* cells.

[0020] In another aspect, the invention provides recombinant cells (such as bacterial cells) capable of increased production of mevalonate, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cells have a higher cell productivity index (CPI) compared to cells (such as bacterial cells) that do not comprise the *mvaE* gene and *mvaS* gene from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*. In one aspect, the *mvaE* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:1. In another aspect, the *mvaS* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:2. In another aspect, the *mvaE* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:3. In another aspect, the *mvaS* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:4. In another aspect, the *mvaE* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:5. In another aspect, the *mvaS* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:6. In another aspect, the *mvaE* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:7. In another aspect, the *mvaS* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:8. In one aspect, the one or more heterologous nucleic acids can be placed under the control of an inducible promoter or under the control of a constitutive promoter. In one aspect, the one or more heterologous nucleic acids are codon optimized. In some aspects, the one or more heterologous nucleic acids are cloned into a multicopy plasmid. In another aspect, the one or more heterologous nucleic acids are integrated into a chromosome of the cell (such as bacterial cell). In one aspect, the cells are bacterial cells which are either gram-positive cells or gram negative cells. In another aspect, the cells are bacterial cells are selected from the group consisting of *E. coli, 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 another aspect, the bacterial cells are *E. coli* cells.

[0021] In another aspect, the invention provides recombinant cells (such as bacterial cells) capable of increased production of mevalonate, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cells produce a higher mass yield of mevalonate compared to cells (such as bacterial cells) that do not comprise the *mvaE* gene and *mvaS* gene from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*. In one aspect, the *mvaE* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:1. In another aspect, the *mvaS* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:2. In another aspect, the *mvaE* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:3. In another aspect, the *mvaS* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:4. In another aspect, the *mvaE* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:5. In another aspect, the *mvaS* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:6. In another aspect, the *mvaE* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:7. In another aspect, the *mvaS* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:8. In one aspect, the one or more heterologous nucleic acids can be placed under the control of an inducible promoter or under the control of a constitutive promoter. In one aspect, the one or more heterologous nucleic acids are codon optimized. In some aspects, the one or more heterologous nucleic acids are cloned into a multicopy plasmid. In another aspect, the one or more heterologous nucleic acids are integrated into a chromosome of the cell (such as bacterial cell). In one aspect, the cells are bacterial cells which are either gram-positive cells or gram negative cells. In another aspect, the cells are bacterial cells are selected from the group consisting of *E. coli, 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 another aspect, the bacterial cells are *E. coli* cells.

[0022] In another aspect, the invention provides methods for increased production of mevalonate, the method comprising: (a) culturing cells comprising one or more heterologous nucleic acids comprising nucleotide sequences selected from the group consisting of (i) an *mvaE* gene and an *mvaS* gene from *L. grayi*; (ii) an *mvaE* gene and an *mvaS* gene from *E. faecium*; (iii) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*; and (iv) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities; and (b) producing mevalonate. In some aspects, the method further comprises the step of recovering the mevalonate. In some aspects, the cells are cultured at 34 ^{o}C. In some aspects, one or more heterologous nucleic acids are expressed on a low to moderate copy plasmid. In some aspects, the one or more heterologous nucleic acids are under the control of a strong promoter.

[0023] In another aspect, the invention provides recombinant cells (such as bacterial cells) capable of increased production of isoprene, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cell further comprises (i) one or more heterologous nucleic acids encoding polypeptides of the lower MVA pathway; and (ii) a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the cells produce greater amounts of isoprene compared to isoprene-producing cells (such as bacterial cells) that do not comprise said *mvaE* gene and *mvaS* gene. In one aspect, the *mvaE* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:1. In another aspect, the *mvaS* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:2. In another aspect, the *mvaE* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:3. In another aspect, the *mvaS* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:4. In another aspect, the *mvaE* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:5. In another aspect, the *mvaS* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:6. In another aspect, the *mvaE* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:7. In another aspect, the *mvaS* gene from *E. casseliflavus* comprises a nucleic acid

corresponding to SEQ ID NO:8. In one aspect, the one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus* are codon optimized. In one aspect, the polypeptides of the lower MVA pathway comprise enzymes selected from: (a) an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate; (b) an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (c) an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. In another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is selected from the group consisting of *M. mazei* mevalonate kinase, *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 another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase. In another aspect, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In one aspect, the isoprene synthase polypeptide is a polypeptide from *Pueraria* or *Populus* or a hybrid, *Populus alba x Populus tremula*. In another aspect, the isoprene synthase polypeptide is selected from the group consisting of *Pueraria montana* or *Pueraria lobata, Populus tremuloides, Populus alba, Populus nigra*, and *Populus trichocarpa*. In another aspect, the plant isoprene synthase polypeptide is a kudzu isoprene synthase polypeptide. In one aspect the cells (such as bacterial cells) further comprise one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide. In another aspect, the nucleic acid encoding an IDI polypeptide is a heterologous nucleic acid encoding an IDI polypeptide. In another aspect, the IDI polypeptide is a yeast IDI polypeptide. In one aspect, the nucleic acid encoding an IDI polypeptide is a copy of an endogenous nucleic acid encoding an IDI polypeptide. In another aspect, the one or more heterologous nucleic acids are placed under an inducible promoter or a constitutive promoter. In some aspects, the one or more heterologous nucleic acids are cloned into a multicopy plasmid. In another aspect, the one or more heterologous nucleic acids are integrated into a chromosome of the cells. In yet another aspect, the cells are gram-positive bacterial cells or gram-negative bacterial cells. In other aspects, the cells are selected from the group consisting of *E. coli, 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 another aspect, the cells are *E. coli.*

[0024] In another aspect, the invention provides recombinant cells (such as bacterial cells) capable of increased production of isoprene, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cell further comprises (i) one or more heterologous nucleic acids encoding polypeptides of the lower MVA pathway; (ii) a heterologous nucleic acid encoding an isoprene synthase polypeptide; and (iii) one or more heterologous nucleic acids encoding polypeptides of the DXP pathway, wherein the cells produce greater amounts of isoprene compared to isoprene-producing cells (such as bacterial cells) that do not comprise said *mvaE* gene and *mvaS* gene. In one aspect, the *mvaE* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:1. In another aspect, the *mvaS* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:2. In another aspect, the *mvaE* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:3. In another aspect, the *mvaS* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:4. In another aspect, the *mvaE* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:5. In another aspect, the *mvaS* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:6. In another aspect, the *mvaE* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:7. In another aspect, the *mvaS* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:8. In one aspect, the one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an mvaS gene from *E. casseliflavus* are codon optimized. In one aspect the polypeptides of the lower MVA pathway comprise enzymes selected from the group consisting of: (a) an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate; (b) an enzyme that converts mevalonate 5-phosphate to

mevalonate 5-pyrophosphate; and (c) an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. In another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is selected from the group consisting of *M. mazei* mevalonate kinase, *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 another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase. In another aspect, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In one aspect, the isoprene synthase polypeptide is a polypeptide from *Pueraria* or *Populus* or a hybrid, *Populus alba x Populus tremula*. In another aspect, the isoprene synthase polypeptide is selected from the group consisting of *Pueraria montana* or *Pueraria lobata, Populus tremuloides, Populus alba, Populus nigra*, and *Populus trichocarpa*. In another aspect, the plant isoprene synthase polypeptide is a kudzu isoprene synthase polypeptide. In one aspect the cells (such as bacterial cells) further comprise one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide. In another aspect, the nucleic acid encoding an IDI polypeptide is a heterologous nucleic acid encoding an IDI polypeptide. In another aspect, the IDI polypeptide is a yeast IDI polypeptide. In one aspect, the nucleic acid encoding an IDI polypeptide is a copy of an endogenous nucleic acid encoding an IDI polypeptide. In one aspect the polypeptides of the DXP pathway comprise enzymes selected from the group consisting of: (a) an enzyme that converts pyruvate and D-glyceraldehyde 3-phosphate into 1-deoxy-d-xylulose 5-phosphate (DXP); (b) an enzyme that converts 1-deoxy-d-xylulose 5-phosphate (DXP) into 2-C-methyl-D-erythritol 4-phosphate (MEP); (c) an enzyme that converts 2-C-methyl-D-erythritol 4-phosphate (MEP) into 4-(cytidine 5'-diphospho)-2-methyl-D-erythritol (CDP-ME); (d) an enzyme that converts 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); (e) an enzyme that converts 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); (f) an enzyme that converts 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate into (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP or HDMAPP); and (g) an enzyme that converts (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In

another aspect, the one or more heterologous nucleic acids are placed under an inducible promoter or a constitutive promoter. In some aspects, the one or more heterologous nucleic acids are cloned into a multicopy plasmid. In another aspect, the one or more heterologous nucleic acids is integrated into a chromosome of the cells. In yet another aspect, the cells are gram-positive bacterial cells or gram-negative bacterial cells. In other aspects, the cells are selected from the group consisting of *E. coli, 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 another aspect, the cells are *E. coli.*

[0025] In another aspect, the invention provides methods for increased production of isoprene, the method comprising: (a) culturing cells (such as bacterial cells) comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cell further comprises (i) one or more heterologous nucleic acids encoding polypeptides of the lower MVA pathway; and (ii) a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the cells produce greater amounts of isoprene compared to isoprene-producing cells (such as bacterial cells) that do not comprise said *mvaE* gene and *mvaS* gene. In some aspects, the cells further comprise one or more heterologous nucleic acids encoding polypeptides of the DXP pathway. In some aspects, the method further comprises the step of recovering the isoprene. In some aspects, the cells are cultured at 34 °C. In some aspects, the one or more heterologous nucleic acids are expressed on an extra-chromosomal plasmid. In some aspects, the one or more heterologous nucleic acids are integrated into a chromosome of a cell (such as a bacterial cell chromosome).

[0026] In another aspect, the invention provides recombinant cells (such as bacterial cells) capable of increased production of isoprenoid precursors and/or isoprenoids, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cell further comprises (i) one or more heterologous nucleic acids encoding polypeptides of the lower MVA pathway; and (ii) a heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide, wherein the cells produce at least greater amounts of isoprenoids and/or isoprenoid precursors, compared to isoprene-producing cells (such as bacterial cells) that do not comprise said *mvaE* gene and *mvaS* gene. In one aspect, the *mvaE* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:1. In another aspect, the *mvaS* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:2. In another aspect, the *mvaE* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:3. In another aspect, the *mvaS* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:4. In another aspect, the *mvaE* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:5. In another aspect, the *mvaS* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:6. In another aspect, the *mvaE* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:7. In another aspect, the *mvaS* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:8. In one aspect, the one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an mvaS gene from *E. casseliflavus* are codon optimized. In another aspect, the one or more heterologous nucleic acids are placed under an inducible promoter or a constitutive promoter. In some aspects, the one or more heterologous nucleic acids are cloned into a multicopy plasmid. In another aspect, the one or more heterologous nucleic acids is integrated into a chromosome of the cells. In yet another aspect, the cells are gram-positive bacterial cells or gram-negative bacterial cells. In other aspects, the cells are selected from the group consisting of *E. coli, 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 another aspect, the cells are *E. coli.* In one aspect the polypeptides of the lower MVA pathway comprise enzymes selected from: (a) an enzyme that phosphorylates mevalonate to mevalonate

5-phosphate; (b) an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (c) an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. In another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is selected from the group consisting of *M. mazei* mevalonate kinase, *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 another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase. In another aspect, the polyprenyl pyrophosphate synthase polypeptide comprises farnesyl pyrophosphate (FPP) synthase. In another aspect, the isoprenoid is selected from group consisting of monoterpenes, diterpenes, triterpenes, tetraterpenes, sequiterpene, and polyterpene. In other aspects, the isoprenoid is a sesquiterpene. In some aspects, the isoprenoid is 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.

[0027] In another aspect, the invention provides recombinant cells (such as bacterial cells) capable of increased production of isoprenoid precursors and/or isoprenoids, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cell further comprises (i) one or more heterologous nucleic acids encoding polypeptides of the lower MVA pathway; (ii) a heterologous nucleic acid encoding a polyprenyl pyrophosphate synthase polypeptide; and (iii) one or more heterologous nucleic acids encoding polypeptides of the DXP pathway, wherein the cells produce greater amounts of isoprene compared to isoprene-producing cells (such as bacterial cells) that do not comprise said *mvaE* gene and *mvaS* gene. In one aspect, the *mvaE* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:1. In another aspect, the *mvaS* gene from *L. grayi* comprises a nucleic acid corresponding to SEQ ID NO:2. In another aspect, the *mvaE* gene

from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:3. In another aspect, the *mvaS* gene from *E. faecium* comprises a nucleic acid corresponding to SEQ ID NO:4. In another aspect, the *mvaE* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:5. In another aspect, the *mvaS* gene from *E. gallinarum* comprises a nucleic acid corresponding to SEQ ID NO:6. In another aspect, the *mvaE* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:7. In another aspect, the *mvaS* gene from *E. casseliflavus* comprises a nucleic acid corresponding to SEQ ID NO:8. In one aspect, the one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an mvaS gene from *E. casseliflavus* are codon optimized. In one aspect the polypeptides of the lower MVA pathway comprise enzymes selected from the group consisting of: (a) an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate; (b) an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (c) an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. In another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is selected from the group consisting of *M. mazei* mevalonate kinase, *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 another aspect, the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase. In another aspect, the polyprenyl pyrophosphate synthase polypeptide comprises farnesyl pyrophosphate (FPP) synthase. In one aspect the cells (such as bacterial cells) further comprise one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide. In another aspect, the nucleic acid encoding an IDI polypeptide is a heterologous nucleic acid encoding an IDI polypeptide. In another aspect, the IDI polypeptide is a yeast IDI polypeptide. In one aspect, the nucleic acid encoding an IDI polypeptide is a copy of an endogenous nucleic acid encoding an IDI polypeptide. In one aspect the polypeptides of the DXP pathway comprise enzymes selected from the group consisting of: (a) an enzyme that converts pyruvate and D-glyceraldehyde 3-phosphate into 1-deoxy-d-xylulose 5-phosphate (DXP); (b) an enzyme that converts 1-deoxy-d-xylulose 5-phosphate (DXP) into 2-C-methyl-D-

erythritol 4-phosphate (MEP); (c) an enzyme that converts 2-C-methyl-D-erythritol 4-phosphate (MEP) into 4-(cytidine 5'-diphospho)-2-methyl-D-erythritol (CDP-ME); (d) an enzyme that converts 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); (e) an enzyme that converts 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); (f) an enzyme that converts 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate into (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP or HDMAPP); and (g) an enzyme that converts (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In another aspect, the one or more heterologous nucleic acids are placed under an inducible promoter or a constitutive promoter. In some aspects, the one or more heterologous nucleic acids are cloned into a multicopy plasmid. In another aspect, the one or more heterologous nucleic acids is integrated into a chromosome of the cells. In yet another aspect, the cells are gram-positive bacterial cells or gram-negative bacterial cells. In other aspects, the cells are selected from the group consisting of *E. coli, 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 another aspect, the cells are *E. coli.*

[0028] In another aspect, the invention provides methods for increased production of isoprenoid and/or isoprenoid precursor molecules, the method comprising: (a) culturing cells comprising one or more heterologous nucleic acids comprising nucleotide sequences 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*; and (d) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*, wherein the *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cell further comprises (i) one or more heterologous nucleic acids encoding polypeptides of the lower MVA pathway; and (ii) a heterologous nucleic acid encoding an polyprenyl pyrophosphate synthase, wherein the cells produce greater amounts of isoprenoid and/or isoprenoid precursor molecules compared to isoprenoid and/or isoprenoid precursor molecules-producing cells (such as bacterial cells) that do not comprise said *mvaE* gene and *mvaS* gene. In some aspects, the cells further comprise one or more heterologous

nucleic acids encoding polypeptides of the DXP pathway. In some aspects, the method further comprises the step of recovering the isoprenoid and/or isoprenoid precursor molecules. In some aspects, the cells are cultured at 34 °C. In some aspects, the one or more heterologous nucleic acids are expressed on an extra chromosomal plasmid. In some aspects, the one or more heterologous nucleic acids are integrated into a cell chromosome (such as a bacterial cell chromosome).

[0029] In another aspect, the invention provides for recombinant host (*e.g.*, bacterial) cells capable of increased production of mevalonate wherein the cells comprise a degradation resistant *mvaE* gene product from one of the following organisms: *E. gallinarum, E. faecium, E. casseliflavus,* or *L. grayi.*

[0030] In another aspect, the invention provides for recombinant host (*e.g.*, bacterial) cells capable of increased production of isoprene wherein the cells comprise a degradation resistant *mvaE* gene product from one of the following organisms: *E. gallinarum, E. faecium, E. casseliflavus,* or *L. grayi* that produces isoprene.

[0031] In another aspect, the invention provides for recombinant host (*e.g.*, bacterial) cells capable of increased production of an isoprenoid wherein the cells comprise a degradation resistant *mvaE* gene product from one of the following organisms: *E. gallinarum, E. faecium, E. casseliflavus,* or *L. grayi* that produces isoprenoids.

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 MVP concentration in *E. faecalis, E. gallinarum,* and *E. casseliflavus* at 40 hours.

[0035] Figure 4 depicts yield of isoprene on glucose achieved in each15-L fermentation over time. All runs using the *E.gallinarum* or *E.casseliflavus* (triangles and squares, respectively) achieved a higher % yield of isoprene on glucose than the two runs using *E.faecalis* upper pathway enzymes (open and closed diamonds). %wt Yield on glucose

calculated as 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.

[0036] Figure 5 depicts volumetric productivity achieved in each15-L fermentation over time. All runs using the *E.gallinarum* or *E.casseliflavus* (triangles and squares, respectively) achieved a higher overall volumetric productivity than the two runs using *E.faecalis* upper pathway enzymes (open and closed diamonds. Volumetric Productivity was calculated using the following formula: Volumetric productivity (g/L/hr) = $[\sum (HGER(t)/1000*68.117)]/[t-t_0]$, where the summation is from t_0 to t. Tank turnaround time is not factored in.

[0037] Figure 6 depicts specific productivity achieved in each15-L fermentation over time. All runs using the *E.gallinarum* or *E.casseliflavus* (triangles and squares, respectively) achieved a higher peak specific productivity than the two runs using *E.faecalis* upper pathway enzymes (open and closed diamonds). 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

[0038] Figure 7 depicts 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.

[0039] Figure 8 depicts depicts expression of *M. mazei* and *M. burtonii* mevalonate kinases in *E. coli* 15-L fermentations.

[0040] Figure 9 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 μM IPTG.

[0041] Figure 10 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.

DETAILED DESCRIPTION

[0042] Microbial cells, such as bacterial cells, are widely used hosts for the production of recombinant proteins. They can also be used to produce other products, such as mevalonate, isoprene, isoprenoid precursor molecules, and isoprenoids. The invention provides, *inter alia,* compositions and methods for the production of increased yields and titers of mevalonate, isoprene, isoprenoid precursor molecules, and isoprenoids using cells (such as bacterial cells) heterologously expressing polypeptides encoded by the *mvaE* and *mvaS* genes from the microorganisms *Listeria grayi_DSM 20601, Enterococcus faecium, Enterococcus gallinarum EG2,* and/or *Enterococcus casseliflavus.*

[0043] The mevalonate-dependent biosynthetic pathway is particularly important for the production of the isoprenoid precursor molecules mevalonate (MVA), 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, the *mvaE* and *mvaS* genes 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.

[0044] The *mvaE* and *mvaS* genes of a different bacterial species, *E. faecalis*, have been incorporated into *E. coli* strains previously to produce mevalonate (*see* U.S. Patent Application Publication No. 2005/0287655 A1; Tabata, K. and Hashimoto, S.-I. *Biotechnology Letters* 26: 1487–1491, 2004). However, the inventors have observed that the mass yield of mevalonate produced in cells (such as bacterial cells) expressing the *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum,* and *E. casseliflavus* is greater than the mass yield of mevalonate produced by *E. coli* strains containing the *mvaE* and *mvaS* genes from *E. faecalis.* 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 as well as in the amount of mevalonate produced by those cells (such as bacterial cells).

General Techniques

[0045] 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

[0046] 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.

[0047] As used herein, the term "polypeptides" includes polypeptides, proteins, peptides, fragments of polypeptides, and fusion polypeptides.

[0048] 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.

[0049] 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.

[0050] 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.

[0051] 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.

[0052] 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 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.

[0053] 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.

[0054] 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.

[0055] 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 cell (such as bacterial cell) growth; (2) various salts, which can vary among cellular species (such as bacterial cellular species) 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.

[0056] 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."

[0057] 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.

[0058] 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.*, mevalonate (MVA), isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP).

[0059] 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.

[0060] 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.

[0061] 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.

[0062] 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.

[0063] 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.

[0064] As used herein, the singular terms "a," "an," and "the" include the plural reference unless the context clearly indicates otherwise.

[0065] 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 Isoprenoid Precursors (e.g. mevalonate)

[0066] 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 MVA, DMAPP and IPP, which serve as the basis for the biosynthesis of terpenes, terpenoids, isoprenoids, and isoprene.

[0067] 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.

Genes encoding mvaE and mvaS polypeptides

[0068] In *L. grayi, E. faecium, E. gallinarum,* and *E. casseliflavus,* 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.

[0069] Accordingly, cells (such as bacterial (*e.g.*, *E. coli*) cells), can be engineered to express one or more *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus,* to increase production, peak titer, and cell productivity of an isoprenoid precursor (e.g., 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.

[0070] Various options of *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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, 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.

[0071] **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***
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. 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. 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. 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*

Exemplary mvaE polypeptides and nucleic acids

[0072] 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.

[0073] 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).

[0074] 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.

[0075] 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*, 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.

[0076] 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.

[0077] 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.

[0078] 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.

[0079] 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:11. 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:11. 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:13. 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:13. 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:9. 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:9. 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:15. 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:15. 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:9-16. In any of the aspects herein, the upper MVA pathway polypeptides may be encoded by a polypeptide with any one of SEQ ID NOs:9-16.

[0080] 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

[0081] 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.

[0082] 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.

[0083] 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 50 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 u1 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 x 10^3 M^{-1} cm^{-1}. By definition, 1 unit of enzyme activity causes 1 umol of acetoacetyl-CoA to be transformed per minute.

[0084] 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, the contents of which is incorporated by reference herein in its entirety) or HPLC (*see* U.S. Patent Application Publication No.: 2011/0159557 A1, the contents of which is incorporated by reference herein in its entirety). 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.

[0085] 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.

[0086] 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:12. 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:12. 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:14. 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:14. The mvaS

polypeptide 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:10. 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:10. 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:16. 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:16.

[0087] 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.

Exemplary host cells

[0088] 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 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.

[0089] 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. Recombinant cells can be made to heterologously express genes can be used to express one or more *mvaE* and *mvaS* genes from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. Bacteria cells, including gram positive or gram negative bacteria can be used to express any of the mvaE and *mvaS* genes described above. In particular, the *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. Additionally, the *mvaE* and *mvaS* genes can be expressed in any *Lactobacillus spp.,* such as *Lactobacillus lactis* or *Lactobacillus plantarum.*

[0090] 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 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.

[0091] 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 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.

[0092] 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 Application Publication No. US 2011/0045563, the contents of which are incorporated by reference herein in its entirety.

[0093] 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, the contents of which are incorporated by reference herein in its entirety). 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 Application Publication No. 2011/0045563, the contents of which are incorporated by reference herein in their entireties.

[0094] 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, the contents of which are incorporated by reference herein in their entireties). In certain embodiments, plasmids or plasmid components for use herein include those described in U.S. Patent Pub. No. US 2011/0045563, the contents of which are incorporated by reference herein in its entirety. 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, the contents of which are incorporated by reference herein in their entireties.

[0095] *E. coli* host cells can be used to express one or more mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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 mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus.* The *E. coli* host cells 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 mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus.* In addition, the one or more heterologously expressed nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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

[0096] 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 cellular (e.g. bacterial) growth; (2) various salts, which can vary among cellular (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.

[0097] 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.

[0098] 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.

[0099] 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).

[0100] 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.

[0101] 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

[0102] 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 mvaE, mvaS, isoprene synthase, DXP pathway (*e.g.*, DXS), IDI, MVA pathway, or PGL polypeptides encoded by a nucleic acid inserted into the host cells.

[0103] 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 (for example, bacterial cells (such as *E. coli* cells)) express one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* under the control of a strong promoter in a low to medium copy plasmid and are cultured at 34°C.

[0104] 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, the contents of each of which are incorporated by reference herein in their entireties. 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.

[0105] 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.

[0106] 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 cells (such as bacterial cells) capable of increased production of Isoprenoid Precursors (e.g. mevalonate)

[0107] The recombinant cells (such as bacterial cells) described herein have the ability to produce isoprenoid precursors (e.g. 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. In one embodiment, 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 lacking one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, optionally when cultured in minimal medium. In some cases, the one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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 or another isoprenoid precursor. 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 or another isoprenoid precursor, inclusive, as well as any numerical value in between these numbers.

[0108] The cells (such as bacterial cells) described herein produce isoprenoid precursors (e.g. 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. In one embodiment, 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, optionally when cultured in minimal medium. The cells (such as bacterial cells) can produce greater than about 105 g/L peak titer of mevalonate (or another isoprenoid precursor) 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 (or another isoprenoid precursor) after 48 hours of fermentation, inclusive, as well as any numerical value in between these numbers.

[0109] The cells (such as bacterial cells) described herein have a higher cell productivity index (CPI) than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. The cells (such as bacterial cells) described herein have a higher cell productivity index (CPI) than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus,* optionally when cultured in minimal medium. The cells (such as bacterial cells) can have a CPI for mevalonate (or another isoprenoid precursor) of at least about 4.5 (g/g). Alternatively, the cells (such as bacterial cells) can have a CPI for mevalonate (or another isoprenoid precursor) 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.

[0110] The cells (such as bacterial cells) described herein have a higher mass yield of isoprenoid precursors (e.g. mevalonate) from glucose than that of the same cells lacking one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. In one embodiment, 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* when cultured in minimal medium. The cells (such as bacterial cells) can produce a mass yield of mevalonate (or another isoprenoid precursor) from glucose of at least about 38%. Alternatively, the cells (such as bacterial cells) can produce a mass yield of mevalonate (or another isoprenoid precursor) 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.

[0111] In some aspects, the cells described herein are mevalonate-producing cells. In one aspect, mevalonate producing cells are wild type cells capable of producing mevalonate. In another aspect, mavalonate producing cells are non-naturally occurring cells engineered to contain one or more non-native upper MVA pathway polypeptides.

Methods of using recombinant cells (such as bacterial cells) to produce high amounts of Isoprenoid precursor (e.g. mevalonate)

[0112] Also provided herein are methods for the production of isoprenoid precursors, such as mevalonate. In some aspects, the method for producing isoprenoid precursors comprises: (a) culturing a composition comprising recombinant cells (including any of the bacterial cells described herein), or progeny thereof, capable of producing isoprenoid precursors; and (b) producing isoprenoid precursor. In some aspects, the method of producing isoprenoid precursor comprises the steps of culturing any of the recombinant cells described herein under conditions suitable for the production of isoprenoid precursor and allowing the recombinant cells to produce isoprenoid precursor. In some aspects, the method of producing isoprenoid precursor further comprises a step of recovering the isoprenoid precursor.

[0113] In some aspects, the method for producing mevalonate comprises: (a) culturing a composition comprising recombinant bacterial cells (including any of 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.

[0114] The method of producing mevalonate (or another isoprenoid precursor) can also 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, optionally in minimal medium, wherein the cells heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*; and (b) producing mevalonate (or another isoprenoid precursor). The cells (such as bacterial cells) can produce mevalonate (or another isoprenoid precursor) 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*, optionally, when the cells are cultured in minimal medium. In some cases, the one or more copies of a heterologous nucleic acid encoding an mvaE and an mvaS polypeptide from *L. grayi, E.*

faecium, E. gallinarum, and/or *E. casseliflavus* is a heterologous nucleic acid that is integrated into the host cell's chromosome.

[0115] The instant methods for the production of isoprenoid precursors can produce greater than about 85 mg/L/hr/OD of isoprenoid precursors. Alternatively, isoprenoid precursors 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 isoprenoid precursors, inclusive, as well as any numerical value in between these numbers. In some aspects, the method of producing isoprenoid precursors further comprises a step of recovering the isoprenoid precursors.

[0116] 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.

[0117] The method of producing isoprenoid precursors 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, wherein the cells (such as bacterial cells) heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*; and (b) producing isoprenoid precursors, wherein the cells (such as bacterial cells) produce isoprenoid precursors 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*. Optionally, the cells described above are cultured in minimal medium.

[0118] The instant methods for the production of isoprenoid prescursors can produce greater than about 105 g/L peak titer of isoprenoid prescursors 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 isoprenoid prescursors after 48 hours of fermentation, inclusive, as well as any numerical value in between these numbers. In some aspects, the method of producing isoprenoid prescursors further comprises a step of recovering the isoprenoid prescursors.

[0119] The method of producing mevalonate 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, optionally 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*; 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*, when the cells are cultured in minimal medium.

[0120] 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.

[0121] The method of producing isoprenoid precursors 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, wherein the cells (such as bacterial cells) heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*; and (b) producing isoprenoid precursors, wherein the

cells (such as bacterial cells) have a CPI for isoprenoid precursors higher than that of the same cells lacking one or more heterologous copies of a gene encoding an mvaE polypeptide and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*. Optionally, the cells above are cultured in minimal medium.

[0122] The instant methods for the production of isoprenoid precursors can produce isoprenoid precursors using cells with a CPI for isoprenoid precursors 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 isoprenoid precursors further comprises a step of recovering the isoprenoid precursors.

[0123] The method of producing mevalonate 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, optionally 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*; 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*, when the cells are cultured in minimal medium.

[0124] 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.

[0125] Provided herein are methods of using any of the cells described above for enhanced mevalonate production and/or production of other isoprenoid precursors. The production of mevalonate (or other isoprenoid precursors) by the cells can be enhanced by the expression of

one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E.* gallinarum, and/or *E. casseliflavus*. The production of mevalonate (or other isoprenoid precursors) 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 (or other isoprenoid precursors) by cells without the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*.

[0126] The production of mevalonate (or other isoprenoid precursors) 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*). The production of mevalonate (or other isoprenoid precursors) 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 (or other isoprenoid precursors) by naturally-occurring cells (*e.g.*, cells not expressing one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*).

[0127] The production of mevalonate (or other isoprenoid precursors) 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 mevalonate (or other isoprenoid precursors) 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,* and/or *E. casseliflavus.*

[0128] 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 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus* 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. In other aspects, the method for the production of isoprenoid precursors 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus* on a low to medium copy plasmid and under the control of a strong promoter; and (b) producing isoprenoid precursors. In some aspects, the method of producing isoprenoid precursors further comprises a step of recovering the isoprenoid precursors.

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

[0129] 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.

[0130] 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

isoprenoid precursors 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 isoprenoid precursors production from glucose translates into higher molar yields of 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. 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.

[0131] Any of the recombinant host cells expressing one or more copies of a heterologous nucleic acid encoding an mvaE and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus* capable of increased production of mevalonate or other isoprenoid precursors 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. In some aspects, these cells further comprise one or more nucleic acids encoding polypeptides of the lower MVA pathway and a heterologous nucleic acid encoding an isoprene synthase polypeptide.

[0132] In some aspects, the cells described herein are isoprene-producing cells. In one aspect, isoprene producing cells are wild type cells capable of producing isoprene. In another aspect, isoprene producing cells are non-naturally occurring cells engineered to contain one or more heterologous upper MVA pathway polypeptides, lower MVA pathway polypeptides, isoprene synthase polypeptides, DXP pathway polypeptides, and/or IDI polypeptides. In a further aspect, the isoprene producing cells may contain both endogenous and heterologous upper MVA pathway polypeptides, lower MVA pathway polypeptides, isoprene synthase polypeptides, DXP pathway polypeptides, and/or IDI polypeptides.

Nucleic acids encoding polypeptides of the lower MVA pathway

[0133] 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.

[0134] 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, *Lactobacillus* mevalonate kinase polypeptide, *Lactobacillus sakei* mevalonate kinase polypeptide, *yeast* mevalonate kinase polypeptide, *Saccharomyces cerevisiae* mevalonate kinase polypeptide, *Streptococcus* mevalonate kinase polypeptide, *Methanococcoides* 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.

[0135] 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, Methanococcoides burtonii,* or *Methanosarcina mazei*. In some aspects, the heterologous lower MVA pathway polypeptide is a mevalonate kinase from *M. burtonii.*

[0136] 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.

[0137] 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.* 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.

[0138] 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.

[0139] 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.

[0140] 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, 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

[0141] 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 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.*

[0142] 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.

[0143] 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.

[0144] 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.

[0145] 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.

[0146] 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.

[0147] 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.

[0148] 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*).

[0149] 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.

[0150] 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.

[0151] 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).

[0152] In some aspects, the variant comprises one or more (i.e. 2, 3, 4, 5, 6, etc.) mutations from the following table (Table 2) corresponding to the amino acid sequence of *P. alba*:

Table 2: Isoprene Synthase Variants of *P. Alba* (MEA)				
A118E	E472R	S510V	K161K	A118P

S22K	K463F	I342I	W392A	A118Q
S21R	K463T	K348F	W392C	A118A
S22K	R71K	K348Y	W392F	E41M
S22R	R71L	K348K	S288Y	G111S
E58L	R71M	C437L	M228Y	S74Q
T481V	R71V	T240C	A3T	S74S
T481Y	R71R	M460M	W392Y	K36D
T502F	K393L	R461A	W392W	S282H
T381L	F542L	H424P	F89D	S282I
T381M	P538K	H424H	F89E	S282W
T381Y	P538R	A448L	F89F	S282Y
T383H	P538P	A448Q	E41Y	S282S
T383L	A503A	A448V	E41E	K36S
E480I	L436I	G389D	R43E	K36T
E480R	L436Y	S444E	R43L	K36W
K393V	L436F	S444S	K36E	K36Y
K393I	E488L	H511Y	K36H	K36K
E415H	E488M	H511H	K36N	
E415V	E488T	R071I	K36P	
E415Y	E488W	R071K	K36Q	
R71H	E488E	R071L	A453I	
R71I	I342Y	K374Y	A453V	
E58Y	C437M	K374K	A453A	
E135G	C437W	L526E	V409I	
A363L	C437Y	L526Q	V409T	
K374Y	C437C	L526L	K161C	
T381I	M460A	R242G	K161E	
L436L	I447T	R242R	K161N	
H254R	I447V	A443G	K161Q	
H254C	I447Y	A443Q	G99E	
E488C	S444D	A443R	G99G	
E488F	G389E	A443S	S288A	
T383Y	L376I	S13S	S288C	
K414I	L376M	V268I	S288T	
K414R	L376L	V268V	W392I	
K414S	I504F	K161A	W392M	
K414W	I504I	V409V	W392S	
E472C	E467W	D323F	W392T	
E472L	S510C	G99D	W392V	

[0153] 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.

[0154] 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.

[0155] 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

[0156] In some aspects of the invention, the cells described in any of the compositions or methods 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.

[0157] 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.

[0158] 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

[0159] 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.

[0160] 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*.

[0161] 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*.

[0162] 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*.

[0163] 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*.

[0164] 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*.

[0165] 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*.

[0166] 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

[0167] 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.

[0168] 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.

[0169] In some aspects, the source organism is a yeast, such as *Saccharomyces sp.*, *Schizosaccharomyces sp.*, *Pichia sp.*, or *Candida sp.*

[0170] 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*.

[0171] 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*.

[0172] 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.*

[0173] 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*.

[0174] In some aspects, the source organism is an algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates.

[0175] 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

[0176] 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.

[0177] 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.

[0178] 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. In some aspects, the phosphoketolase nucleic acid is a heterologous nucleic acid encoding a phosphoketolase polypeptide.

[0179] 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.

[0180] 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

[0181] 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).

[0182] 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.

[0183] 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.

[0184] 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%.

[0185] 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

[0186] *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).

[0187] 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

[0188] 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).

[0189] 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.

Host cell mutations

[0190] The invention also contemplates additional host cell mutations that increase carbon flux through the MVA pathway. By increasing the carbon flow, more isoprene, mevalonate, isoprenoid precursor molecules, and/or isoprenoids can be produced. The recombinant cells as described herein can also be engineered for increased carbon flux towards mevalonate, isoprene, isoprenoid, and/or isoprenoid precursor 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 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

[0191] 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. & 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).

[0192] 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 and isoprene. 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

[0193] 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, isorpenoids, isoprenoid precursor molecules, and/or isoprene. In some aspects, the *eutD* gene, which exhibits phosphotransacetylase-like enzymatic activity (*e.g.,* the *eutD* gene in organisms such as, but not limited to, *E. coli* and *Saccharomyces cerevisiae*) is attenuated or deleted.

[0194] 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.

[0195] 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 the specific activity or total activity that occurs 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%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, inclusive, including any values in between these percentages.

[0196] 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

[0197] 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 molecule, and/or isoprenoid 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.

[0198] 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.

[0199] 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%, inclusive, including any percentage in between these values.

[0200] 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

[0201] 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:

(S)-malate + $NAD(P)^+$ ⇌ pyruvate + CO_2 + NAD(P)H

[0202] Thus, the two substrates of this enzyme are (S)-malate and $NAD(P)^+$, whereas its 3 products are pyruvate, CO_2, and NADPH.

[0203] Expression of the NADP-dependent malic enzyme (maeB – Figure 5) (Iwikura, M. et al. 1979, *J. Biochem.* 85: 1355-1365) can help increase mevalonate and/or isoprene 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).

[0204] As such, more starting substrate (pyruvate or acetyl-CoA) for the downstream production of mevalonate, isoprenoid precursor molecules, isoprenoids, and/or isoprene 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.

[0205] 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.

[0206] 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.

[0207] 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

[0208] 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 (aattcatataaaaaacatacagataaccatctgcggtgataaattatctctggcggtgttgacataaataccactggcggtgatactgagcac atcagcaggacgcactgaccaccatgaaggtg - lambda promoter, GenBank NC_001416), in front of the operon or using one or more synthetic constitutively expressing promoters.

[0209] 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.

[0210] 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.

[0211] 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

[0212] 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, and pyruvate decarboxylase (*aceE*, *aceF*, and/or *lpdA*) is designated as F. 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.

[0213] Accordingly, for combinations of any two of the enzymes A-F, non-limiting combinations that can be used are: AB, AC, AD, AE, AF, BC, BD, BE, BF, CD, CE, CF, DE, DF and EF. For combinations of any three of the enzymes A-F, non-limiting combinations that can be used are: ABC, ABD, ABE, ABF, BCD, BCE, BCF, CDE, CDF, DEF, ACD, ACE, ACF, ADE, ADF, AEF, BDE, BDF, BEF, and CEF. For combinations of any four of the enzymes A-F, non-limiting combinations that can be used are: ABCD, ABCE, ABCF, ABDE, ABDF, ABEF, BCDE, BCDF, CDEF, ACDE, ACDF, ACEF, BCEF, BDEF, and ADEF. For

combinations of any five of the enzymes A-F, non-limiting combinations that can be used are: ABCDE, ABCDF, ABDEF, BCDEF, ACDEF, and ABCEF. In another aspect, all six enzyme combinations are used: ABCDEF.

[0214] 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 isoprene production

[0215] Other molecular manipulations can be used to increase the flow of carbon towards isoprene 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 mevalonate and/or isoprene.

[0216] 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 and/or isoprene. PGL may be introduced using chromosomal integration or extra-chromosomal vehicles, such as plasmids. In other aspects, PGL may be deleted from the genome of cells (for example, microorganisms, such as various *E. coli* strains) which express an endogenous PGL to improve production of mevalonate and/or isoprene. In some 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 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.

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

[0217] The recombinant cells (such as bacterial cells) 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 mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, 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, optionally when cultured in minimal media. In some cases, the one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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 that are integrated into the host cell's chromosome. 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. 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.

[0218] In one aspect of the invention, there are provided cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from

L. grayi, E. faecium, E. gallinarum, and/or *E. casseliflavus*, 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, 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.

[0219] 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*). 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus.* 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 mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus.*

[0220] 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

[0221] Also 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus,* 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.

[0222] Thus, also 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus,* in a suitable condition for producing isoprene and (b) producing isoprene. 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 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 (such as bacterial) strains or plant cells 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.

[0223] 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. In some aspects, the amount of isoprene produced is measured at the peak volumetric productivity time point. In some aspects, the peak volumetric 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 cumulative percent yield on glucose time point. In some aspects, the cumulative percent yield on glucose is about any of the amounts of isoprene disclosed herein.

[0224] In some aspects, any of the cells described herein (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.

[0225] In some aspects, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 100,000, or more ng of isoprene/gram of cells for the wet weight of the cells/hr (ng/g_{wcm}/h). In some 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.

[0226] In some aspects, the cells 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}.

[0227] In some aspects, the isoprene produced by the cells 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 %.

[0228] 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 mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, 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, increased volumetric productivity, 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus.* 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 mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*.

[0229] The production of isoprene 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 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,* and/or *E. casseliflavus*.

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

[0230] Isoprenoids can be produced in many organisms from the synthesis of the isoprenoid precursor molecules which are produced by 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.

[0231] 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.

[0232] 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).

[0233] Any of the recombinant host cells expressing one or more copies of a heterologous nucleic acid encoding an mvaE and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus* capable of increased production of mevalonate or isoprenoid precursors 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, increasing the cellular

production of isoprenoid precursors in cells (such as bacterial cells) by any of the compositions and methods described above can result in the production of higher amounts of isoprenoids. Increasing the molar yield of isoprenoid precursor production from glucose translates into higher molar yields of isoprenoids 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 isoprenoid production. Also 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.

[0234] In some aspects, the cells described herein are isoprenoid-producing cells. In one aspect, isoprenoid producing cells are wild type cells capable of producing isoprenoid. In another aspect, isoprenoid producing cells are non-naturally occurring cells engineered to contain one or more heterologous upper MVA pathway polypeptides, lower MVA pathway polypeptides, polyprenyl pyrophosphate synthase polypeptides, DXP pathway polypeptides, and/or IDI polypeptides. In a further aspect, the isoprene producing cells may contain both endogenous and heterologous upper MVA pathway polypeptides, lower MVA pathway polypeptides, polyprenyl pyrophosphate synthase polypeptides, DXP pathway polypeptides, and/or IDI polypeptides.

Types of isoprenoids

[0235] The cells (such as bacterial cells) of the present invention are capable of increased production of isoprenoids. 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.

[0236] 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

[0237] In some aspects of the invention, the cells 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 polypeptide(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.

[0238] 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.

[0239] 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.

[0240] 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.

[0241] In some aspects of the invention, the cells 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.

[0242] 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.

[0243] 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.

[0244] 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 isoprenoids

[0245] The recombinant cells (such as bacterial cells) described herein have the ability to produce isoprenoids 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 from *L. grayi, E.*

faecium, E. gallinarum, and/or *E. casseliflavus*, 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, optionally when cultured in minimal media. In some cases, the one or more copies of a heterologous nucleic acid encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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 (such as bacterial cells) can produce at least 5% greater amounts of isoprenoids when compared to isoprenoids-producing cells (such as bacterial cells) that do not comprise the mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. 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 isoprenoids , inclusive, as well as any numerical value in between these numbers.

[0246] In one aspect of the invention, there are provided cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, 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 mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, 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.

[0247] Provided herein are methods of using any of the cells described above for enhanced isoprenoid production. The production of isoprenoids by the cells can be enhanced by the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, 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 production refers to an increased cell productivity index (CPI) for isoprenoid production, an increased titer of isoprenoids, an increased mass yield of isoprenoids, and/or an increased specific productivity of 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 the mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus.* The production of isoprenoids can be enhanced by about 5% to about 1,000,000 folds. The production of 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 by cells without the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus..*

[0248] The production of 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus,* 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 isoprenoids can be enhanced by about 5% to about 1,000,000 folds. The production of 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 isoprenoids by naturally-occurring cells (*e.g.*, cells without the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*).

[0249] The production of 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 isoprenoids 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,* and/or *E. casseliflavus.*

Methods of using the recombinant cells to produce isoprenoid molecules

[0250] Also provided herein are methods of producing isoprenoids comprising culturing cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding an mvaE and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus,* a lower MVA pathway polypeptide, and an polyprenyl pyrophosphate synthase polypeptide. The 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 isoprenoids from carbohydrates, including six carbon sugars such as glucose.

[0251] Thus, provided herein are methods of producing isoprenoids comprising culturing cells (such as bacterial cells) comprising one or more heterologous nucleic acids encoding an mvaE and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus,* 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 (such as bacterial) strains or plant cells described herein, any of the promoters described herein, and/or any of the vectors described herein can also be used to produce 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 isoprenoids further comprises a step of recovering the isoprenoids.

[0252] The method of producing 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 from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*, wherein the cells (such as bacterial cells) heterologously express one or more copies of a gene encoding a mvaE polypeptide and an mvaS polypeptide from *L. grayi, E. faecium, E.* gallinarum, or *E. casseliflavus*; and (b) producing isoprenoids , wherein the cells (such as bacterial cells) produce greater amounts of isoprenoids when compared to isoprenoid-producing cells (such as bacterial cells) that do not comprise the mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*.

[0253] The instant methods for the production of isoprenoids can produce at least 5% greater amounts of isoprenoids when compared to isoprenoids-producing cells (such as bacterial cells) that do not comprise the mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*. 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 isoprenoids, inclusive. In some aspects, the method of producing isoprenoids further comprises a step of recovering the isoprenoids.

[0254] Provided herein are methods of using any of the cells described above for enhanced isoprenoid production. The production of isoprenoids by the cells can be enhanced by the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E.* casseliflavus, 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 production refers to an increased cell productivity index (CPI) for isoprenoid production, an increased titer of isoprenoids, an increased mass yield of isoprenoids, and/or an increased specific productivity of 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 the mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus.* The production of isoprenoids can be enhanced by about 5% to about 1,000,000 folds. The production of 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 isoprenoids by cells without the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*.

[0255] The production of 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 isoprenoids by cells without the expression of one or more heterologous nucleic acids encoding mvaE and mvaS polypeptides from *L. grayi, E. faecium, E. gallinarum,* and/or *E. casseliflavus*.

[0256] 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 isoprenoids 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 from *L.* grayi, *E. faecium, E. gallinarum,* and/or *E. casseliflavus* 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 from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus* on a low to medium copy plasmid and under the control of a strong promoter; and (b) producing isoprenoid, such as mevalonate. In some aspects, the method of producing

mevalonate further comprises a step of recovering the isoprenoid precursor molecules and/or isoprenoids.

Vectors

[0257] 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 mvaE polypeptide and an mvaS polypeptide from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*, 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 mvaE and an mvaS nucleic acid from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus*, an isoprene synthase, a polyprenyl pyrophosphate synthase, and/or one or more MVA pathway polypeptides nucleic acid(s) integrate into the genome of host cells without a selective marker.

[0258] Any one of the vectors characterized or used in the Examples of the present disclosure can be used.

Transformation methods

[0259] Nucleic acids encoding one or more copies of an mvaE and an mvaS nucleic acid from *L. grayi, E. faecium, E. gallinarum,* or *E. casseliflavus,* 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.

Exemplary Purification Methods

[0260] 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 Publ. No. 2011/0178261, the contents of which is incorporated by reference, especially with respect to the absorption stripping and purification techniques disclosed therein). In some aspects, any of the methods described herein further include a step of recovering the heterologous polypeptide. In some aspects, any of the methods described herein further include a step of recovering the terpenoid or carotenoid.

[0261] Suitable purification methods are described in more detail in U.S. Patent Application Publication US2010/0196977 A1.

[0262] 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

[0263] 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'-

TATTTAATTTTTAATCATCTAATTTGACAATCATTCAACAAAGTTGTTACAATTAACC CTCACTAAAGGGCGG-3') and DngltA1.xgiCm-R (5'-TCAACAGCTGTATCCCCGTTGAGGGTGAGTTTTGCTTTTGTATCAGCCATATATTCCA CCAGCTATTTGTTAGTGAATAAAAGTGGTTGAATTATTTGCTCAGGATGTGGCATHG TCAAGGGCTAATACGACTCACTATAGGGCTCG-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 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 (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 gltA	MD09-314
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

[0264] 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 Publication No. 2011/0159557) 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.

[0265] 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)

[0266] 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 (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)

[0267] 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)

[0268] 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'-AAAATTTTCATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTCACATG GAGTTGGCAGGATGTTTGATTACATGGGAATTAGCCATGGTCC-3') and CMP172 (5'-GACCAGCCGCGTAACCTGGCAAAATCGGTTACGGTTGAGTAATAAATGGATGCCCT GCGTAAGCGGGGCATTTTTCTTGGTGTAGGCTGGAGCTGCTTCG-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

Plasmid pCHL276 (see example 6 (iii)) was 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*

[0269] 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:*

[0270] 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.

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*

(i) *Gene identification and selection*

[0271] 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.

[0272] 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*

[0273] 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 *Bgl*II 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'

[0274] 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 (*Enterococcus gallinarum* EG2)	*E. gallinarum* EG2	gi|257869528|ref|ZP_05649181.1| acetyl-CoA acetyltransferase/hydroxymethylglutaryl-CoA reductase [*Enterococcus gallinarum EG2*]	gi|257869527|ref|ZP_05649180.1| hydroxymethylglutaryl-CoA synthase [*Enterococcus gallinarum EG2*]	pSC101, Spectinomycin (50ug/mL)

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

[0275] 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)*

[0276] 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 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 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*

[0277] 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):

[0278] 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 (123°C 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):

[0279] 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):

[0280] $MgSO_4 * 7H_2O$ 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):

[0281] 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):

[0282] 50g spectinomycin was q.s. to volume with deionized water and filter sterilized with 0.22 micron filter.

Feed solution #1 (per kilogram):

[0283] 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*

[0284] 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

[0285] 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.

[0286] 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.

[0287] 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 uM 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*

[0288] 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:

[0289] 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.

[0290] 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

[0291] 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%

[0292] 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%

[0293] 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

[0294] 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.

Example 9: Increased MVP levels utilizing the upper MVA pathway from *E. casseliflavus* or *E. gallinarum*

[0295] These experiments highlight increase in mevalonate-5-phosphate (MVP) levels when utilizing either the upper MVA pathway from *E. casseliflavus* or *E. gallinarum* compared to an upper MVA pathway from *E. faecalis*. Mevalonate 5-phosphate is a substrate for phosphomevalonate kinase (PMK). Accordingly, without being bound to theory, increased MVP concentrations in cells indicate increased carbon flux through the upper MVA pathway.

(i) Materials and Methods

[0296] **Metabolite Extraction from *E. coli*:** The metabolism of bacterial cells grown in fermentors was rapidly inactivated by withdrawing approximately 3 mL of culture into a tube filled with 9 mL of dry ice-cold methanol. The resulting samples were weighed to calculate the amount of sampled broth and then stored at −80° C until further analysis. For metabolite extraction and concentration, 0.25 mL aliquots of cell suspension (0.4 mL aliquot was used if cell density of the culture measured as OD_{600} was below 50) were diluted with 1.5 mL of methanol/ammonium acetate buffer (5 mM, pH=8.0) mixture (6:1, v/v) and cell debris was pelleted by a 4 minute centrifugation. The supernatant was collected and loaded onto Strata-X-AW columns from Phenomenex (33 µm 30mg/3mL Polymeric Weak Anion Exchange). The cell pellet was extracted two more times, first with 1.5 mL of the methanol/ammonium acetate buffer (5 mM, pH=8.0) mixture (6:1 v/v), and then with 1.5 mL of methanol/ammonium acetate buffer (5 mM, pH=8.0) mixture (1:1 v/v). Both times the cells were pelleted by centrifugation, and the resulting supernatants were consecutively loaded onto the same Strata-X-AW columns. During the extraction-centrifugation, samples with cells were kept below 4° C. After washing the columns with 1 mL of water and 1 mL of methanol, metabolites of interest were eluted from the

columns first with 0.3 mL of concentrated NH_4OH/methanol (1:14, v/v) mixture and then with 0.3 mL of concentrated NH_4OH/methanol/water (1:12:2, v/v/v) mixture. The resulting eluant was neutralized by adding 20 μL of glacial acetic acid, and then cleared by centrifugation.

[0297] **Metabolite Quantification.** Analysis of metabolites was carried out by mass spectrometry using a TSQ Quantum Access TSQ system (Thermo Scientific). All system control, data acquisition, and mass spectral data evaluation were performed using XCalibur and LCQuan software (Thermo Scientific). For the LC-ESI-MS/MS method, a chiral Nucleodex β-OH 5 μM HPLC column (100×2 mm, Macherey-Nagel, Germany) was used with a CC 8/4 Nucleodex beta-OH guard cartridge. A mobile phase gradient was applied as described in Table 14 in which mobile phase A was 100 mM ammonium acetate (SigmaUltra grade, Sigma) buffer (pH=8) in MilliQ-grade water, mobile phase B was MilliQ-grade water, and mobile phase C was LC-MS grade acetonitrile (Chromasolv, Riedel-de Haën). The column and sample tray temperatures were reduced to 5° C. and 4° C, respectively. The injection volume was 10 μL.

Table 14: HPLC gradient used to elute metabolites in the MVA pathway.

Time	Solvent A	Solvent B	Solvent C	Flow rate
0.0 min	20 %	0%	80 %	0.4 mL/min
0.5 min	20%	0%	80%	0.4 mL/min
4.0 min	60%	0%	40%	0.4 mL/min
6.5 min	60%	0%	40%	0.4 mL/min
7.0 min	0.5%	59.5%	40%	0.5 mL/min
13.0 min	0.1%	34.9%	65%	0.5 mL/min
13.5 min	20%	0%	80%	0.5 mL/min
14.0 min	20%	0%	80%	0.5 mL/min

[0298] Mass detection was carried out using electrospray ionization in the negative mode (ESI spray voltage of 3.0 kV and ion transfer tube temperature of 390° C). The following m/z values for precursor ions were selected to detect the metabolites of interest in SRM mode: 245.0

for IPP and DMAPP, 313.1 for GPP, 381.1 for FPP, 227.0 for MVP, and 307.1 for MVPP. To account for small variations in sensitivity while running the mass spectrometer, uniformly labeled $^{13}C_{10}$-ADP was also added in equal amounts (final concentration of 19.6 uM) to both samples and calibrants as an internal standard ($^{13}C_{10}$-ADP was prepared enzymatically from $^{13}C_{10}$-ATP obtained from Isotec, Sigma-Aldrich; m/z = 436.1). Concentrations of metabolites were determined based on the sample/internal standard response ratio of integrated intensities of peaks generated by PO_3^- product ion (m/z =79.0). Calibration curves obtained by injection of standards were used to calculate concentrations of metabolites in cell extracts. IPP, DMAPP, GPP, and FPP standards were purchased from Echelon Biosciences Inc and MVP and MVPP (R-forms) were purchased from Sigma-Aldrich.

Results

[0299] MVP concentration using the upper MVA pathway from both *E. gallinarum* and *E. casselflavis* were higher than *E. faecalis* at 40 hours (**Figure 3).**

Example 10**:** Increased production of isoprene in strains containing the plasmids with alternative Upper mevalonate pathways compared to a pathway with *E. faecalis* Upper pathway.

(i) *Materials*

TM3 media recipe (per liter fermentation media):

[0300] 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)

[0301] 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*

[0302] 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*:

[0303] When the strains containing pMCM1223 (*L. grayi*), pMCM1224 (*E. faecium*), pMCM1225 (*E.* gallinarum), or pCHL277 (*E. casseliflavus*) are compared to the same background containing pCHL276 (*E. faecalis*), increased specific productivity, yield, CPI and/or titer of isoprene are observed.

Example 11: Isoprene production from *E. coli* expressing upper MVA pathway genes

[0304] This example evaluated isoprene production in *E. coli (BL21)* expressing introduced genes from the mevalonate pathway and grown in fed-batch culture at the 15-L scale. The genes for the upper MVA pathway enzymes came from either *E. faecalis* (strain DW709 and DW717), *E.casseliflavus* (DW718) or *E.gallinarum* (DW719, MCM2158).

(i) Materials and Methods

[0305] **Strain construction:** Strains DW709, DW717, DW718, and DW719 were generated by co-transformation of a plasmid harboring an isoprene synthase (IspS) variant and one of four plasmids harboring different upper MVA pathways into a production host strain of *Escherichia coli*. Following standard molecular biology techniques, the host strain CMP1133 (BL21 Δpgl PL.2mKKDyI GI1.2gltA yhfSFRTPyddVIspAyhfS thiFRTtruncIspA) was

electroporated with pDW240 (pTrc P. alba IspS MEA -mMVK (Carb50)), carrying an *IspS* variant, and either pMCM82 (U.S. Patent Application Publication No.: 2009/0203102), pCHL276 (*pCL_pTrc-Upper(E. faecalis)-leaderless)*, pCHL277 (*pCL_pTrc-Upper(E. casseliflavus)-leaderless)*, or pMCM1225 (*see* Table 7). Cells were recovered and plated on selective medium, and individual transformants, resistant to spectinomycin and carbenicillin, resulted in strains DW709, DW717, DW718, and DW719. These isoprene production strains expressed an IspS variant and either the upper MVA pathway from *Enterococcus faecalis*, the leaderless upper MVA pathway from *Enterococcus faecalis*, the upper MVA pathway from *Enterococcus casseliflavus*, or the upper MVA pathway from *Enterococcus gallinarum*, respectively (*see* Table 15).

Table 15: isoprene-producing strains

Strain name	**genotype**	**Host parent**	**plasmids**
DW709	BL21 GI1.2gltA PL.2 MKKDyI t pgl pgl-, yhfSFRTPyddVIspAyhfS thiFRTtruncIspA, pTrc(IspS variant)_mMVK, pCLPtrcUpper_E.faecalis	CMP1133	pDW240, pMCM82
DW717	BL21 GI1.2gltA PL.2 MKKDyI t pgl pgl-, yhfSFRTPyddVIspAyhfS thiFRTtruncIspA, pTrc(IspS variant)_mMVK, pCLPtrcUpper_E.faecalis_leaderless	CMP1133	pDW240, pCHL276
DW718	BL21 GI1.2gltA PL.2 MKKDyI t pgl pgl-, yhfSFRTPyddVIspAyhfS thiFRTtruncIspA, pTrc(IspS variant)_mMVK, pCLPtrcUpper_E.casseliflavus	CMP1133	pDW240, pCHL277
DW719	BL21 GI1.2gltA PL.2 MKKDyI t pgl pgl-, yhfSFRTPyddVIspAyhfS thiFRTtruncIspA, pTrc(IspS variant)_mMVK, pCLPtrcUpper_E.gallinarum	CMP1133	pDW240, pMCM1225
MCM2158	pgl- FRT-PL.2-2cis-RBS10000-MVK(burtonii) + pTrcAlba-MVKdel2 + pCL-Ptrc-Upper_Egallinarum	CMP1133	pDW240

[0306] **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.

[0307] **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.

[0308] **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.

[0309] **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.

[0310] **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.

[0311] 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. The isoprene producing strains were run in a fed-batch fermentation process.

[0312] 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 10 g/min. The fermentation was run long enough to determine the maximum isoprene mass yield on glucose, a total of 64 to 68 hrs elapsed fermentation time.

[0313] **Analysis:** 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. Dissolved Oxygen in the fermentation broth is measured by sanitary, sterilizable probe with an optical sensor provided Hamilton Company.

[0314] 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.

(ii) Results

Table 16: Isoprene productivity metrics

Strain description / Run Number	Overall Isoprene Volumetric Productivity (g/L/hr) (at peak yield)	Peak Overall % Yield of Isoprene on glucose (g/g)	Peak Specific Productivity (mg isoprene /L/hr/OD)
DW709 / 20120108	1.89	16.35	26.0
DW717 / 20120131	1.97	16.46	27.7
DW718 / 20120132	2.44	17.54	37.6

DW719 / 20120133	2.38	18.16	34.3
MCM2158 / 20120409	2.11	17.35	38.6

[0315] As summarized in Table 16, compared to fermentations using the upper MVA pathway of *E. faecalis,* fermentations using either *E. gallinarum* or *E. casseliflavus* upper MVA pathway enzymes exhibited overall higher mass yield (**Figure 4),** higher peak volumetric productivity (**Figure 5**), higher peak specific productivity (**Figure 6).** Additionally, acetyl Co-A levels in the cells were lower when the strain harbored an *E. casseliflavus* or an *E. gallinarum* pathway (Table 17). This reduction is acetyl-CoA levels is indicative of increased carbon flux into the MVA pathway in cells.

Table 17: Acetyl-CoA levels (mM) at around 24h of Elapsed Fermentation Time (EFT) in strains of identical background but with different Upper mevalonate pathway having upper MVA pathways from *E. gallinarum* or *E. casseliflavus*.

Upper	*E. faecalis* (DW717) – 20 h	*E. casseliflavus* (DW718) -24 h	*E. gallinarum* (DW719) – 24 h
Acetyl-CoA (mM)	6.34	3.57	3.56

Example 12: Growth and isoprene productivity of *E. coli* strains expressing *M. burtonii* or *M. mazei* mevalonate kinase on the *E. coli* chromosome

[0316] 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

[0317] **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 18: List of the engineered *E. coli* strains examined at small scale

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

[0318] **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.

[0319] **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

[0320] 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 it is able to support high carbon flux from the upper pathways.

[0321] 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 7**).

Example 13: Plasmid and chormosomal expression of *M. mazei* and *M. burtonii* mevalonate kinases in *E. coli.*

[0322] Strains MCM2126 and MCM2127 were run to determine the effect of expressing the *Mazei* MVK off of the chromosome only.

Materials and Methods

(i) Solutions

[0323] **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.

[0324] **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.

[0325] **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.

[0326] **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.

[0327] **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

[0328] 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^{TM} 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

[0329] Expression of *M. burtonii* mevalonate kinase in MCM2125 is at least 15 fold lower than expression of *M. mazei* mevalonate kinase in DW708 strain, based on protein quantitation by western blot analysis (**Figure 8**).

Example 14: Expression Constructs and *Lactobacillus* Strains Producing Mevalonate

I. Construction of plasmids encoding the Upper MVA pathway

[0330] The vector pDM20 is an *E. coli-Lactobacillus* shuttle vector (U.S. Patent Appl. Publication No. 2010/0081182) which is herein incorporated by reference. The vector contains a minimal pLF1 replicon (~0.7 Kbp) and pemK-pemI toxin-antitoxin(TA) from *Lactobacillus plantarum* ATCC14917 plasmid pLF1, a P15A replicon from pACYC184, a chloramphenicol resistance marker for selection in both *E. coli* and *L. plantarum*, and a P30 synthetic promoter (Rud et al., *Microbiology* (2006) 152:1011-1019).

[0331] The pDM20 plasmid is modified by adding the rrnBT1T2 terminator from pTrc99a into the multiple cloning site downstream of the P30 promoter. The terminator region is amplified from pTrc99a with Phusion High Fidelity DNA Polymerase (New England Biolabs, Beverly, MA) using primers T1T2_F_Hind3_Sal (SEQ) and T1T2 R_Pst (SEQ)

[0332] Amplification is according to the manufacturer's protocol with HF Buffer in a 50 µl reaction. The cycle parameters are 98 °C for 30 seconds, then 30 cycles of 98 °C for 10 seconds, 55 °C for 30 seconds, 72 °C for 10 seconds, and a final extension at 72 °C for 10 minutes. The PCR product of the terminator is purified with DNA Clean and Concentrator-5 Kit (Zymo Research Corp., Irvine, CA) following the manufacturer's protocol.

[0333] The PCR product and pDM20 are each sequentially digested with HindIII and then PstI (NEB). The digested insert and vector are purified with a DNA Clean and Concentrator-5 Kit (Zymo Research Corp)

[0334] The insert and vector are ligated in a 20 µl volume using a Quick Ligation kit (NEB) according to manufacturer's instructions. The ligation is transformed into TOP10 chemically competent cells (Invitrogen Corp, Carlsbad, CA) according to manufacturer's protocols. The cells and ligation are mixed and incubated on ice for 30 minutes, then the cells are heat-shocked 42°C for 45 seconds, followed by a 2 minute incubation on ice. SOC medium is added to the cells and the cells are then placed at 37 °C with shaking (220 rpm) for 1 hour. Cells are plated onto LB plates containing 25 µg/mL chloramphenicol (Sigma-Aldrich, St. Louis, MO). Transformant colonies are sequenced. After confirmation of the sequence, the plasmid pDM20_T is prepared using a Qiaprep Mini Kit (Qiagen Inc, Valencia, CA).

[0335] The upper mevalonate pathway comprising two genes, *mvaE* and *mvaS* from *Enterococcus faecalis* is PCR amplified using primers UP_EF_BamHI (SEQ) and UP_EF R Xho (SEQ) from template pCL-PtrcUpper (pCHL276). The resulting PCR product is digested with BamHI and XhoI and gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corp).

[0336] The genes encoding *mvaE* and *mvaS* from *Enterococcus gallinarum* from template pCL-Ptrc-upper Gc-MM163 are PCR amplified using primers UP_EF_BamHI (SEQ) and UP_EG R Xho (SEQ). The resulting PCR product is digested with BamHI and XhoI and gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corp).

[0337] Vector pDM20_T is double-digested with BamHI and SalI and purified with DNA Clean and Concentrator-5 Kit (Zymo Research Corp).

[0338] The digested vector pDM20_T and the UP_EF fragment are ligated using the Quick Ligation Kit (NEB) according to kit instructions. The ligation is transformed into TOP10 chemically competent cells (Invitrogen) and plated onto LB plates containing 25 µg/mL chloramphenicol. Transformants are sequenced. The resulting plasmid is designated pDM20_T_EF

[0339] The digested vector pDM20_T and the UP_EG fragment are ligated using the Quick Ligation Kit (NEB) according to kit instructions. The ligation is transformed into TOP10 chemically competent cells (Invitrogen) and plated onto LB plates containing 25 µg/mL

chloramphenicol. Transformants are sequenced. The resulting plasmid is designated pDM20_T_EG.

II. Creation of *Lactobacillus* Strains Expressing the Upper Mevalonate Pathway

[0340] Plasmids pDM20_T, pDM20_T_UP_EF and pDM20_T_UP_EG are transformed into *Lactobacillus plantarum* PN0512 (ATCC strain # PTA-7727; (U.S. Patent Applciation Publication No.: 2008/0124774 A1) by the following procedure as described in U.S. Patent Applciation Publication No.: 2011/0244536 A1. 5 ml of *Lactobacilli* MRS medium (Becton Dickenson, Sparks, MD) containing 1% glycine (Sigma-Aldrich, St. Louis, MO) is inoculated with PN0512 cells and grown overnight at 30°C. 100 ml MRS medium with 1% glycine is inoculated with overnight culture to an OD600 of 0.1 and grown to an OD600 of 0.7 at 30°C. Cells are harvested at 3700xg for 8 min at 4 °C, washed with 100 ml cold 1 mM $MgCl_2$ (Sigma-Aldrich, St. Louis, MO), centrifuged at 3700xg for 8 min at 4 °C, washed with 100 ml cold 30% PEG-1000 (Sigma-Aldrich, St. Louis, MO), then centrifuged at 3700xg for 20 min at 4 °C, then resuspended in 1 ml cold 30% PEG-1000. 60 µl cells are mixed with ~100 ng plasmid DNA in a cold 1 mm gap electroporation cuvette and electroporated in a BioRad Gene Pulser (Hercules, CA) at 1.7 kV, 25 µF, and 400 Ω. Cells are resuspended in 1 ml MRS medium containing 500 mM sucrose (Sigma-Aldrich, St. Louis, MO) and 100 mM $MgCl_2$, incubated at 30°C for 2 hrs, plated on MRS medium plates containing 10 µg/ml of chloramphenicol (Sigma-Aldrich, St. Louis, MO), then incubated at 30°C.

III. Testing for Mevalonate

[0341] The purpose of this example is to demonstrate the increased production of mevalonate in *Lactobacillus* strains with the *E. gallinarum* upper mevalonate pathway compared to strains carrying the upper mevalonate pathway from *Enterococcus faecalis.*

(i) Materials and Methods

[0342] **Cell culture:** *L. plantarum* PN0512 carrying, pDM20_T_UP_EF and pDM20_T_UP_EG as well as an empty control plasmid, pDM20_T are grown overnight in 20 mls of Lactobacilli MRS medium supplemented with 10 µg/ml chloramphenicol. Cultures are incubated for 14 hours overnight at 30°C. The overnight cultures are diluted into a 5 mL 48-well plate (Axygen Scientific) containing 2 mL MRS supplemented with 10 µg/ml chloramphenico to

final OD of 0.2. The plate is sealed with a Breath Easier membrane (Diversified Biotech) and incubated at 30°C in a Shel Lab shaker/incubator at 600 rpm for 24 hours. One mL of each culture is centrifuged at 3,000 x g for 5 min. 250 µl of supernatant is added to 19 µL of 20% sulfuric acid and incubates on ice for 5 min. The mixture is then centrifuged for 5 min at 3000 x g and the supernatant collected for HPLC analysis. 200 µl of supernatant is transferred to a HPLC compatible 96-well conical bottom polypropylene plate (Nunc). The concentration of mevalonate in samples is determined by comparison to a standard curve of mevalonate (Sigma). The glucose concentration is measured by performing a glucose oxidase assay according to the manufacturer's specifications (Pointe Scientific, Inc.).

[0343] **HPLC detection of mevalonate:** HPLC analysis is 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 are run at a flow rate of 0.6 ml/min in 0.01 N sulfuric acid running buffer. Broth levels of mevalonate are quantified by comparing the refractive index response of each sample versus a calibration curve generated by running various mevalonate containing solutions of known concentration.

(ii) Results

[0344] The specific productivity of mevalonate from *Lactobacillus* strains expressing the upper mevalonic pathway encoded by genes from *E. gallinarum* is compared to a strain that expresses the upper mevalonate pathway encoded by genes from *Enterococcus faecalis*. The bacteria were grown under identical conditions. HPLC analysis will show that the strain with the *E. gallinarum* upper pathway has higher specific productivity of isoprene compared to the strain with the upper pathway from *Enterococcus faecalis.* Both strains expressing the upper pathway from *E gallinarum* or *Enterococcus faecalis* will produce more mevalonate than the strain with an empty control plasmid.

Example 15: Construction of Strains for Producing Isoprene in *Lactobacillus*

[0345] Production of isoprene in *Lactobacillus* requires integrating the lower mevalonate pathway consisting of MVK, yPMK, MVD genes into the chromosome of *Lactobacillus*. The

genes encoding isoprene synthase and IDI are cloned as an operon under the control of the PldhL promoter onto a plasmid. The Upper pathway genes (*mvaE* and *mvaS*) are cloned as an operon onto the same plasmid under the control of the P30 promoter. The plasmid carrying the IspS-IDI operon and Upper pathway operon is transformed into *Lactobacillus* with an integrated lower mevalonate pathway.

I. Cloning IspS_IDI operon into *E.coli-Lactobacillus* shuttle vector

[0346] The PldhL promoter is amplified from template pDM5-PldhL1-ilvC (United States Patent Application Publication No.: 2011/0136192) with primers PldhL F (SEQ) and PldhL R (SEQ) using Phusion High Fidelity DNA Polymerase in a 50 µl reaction with HF buffer according to kit instructions. The annealing temperature is 55 °C and extension at 72 °C is for 10 seconds. The PCR reaction is cleaned with the DNA Clean and Concentrator-5 Kit (Zymo Research Corp) following kit protocol. The purified PCR product and the vector pDM20_T1 are digested with PstI at 37 °C for 2 hours. The vector and insert are incubated at 80 °C for 20 minutes to inactivate PstI. The PstI digested vector is treated with Shrimp Alkaline Phosphatase (Affymetrix Inc, Santa Clara, CA) at 37 °C for 30 minutes. The phosphatase reaction is stopped by heating at 65 °C for 15 minutes. Both the treated vector and digested PCR product are cleaned with DNA Clean and Concentrator-5 Kit (Zymo Research Corp) following kit protocol.

[0347] The digested PCR product and vector are ligated using the Quick Ligation kit (NEB). The ligation mixture is transformed into chemically competent E. coli Top10 cells (Invitrogen Corp, Carlsbad, CA). Transformants are selected on LB plates containing 25 µg/mL chloramphenicol at 37 °C. Transformants are screened by DNA sequencing. The resulting plasmid is called pDM20_T_PldhL.

[0348] The *Populus alba ispS* gene and *yIDI* gene are synthesized codon optimized for *Lactobacillus* by Gene Oracle Inc. (Mountain View, CA). The genes are synthesized as an operon with *Lactobacillus* ribosome binding sites preceding each of the ATG starts of the genes. The *IspS-yIDI* operon is cloned into pCR Blunt II TOPO (Invitrogen) creating pCR Blunt II TOPO-II.

[0349] The *ispS-yIDI* operon is amplified from pCR Blunt II TOPO-II with primers II F Avr2 (SEQ) and II R Nde (SEQ) with Phusion High Fidelity DNA Polymerase using an annealing of 55 C and an extension of 1 minute.

[0350] Vector pDM20_T_PldhL and ispS-yIDI PCR product are digested with AvrII and NdeI. The vector and insert is ligated and transformed into TOP10 cells. Transformants are selected on LB plates containing 25 μg/mL chloramphenicol at 37 °C. Transformants are verified by DNA sequencing. The resulting plasmid is called pDM20_T_Pldh-II.

II. Addition of Upper pathway to Plasmid with *IspS-yIDI*

[0351] Vector pDM20_T_Pldh-II is digested with BamHI and SalI. The digested vector is ligated with the BamHI and XhoI digested EF_UP PCR product (see Example 14). The digested vector is also ligated with the BamHI and XhoI digested EG_UP PCR product (see Example 14). The ligations are transformed into Top10 cells and plated on LB plates containing 25 μg/mL chloramphenicol for selection at 37 °C. The resulting plasmids are named pDM20T-EF-Pldh-II and pDM20T-EG-Pldh-II.

III. Construction of the lower pathway integration vector and PN0512ΔldhL1:: MVK-yPMK-MVD integration strain

[0352] This describes integration of the lower MVA pathway genes into the chromosome of *L. plantarum* strain PN0512 for expression of MVK, yPMK, MVD. Genes may be integrated into different locations in the chromosome, including neutral locations that have no effect on cellular metabolism or integrations may be designed to change the physiology of the cell.

[0353] Two DNA segments (homologous arms) are designed to provide regions of homology for the two genetic cross-overs such that integration would place the MVK, yPMK, MVD, coding region downstream of the *ldhL1* promoter in strain PN0512. The left and right homologous arms cloned into the plasmid are each approximately 1200 base pairs. The left and right homologous arms are amplified from *L. plantarum* PN0512 genomic DNA. The construction of integration vector pFP996-ldhL1-arms is described in U.S. Patent Application Publication No.: 2011/0244536 A1, which is herein incorporated by reference.

[0354] The lower pathway genes are PCR amplified using genomic DNA from *E. coli* MVKCMP451, (which contains the coding sequence for MVK, yPMK, MVD, and yIDI) as template to amplify MPM operon. The genomic DNA is purified from a 1 ml cell pellet of culture grown in LB to stationary phase at 37°C using Gentra Pure Gene Kit (Qiagen Inc., Valencia, Ca). The MPM operon is created by primers MPMI Xho Spe For (Table 23) containing a Xho I site, a Spe I site, and ribosome binding sequence and MPM Pme Xho Rev1 (Table 23) containing a Pme I and Xho I site, using Phusion High Fidelity PCR Kit (New England Biolabs). A typical PCR reaction (50 µl) contains 1X HF Buffer, 1 µl 10mM dNTPs, 2.5 µl 10 µM each primer, 0.5 µl Phusion polymerase, and 250 ng genomic DNA. The cycling conditions are: 98°C. for 30 seconds for one cycle, followed by 30 cycles of 98°C for 10 seconds, 56°C for 30 seconds, 72°C for 2 minutes 20 seconds. Following cycling, the reaction mixtures are held at 72°C for 10 minutes. Reaction is cleaned using Zymo Clean and Concentrate-5 kit (Zymo Research). The resulting PCR fragment is restriction endonuclease digested with XhoI (New England Biolabs) at 37°C. The reaction is cleaned using Zymo Clean and Concentrate-5 kit (Zymo Reseach).

Table 18: Primers

Name	Sequence
T1T2_F_Hind3_Sal	CATAAGCTTGTCGACCCATGCGAGAGTAGGGAACTGCC
T1T2 R_Pst	CATCTGCAGTCTCATGAGCGGATACATATTTGAA
UP_EF_BamHI	CATGGATCCCGATTAAATAAGGAGGAATAAACC
UP_EF R Xho	GTCACTCGAGGGTACCAGCTGCAGATCTCTTAG
UP_EG R Xho	GTCACTCGAGCATATGGTACCAGCTGCAGTCA
PldhL F	CATCTGCAGTAAGTCGTATTGGCACCACTACTCAC
PldhL R	CATCTGCAGCATATGATCCTAGGGCTTGACAAAATAAGT CATCCTCTC
II F Avr2	CATCCTAGGAGGAGGAGAAAAAAAACCATG
II R Nde	CATCATATGTTACAACATTCTGTGAATTTGTCG
MPMI Xho Spe For	CAATCTCGAGACTAGTCAAAGGAGGTAAAAAAACATGG TATC
MPM Pme Xho Rev1	GTTACTCGAGGTTTAAACTTATTCCTTTGGTAGACCAGTC TTTG
MPMIseqF5	GTGGCCTGGGAAATGGGAAAAGCTG
ldhseqR3	CCCCCAATCATAAGTCCACGTTTA
MPMIseqF3	CAGATATTGGAAGTGCTACTTACGGC
MPMIseqR4	TGCGGTAACGGATGCTGTGTAAACGG

ldhL left arm check UP	CAACCGAGGTCACGACCACTGCCG
MPMIseqR8	GAACACGGGTACGCAGTTCCACCG
MPMIseqF6	GATGTTGCCAGAGTGATTTTAACTC
ldhL right arm check DN	GAAACTGGTTGGGAATAACTTGAGCC

[0355] The pFP996-ldhL1arms vector is restriction digested with XhoI (New England Biolabs). After digest, XhoI is heat inactivated at 65°C for 20 min. Vector ends are then dephosphorylated using Shrimp Alkaline Phosphatase (Affymetrix). The reaction is incubated at 37°C for 45 minutes, then the phosphatase is heat inactivated at 65°C for 15 min. Vector is purified from an agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research Corp.)

[0356] The resulting Xho-I digested, dephosphorylated vector pFP996ldhL1arms and the XhoI-digested MPM fragment are ligated using Quick Ligation Kit (NEB) at 25°C for 5 min. Chemically competent *E. coli* Stbl3 (Invitrogen) cells are transformed with ligation mix. A typical transformation includes incubation of cells and ligation mix for 30 minutes on ice, a heat shock at 42°C for 45 seconds, 2 minute incubation on ice, and recovery in SOC media for 1 hour at 30 C. The transformation is spread onto LB agar containing ampicillin (100 µg/ml) for selection. Incubate plates overnight at 30°C.

[0357] Transformants are PCR colony screened with primers MPMIseqF5 (Table 18) and ldhseqR3 (Table 18) using JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma-Aldrich, Inc., St. Louis Mo). Several positive transformants are verified by DNA sequencing. The resulting integration plasmid is designated pFP996-ldhL1arms::MPM.

[0358] Plasmid DNA is isolated from cell pellets of overnight growth in LB containing ampicillin (100 µg/ml) from the *E. coli* Stbl3 /pFP996-ldhL1arms::MPM strains using Qiaprep Mini Kit (Qiagen Inc, Valencia, CA).

[0359] The MPM operon is integrated into the chromosome of the *Lactobacillus plantarum* PN0512 strain such that it would be expressed from the *ldhL1* promoter and *ldhL1* would be deleted. The chromosomal integration of a single copy of MVK, yPMK, MVD, coding region expressed from the *ldhL1* promoter is constructed by the same two-step homologous

recombination procedure used for unmarked deletions, as described (Ferain et al., 1994, *J. Bact.* 176:596), except that the second crossover event yields either the wild type sequence or the intended integration rather than a deletion.

[0360] Integration of the MVK, yPMK, MVD coding region is obtained by transforming *L. plantarum* PN0512 with pFP996-ldhL1arms:: MPM . A culture with 5 ml of Lactobacilli MRS medium containing 0.5% glycine is inoculated with PN0512 and grown overnight at 30°C. 100 ml MRS medium with 0.5% glycine is inoculated with overnight culture to an OD_{600} of 0.1 and grown to an OD_{600} of 0.7 at 30°C. Cells are harvested at 3700xg for 8 min at 4 °C, washed with 100 ml cold 1 mM $MgCl_2$, centrifuged at 3700xg for 8 min at 4 °C, washed with 100 ml cold 30% PEG-1000, recentrifuged at 3700xg for 20 min at 4 °C, and then resuspended in 1 ml cold 30% PEG-1000. 60 µl of cells are mixed with ~100 ng of plasmid DNA in a cold 1 mm gap electroporation cuvette and electroporated in a BioRad Gene Pulser at 1.7 kV, 25 µF, and 400 Ω. Cells are resuspended in 1 ml MRS medium containing 500 mM sucrose and 100 mM $MgCl_2$, incubated at 30°C for 2 hrs, and then spread on MRS medium plates containing 2 µg/ml of erythromycin (Sigma-Aldrich, St. Louis, MO).

[0361] Transformants are screened by PCR using operon specific primers MPMIseqF3 (Table 18) and MPMIseqR4 (Table 18). Transformants are grown at 30°C in *Lactobacilli* MRS medium containing erythromycin (1 µg/ml) for approximately 10 generations and then at 37°C for approximately 40 generations by serial inoculations in Lactobacilli MRS medium. The culture is spread on Lactobacilli MRS medium with erythromycin (0.5 µg/ml). The isolates are screened by colony PCR for a single crossover with chromosomal specific primer *ldhL* left arm check DN (Table 18) and plasmid specific primer MPMIseqR8 (Table 18).

[0362] Single crossover integrants are then grown at 37°C for approximately 40 generations by serial inoculations in *Lactobacilli* MRS medium. The cultures are spread on MRS medium. Colonies are patched to MRS plates and grown at 37°C. The isolates are then patched onto MRS medium with erythromycin (0.5 µg/ml). Erythromycin sensitive isolates are screened by colony PCR for the presence of a wild-type or integration second crossover using chromosomal specific primers and gene specific primer pairs, ldhL left arm check UP and MPMIseqR8 will yield an approximately 1400-bp product; MPMIseqF6 and *ldhL* right arm check DN. will yield an approximately 1600-bp product; The integration is confirmed by

sequencing the PCR product and an identified integration strain is designated PN0512ΔldhL1:: MPM.

IV. Creation of LAB Strains Producing Isoprene

[0363] *Lactobacillus plantarum* PN0512ΔldhL1:: MPM is made electrocompetent as described above and transformed with either pDM20T- EF-Pldh-II and pDM20T EG-Pldh-II. Cells are plated onto MRS with 10 μg/ml chloramphenicol.

Example 16: Testing for Isoprene Production in *Lactobacillus*

[0364] The purpose of this example is to demonstrate the increased production of isoprene in *Lactobacillus* strains with the *E. gallinarum* upper mevalonate pathway compared to strains carrying the upper mevalonate pathway from *Enterococcus faecalis.*

(i) Materials and Methods

[0365] *L. plantarum* PN0512ΔldhL1:: MPM containing pDM20T- EF-Pldh-II or pDM20T EG-Pldh-II is inoculated in MRS medium supplemented with 10 μg/mL chloramphenicol and grown at 30 °C for 14 h. Isoprene production is analyzed by growing the strains in a Cellerator TM from MicroReactor Technologies, Inc. The working volume in each of the 24 wells is 4.5 ml. The overnight cultures are diluted into 4.5 ml of MRS with 10 μg/mL chloramphenicol to reach an optical density of 0.05 measured at 550 nm. The temperature is maintained at 30 °C, the pH setpoint was 7.0, oxygen flow setpoint is 20 sccm and the agitation rate is 800 rpm.

[0366] Off-gas analysis of isoprene is performed using a gas chromatograph-mass spectrometer (GC-MS) (Agilent) headspace assay. Sample preparation is as follows: 100 μL of whole broth is placed in a sealed GC vial and incubated at 30° C. for a fixed time of 30 minutes. Following a heat kill step, consisting of incubation at 70° C. for 5 minutes, the sample is loaded on the GC.

[0367] Optical density (OD) at a wavelength of 550 nm is obtained using a microplate reader (Spectramax) during the course of the run. Specific productivity is obtained by dividing the isoprene concentration (μg/L) by the OD reading. Samples are taken at three time points for each of the 24-wells over the course of the mini-fermentations.

(ii) Results

[0368] The specific productivity of isoprene from strains expressing the full mevalonic acid pathway with the upper pathway encoded by genes from *E. gallinarum* is compared to a strain that is expressing the full mevalonic acid pathway that carries the upper mevalonate pathway from *Enterococcus faecalis.* The bacteria were grown under identical conditions in mini-fermentations. Headspace measurements over time (*see* U.S. Patent Application Publication No.: 2010/0086978) show that the strain with the *E. gallinarum* upper pathway has higher specific productivity of isoprene compared to the strain with the upper pathway from *Enterococcus faecalis.*

Example 17: Construction of amorphadiene- or farnesene-producing strains

[0369] 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.

Example 18: Increased production of amorphadiene or farnesene in strains containing the plasmids with alternative Upper mevalonate pathways compared to a pathway with E. faecalis Upper pathway.

(i) *Materials*

TM3 media recipe (per liter fermentation media): K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, $MgSO_4*7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 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): 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*

[0370] 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).

[0371] 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*

[0372] When the strains containing pMCM1223 (*L. grayi*), pMCM1224 (*E. faecium*), pMCM1225 (*E. gallinarum*), or pCHL277 (*E. casseliflavus*) 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*

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.

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

[0373] 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 (**Figure 9**). Cleaved fragments of *E. faecalis* MvaE are also identified by Safestain staining of His-tagged purified material run on an SDS-PAGE gel (**Figure 10**). Identification and use of degradation resistant *mvaE* gene products are beneficial for the increased production of mevalonate, isoprene and isoprenoids.

[0374] 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*:

[0375] 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*

[0376] Enfors, S.O., Scheper, T. Physiological Stress Responses in Bioprocesses. Springer-Verlag Berlin Heidelberg 2004.

SEQUENCES

L.grayi mvaE:

atggttaaagacattgtaataattgatgccctccgtactcccatcggtaagtaccgcggtcagctctcaaagatgacggcggtggaattgggaaccgcagttacaaaggct
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L.grayi mvaS:

atgaccatgaacgttggaatcgataaaatgtcattctttgttccaccttactttgtggacatgactgatctggcagtagcacgggatgtcgatcccaataagtttctgattggtat
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E. faecium mvaE:

atgaaagaagtggttatgattgatgcggctcgcacacccattgggaaatacagaggtagtcttagtccttttacagcggtggagctggggacactggtcacgaaagggct
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ttctttagcgatttcgataggtgccatcggtgaggagatagagcaagtcgcgaaaaaactgcgtgaagctgaaaaaatgaatcagcaaacggcaatacagattttagaaa
aaattcgcgagaaatga **SEQ ID NO:3**

E. faecium mvaS:
atgaaaatcggtattgaccgtctgtccttcttcatcccgaatttgtatttggacatgactgagctggcagaatcacgcggggatgatccagctaaatatcatattggaatcgga
caagatcagatggcagtgaatcgcgcaaacgaggacatcataacactgggtgcaaacgctgcgagtaagatcgtgacagagaaagaccgcgagttgattgatatggta
atcgttggcacggaatcaggaattgaccactccaaagcaagcgccgtgattattcaccatctccttaaaattcagtcgttcgcccgttctttcgaggtaaaagaagcttgcta
tggcggaactgctgccctgcacatggcgaaggagtatgtcaaaaatcatccggagcgtaaggtcttggtaattgcgtcagacatcgcgcgttatggtttggccagcgga
ggagaagttactcaaggcgtgggggccgtagccatgatgattacacaaaaccccccggattctttcgattgaagacgatagtgtttttctcacagaggatatctatgatttctg
gcggcctgattactccgagttccctgtagtggacgggcccctttcaaactcaacgtatatagagagttttcagaaagtttggaaccggcacaaggaattgtccggaagag
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gctggatagccggactcggattacggtcgatgaatacgagaccatcttttcagagactctgccagaacatggtgaatgcgccgaatatacgagcgacgtccccttttctat
aaccaagattgagaacgacattcgttattataaaatctga **SEQ ID NO:4**

E. gallinarum mvaE:
atggaagaagtggtaattatagatgcacgtcggactccgattggtaaatatcacgggtcgttgaagaagttttcagcggtggcgctggggacggccgtggctaaagacat
gttcgaacgcaaccagaaaatcaaagaggagatcgcgcaggtcataattggtaatgtcttgcaggcaggaaatggccagaaccccgcgcggcaagttgctcttcaatc
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ggtggtgcaacggtggcccagaaaattgtgcaggcgtcgctcttcgcaaagacagacccataccgcgcagtgacccacaacaaagggattatgaacggtgtagaggc
tgttatgcttgccacaggcaacgacacgcgcgcagtctcagccgcttgtcatggatacgcagcgcgcaccggtagctatcagggtctgactaactggacgattgagtcg
gatcgcctggtaggcgagataacactgccgctggccatcgctacagttggaggcgctaccaaagtgttgcccaaagctcaagcggcactggagattagtgatgttcact
cttctcaagagcttgcagccttagcggcgtcagtaggtttagtacaaaatctcgcggccctgcgcgcactggtttccgaaggtatacaaaaagggcacatgtccatgcaa
gcccggtctctcgcaatcgcggtcggtgctgaaaaagccgagatcgagcaggtcgccgaaaagttgcggcagaacccgccaatgaatcagcagcaggcgctccgttt
tcttggcgagatccgcgaacaatga **SEQ ID NO:5**

E. gallinarum mvaS:
atgaacgtcggcattgacaaaattaatttttttcgttccaccgtattatctggatatggtcgacctggcccacgcacgcgaagtggacccgaacaaatttacaattggaattgg
acaggatcagatggctgtgagcaaaaagacgcacgatatcgtaacattcgcggctagtgccgcgaaggaaatttttagaacctgaggacttgcaagctatagacatggtt
atagttggtaccgaatcgggcattgacgagagcaaagcatccgcggtcgttttacatcgtttgttgggcgtacaaccttttcgctcgcagttttgaaattaaagaagcctgtta
cggggcaaccgcaggcattcagtttgccaagactcatatacaagcgaacccggagagcaaggtcctggtaattgcaagcgatatagctcggtatggtcttcggtcaggt
ggagagcccacacaaggcgcaggggcagttgctatgcttctcacggcaaatcccagaatcctgaccttcgaaaacgacaatctgatgttaacgcaggatatttatgactt
ctggagaccacttggtcacgcttaccctatggtagatggccacctttccaatcaagtctatattgacagttttaagaaggtctggcaagcacattgcgaacgcaatcaagctt
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gcacggtatgaagagtctatcgtatattcacgccggatcggcaacttgtatacgggatcattgtacctggggctgatatccttattggaaaacagttctcacctgtcggcgg
gcgaccggataggattgtttagttatgggagtggcgctgtcagcgaatttttctccggtcgtttagtggcaggctatgaaaatcaattgaacaaagaggcgcataccagct

cctggatcagcgtcagaagctttccatcgaagagtatgaggcgatttttacagattccttagaaattgatcaggatgcagcgttctcggatgacctgccatattccatccgcg
agataaaaaacacgattcggtactataaggagagctga **SEQ ID NO:6**

E. casseliflavus mvaE:

atggaagaagttgtcatcattgacgcactgcgtactccaataggaaagtaccacggttcgctgaaagattacacagctgttgaactggggacagtagcagcaaaggcgtt
gctggcacgaaatcagcaagcaaaagaacacatagcgcaagttattattggcaacgtcctgcaagccggaagtgggcagaatccaggccgacaagtcagtttacagtc
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ttaacaggcggaattgaaagcatgaccaacgcgccgctgtttagttattacaacaaggctgaggatcaatattcggcgccggttagcacaatgatgcacgatggtctaac
agatgctttcagttccaaaccaatgggcttaaccgcagagaccgtcgctgagagatatggaattacgcgtaaggaacaagatgaatttgcttatcactctcaaatgaaggc
ggccaaagcccaggcggcgaaaaagtttgatcaggaaattgtacccctgacggaaaaatccggaacggttctccaggacgaaggcatcagagccgcgacaacagtc
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gaaaaatcaagcactgaccatagaggatataggaatatttgagattaatgaagcctttgctgcgagttcgattgtggtagaacgcgagttgggcctggaccccaaaaaagt
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gaagaagaagttttatgcgctagctcctaacgaacgcttagcgtttttggaagcccaaggcgctattaccgctgctgaaaccctggtcttccaggagatgaccttaaacaa
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ggaggaaccgtccgttatcgctgcgatgtcgaatggcgccaaaatggctggtcctattacaacaacaagtcaggagaggctgttacggggtcagattgtcttcatggacg
tacaggacccagaagcaatattagcgaaagttgaatccgagcaagctaccattttcgcggtggcaaatgaaacatacccgtctatcgtgaaaagaggaggaggtctgcg
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cagttccgtttgataaattgtccaaaactgggaatggtcgacaagtagctggtaaaatagtgcacgcggcggacttttgctaagatagatccatacagagctgccacacaca
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cgccctggcgctaactggcgttgagacggcgtcggaactggccagcctggcggcgagtgtgggattagttcaaaatttggccgctttacgagcactagtgagcgaggg
cattcagcaagggcacatgagtatgcaagctagatccctggccattagcgtaggtgcgaaaggtactgaaatagagcaactagctgcgaagctgagggcagcgacgc
aaatgaatcaggagcaggctcgtaaatttctgaccgaaataagaaattaa **SEQ ID NO:7**

E. casseliflavus mvaS:

atgaacgttggaattgataaaatcaatttttcgttccgccctatttcattgatatggtggatctcgctcatgcaagagaagttgaccccaacaagttcactataggaataggcc
aagatcagatggcagtaaacaagaaaacgcaagatatcgtaacgttcgcgatgcacgccgcgaaggatattctgactaaggaagatttacaggccatagatatggtaat
agtggggactgagtctgggatcgacgagagcaaggcaagtgctgtcgtattgcatcggcttttaggtattcagccttttgcgcgctcctttgaaattaaggaggcatgctat
ggggccactgccggccttcagtttgcaaaagctcatgtgcaggctaatccccagagcaaggtcctggtggtagcttccgatatagcacgctacggactggcatccggag
gagaaccgactcaaggtgtaggtgctgtggcaatgttgatttccgctgatccagctatcttgcagttagaaaatgataatctcatgttgacccaagatatatacgatttttggc
gcccggtcgggcatcaatatcctatggtagacggccatctgtctaatgccgtctatatagacagctttaaacaagtctggcaagcacattgcgagaaaaaccaacggact
gctaaagattatgctgcattgtcgttccatattccgtacacgaaaatgggtaagaaagctctgttagcggtttttgcggaggaagatgagacagaacaaaagcggttaatgg
cacgttatgaagaatcaattgtatacagtcgtcggactggaaatctgtatactggctcactctatctgggcctgatttccttactggagaatagtagcagtttacaggcgaacg
atcgcataggtctgtttagctatggttcaggggccgttgcggaatttttcagtggcctcttggtaccgggttacgagaaacaattagcgcaagctgcccatcaagctcttctg
gacgaccggcaaaaactgactatcgcagagtacgaagccatgtttaatgaaaccattgatattgatcaggaccagtcatttgaggatgacttactgtactccatcagagag
atcaaaaacactattcgctactataacgaggagaatgaataa **SEQ ID NO:8**

E. gallinarum EG2 (mvaE):

MEEVVIIDARRTPIGKYHGSLKKFSAVALGTAVAKDMFERNQKIKEEIAQVIIGNVLQAGNGQNPARQVA
LQSGLSVDIPASTINEVCGSGLKAILMGMEQIQLGKAQVVLAGGIESMTNAPSLSHYNKAEDTYSVPVSS
MTLDGLTDAFSSKPMGLTAENVAQRYGISREAQDQFAYQSQMKAAKAQAENKFAKEIVPLAGETKTITAD
EGIRSQTTMEKLASLKPVFKTDGTVTAGNASTINDGAALVLLASKTYCETNDIPYLATIKEIVEVGIDPE
IMGISPIKAIQTLLQNQKVSLEDIGVFEINEAFAASSIVVESELGLDPAKVNRYGGGISLGHAIGATGAR
LATSLVYQMQEIQARYGIASLCVGGGLGLAMLLERPTIEKAKPTDKKFYELSPAERLQELENQQKISSET
KQQLSQMMLAEDTANHLIENQISEIELPMGVGMNLKVDGKAYVVPMATEEPSVIAAMSNGAKMAGEIHTQ
SKERLLRGQIVFSAKNPNEIEQRIAENQALIFERAEQSYPSIVKREGGLRRIALRHFPADSQQESADQST
FLSVDLFVDVKDAMGANIINAILEGVAALFREWFPNEEILFSILSNLATESLVTAVCEVPFSALSKRGGA
TVAQKIVQASLFAKTDPYRAVTHNKGIMNGVEAVMLATGNDTRAVSAACHGYAARTGSYQGLTNWTIESD
RLVGEITLPLAIATVGGATKVLPKAQAALEISDVHSSQELAALAASVGLVQNLAALRALVSEGIQKGHMS
MQARSLAIAVGAEKAEIEQVAEKLRQNPPMNQQQALRFLGEIREQ **SEQ ID NO:9**

E. gallinarum EG2 (mvaS)

MNVGIDKINFFVPPYYLDMVDLAHAREVDPNKFTIGIGQDQMAVSKKTHDIVTFAASAAKEILEPEDLQA
IDMVIVGTESGIDESKASAVVLHRLLGVQPFARSFEIKEACYGATAGIQFAKTHIQANPESKVLVIASDI
ARYGLRSGGEPTQGAGAVAMLLTANPRILTFENDNLMLTQDIYDFWRPLGHAYPMVDGHLSNQVYIDSFK
KVWQAHCERNQASISDYAAISFHIPYTKMGKKALLAVFADEVETEQERVMARYEESIVYSRRIGNLYTGS
LYLGLISLLENSSHLSAGDRIGLFSYGSGAVSEFFSGRLVAGYENQLNKEAHTQLLDQRQKLSIEEYEAI
FTDSLEIDQDAAFSDDLPYSIREIKNTIRYYKES **SEQ ID NO:10**

L. grayi (mvaE):

MVKDIVIIDALRTPIGKYRGQLSKMTAVELGTAVTKALFEKNDQVKDHVEQVIFGNVLQAGNGQNPARQI
ALNSGLSAEIPASTINQVCGSGLKAISMARQQILLGEAEVIVAGGIESMTNAPSITYYNKEEDTLSKPVP
TMTFDGLTDAFSGKIMGLTAENVAEQYGVSREAQDAFAYGSQMKAAKAQEQGIFAAEILPLEIGDEVITQ
DEGVRQETTLEKLSLLRTIFKEDGTVTAGNASTINDGASAVIIASKEFAETNQIPYLAIVHDITEIGIDP
SIMGIAPVSAINKLIDRNQISMEEIDLFEINEAFAASSVVVQKELSIPDEKINIGGSGIALGHPLGATGA
RIVTTLAHQLKRTHGRYGIASLCIGGGLGLAILIEVPQEDQPVKKFYQLAREDRLARLQEQAVISPATKH
VLAEMTLPEDIADNLIENQISEMEIPLGVALNLRVNDKSYTIPLATEEPSVIAACNNGAKMANHLGGFQS
ELKDGFLRGQIVLMNVKEPATIEHTITAEKAAIFRAAAQSHPSIVKRGGGLKEIVVRTFDDDPTFLSIDL
IVDTKDAMGANIINTILEGVAGFLREILTEEILFSILSNYATESIVTASCRIPYEALSKKGDGKRIAEKV
AAASKFAQLDPYRAATHNKGIMNGIEAVVLASGNDTRAVAAAAHAYASRDQHYRGLSQWQVAEGALHGEI
SLPLALGSVGGAIEVLPKAKAAFEIMGITEAKELAEVTAAVGLAQNLAALRALVSEGIQQGHMSLQARSL
ALSVGATGKEVEILAEKLQGSRMNQANAQTILAEIRSQKVEL **SEQ ID NO:11**

L. grayi (mvaS):

MTMNVGIDKMSFFVPPYFVDMTDLAVARDVDPNKFLIGIGQDQMAVNPKTQDIVTFATNAAKNILSAEDL
DKIDMVIVGTESGIDESKASAVVLHRLLGIQKFARSFEIKEACYGGTAALQFAVNHIRNHPESKVLVVAS
DIAKYGLASGGEPTQGAGAVAMLVSTDPKIIAFNDDSLALTQDIYDFWRPVGHDYPMVDGPLSTETYIQS
FQTVWQEYTKRSQHALADFAALSFHIPYTKMGKKALLAILEGESEEAQNRILAKYEKSIAYSRKAGNLYT
GSLYLGLISLLENAEDLKAGDLIGLFSYGSGAVAEFFSGRLVEDYQEQLLKTKHAEQLAHRKQLTIEEYE
TMFSDRLDVDKDAEYEDTLAYSISSVRNTVREYRS **SEQ ID NO:12**

E. faecium (mvaE):

MKEVVMIDAARTPIGKYRGSLSPFTAVELGTLVTKGLLDKTKLKKDKIDQVIFGNVLQAGNGQNVARQIA
LNSGLPVDVPAMTINEVCGSGMKAVILARQLIQLGEAELVIAGGTESMSQAPMLKPYQSETNEYGEPISS
MVNDGLTDAFSNAHMGLTAEKVATQFSVSREEQDRYALSSQLKAAHAVEAGVFSEEIIPVKISDEDVLSE
DEAVRGNSTLEKLGTLRTVFSEEGTVTAGNASPLNDGASVVILASKEYAENNNLPYLATIKEVAEVGIDP
SIMGIAPIKAIQKLTDRSGMNLSTIDLFEINEAFAASSIVVSQELQLDEEKVNIYGGAIALGHPIGASGA
RILTTLAYGLLREQKRYGIASLCIGGGLGLAVLLEANMEQTHKDVQKKKFYQLTPSERRSQLIEKNVLTQ
ETALIFQEQTLSEELSDHMIENQVSEVEIPMGIAQNFQINGKKKWIPMATEEPSVIAAASNGAKICGNIC
AETPQRLMRGQIVLSGKSEYQAVINAVNHRKEELILCANESYPSIVKRGGGVQDISTREFMGSFHAYLSI
DFLVDVKDAMGANMINSILESVANKLREWFPEEEILFSILSNFATESLASACCEIPFERLGRNKEIGEQI
AKKIQQAGEYAKLDPYRAATHNKGIMNGIEAVVAATGNDTRAVSASIHAYAARNGLYQGLTDWQIKGDKL
VGKLTVPLAVATVGGASNILPKAKASLAMLDIDSAKELAQVIAAVGLAQNLAALRALVTEGIQKGHMGLQ
ARSLAISIGAIGEEIEQVAKKLREAEKMNQQTAIQILEKIREK **SEQ ID NO:13**

E. faecium (mvaS)

MKIGIDRLSFFIPNLYLDMTELAESRGDDPAKYHIGIGQDQMAVNRANEDIITLGANAASKIVTEKDREL
IDMVIVGTESGIDHSKASAVIIHHLLKIQSFARSFEVKEACYGGTAALHMAKEYVKNHPERKVLVIASDI
ARYGLASGGEVTQGVGAVAMMITQNPRILSIEDDSVFLTEDIYDFWRPDYSEFPVVDGPLSNSTYIESFQ
KVWNRHKELSGRGLEDYQAIAFHIPYTKMGKKALQSVLDQTDEDNQERLMARYEESIRYSRRIGNLYTGS
LYLGLTSLLENSKSLQPGDRIGLFSYGSGAVSEFFTGYLEENYQEYLFAQSHQEMLDSRTRITVDEYETI
FSETLPEHGECAEYTSDVPFSITKIENDIRYYKI **SEQ ID NO:14**

E. casseliflavus (mvaE):

MEEVVIIDALRTPIGKYHGSLKDYTAVELGTVAAKALLARNQQAKEHIAQVIIGNVLQAGSGQNPGRQVS
LQSGLSSDIPASTINEVCGSGMKAILMGMEQIQLNKASVVLTGGIESMTNAPLFSYYNKAEDQYSAPVST
MMHDGLTDAFSSKPMGLTAETVAERYGITRKEQDEFAYHSQMKAAKAQAAKKFDQEIVPLTEKSGTVLQD
EGIRAATTVEKLAELKTVFKKDGTVTAGNASTINDGAAMVLIASKSYCEEHQIPYLAVIKEIVEVGFAPE
IMGISPIKAIDTLLKNQALTIEDIGIFEINEAFAASSIVVERELGLDPKKVNRYGGGISLGHAIGATGAR
IATTVAYQLKDTQERYGIASLCVGGGLGLAMLLENPSATASQTNFDEESASEKTEKKKFYALAPNERLAF
LEAQGAITAAETLVFQEMTLNKETANHLIENQISEVEIPLGVGLNLQVNGKAYNVPLATEEPSVIAAMSN
GAKMAGPITTTSQERLLRGQIVFMDVQDPEAILAKVESEQATIFAVANETYPSIVKRGGGLRRVIGRNFS
PAESDLATAYVSIDLMVDVKDAMGANIINSILEGVAELFRKWFPEEEILFSILSNLATESLVTATCSVPF
DKLSKTGNGRQVAGKIVHAADFAKIDPYRAATHNKGIMNGVEALILATGNDTRAVSAACHGYAARNGRMQ
GLTSWTIIEDRLIGSITLPLAIATVGGATKILPKAQAALALTGVETASELASLAASVGLVQNLAALRALV
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E. casseliflavus (mvaS)

MNVGIDKINFFVPPYFIDMVDLAHAREVDPNKFTIGIGQDQMAVNKKTQDIVTFAMHAAKDILTKEDLQA
IDMVIVGTESGIDESKASAVVLHRLLGIQPFARSFEIKEACYGATAGLQFAKAHVQANPQSKVLVVASDI
ARYGLASGGEPTQGVGAVAMLISADPAILQLENDNLMLTQDIYDFWRPVGHQYPMVDGHLSNAVYIDSFK
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LYLGLISLLENSSSLQANDRIGLFSYGSGAVAEFFSGLLVPGYEKQLAQAAHQALLDDRQKLTIAEYEAM
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Isoprene synthase:

Atggaagctcgtcgttctgcgaactacgaacctaacagctgggactatgattacctgctgtcctccgacacggacgagtccatcgaagtatacaaagacaaagcgaaaa
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ispA:

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

ATGAGCCTGACCGAAGAAAAACCGATTCGTCCGATTGCAAATTTTCCGCCTAGCATTTGGGGTGATCA
GTTTCTGATTTATGAGAAACAGGTTGAACAGGGCGTTGAGCAGATTGTTAATGATCTGAAAAAAGAA
GTTCGCCAGCTGCTGAAAGAAGCACTGGATATTCCGATGAAACATGCCAATCTGCTGAAACTGATTGA
TGAAATTCAGCGTCTGGGTATCCCGTATCATTTTGAACGTGAAATTGATCATGCCCTGCAGTGCATTTA
TGAAACCTATGGTGATAATTGGAATGGTGATCGTAGCAGCCTGTGGTTTCGTCTGATGCGTAAACAGG
GTTATTATGTTACCTGCGACGTGTTTAACAACTATAAAGATAAAAACGGTGCCTTTAAACAGAGCCTG
GCAAATGATGTTGAAGGTCTGCTGGAACTGTATGAAGCAACCAGCATGCGTGTTCCGGGTGAAATTAT
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ATCCGGCACTGTTTACCGAAATCCAGCGTGCACTGAAACAGCCGCTGTGGAAACGTCTGCCTCGTATT
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AAAGCCTTCGACATCAAAAAAAACGCACCGTGTCTGCGTGATCGTATTGTTGAATGTTATTTTTGGGG
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TAAAGAATTTGTGAAAGAATTCGTGCGTAACCTGATGGTTGAAGCAAAATGGGCCAATGAAGGTCAT
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TGTTTCGTTATAGCGGTATTCTGGGTCGTCGTCTGAACGATCTGATGACCCATAAAGCAGAACAAGAA
CGTAAACATAGCAGCAGCAGCCTGGAAAGCTATATGAAAGAATATAACGTGAACGAAGAGTATGCAC
AGACCCTGATTTACAAAGAAGTTGAGGACGTTTGGAAAGATATCAACCGTGAATATCTGACCACGAA
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TAAAGATAACTTTACGCGTATGGGCGACGAATATAAACATCTGATTAAAAGCCTGCTGGTGTATCCGA
TGAGCATTTAA

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

ATGAGCACCCTGCCGATTAGCAGCGTTAGCTTTAGCAGCAGCACCAGTCCGCTGGTTGTTGATGATAA
AGTTAGCACCAAACCGGATGTTATTCGTCACACCATGAACTTTAATGCAAGCATTTGGGGTGATCAGT
TTCTGACCTATGATGAACCGGAAGATCTGGTGATGAAAAAACAGCTGGTTGAAGAACTGAAAGAAGA
AGTTAAAAAAGAGCTGATCACCATCAAAGGTAGCAATGAACCGATGCAGCATGTTAAACTGATTGAA
CTGATCGATGCCGTTCAGCGTCTGGGTATTGCATATCATTTTGAAGAAGAAATCGAAGAAGCCCTGCA
GCATATTCATGTTACCTATGGTGAACAGTGGGTGGATAAAGAAAATCTGCAGAGCATTAGCCTGTGGT
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gttgctttgttttactggtttcacctgttctattaggtgttacatgctgttcatctgttacattgtcgatctgttcatggtgaacagctttgaatgcaccaaaaactcgtaaaagctct
gatgtatctatctttttttacaccgttttcatctgtgcatatggacagttttcccttttgatatgtaacggtgaacagttgttctacttttgtttgttagtcttgatgcttcactgatagatac
aagagccataagaacctcagatccttccgtatttagccagtatgttctctagtgtggttcgttgtttttgcgtgagccatgagaacgaaccattgagatcatacttactttgcatg
tcactcaaaaattttgcctcaaaactggtgagctgaatttttgcagttaaagcatcgtgtagtgttttttcttagtccgttatgtaggtaggaatctgatgtaatggttgttggtatttt
gtcaccattcatttttatctggttgttctcaagttcggttacgagatccatttgtctatctagttcaacttggaaaatcaacgtatcagtcgggcggcctcgcttatcaaccaccaa
tttcatattgctgtaagtgtttaaatctttacttattggtttcaaaacccattggttaagccttttaaactcatggtagttatttttcaagcattaacatgaacttaaattcatcaaggcta
atctctatatttgccttgtgagttttcttttgtgttagttctttttaataaccactcataaatcctcatagagtatttgttttcaaaagacttaacatgttccagattatattttatgaatttttt
aactggaaaagataaggcaatatctcttcactaaaaactaattctaatttttcgcttgagaacttggcatagtttgtccactggaaaatctcaaagcctttaaccaaaggattcct
gatttccacagttctcgtcatcagctctctggttgctttagctaatacaccataagcattttccctactgatgttcatcatctgagcgtattggttataagtgaacgataccgtccg
ttctttccttgtaggttttcaatcgtggggttgagtagtgccacacagcataaaattagcttggtttcatgctccgttaagtcatagcgactaatcgctagttcatttgctttgaa
aacaactaattcagacatacatctcaattggtctaggtgattttaatcactataccaattgagatgggctagtcaatgataattactagtccttttccttgagttgtgggtatctgt
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aaacagaccttaaaaccctaaaggcttaagtagcaccctcgcaagctcgggcaaatcgctgaatattccttttgtctccgaccatcaggcacctgagtcgctgtctttttcgt
gacattcagttcgctgcgctcacggctctggcagtgaatgggggtaaatggcactacaggcgccttttatggattcatgcaaggaaactacccataatacaagaaaagcc
cgtcacgggcttctcagggcgttttatggcgggtctgctatgtggtgctatctgacttttgctgttcagcagttcctgccctctgattttccagtctgaccacttcggattatccc
gtgacaggtcattcagactggctaatgcacccagtaaggcagcggtatcatcaacaggctta

CLAIMS

What is claimed is:

1. Recombinant cells capable of increased production of isoprene, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences selected from the group consisting of:

a. a *mvaE* gene and a *mvaS* gene from *E. gallinarum*;

b. a *mvaE* gene and a *mvaS* gene from *E. casseliflavus*;

c. a *mvaE* gene and a *mvaS* gene from *E. faecium*; and

d. a *mvaE* gene and a *mvaS* gene from *L. grayi*

wherein said mvaE gene and mvaS gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cells further comprise:

i. one or more nucleic acids encoding polypeptides of the lower MVA pathway; and

ii. a heterologous nucleic acid encoding an isoprene synthase polypeptide,

wherein the cells produce increased amounts of isoprene compared to isoprene-producing cells that do not comprise said *mvaE* gene and *mvaS* gene.

2. The cells of claim 1, wherein the nucleic acids encoding polypeptides of the lower MVA pathway comprise enzymes selected from: (a) an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate; (b) an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (c) an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate.

3. The cells of claim 1 or 2, wherein the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate 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.

4. The cells of claim 3, wherein the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase.

5. The cells of any one of claims 1-4, wherein the isoprene synthase polypeptide is a plant isoprene synthase polypeptide or variants thereof.

6. The cells of claim 5, wherein the isoprene synthase polypeptide is a polypeptide from *Pueraria* or *Populus* or a hybrid, *Populus alba x Populus tremula*, or variants thereof.

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

8. The cells of claim 5, wherein the plant isoprene synthase polypeptide is a *Populus alba* isoprene synthase polypeptide.

9. The cells of any one of claims 1-8, further comprising one or more nucleic acids encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide.

10. The cells of claim 9, wherein the nucleic acid encoding an IDI polypeptide is a heterologous nucleic acid encoding an IDI polypeptide.

11. The cells of claim 10, wherein the IDI polypeptide is a yeast IDI polypeptide.

12. The cells of claim 9, wherein the nucleic acid encoding an IDI polypeptide is a copy of an endogenous nucleic acid encoding an IDI polypeptide.

13. The cells of any one of claims 1-12, wherein the one or more nucleic acids is placed under an inducible promoter or a constitutive promoter.

14. The cells of any one of claims 1-13, wherein the one or more nucleic acids is cloned into a multicopy plasmid.

15. The cells of any one of claims 1-13, wherein the one or more nucleic acids is integrated into a chromosome of the cells.

16. The cells of any one of claims 1-15, wherein the cells are gram-positive bacterial cells or gram-negative bacterial cells, *Escherichia* cells, *Pantoea* cells, fungal cells, filamentous fungal cells, *Trichoderma* cells, *Aspergillus* cells, or yeast cells.

17. The cells of claim 16, wherein the cells are selected from the group consisting of *E. coli, 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*.

18. The cells of claim 17, wherein the cells are *E. coli*.

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

20. The method of claim 19, further comprising (c) recovering the isoprene.

21. Recombinant cells capable of increased production of isoprenoid precursors, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences selected from the group consisting of:

(a) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*;

(b) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*;

(c) an *mvaE* gene and an *mvaS* gene from *E. faecium*; and

(d) an *mvaE* gene and an *mvaS* gene from *L. grayi*,

wherein the mvaE gene and mvaS gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cells produce increase amounts of isoprenoid precursors compared to isoprenoid precursor-producing cells that do not comprise said *mvaE* gene and *mvaS* gene.

22. The cells of claim 21, wherein the one or more nucleic acids is placed under an inducible promoter or a constitutive promoter.

23. The cells of any one of claims 21-22, wherein the one or more nucleic acids is cloned into a multicopy plasmid.

24. The cells of any one of claim 21-22 wherein the one or more nucleic acids is integrated into a chromosome of the cells.

25. The cells of any one of claims 21-24, wherein the cells are gram-positive bacterial cells, gram-negative bacterial cells, *Escherichia* cells, *Pantoea* cells, fungal cells, filamentous fungal cells, *Trichoderma* cells, *Aspergillus* cells, or yeast cells.

26. The cells of any one of claims 25, wherein the cells are selected from the group consisting of *E. coli, 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.

27. The cells of claim 26, wherein the cells are *E. coli*.

28. The cells of any one of claims 21-27, wherein the isoprenoid precursor is mevalonate (MVA).

29. A method of producing isoprenoid precursors, comprising: (a) culturing the host cells of claim 21 under suitable culture conditions for production of isoprenoid precursors; and (b) producing the isoprenoid precursors.

30. The method of claim 29, further comprising (c) recovering the isoprenoid precursors.

31. Recombinant cells capable of increased production of isoprenoids, the cells comprising one or more heterologous nucleic acids comprising nucleotide sequences selected from the group consisting of:

(a) an *mvaE* gene and an *mvaS* gene from *E. gallinarum*;

(b) an *mvaE* gene and an *mvaS* gene from *E. casseliflavus*;

(c) an *mvaE* gene and an *mvaS* gene from *E. faecium*; and

(d) an *mvaE* gene and an *mvaS* gene from *L. grayi*,

wherein said *mvaE* gene and *mvaS* gene encode polypeptides having thiolase, HMG-CoA synthase, and HMG-CoA reductase catalytic activities, and wherein the cell further comprise:

i. one or more nucleic acids encoding polypeptides of the lower MVA pathway; and

ii. one or more nucleic acids encoding polyprenyl pyrophosphate synthases,

wherein the cells produce increased amounts of isoprenoids compared to isoprenoid-producing cells that do not comprise said *mvaE* gene and *mvaS* gene.

32. The cells of claim 31, wherein the nucleic acids encoding polypeptides of the lower MVA pathway comprise enzymes selected from: (a) an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate; (b) an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (c) an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate.

33. The cells of claim 31 or 32, wherein the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate 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.

34. The cells of claim 33, wherein the enzyme that phosphorylates mevalonate to mevalonate 5-phosphate is *M. mazei* mevalonate kinase.

35. The cells of any one of claims 31-33, wherein the one or more nucleic acids is placed under an inducible promoter or a constitutive promoter.

36. The cells of any one of claims 31-35, wherein the one or more nucleic acids is cloned into a multicopy plasmid.

37. The cells of any one of claim 31-35, wherein the one or more nucleic acids is integrated into a chromosome of the cells.

38. The cells of any one of claims 31-37, wherein the cells are gram-positive bacterial cells, gram-negative bacterial cells, *Escherichia* cells, *Pantoea* cells, fungal cells, filamentous fungal cells, *Trichoderma* cells, *Aspergillus* cells, or yeast cells.

39. The cells of claim 36, wherein the cells are selected from the group consisting of *E. coli, 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.

40. The cells of claim 39, wherein the cells are *E. coli*.

41. The cells of any one of claims 31-40, wherein the isoprenoid is selected from group consisting of monoterpenes, diterpenes, triterpenes, tetraterpenes, sequiterpenes, and polyterpenes.

42. The cells of claim 41, wherein the isoprenoid is a sesquiterpene.

43. The cells of any one of claims 31-41, 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.

44. A method of producing isoprenoids, comprising: (a) culturing the host cells of claim 31 under suitable culture conditions for production of isoprenoids; and (b) producing the isoprenoids.

45. The method of claim 44, further comprising (c) recovering the isoprenoids.

FIGURE 1:

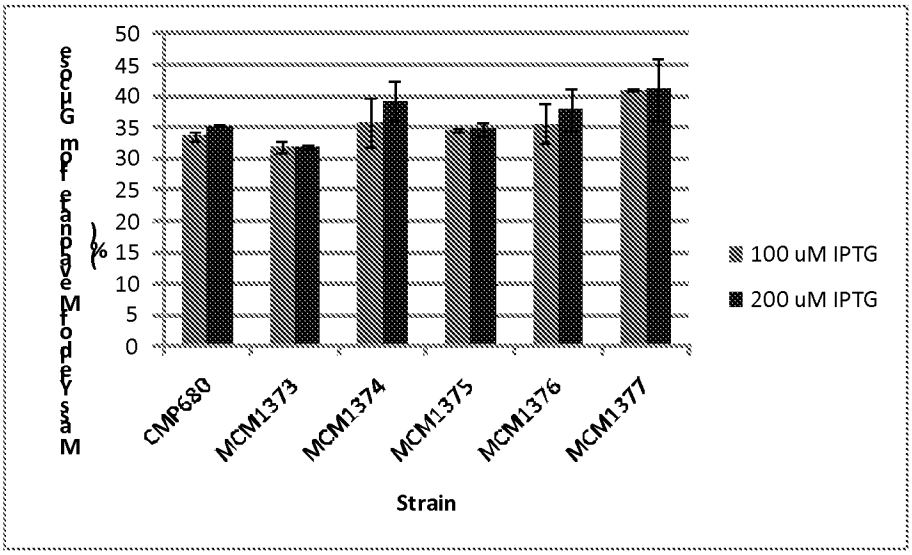

50
45
40
35
30
25
20
15
10
5
0
CMP680
MCM1373
MCM1374
MCM1375
MCM1376
MCM1377
Strain
100 uM IPTG
200 uM IPTG

FIGURE 2:

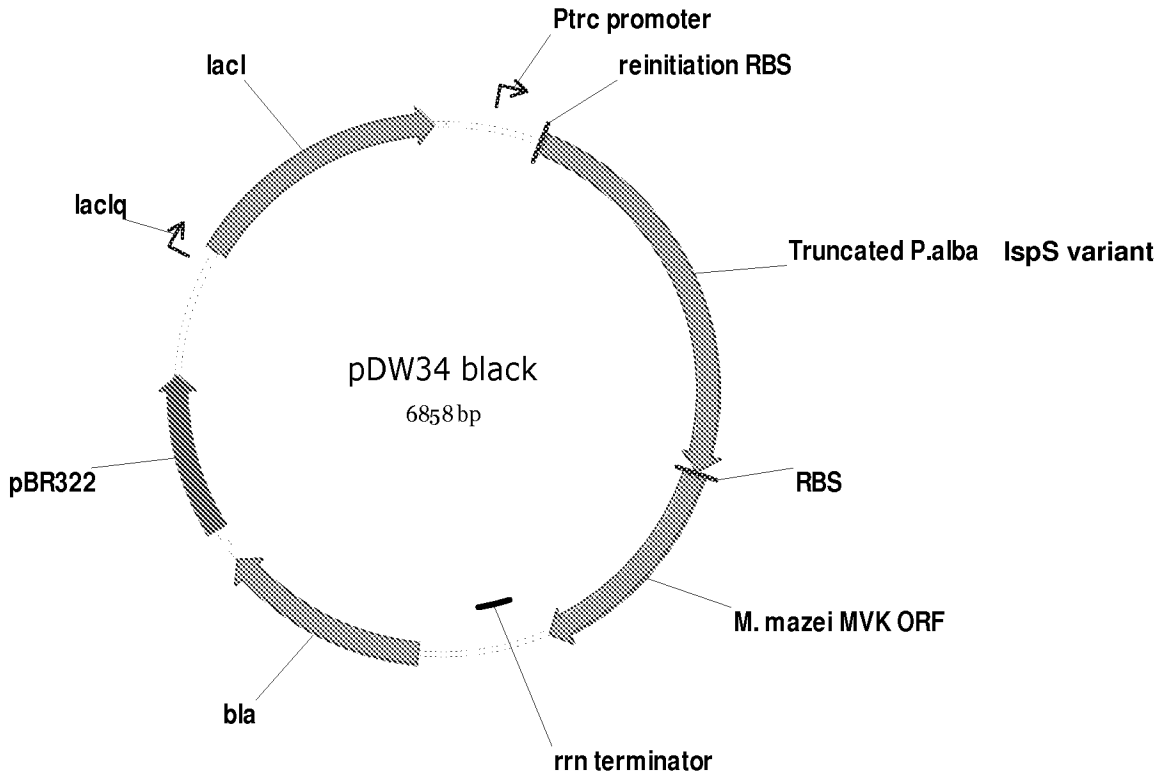

Ptrc promoter
lacI
reinitiation RBS
lacIq
Truncated P.alba IspS variant
pDW34 black
6858 bp
pBR322
RBS
M. mazei MVK ORF
bla
rrn terminator

FIGURE 3:

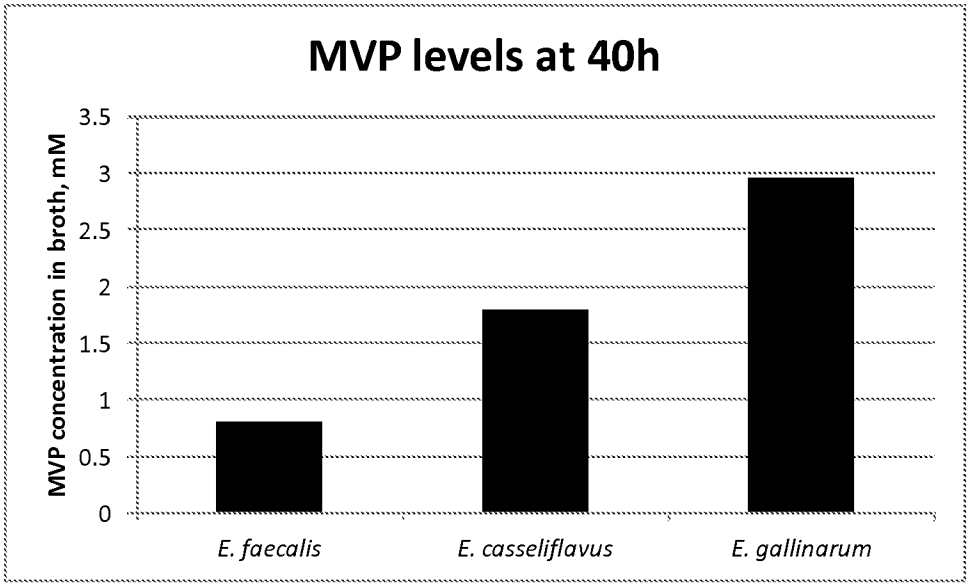

MVP levels at 40h
MVP concentration in broth, mM
3.5
3
2.5
2
1.5
1
0.5
0
E. faecalis
E. casseliflavus
E. gallinarum

FIGURE 4:

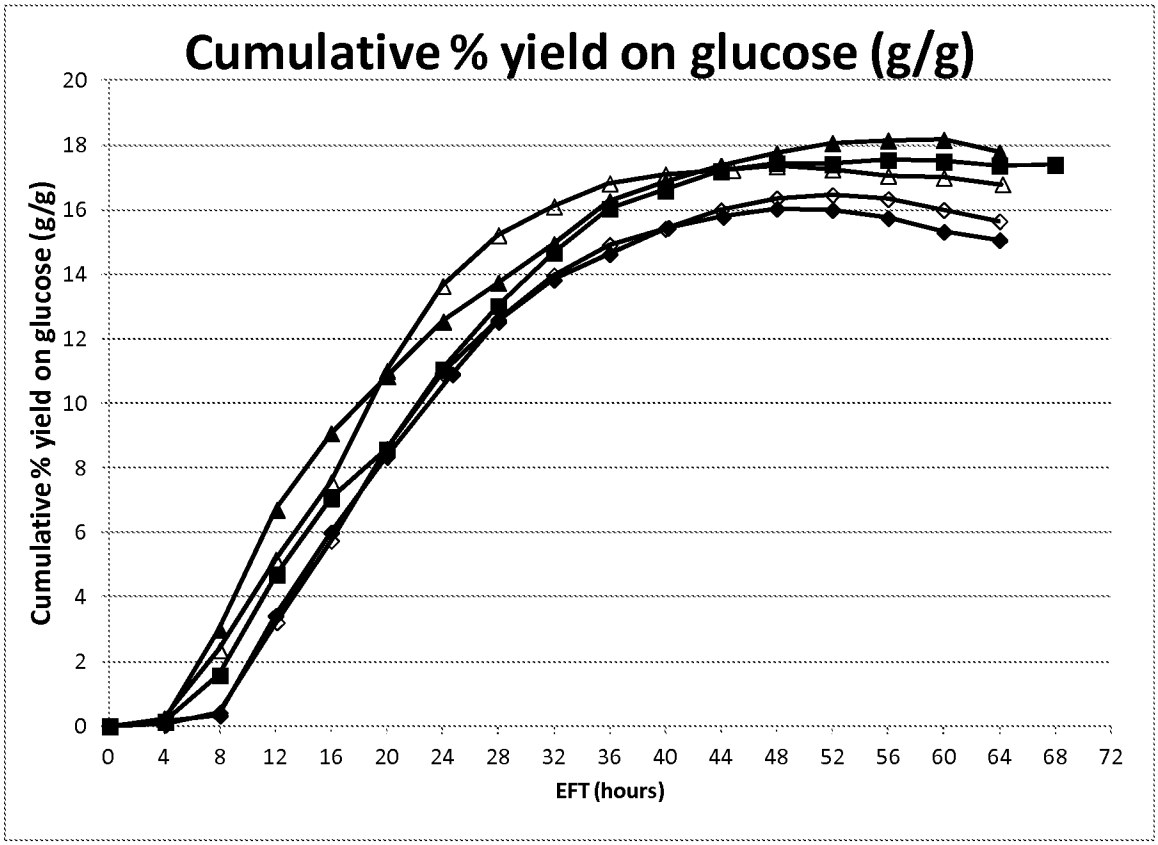

Cumulative % yield on glucose (g/g)
Cumulative % yield on glucose (g/g)
EFT (hours)
0
2
4
6
8
10
12
14
16
18
20
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72

FIGURE 5:

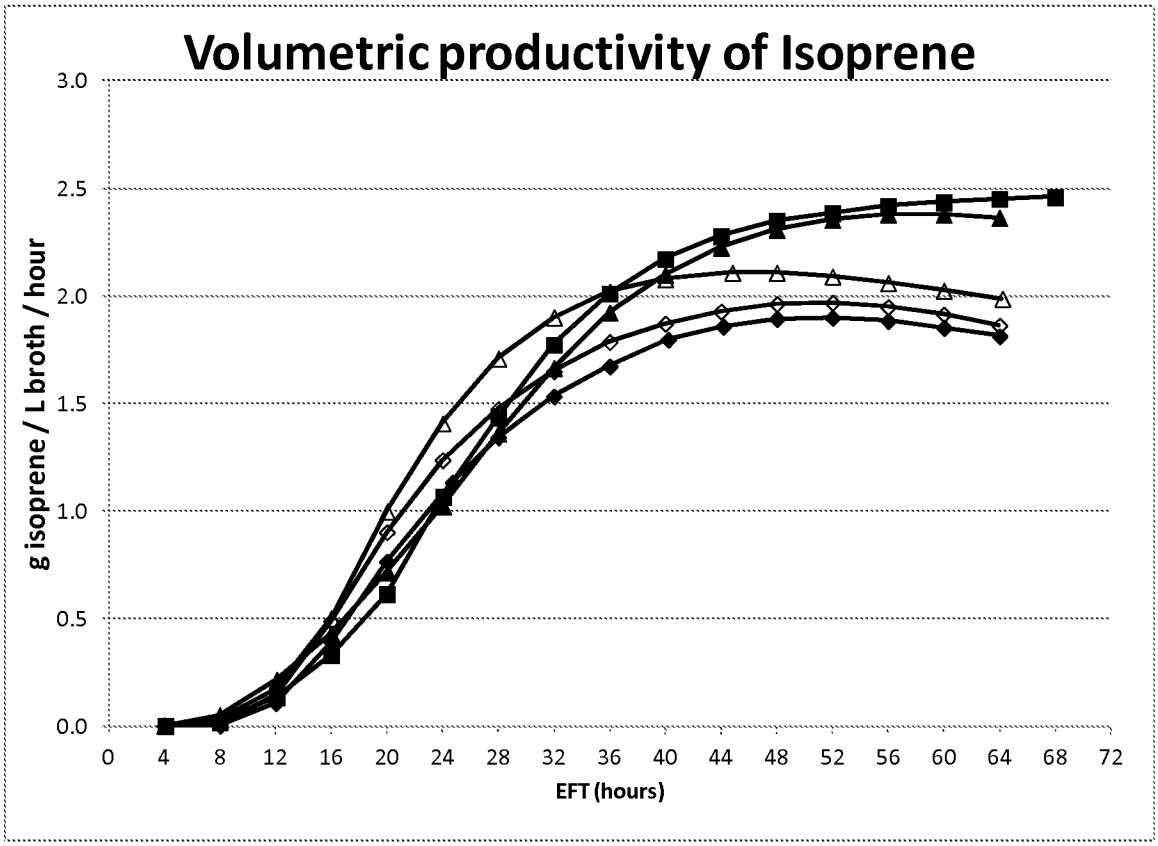

Volumetric productivity of Isoprene
g isoprene / L broth / hour
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
EFT (hours)

FIGURE 6:

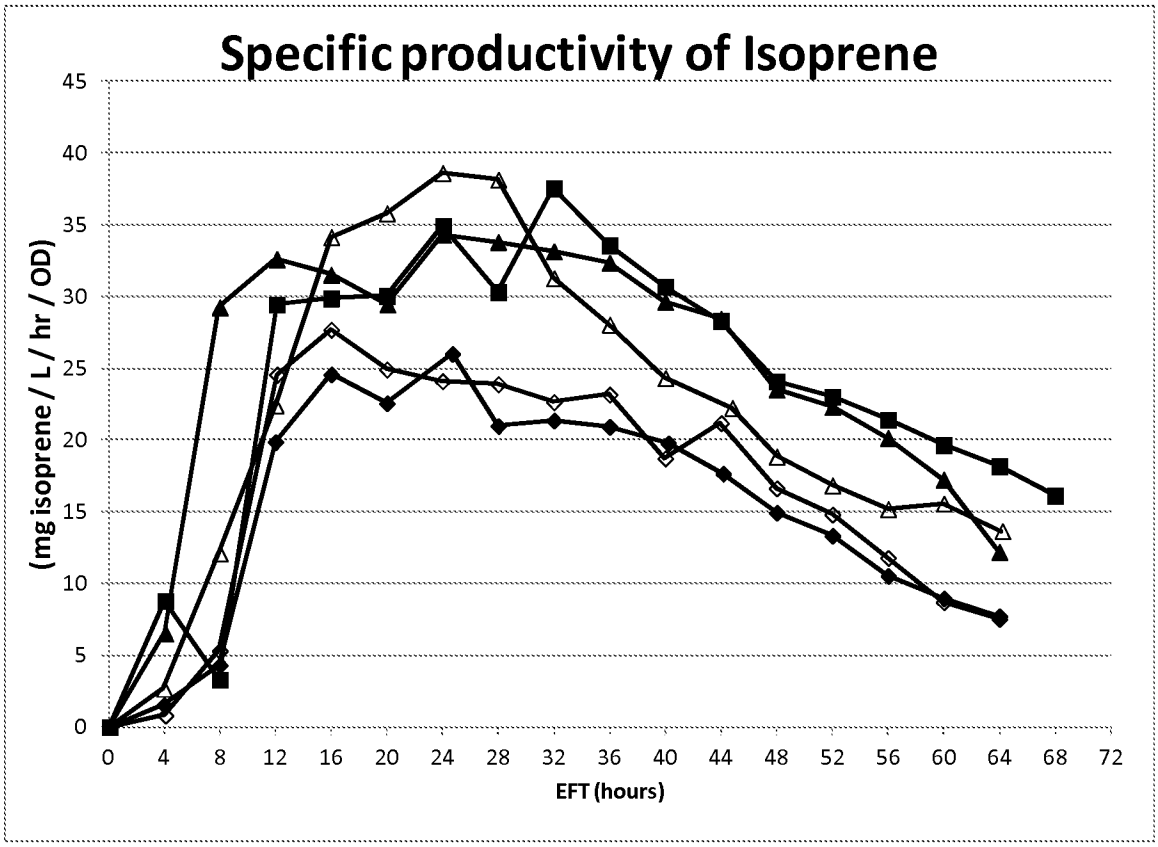

Specific productivity of Isoprene
(mg isoprene / L / hr / OD)
EFT (hours)
0
5
10
15
20
25
30
35
40
45
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72

FIGURE 7:

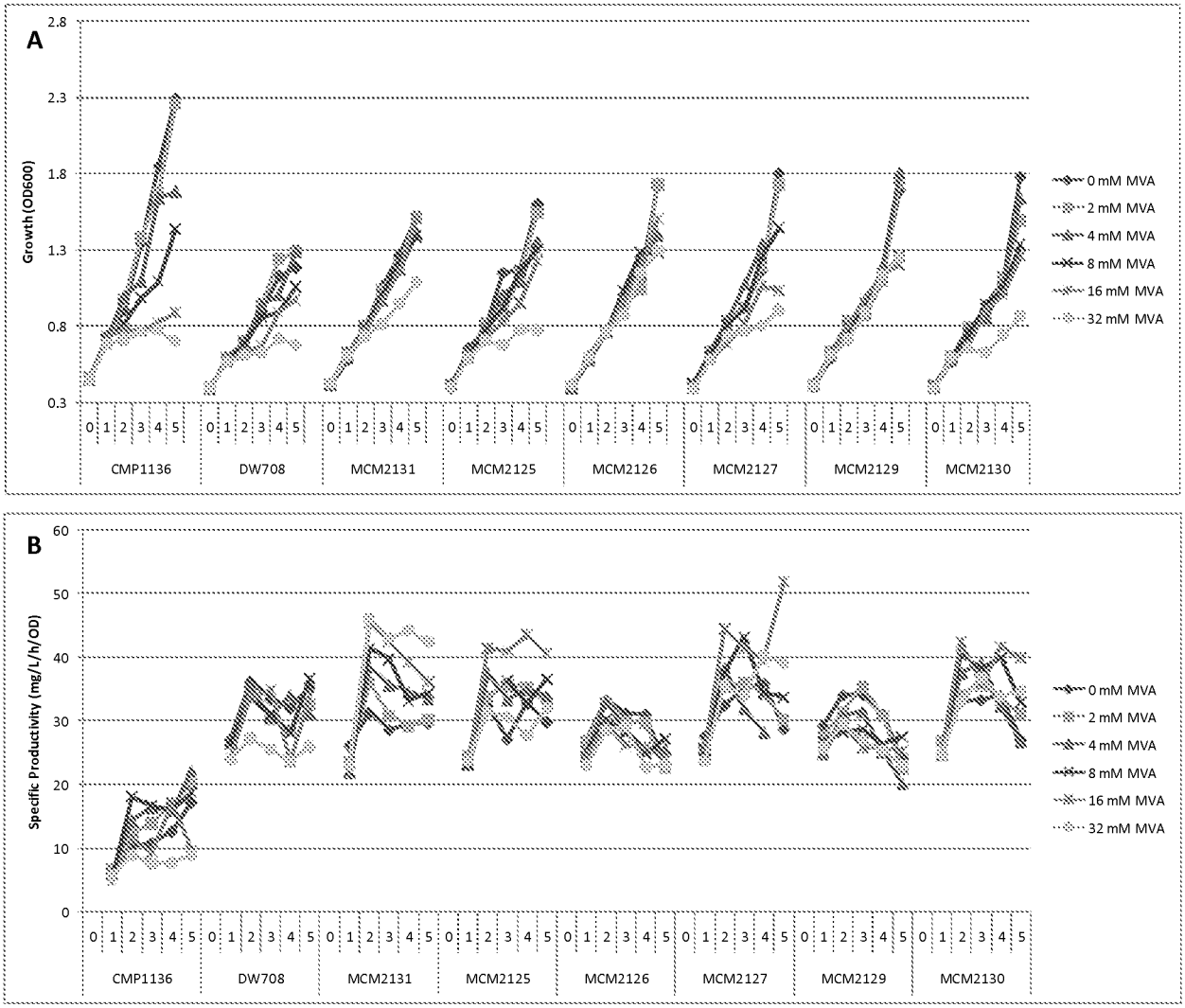

A
Growth (OD600)
2.8
2.3
1.8
1.3
0.8
0.3
0 1 2 3 4 5
CMP1136
DW708
MCM2131
MCM2125
MCM2126
MCM2127
MCM2129
MCM2130
0 mM MVA
2 mM MVA
4 mM MVA
8 mM MVA
16 mM MVA
32 mM MVA
B
Specific Productivity (mg/L/h/OD)
60
50
40
30
20
10
0
0 1 2 3 4 5
CMP1136
DW708
MCM2131
MCM2125
MCM2126
MCM2127
MCM2129
MCM2130
0 mM MVA
2 mM MVA
4 mM MVA
8 mM MVA
16 mM MVA
32 mM MVA

FIGURE 8:

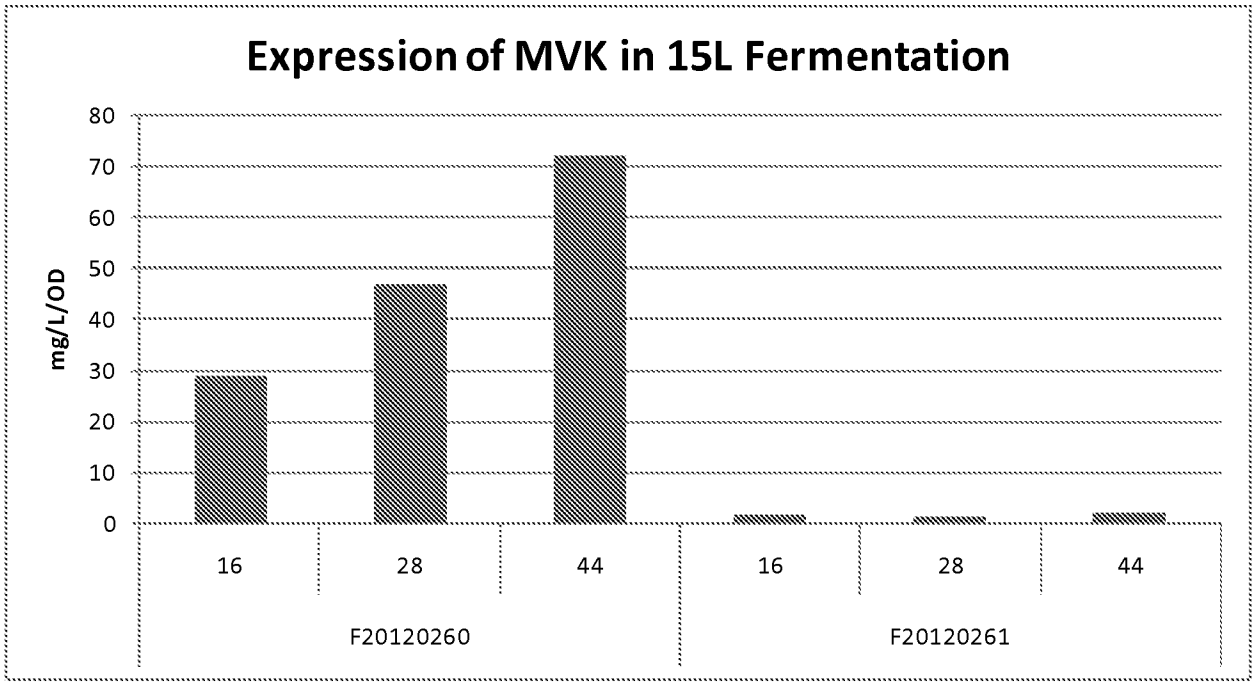

Expression of MVK in 15L Fermentation
mg/L/OD
80
70
60
50
40
30
20
10
0
16
28
44
16
28
44
F20120260
F20120261

FIGURE 9:

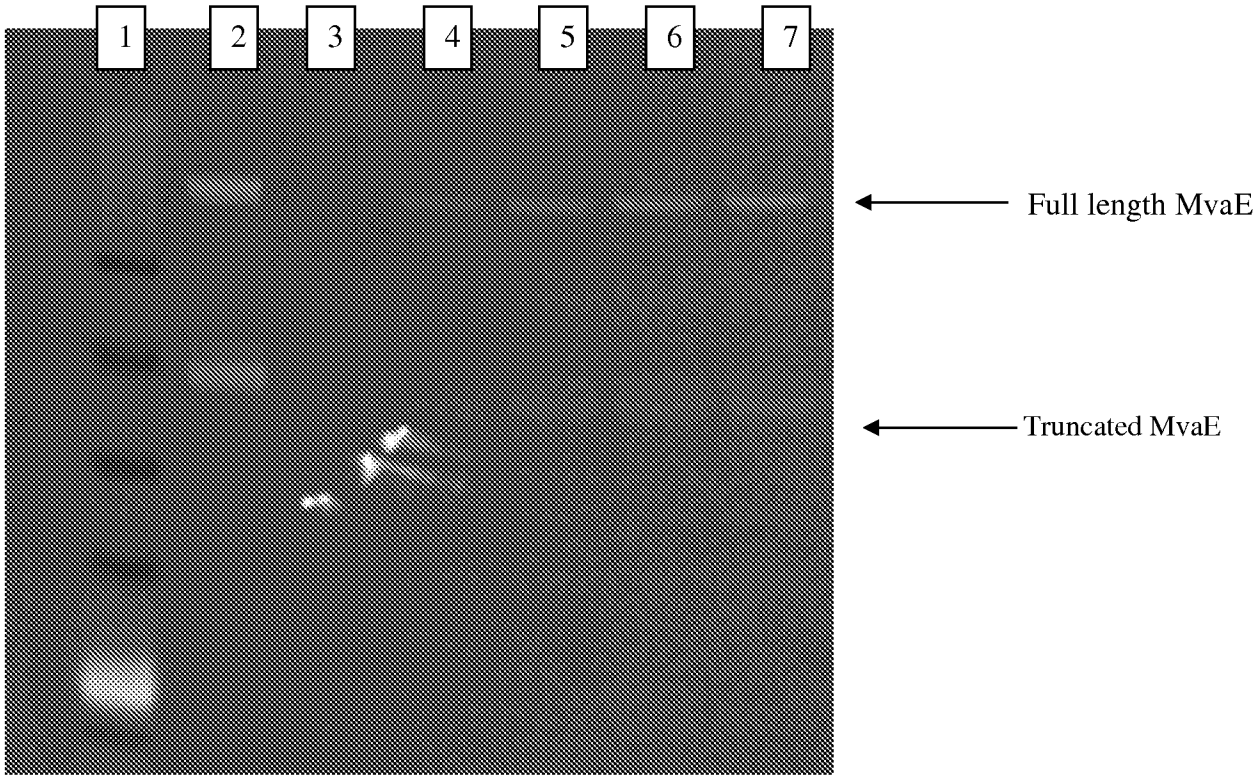
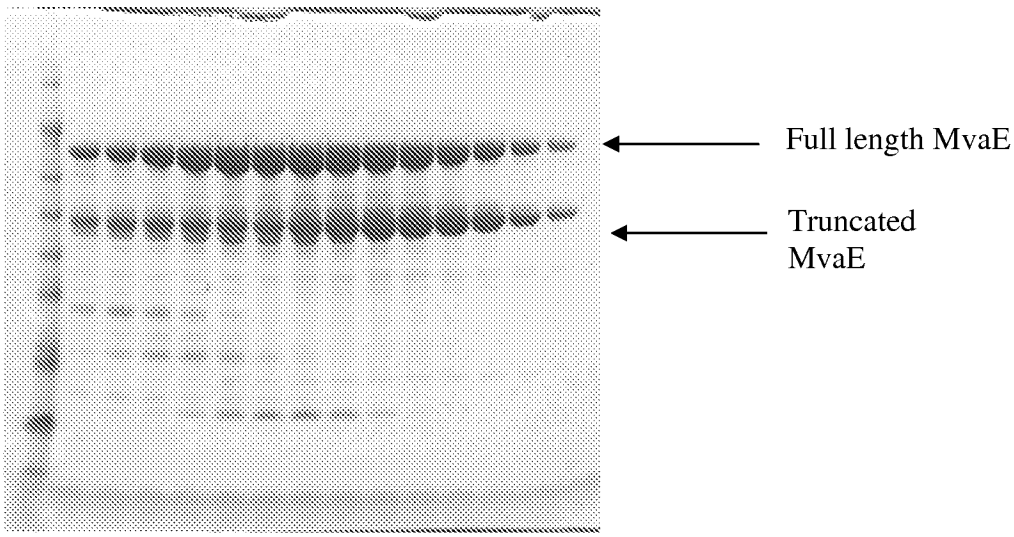


FIGURE 10:



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/035655

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/21 C12N9/00 C12N9/10 C12P5/00 C12P7/02
C12P7/04 C12P7/42
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P C12Y

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 510 583 A1 (KYOWA HAKKO KOGYO KK [JP]) 2 March 2005 (2005-03-02) cited in the application the whole document page 4, lines 23-26 page 5, lines 45,46 page 6, lines 8,11 page 9, lines 51-55 examples 1-5 ----- -/--	21-30

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 August 2012	28/08/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van de Kamp, Mart

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/78935 A1 (SMITHKLINE BEECHAM CORP [US]; SMITHKLINE BEECHAM PLC [GB]) 28 December 2000 (2000-12-28) the whole document SEQ ID NO:61,62; page 30, line 3 - page 31, line 31 SEQ ID NO:11,12; page 9, lines 1-39 claims 4, 15,16,20,21,22	21-28
A	WO 2009/076676 A2 (GENENCOR INT [US]; GOODYEAR TIRE & RUBBER [US]; CERVIN MARGUERITE [US]) 18 June 2009 (2009-06-18) cited in the application the whole document example 8	1,19,21, 29,31,44
A	WO 2007/140339 A2 (AMYRIS BIOTECHNOLOGIES INC [US]; RENNINGER NEIL STEPHEN [US]; NEWMAN J) 6 December 2007 (2007-12-06) cited in the application the whole document examples 3,6,8,10 claims 1,2,13,14,36-39	1,19,21, 29,31,44
A	WO 2010/031077 A1 (DANISCO US INC [US]; GOODYEAR TIRE & RUBBER [US]; BECK ZACHARY QUINN [] 18 March 2010 (2010-03-18) the whole document paragraph [0242] example 20	1,19,21, 29,31,44
A	PITERA ET AL: "Balancing a heterologous mevalonate pathway for improved isoprenoid production in Escherichia coli", METABOLIC ENGINEERING, vol. 9, no. 2, 16 February 2007 (2007-02-16), pages 193-207, XP005888126, ISSN: 1096-7176 abstract	1,19,21, 29,31,44
A	HISASHI HARADA ET AL: "Novel approaches and achievements in biosynthesis of functional isoprenoids in Escherichia coli", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 84, no. 6, 12 August 2009 (2009-08-12), pages 1021-1031, XP019757474, ISSN: 1432-0614 the whole document	1,19,21, 29,31,44

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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