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(71) Applicant *(for all designated States except US)*: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94607-5200 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **KIZER, Jeffery, Lance** [US/US]; 3545A 23rd Street, San Francisco, CA 94110 (US).

(74) Agent: **BORDEN, Paula, A.**; Bozicevic, Field & Francis LLP, 1900 University Avenue, Suite 200, East Palo Alto, CA 94303 (US).

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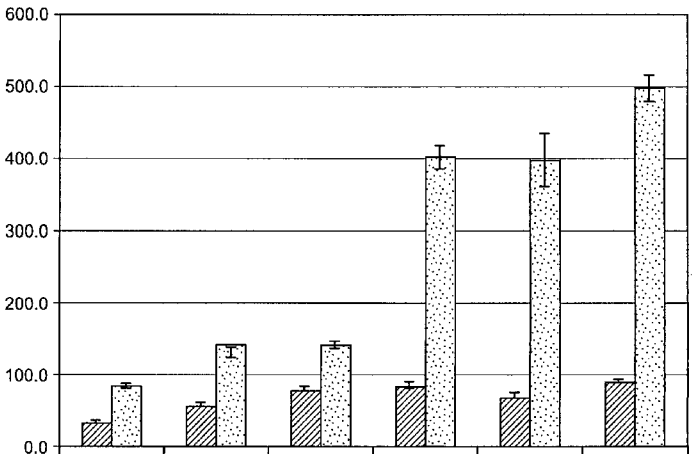
(54) **Title:** METHODS OF INCREASING ISOPRENOID OR ISOPRENOID PRECURSOR PRODUCTION



	MGL	MOPS-Min	Shikimate	MOPS-Rich	8 AA	C1
▨ Specific Production (mg/L-OD)	27.2	45.3	63.7	68.8	73.3	92.1
□ Total Producrion (mg/L)	87.2	134.4	134.4	402.1	398.6	498.5
Final Cell Density (OD600)	**3.5**	**3.0**	**3.0**	**6.4**	**5.7**	**5.9**

FIG. 5

(57) **Abstract:** The present invention provides methods of producing an isoprenoid or isoprenoid precursor in an isoprenoid-producing host cell, generally involving culturing isoprenoid-producing cells in a defined culture medium that includes serine.

WO 2009/005704 A1

METHODS OF INCREASING ISOPRENOID OR ISOPRENOID PRECURSOR PRODUCTION

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 60/947,918, filed July 3, 2007, which application is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Engineering synthetic metabolic pathways in microbes for the production of pharmaceuticals or complex chemicals is an attractive alternative to traditional chemical synthesis. However, the incorporation of an exogenous biosynthetic pathway into a host organism often abolishes the regulation of carbon flux through the pathway, leading to the accumulation of toxic intermediates or altered levels of key endogenous metabolites.

[0003] Microbes have been designed to efficiently produce a wide range of industrially relevant compounds via heterologous pathway engineering including isoprenoids, bioplastics, polyketides, amino acids, and polymer precursors. It can be exceedingly difficult, in such complexly engineered systems, to predict what aspect of heterologous host metabolism limits final product titers. The unregulated consumption of cellular resources, metabolic burden of heterologous protein production or the accumulation of pathway intermediates/products that are inhibitory or toxic to the heterologous host are all significant issues that arise from novel pathway engineering.

[0004] There is a need in the art for culture conditions that improve production levels of isoprenoid compounds in *in vitro* cell culture of isoprenoid-producing cells.

Literature

[0005] Newman et al. (2006) *Biotechnol. Bioeng.* 95:684-691; Martin et al. (2003) *Nature Biotechnol.* 21:796-802; U.S. Patent No. 7,172,886.

SUMMARY OF THE INVENTION

[0006] The present invention provides methods of producing an isoprenoid or isoprenoid precursor in an isoprenoid-producing host cell, generally involving culturing isoprenoid-producing cells in a defined culture medium that includes serine.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] Figure 1 is a schematic depiction of mevalonate and 1-deoxy-D-xylulose 5-diphosphate (DXP) pathways.

[0008] Figure 2 is a schematic depiction of synthesis of various isoprenoid compounds from the precursors isopentenyl pyrophosphate and dimethylallyl pyrophosphate.

[0009] Figure 3 is a schematic depiction of serine, glycine and single-carbon unit pathways.

[0010] Figure 4 depicts a heterologous amorpha-4-11-diene production pathway.

[0011] Figure 5 depicts specific amorpha-4-11-diene production (mg/L-OD) and total amorpha-4-11-diene production in various media formulations.

DEFINITIONS

[0012] The terms "isoprenoid," "isoprenoid compound," "terpene," "terpene compound," "terpenoid," and "terpenoid compound" are used interchangeably herein, and refer to any compound that is capable of being derived from isopentenyl pyrophosphate (IPP). The number of C-atoms present in the isoprenoids is typically evenly divisible by five (e.g., C5, C10, C15, C20, C25, C30 and C40). Irregular isoprenoids and polyterpenes have been reported, and are also included in the definition of "isoprenoid." Isoprenoid compounds include, but are not limited to, hemiterpenes, monoterpenes, diterpenes, triterpenes, sesquiterpenes, and polyterpenes. Isoprenoid compounds include, e.g., C_5-C_{10} isoprenoids, C_5-C_{15} isoprenoids, C_5-C_{20} isoprenoids, C_{10}-C_{20} isoprenoids, C_{10}-C_{25} isoprenoids, C_{10}-C_{30} isoprenoids, C_{10}-C_{40} isoprenoids, and the like.

[0013] As used herein, the term "prenyl diphosphate" is used interchangeably with "prenyl pyrophosphate," and includes monoprenyl diphosphates having a single prenyl group (e.g., IPP and DMAPP), as well as polyprenyl diphosphates that include 2 or more prenyl groups. Monoprenyl diphosphates include isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP).

[0014] As used herein, the term "terpene synthase" refers to any enzyme that enzymatically modifies IPP, DMAPP, or a polyprenyl pyrophosphate, such that a terpene or a terpenoid precursor compound is produced.

[0015] The word "pyrophosphate" is used interchangeably herein with "diphosphate." Thus, e.g., the terms "prenyl diphosphate" and "prenyl pyrophosphate" are interchangeable; the terms "isopentenyl pyrophosphate" and "isopentenyl diphosphate" are interchangeable; the terms farnesyl diphosphate" and farnesyl pyrophosphate" are interchangeable; etc.

[0016] The term "mevalonate pathway" or "MEV pathway" is used herein to refer to the biosynthetic pathway that converts acetyl-coenzyme A (acetyl-CoA) to IPP. The mevalonate pathway comprises enzymes that catalyze the following steps: (a) condensing two molecules of acetyl-CoA to acetoacetyl-CoA; (b) condensing acetoacetyl-CoA with acetyl-CoA to form hydroxymethylglutaryl-CoA (HMG-CoA); (c) converting HMG-CoA to mevalonate; (d) phosphorylating mevalonate to mevalonate 5-phosphate; (e) converting mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (f) converting mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. The mevalonate pathway is illustrated schematically in Figure 1. The "top half" of the mevalonate pathway refers to the enzymes responsible for the conversion of acetyl-CoA to mevalonate through a MEV pathway intermediate.

[0017] The term "1-deoxy-D-xylulose 5-diphosphate pathway" or "DXP pathway" is used herein to refer to the pathway that converts glyceraldehyde-3-phosphate and pyruvate to IPP and DMAPP

through a DXP pathway intermediate, where DXP pathway comprises enzymes that catalyze the reactions depicted schematically in Figure 1.

[0018] As used herein, the term "prenyl transferase" is used interchangeably with the terms "isoprenyl diphosphate synthase" and "polyprenyl synthase" (e.g., "GPP synthase," "FPP synthase," "GGPP synthase," etc.) to refer to an enzyme that catalyzes the consecutive 1'-4 condensation of isopentenyl diphosphate with allylic primer substrates, resulting in the formation of prenyl diphosphates of various chain lengths.

[0019] The terms "polynucleotide" and "nucleic acid," used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides. Thus, this term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases.

[0020] The terms "peptide," "polypeptide," and "protein" are used interchangeably herein, and refer to a polymeric form of amino acids of any length, which can include coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones.

[0021] As used herein, "standard amino acid" refers to the twenty encoded amino acids including phenylalanine, leucine, isoleucine, methionine, valine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, lysine, aspartic acid, glutamic acid, cysteine, tryptophan, arginine, serine, and glycine.

[0022] As used herein, the term "exogenous nucleic acid" refers to a nucleic acid that is not normally or naturally found in and/or produced by a given bacterium, organism, or cell in nature. As used herein, the term "endogenous nucleic acid" refers to a nucleic acid that is normally found in and/or produced by a given bacterium, organism, or cell in nature. An "endogenous nucleic acid" is also referred to as a "native nucleic acid" or a nucleic acid that is "native" to a given bacterium, organism, or cell.

[0023] The term "heterologous nucleic acid," as used herein, refers to a nucleic acid wherein at least one of the following is true: (a) the nucleic acid is foreign ("exogenous") to (i.e., not naturally found in) a given host microorganism or host cell (e.g., the nucleic acid encodes a polypeptide that is heterologous to the host cell, e.g., the encoded polypeptide is not normally produced by the host cell); (b) the nucleic acid comprises a nucleotide sequence that is naturally found in (e.g., is "endogenous to") a given host microorganism or host cell (e.g., the nucleic acid comprises a nucleotide sequence that is endogenous to the host microorganism or host cell) but is either produced in an unnatural (e.g., greater than expected or greater than naturally found) amount in the cell, or differs in sequence from the endogenous nucleotide sequence such that the same encoded protein (having the same or substantially the same amino acid sequence) as found endogenously is produced in an unnatural (e.g., greater than expected or greater than naturally found) amount in the cell; (c) the nucleic acid comprises two or more

nucleotide sequences or segments that are not found in the same relationship to each other in nature, e.g., the nucleic acid is recombinant.

[0024] The term "heterologous polypeptide," as used herein, refers to a polypeptide that is not naturally associated with a given polypeptide. For example, an isoprenoid precursor-modifying enzyme that comprises a "heterologous transmembrane domain" refers to an isoprenoid precursor-modifying enzyme that comprises a transmembrane domain that is not normally associated with (e.g., not normally contiguous with; not normally found in the same polypeptide chain with) the isoprenoid precursor-modifying enzyme in nature.

[0025] By "construct" or "vector" is meant a recombinant nucleic acid, generally recombinant DNA, which has been generated for the purpose of the expression and/or propagation of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences.

[0026] The terms "DNA regulatory sequences," "control elements," and "regulatory elements," used interchangeably herein, refer to transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, protein degradation signals, and the like, that provide for and/or regulate expression of a coding sequence and/or production of an encoded polypeptide in a host cell.

[0027] The term "transformation" is used interchangeably herein with "genetic modification" and refers to a permanent or transient genetic change induced in a cell following introduction of new nucleic acid (i.e., DNA exogenous to the cell). Genetic change ("modification") can be accomplished either by incorporation of the new DNA into the genome of the host cell, or by transient or stable maintenance of the new DNA as an episomal element. Where the cell is a eukaryotic cell, a permanent genetic change can be achieved by introduction of the DNA into the genome of the cell. In prokaryotic cells, permanent changes can be introduced into the chromosome or via extrachromosomal elements such as plasmids and expression vectors, which may contain one or more selectable markers to aid in their maintenance in the recombinant host cell. Suitable methods of genetic modification include viral infection, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate precipitation, direct microinjection, and the like. The choice of method is generally dependent on the type of cell being transformed and the circumstances under which the transformation is taking place (i.e. *in vitro, ex vivo,* or *in vivo*). A general discussion of these methods can be found in Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed., Wiley & Sons, 1995.

[0028] "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression. As used herein, the terms "heterologous promoter" and "heterologous control regions" refer to promoters and other control regions that are not normally associated with a particular nucleic acid in nature. For example, a "transcriptional control region heterologous to a coding region" is a transcriptional control region that is not normally associated with the coding region in nature.

[0029] A "host cell," as used herein, denotes an *in vivo* or *in vitro* eukaryotic cell, a prokaryotic cell, or a cell from a multicellular organism (e.g., a cell line) cultured as a unicellular entity, which eukaryotic or prokaryotic cells can be, or have been, used as recipients for a nucleic acid (e.g., an expression vector that comprises a nucleotide sequence encoding one or more biosynthetic pathway gene products such as mevalonate pathway gene products), and include the progeny of the original cell which has been genetically modified by the nucleic acid. It is understood that the progeny of a single cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. A "recombinant host cell" (also referred to as a "genetically modified host cell") is a host cell into which has been introduced a heterologous nucleic acid, e.g., an expression vector. For example, a subject prokaryotic host cell is a genetically modified prokaryotic host cell (e.g., a bacterium), by virtue of introduction into a suitable prokaryotic host cell a heterologous nucleic acid, e.g., an exogenous nucleic acid that is foreign to (not normally found in nature in) the prokaryotic host cell, or a recombinant nucleic acid that is not normally found in the prokaryotic host cell; and a subject eukaryotic host cell is a genetically modified eukaryotic host cell, by virtue of introduction into a suitable eukaryotic host cell a heterologous nucleic acid, e.g., an exogenous nucleic acid that is foreign to the eukaryotic host cell, or a recombinant nucleic acid that is not normally found in the eukaryotic host cell.

[0030] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0031] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0032] Unless defined otherwise, 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 belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0033] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a host cell" includes a plurality of host cells and reference to "the culture medium" includes reference to one or more culture media and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0034] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

[0035] The present invention provides methods of producing an isoprenoid or isoprenoid precursor *in vitro* in a cell that is capable of producing the isoprenoid or isoprenoid compound, generally involving culturing the cell in a defined culture medium. The defined culture medium includes at least one supplement (e.g., an amino acid) that feeds into and increases single-carbon metabolism in the isoprenoid-producing cell, e.g., where the amino acid provides a single-carbon unit, such as a methyl group, that is essential to a metabolic pathway in the cell.

[0036] The biosynthetic pathways, discussed herein, for generating isoprenoid or isoprenoid precursors are depicted schematically in Figures 1 and 2. Isoprenoid compounds are synthesized from a universal five carbon precursor, isopentenyl pyrophosphate (IPP). IPP is synthesized via two different pathways: the mevalonate (MEV) pathway and the 1-deoxyxylulose-5-phosphate (DXP) or non-mevalonate pathway. The MEV pathway and the DXP pathway are depicted schematically in Figure 1.

[0037] The mevalonate pathway comprises the following enzymatic reactions: (a) condensing two molecules of acetyl-CoA to acetoacetyl-CoA; (b) condensing acetoacetyl-CoA with acetyl-CoA to form HMG-CoA; (c) converting HMG-CoA to mevalonate; (d) phosphorylating mevalonate to mevalonate 5-phosphate; (e) converting mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (f) converting mevalonate 5-pyrophosphate to isopentenyl pyrophosphate.

[0038] The DXP pathway produces IPP and DMAPP from pyruvate and glyceraldehyde-3-phosphate, as depicted schematically in Figure 1. The pathway begins with the formation of 1-deoxy-D-xylulose-5-phosphate (DXP) from pyruvate and glyceraldehyde-3-phosphate by DXP synthase (Dxs). DXP is then isomerized and reduced to 2-C-methyl-D-erythritol-4-phosphate (MEP), the first committed step of the non-mevalonate pathway, by DXP reductoisomerase (IspC or Dxr). In the next step, a cytidylic acid moiety is added to MEP by the action 2-C-methylerythritol-4-phosphate cytidyltransferase (IspD) to

produce 4-diphosphocytidyl-2C-methyl-D-erythritol. 4-diphosphocytidyl-2C-methyl-D-erythritol is then phosphorylated by 4-diphosphocytidyl-2C-methyl-D-erythritol kinase (IspE) and further converted to 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate by the sequential action of 2-C-methylerythritol-2,4-cyclodiphosphate synthase (IspF) and 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (IspG). The terminal enzyme of the DXP pathway in *E. coli* has recently been identified as the product of *ispH* (formerly *lytB*), and has been shown to convert 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate to both IPP and DMAPP at a 5:1 ratio.

[0039] As depicted schematically in Figure 2, DMAPP acts as a primer for the sequential additions of IPP by the isoprenyl pyrophosphate synthases (also known as the prenyl transferases) to form C_{10} geranyl pyrophosphate (GPP), C_{15} farnesyl pyrophosphate (FPP), C_{20} geranylgeranyl pyrophosphate (GGPP), and larger isoprenyl pyrophosphates. The isoprenyl pyrophosphates are then cyclized by the terpene cyclases (synthases) to form the various terpene classes. Carotenoids are synthesized by a series of enzymatic reactions beginning with the condensation of two GGPP molecules.

[0040] An isoprenoid compound (and/or an isoprenoid precursor such as mevalonate, IPP, a DXP pathway intermediate, and a polyprenyl diphosphate) can be produced *in vitro* in cells that are capable of producing the isoprenoid or isoprenoid precursor (also referred to herein as "isoprenoid-producing cells"). It has now been found that the composition of the culture medium in which the cells are cultured can be modified in specific ways to increase the amount of isoprenoid or isoprenoid precursor recoverable from the cells and/or the culture medium.

[0041] Thus, the present invention provides methods for producing an isoprenoid or an isoprenoid precursor, the methods generally involving culturing *in vitro* a plurality of isoprenoid-producing cells in a defined culture medium comprising at least one supplement that feeds into and increases single-carbon metabolism in the genetically modified host cell. The cells have a biosynthetic pathway for converting a substrate(s) (e.g., acetoacetyl-CoA; glyceraldehyde-3-phosphate and pyruvate; mevalonate) to IPP (also referred to herein as an "IPP biosynthetic pathway"). Illustrative examples include: 1) cells that have an endogenous IPP biosynthetic pathway (e.g., cells that have an endogenous mevalonate pathway, such as eukaryotic cells such as yeast cells, fungal cells, etc.; and cells that have an endogenous DXP pathway, e.g., a prokaryotic cell that normally produces IPP via a DXP pathway), and that synthesize the isoprenoid or isoprenoid precursor compound via the endogenous IPP biosynthetic pathway; 2) cells that have been genetically modified with one or more exogenous nucleic acids comprising nucleotide sequences encoding one or more heterologous IPP biosynthetic pathway enzymes (thus having an exogenous IPP biosynthetic pathway); and 3) cells that have a modified endogenous IPP biosynthetic pathway, e.g., where the endogenous pathway is genetically modified, where the cells synthesize the isoprenoid or isoprenoid precursor via the modified endogenous IPP biosynthetic pathway.

[0042] Culturing of the isoprenoid-producing cells *in vitro* in the defined culture medium provides for production of the isoprenoid or isoprenoid precursor by the cells in a recoverable amount. The

isoprenoid or isoprenoid precursor can then be recovered (e.g., isolated) from the cells, the culture medium, or both.

METHODS FOR ENHANCING PRODUCTION ISOPRENOIDS AND ISOPRENOID PRECURSORS

[0043] The present invention provides methods for producing an isoprenoid or an isoprenoid precursor. The methods generally involve culturing a plurality of cells *in vitro* in a defined culture medium comprising a supplement (e.g., serine) that is a substrate for a single-carbon metabolic pathway. The cells are capable of synthesizing the isoprenoid or isoprenoid precursor. In some embodiments, the cells synthesize the isoprenoid or isoprenoid precursor via an endogenous IPP biosynthetic pathway. In other embodiments, the cells synthesize the isoprenoid or isoprenoid precursor via an exogenous IPP biosynthetic pathway. In other embodiments, the cells synthesize the isoprenoid or isoprenoid precursor via a modified endogenous IPP biosynthetic pathway.

[0044] As noted above, a cell that is capable of synthesizing an isoprenoid or isoprenoid precursor is cultured in a defined culture medium comprising a supplement (e.g., serine) that is a substrate for a single-carbon metabolic pathway, e.g., a metabolic pathway that requires one-carbon units (e.g., methyl groups). Such metabolic pathways include, e.g., metabolic pathways that depend on serine, e.g., where serine or a serine metabolite is a substrate for the pathway; and metabolic pathways that depend on methionine, e.g., where methionine is a substrate for the pathway.

[0045] For example, serine is an important source of single carbon units in *Escherichia coli* during growth. Serine is converted to glycine by action of serine hydroxymethyltransferase (SHMT, encoded by the *glyA* gene), in a reaction that transfers a methyl group to the C1 carrier molecule, tetrahydrofolate. The conversion of serine to glycine provides the majority of single-carbon units required during growth. As another example, methionine is a key precursor to the primary methyl-carrier cofactor in *E. coli*, S-adenosyl-methionine (SAM).

[0046] In one aspect, a subject method comprises culturing *in vitro* a cell that is capable of synthesizing an isoprenoid or isoprenoid precursor in a culture medium comprising: a) serine; and b) a subset of amino acids selected from the group consisting of alanine, glutamine, glutamic acid, isoleucine, leucine, valine, and methionine, where the subset consists of zero to seven amino acids, and where the medium does not comprise one or more amino acids that are not part of the subset. In certain embodiments, the subset consists of zero amino acid. In other embodiments, the subset consists of one amino acid. In other embodiment, the subset consists of two amino acids. In other embodiments, the subset consists of three amino acids. In other embodiments, the subset consists of four amino acids. In other embodiments, the subset consists of five amino acids. In other embodiments, the subset consists of six amino acids. In still other embodiments, the subset consists of seven amino acids. In certain embodiments, the medium does not additionally comprise an amino acid (e.g., a standard amino acid) other than serine and the one or more amino acids that are in the subset.

[0047] As an example, in some embodiments, the culture medium comprises: a) serine; and b) a subset of amino acids consisting of glutamine, glutamic acid, isoleucine, leucine, valine, and methionine;

where the culture medium does not comprise alanine. In another example, the culture medium comprises: a) serine; and b) a subset of amino acids consisting of glutamic acid, isoleucine, leucine, valine, and methionine; where the culture medium does not comprise alanine and glutamine. In another example, the culture medium comprises: a) serine; and b) a subset of amino acids consisting of isoleucine, leucine, valine, and methionine; where the culture medium does not comprise alanine, glutamine, and glutamic acid. In another example, in some embodiments, the culture medium comprises: a) serine; and b) a subset of amino acids consisting of leucine, valine, and methionine; where the culture medium does not comprise alanine, glutamine, glutamic acid, and isoleucine. In another example, the culture medium comprises: a) serine; and b) a subset of amino acids consisting of alanine, glutamic acid, and glutamine; where the culture medium does not comprise isoleucine, leucine, valine, and methionine. In another example, the culture medium comprises: a) serine; and b) a subset of amino acids consisting of valine and methionine; where the culture medium does not comprise alanine, glutamine, glutamic acid, isoleucine, and leucine. In another example, the culture medium comprises: a) serine; and b) a subset of amino acids consisting of alanine and methionine; where the culture medium does not comprise glutamic acid, glutamine, isoleucine, leucine, and valine. In yet another example, the culture medium comprises: a) serine; and b) methionine; and does not include any of alanine, glutamine, glutamic acid, isoleucine, leucine, and valine. In still another example the culture medium comprises serine; does not include any of alanine, glutamine, glutamic acid, isoleucine, leucine, valine, and methionine.

[0048] In certain embodiments, the culture medium does not comprise one or more of the other standard amino acids selected from the group consisting of: arginine, cysteine, glycine, histidine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, aspartic acid, and asparagine. For example, if the defined culture medium comprises serine and methionine, the defined culture medium does not comprise one or more of alanine, glutamine, glutamic acid, isoleucine, leucine, valine, arginine, cysteine, glycine, histidine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, aspartic acid, and asparagine. As another example, if the defined culture medium comprises serine, alanine, glutamine, glutamic acid, isoleucine, leucine, methionine, and valine, the defined culture medium does not comprise one or more of arginine, cysteine, glycine, histidine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, aspartic acid, and asparagine. In some embodiments, the defined culture medium does not additionally comprise an amino acid (e.g., a standard amino acid) other than serine and the one or more amino acids that are in the subset. For example, if the defined culture medium comprises serine and methionine, the defined culture medium does not comprise any of alanine, glutamine, glutamic acid, isoleucine, leucine, valine, arginine, cysteine, glycine, histidine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, aspartic acid, and asparagine. As another example, if the defined culture medium comprises serine, alanine, glutamine, glutamic acid, isoleucine, leucine, methionine, and valine, the defined culture medium does not comprise any of arginine,

cysteine, glycine, histidine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, aspartic acid, and asparagine.

[0049] In some embodiments, the culture medium further comprises a purine. In certain embodiments, the purine is adenine. In other embodiments, the purine is guanine. In still other embodiments, the purine is both adenine and guanine.

[0050] In one aspect, a subject method comprises culturing *in vitro* a cell that is capable of synthesizing an isoprenoid or isoprenoid precursor in a culture medium comprising: a) serine; and b) a subset of amino acids selected from the group consisting of glycine, alanine, glutamine, glutamic acid, isoleucine, leucine, valine, and methionine, where the subset consists of zero to eight amino acids, and where the medium does not comprise one or more amino acids that are not part of the subset. In certain embodiments, the subset consists of zero amino acid. In other embodiments, the subset consists of one amino acid. In other embodiment, the subset consists of two amino acids. In other embodiments, the subset consists of three amino acids. In other embodiments, the subset consists of four amino acids. In other embodiments, the subset consists of five amino acids. In other embodiments, the subset consists of six amino acids. In still other embodiments, the subset consists of seven amino acids. In still other embodiments, the subset consists of eight amino acids. In certain of these embodiments, the culture medium does not comprise one or more of the other standard amino acids selected from the group consisting of: arginine, cysteine, glycine, histidine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, aspartic acid, and asparagine. For example, if the defined culture medium comprises serine and a subset of amino acids, where the subset is glycine and methionine, the defined culture medium does not additionally comprise one or more of alanine, glutamine, glutamic acid, isoleucine, leucine, valine, arginine, cysteine, histidine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, aspartic acid, and asparagine. In some embodiments, the defined culture medium does not additionally comprise an amino acid other than serine and the one or more amino acids that are in the subset. For example, if the defined culture medium comprises serine and a subset of amino acids, where the subset is glycine and methionine, the defined culture medium does not additionally comprise an amino acid other than serine, glycine, and methionine, e.g., the defined culture medium does not comprise any of alanine, glutamine, glutamic acid, isoleucine, leucine, valine, arginine, cysteine, histidine, lysine, phenylalanine, proline, threonine, tryptophan, tyrosine, aspartic acid, and asparagine.

[0051] In another aspect, a subject method comprises culturing *in vitro* a cell that is capable of synthesizing an isoprenoid or isoprenoid precursor in a culture medium comprising: a) serine; and b) methionine and/or glycine; and c) a purine wherein the culture medium does not include any amino acid other than serine, and methionine and/or glycine. In some embodiments, the purine is adenine. In other embodiments, the purine is guanine. In still other embodiments, the purine is both adenine and guanine.

[0052] In another aspect, a subject method comprises culturing *in vitro* a cell that is capable of synthesizing an isoprenoid or isoprenoid precursor in a culture medium comprising: a) serine; b)

methionine; (c) glycine; and d) a purine wherein the culture medium does not include any amino acid other than serine, methionine, and glycine. In some embodiments, the purine is adenine. In other embodiments, the purine is guanine. In still other embodiments, the purine is both adenine and guanine.

[0053] Serine can be present in the defined culture medium in a concentration range of from about 5 mM to about 20 mM, e.g., from about 5 mM to about 10 mM, from about 10 mM to about 15 mM, or from about 15 mM to about 20 mM; and in some embodiments is present in the defined culture medium at a concentration of 10 mM. Where the culture medium includes methionine, methionine can be present in the defined culture medium in a concentration range of from about 0.05 mM to about 0.5 mM, e.g., from about 0.05 mM to about 0.1 mM, from about 0.1 mM to about 0.15 mM, from about 0.15 mM to about 0.20 mM, from about 0.20 mM to about 0.30 mM, from about 0.30 mM to about 0.40 mM, or from about 0.40 mM to about 0.50 mM; and in some embodiments is present in the defined culture medium at a concentration of 0.2 mM. Where the culture medium includes adenine and/or guanine, adenine and/or guanine can each be present in the defined culture medium in a concentration range of from about 0.05 mM to about 0.4 mM, e.g., from about 0.05 mM to about 0.1 mM, from about 0.1 mM to about 0.15 mM, from about 0.15 mM to about 0.2 mM, from about 0.2 mM to about 0.3 mM, or from about 0.3 mM to about 0.4 mM; and in some embodiments are present in the defined culture medium at a concentration of 0.2 mM each.

[0054] Where the culture medium includes one or more of alanine, glutamine, glutamic acid, isoleucine, leucine, and valine, each of these amino acids can each be present in the defined medium in a concentration range of from about 0.2 mM to about 1.2 mM, e.g., from about 0.2 mM to about 0.4 mM, from about 0.4 mM to about 0.6 mM, from about 0.6 mM to about 0.8 mM, from about 0.8 mM to about 1.0 mM, or from about 1.0 mM to about 1.2 mM; and in some embodiments are present in the defined medium at a concentration of 0.8 mM (alanine and leucine), 0.6 mM (glutamine, glutamic acid, and valine), and 0.4 mM (isoleucine).

[0055] In some embodiments, the culture medium can further comprise glycine. Glycine can be present in the defined culture medium in a concentration range of from about 0.4 mM to about 1.5 mM, e.g., from about 0.4 mM to about 0.5 mM, from about 0.5 mM to about 0.6 mM, from about 0.6 mM to about 0.8 mM, from about 0.8 mM to about 1.0 mM, from about 1.0 mM to about 1.25 mM, or from about 1.25 mM to about 1.5 mM; and in some embodiments is present in the defined culture medium at a concentration of 0.8 mM.

[0056] In some embodiments, the culture medium further includes an aromatic amino acid, e.g., one or more of tryptophan, tyrosine, and phenylalanine. In other embodiments, the culture medium further includes para-amino benzoic acid. In other embodiments, the culture medium further includes para-hydroxy benzoic acid. In other embodiments, the culture medium further includes 2,3-dihydroxybenzoic acid. In other embodiments, the culture medium further includes tryptophan,

tyrosine, phenylalanine, para-amino benzoic acid, para-hydroxy benzoic acid, and 2,3-dihydroxybenzoic acid.

[0057] Where the culture medium includes an aromatic amino acid (e.g., one or more of tryptophan, tyrosine, and phenylalanine), the aromatic amino acid can be present in the defined medium in a concentration range of from about 0.05 mM to about 0.6 mM, e.g., from about 0.05 mM to about 0.1 mM, from about 0.1 mM to about 0.2 mM, from about 0.2 mM to about 0.4 mM, or from about 0.4 mM to about 0.6 mM; and in some embodiments are present in the defined culture medium at a concentration of 0.1 mM (tryptophan), 0.2 mM (tyrosine), and 0.4 mM (phenylalanine). Para-amino benzoic acid, para-hydroxy benzoic acid, and 2,3-dihydroxybenzoic acid can each be present in the defined medium in a concentration range of from about 0.005 mM to about 0.1 mM, e.g., from about 0.005 mM to about 0.01 mM, from about 0.01 mM to about 0.05 mM, or from about 0.05 mM to about 0.1 mM; and in some embodiments are present in the defined culture medium at a concentration of 0.01 mM each.

[0058] In some embodiments, the defined culture medium further comprises a C_{12}-C_{22} fatty acid. In these embodiments, the genetically modified host cell includes an endogenous type II fatty acid biosynthetic pathway. In some embodiments, the C_{12}-C_{22} fatty acid is a C_{12} saturated fatty acid. In other embodiments, the C_{12}-C_{22} fatty acid is a C_{14} saturated fatty acid. In other embodiments, the C_{12}-C_{22} fatty acid is a C_{16} saturated fatty acid. In other embodiments, the C_{12}-C_{22} fatty acid is a C_{18} saturated fatty acid. In other embodiments, the C_{12}-C_{22} fatty acid is a C_{20} saturated fatty acid. In other embodiments, the C_{12}-C_{22} fatty acid is a C_{22} saturated fatty acid. In some embodiments, the fatty acid is palmitic acid.

[0059] Suitable saturated fatty acids include, but are not limited to, C_{12}-C_{22} saturated fatty acids, e.g., C_{12} saturated fatty acids, C_{14} saturated fatty acids, C_{16} saturated fatty acids, C_{18} saturated fatty acids, C_{20} saturated fatty acids, and C_{22} saturated fatty acids. Suitable saturated fatty acids include, but are not limited to, myristic acid (tetradecanoic acid), pentadecanoic acid, palmitic acid (hexadecanoic acid), stearic acid (octadecanoic acid), arachidic acid (eicosanoic acid), docsosanoic acid, and tetracosanoic acid. Also suitable for use are salts of a saturated fatty acid, derivatives of a saturated fatty acid, and salts of a derivative of a saturated fatty acid. Suitable salts include, but are not limited to, lithium salts, potassium salts, sodium salts, and the like.

[0060] Suitable unsaturated fatty acids include, but are not limited to, oleic acid, vaccenic acid, linoleic acid, palmitelaidic acid, and arachidonic acid. Also suitable for use are salts of an unsaturated fatty acid, derivatives of an unsaturated fatty acid, and salts of a derivative of an unsaturated fatty acid. Suitable salts include, but are not limited to, lithium salts, potassium salts, sodium salts, and the like.

[0061] The fatty acid (or compound that yields a fatty acid) will in some embodiments be present in the culture medium in an amount or a concentration that is effective to reduce HMG-CoA accumulation-induced growth inhibition of the cell. In some embodiments, the culture medium comprises a fatty acid in a concentration range of from about 0.10 mM to about 0.50 mM, e.g., from

about 0.1 mM to about 0.15 mM, from about 0.15 mM to about 0.2 mM, from about 0.2 mM to about 0.25 mM, from about 0.25 mM to about 0.3 mM, from about 0.3 mM to about 0.35 mM, from about 0.3 M to about 0.4 mM, from about 0.35 mM to about 0.4 mM, from about 0.35 mM to about 0.45 mM, or from about 0.45 mM to about 0.5 mM.

[0062] As noted above, in these embodiments, where the defined medium includes as a supplement a C_{12}-C_{22} fatty acid, the genetically modified host cell has an endogenous type II fatty acid biosynthetic pathway. Type II fatty acid biosynthetic pathway enzymes include, but are not limited to, malonyl-CoA:ACP transacylase, β-ketoacyl-ACP synthase I, β-ketoacyl-ACP synthase II, β-ketoacyl-ACP synthase III, acetyl-CoA:ACP transacylase, malonyl-ACP decarboxylase, β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydratase, β-hydroxydecanoyl-ACP dehydrase, trans-2-decenoyl-ACP isomerase, and enoyl-ACP reductase. "ACP" is "acetyl carrier protein."

[0063] In other embodiments, the culture medium further comprises a supplement that increases the osmolarity compared to the osmolarity of the control culture medium. In some embodiments, the supplement is a salt in a concentration range of from about 50 mM to about 500 mM. An agent(s) that increases osmolarity is present in the culture medium at a concentration that increases the osmolarity of the culture medium by at least about 10%, at least about 20%, at least about 25%, at least about 50%, at least about75%, at least about 100% (or 2-fold), at least about 2.5 fold, at least about 3 fold, at least about 3.5 fold, at least about 4 fold, at least about 5 fold, at least about 10 fold, at least about 25 fold, at least about 50 fold, at least about 100 fold, at least about 200 fold, at least about 300 fold, at least about 400 fold, or at least about 500 fold, or more, compared to the osmolarity of a control culture medium not containing the agent (e.g., compared to the defined medium without the agent, or, where the agent is a salt, with the salt but at a concentration below 50 mM).

[0064] For example, in some embodiments the osmolarity of control culture medium is from about 50 mOsM to about 100 mOsM; and a supplemented culture medium comprising one or more agents that increase osmolarity has an osmolarity of from about 100 mOsM to about 500 mOsM, e.g., from about 100 mOsM to about 110 mOsM , from about 100 mOsM to about 125 mOsM, from about 125 mOsM to about 150 mOsM, from about 150 mOsM to about 200 mOsM, from about 200 mOsM to about 250 mOsM, from about 250 mOsM to about 300 mOsM, from about 300 mOsM to about 350 mOsM, from about 350 mOsM to about 400 mOsM, from about 400 mOsM to about 450 mOsM, or from about 450 mOsM to about 500 mOsM, or greater than 500 mOsM.

[0065] Agents that increase osmolarity include, but are not limited to, salts, sugars (e.g. monosaccharides such as glucose; disaccharides; etc.), sugar alcohols, starches, polysaccharides, glycerol, and the like. Suitable salts include, but are not limited to, NaCl, sodium citrate, Na_2HPO_4, $CaCl_2$, KCl, KH_2PO_4, K_2HPO_4, NH_4Cl, $MgSO_4$, and the like. Suitable sugars and sugar alcohols include sorbitol, trehalose, and the like.

[0066] In some embodiments, the culture medium comprises a salt, a sugar, or a sugar alcohol in a concentration range of from about 50 mM to about 500 mM, e.g., from about 50 mM to about 75 mM,

from about 75 mM to about 100 mM, from about 100 mM to about 125 mM, from about 125 mM to about 150 mM, from about 150 mM to about 175 mM, from about 175 mM to about 200 mM, from about 200 mM to about 250 mM, from about 250 mM to about 300 mM, from about 300 mM to about 350 mM, from about 350 mM to about 400 mM, from about 400 mM to about 450 mM, or from about 450 mM to about 500 mM.

[0067] In some embodiments, the defined culture medium comprises a minimal medium that is supplemented with serine and a subset of amino acids, as described above. For example, in some embodiments, the defined culture medium comprises: a) a minimal culture medium; b) serine; and c) a subset of amino acids selected from the group consisting of alanine, glutamine, glutamic acid, isoleucine, leucine, valine, and methionine, where the subset consists of zero to seven amino acids, and where the medium does not comprise one or more amino acids that are not part of the subset.

[0068] As used herein, a "minimal culture medium" is a culture medium that provides the minimum nutrients possible for bacterial growth; for example, a minimal culture medium includes a carbon source (e.g., citrate, succinate, glucose), magnesium, nitrogen, phosphorus, and sulfur, which components allow the bacterium to synthesize proteins and nucleic acids. A minimal culture medium does not include amino acids or nucleotides.

[0069] Minimal culture media suitable for *in vitro* culture (e.g., for bacterial culture; for yeast culture) are known in the art, and any minimal culture medium can be used to generate a defined culture medium suitable for use in a subject method. A suitable minimal culture medium comprises: a buffer that is compatible with the isoprenoid-producing cells (e.g., is not substantially toxic or growth-inhibiting to the isoprenoid-producing cells; e.g., is not substantially bacteriostatic or bactericidal to the isoprenoid-producing cells at the concentration used in the minimal culture medium); salts that provide sources of magnesium, nitrogen, phosphorus, and sulfur (e.g., a potassium salt, a sodium salt, an ammonium salt, a magnesium salt); and micronutrients (e.g., zinc, manganese, copper, cobalt, boron, and molybdenum). Suitable buffers include, e.g., morpholinopropane sulfonate (MOPS), phosphate buffers, Tris buffers, and the like.

[0070] Minimal media are known in the art; and any known minimal medium can be used. For example, M9 minimal medium is described in, e.g., Miller (1972) Experiments in molecular genetics, p. 431-432, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; and Sambrook, J., E. F. Fritsch, and T. Maniatis (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. An exemplary, non-limiting minimal culture medium suitable for use is a culture medium comprising MOPS, tricine, a carbon source, nitrogen, phosphorus, sulfur, and micronutrients. For example, a suitable minimal culture medium can include MOPS (e.g., in a concentration range of from about 20 mM to about 80 mM), potassium phosphate (e.g., in a concentration range of from about 0.5 mM to about 2 mM), ammonium chloride (e.g., in a concentration range of from about 5 mM to about 15 mM), magnesium chloride (e.g., in a concentration range of from about 0.2 mM to about 1 mM), potassium sulfate (e.g., in a concentration range of from

about 0.1 mM to about 1 mM), iron sulfate (e.g., in a concentration range of from about 0.005 mM to about 0.1 mM), calcium chloride (e.g., in a concentration range of from about 10^{-4} mM to about 10^{-3} mM), sodium chloride (e.g., in a concentration range of from about10 mM to about 100 mM), *N*-Tris(hydroxymethyl)-methyl glycine (also known as Tricine) (e.g., in a concentration range of from about 1 mM to about 10 mM), and micronutrients (e.g., zinc, manganese, copper, cobalt, boron, and molybdenum; each in a concentration range of from about 10^{-6} mM to about 10^{-3} mM).

[0071] One example of a suitable minimal medium is the MOPS minimal medium described in Neidhardt et al. (1974) *J. Bacteriol.* 119:736. "MOPS minimal medium" includes potassium phosphate (1.32 mM); ammonium chloride (9.52 mM); $MgCl_2$ (0.523 mM); potassium sulfate (0.276 mM); iron sulfate (0.010 mM); $CaCl_2$ (5×10^{-4} mM); NaCl (50 mM); MOPS (40 mM); Tricine (4 mM); and micronutrients (ammonium molybdate, 2×10^{-6} mM; borate, 4×10^{-4} mM; cobalt chloride, 2×10^{-5} mM; copper sulfate, 10^{-5} mM; manganese chloride, 8×10^{-5} mM; and zinc sulfate, 10^{-5} mM).

Isoprenoid-producing cells

[0072] Suitable cells include: 1) cells that have an endogenous mevalonate pathway, e.g., eukaryotic cells such as yeast cells, fungal cells, etc., and that synthesize the isoprenoid or isoprenoid precursor compound at least in part via the endogenous mevalonate pathway; 2) cells that have an endogenous DXP pathway, e.g., eukaryotic cells such as a prokaryotic cell that normally produces IPP via a DXP pathway, and that synthesize the isoprenoid or isoprenoid precursor compound at least in part via the endogenous DXP pathway; 3) cells that have been genetically modified with one or more exogenous nucleic acids comprising nucleotide sequences encoding one or more heterologous mevalonate pathway enzymes, and that synthesize the isoprenoid or isoprenoid precursor at least in part via the exogenous mevalonate pathway, e.g., a prokaryotic cell that does not normally produce IPP via a mevalonate pathway, and that has been genetically modified with one or more nucleic acids comprising nucleotide sequences encoding one or more heterologous mevalonate pathway enzymes; 4) cells that have been genetically modified with one or more exogenous nucleic acids comprising nucleotide sequences encoding one or more heterologous DXP pathway enzymes, and that synthesize the isoprenoid or isoprenoid precursor at least in part via the exogenous DXP pathway, e.g., a eukaryotic cell that does not normally produce IPP via a DXP pathway, and that has been genetically modified with one or more nucleic acids comprising nucleotide sequences encoding one or more heterologous DXP pathway enzymes ; 5) cells that have a modified endogenous mevalonate pathway, e.g., where the endogenous mevalonate pathway is genetically modified, where the cells synthesize the isoprenoid or isoprenoid precursor at least in part via the modified endogenous mevalonate pathway, e.g., a eukaryotic host cell that normally synthesizes IPP via an endogenous mevalonate pathway, where the endogenous mevalonate pathway has been genetically modified; and 6) cells that have a modified endogenous DXP pathway, e.g., where the endogenous DXP pathway is genetically modified, where the cells synthesize the isoprenoid or isoprenoid precursor at least in part via the modified endogenous DXP pathway, e.g., a

prokaryotic host cell that normally synthesizes IPP via an endogenous DXP pathway, where the endogenous DXP pathway has been genetically modified.

[0073] Isoprenoid-producing cells can be unicellular organisms, or can be cells that grown in culture as single cells. In some embodiments, the isoprenoid-producing cell is a eukaryotic cell. Suitable eukaryotic host cells include, but are not limited to, yeast cells, insect cells, plant cells, fungal cells, and algal cells. Suitable eukaryotic host cells include, but are not limited to, *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorpha*, *Kluyveromyces* sp., *Kluyveromyces lactis*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*, *Chrysosporium lucknowense*, *Fusarium* sp., *Fusarium gramineum*, *Fusarium venenatum*, *Neurospora crassa*, *Chlamydomonas reinhardtii*, and the like. In some embodiments, the host cell is a eukaryotic cell other than a plant cell.

[0074] In other embodiments, the isoprenoid-producing cell is a prokaryotic cell. Suitable prokaryotic cells include, but are not limited to, any of a variety of laboratory strains of *Escherichia coli*, *Lactobacillus* sp., *Salmonella* sp., *Shigella* sp., and the like. See, e.g., Carrier et al. (1992) *J. Immunol.* 148:1176-1181; U.S. Patent No. 6,447,784; and Sizemore et al. (1995) *Science* 270:299-302. Examples of Salmonella strains which can be employed in the present invention include, but are not limited to, *Salmonella typhi* and *S. typhimurium*. Suitable Shigella strains include, but are not limited to, *Shigella flexneri*, *Shigella sonnei*, and *Shigella disenteriae*. Typically, the laboratory strain is one that is non-pathogenic. Non-limiting examples of other suitable bacteria include, but are not limited to, *Bacillus subtilis*, *Pseudomonas pudita*, *Pseudomonas aeruginosa*, *Pseudomonas mevalonii*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Rhodococcus* sp., and the like. In some embodiments, the host cell is *Escherichia coli*.

Genetically modified host cells

[0075] As noted above, in some embodiments, an isoprenoid-producing cell can be a cell that produces an isoprenoid or isoprenoid precursor via a genetically modified (heterologous) IPP biosynthetic pathway. An isoprenoid-producing cell that produces an isoprenoid or isoprenoid precursor via a genetically modified (heterologous) IPP biosynthetic pathway is referred to as a "genetically modified isoprenoid-producing host cell" or simply a "genetically modified host cell." Suitable genetically modified host cells include: 1) cells that have been genetically modified with one or more exogenous nucleic acids comprising nucleotide sequences encoding one or more heterologous mevalonate pathway enzymes, and that synthesize the isoprenoid or isoprenoid precursor at least in part via the exogenous mevalonate pathway, e.g., a prokaryotic cell that does not normally produce IPP via a mevalonate pathway, and that has been genetically modified with one or more nucleic acids comprising nucleotide sequences encoding one or more heterologous mevalonate pathway enzymes; 2) cells that have been genetically modified with one or more exogenous nucleic acids comprising nucleotide sequences

encoding one or more heterologous DXP pathway enzymes, and that synthesize the isoprenoid or isoprenoid precursor at least in part via the exogenous DXP pathway, e.g., a eukaryotic cell that does not normally produce IPP via a DXP pathway, and that has been genetically modified with one or more nucleic acids comprising nucleotide sequences encoding one or more heterologous DXP pathway enzymes ; 3) cells that have a modified endogenous mevalonate pathway, e.g., where the endogenous mevalonate pathway is genetically modified, where the cells synthesize the isoprenoid or isoprenoid precursor at least in part via the modified endogenous mevalonate pathway, e.g., a eukaryotic host cell that normally synthesizes IPP via an endogenous mevalonate pathway, where the endogenous mevalonate pathway has been genetically modified; and 4) cells that have a modified endogenous DXP pathway, e.g., where the endogenous DXP pathway is genetically modified, where the cells synthesize the isoprenoid or isoprenoid precursor at least in part via the modified endogenous DXP pathway, e.g., a prokaryotic host cell that normally synthesizes IPP via an endogenous DXP pathway, where the endogenous DXP pathway has been genetically modified.

[0076] Thus, for example, in some embodiments, a genetically modified isoprenoid-producing host cell comprises one or more nucleic acids comprising nucleotide sequences encoding one or more heterologous MEV pathway enzymes; and may further comprise nucleotide sequences encoding one or more of a heterologous IPP isomerase, a heterologous prenyl transferase, and a heterologous terpene synthase. In other embodiments, a genetically modified isoprenoid-producing host cell comprises one or more nucleic acids comprising nucleotide sequences encoding one or more heterologous DXP pathway enzymes; and may further comprise nucleotide sequences encoding one or more of a heterologous IPP isomerase, a heterologous prenyl transferase, and a heterologous terpene synthase.

[0077] To generate a genetically modified isoprenoid-producing host cell, one or more exogenous nucleic acids is introduced stably or transiently into a parent host cell, using established techniques, including, but not limited to, electroporation, calcium phosphate precipitation, DEAE-dextran mediated transfection, liposome-mediated transfection, and the like. For stable transformation, a nucleic acid will generally further include a selectable marker, e.g., any of several well-known selectable markers such as neomycin resistance, ampicillin resistance, tetracycline resistance, chloramphenicol resistance, kanamycin resistance, and the like.

Mevalonate pathway enzymes

[0078] The mevalonate pathway comprises: (a) condensing two molecules of acetyl-CoA to acetoacetyl-CoA; (b) condensing acetoacetyl-CoA with acetyl-CoA to form HMG-CoA; (c) converting HMG-CoA to mevalonate; (d) phosphorylating mevalonate to mevalonate 5-phosphate; (e) converting mevalonate 5-phosphate to mevalonate 5-pyrophosphate; and (f) converting mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. The mevalonate pathway enzymes required for production of IPP vary, depending on the culture conditions.

[0079] In some embodiments, a genetically modified host cell is one that has been genetically modified with one or more heterologous nucleic acids comprising nucleotide sequences encoding

mevalonate kinase (MK), phosphomevalonate kinase (PMK), and mevalonate pyrophosphate decarboxylase (MPD). In some embodiments, a genetically modified host cell is one that has been genetically modified with one or more heterologous nucleic acids comprising nucleotide sequences encoding MK, PMK, MPD, and isopentenyl pyrophosphate isomerase (IDI). In some embodiments, a genetically modified host cell is one that has been genetically modified with one or more heterologous nucleic acids comprising nucleotide sequences encoding MK, PMK, MPD, IDI, and a prenyltransferase.

[0080] In other embodiments, a genetically modified host cell is one that has been genetically modified with one or more heterologous nucleic acids comprising nucleotide sequences encoding acetoacetyl-CoA thiolase, hydroxymethylglutaryl-CoA synthase (HMGS), hydroxymethylglutaryl-CoA reductase (HMGR) HMGS, HMGR, MK, PMK, and MPD. In other embodiments, a genetically modified host cell is one that has been genetically modified with one or more heterologous nucleic acids comprising nucleotide sequences encoding acetoacetyl-CoA thiolase, HMGS, HMGR, MK, PMK, MPD, and IDI. In other embodiments, a genetically modified host cell is one that has been genetically modified with one or more heterologous nucleic acids comprising nucleotide sequences encoding acetoacetyl-CoA thiolase, HMGS, HMGR, MK, PMK, MPD, IDI, and a prenyl transferase.

[0081] Suitable prokaryotic host cells include, but are not limited to, any of a variety of laboratory strains of *Escherichia coli*, *Lactobacillus* sp., *Salmonella* sp., *Shigella* sp., and the like. See, e.g., Carrier et al. (1992) *J. Immunol.* 148:1176-1181; U.S. Patent No. 6,447,784; and Sizemore et al. (1995) *Science* 270:299-302. Examples of Salmonella strains which can be employed in the present invention include, but are not limited to, *Salmonella typhi* and *S. typhimurium*. Suitable Shigella strains include, but are not limited to, *Shigella flexneri*, *Shigella sonnei*, and *Shigella disenteriae*. Typically, the laboratory strain is one that is non-pathogenic. Non-limiting examples of other suitable bacteria include, but are not limited to, *Bacillus subtilis*, *Pseudomonas pudita*, *Pseudomonas aeruginosa*, *Pseudomonas mevalonii*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Rhodococcus* sp., and the like. In some embodiments, the host cell is *Escherichia coli*.

[0082] In some embodiments, the nucleic acid with which the host cell is genetically modified such that it produces IPP and/or mevalonate via a mevalonate pathway is an expression vector that includes a nucleic acid comprising a nucleotide sequence that encodes a mevalonate pathway enzyme(s). Suitable expression vectors include, but are not limited to, baculovirus vectors, bacteriophage vectors, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral vectors (e.g. viral vectors based on vaccinia virus, poliovirus, adenovirus, adeno-associated virus, SV40, herpes simplex virus, and the like), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as *E. coli* and yeast). Thus, for example, a nucleic acid encoding a mevalonate pathway gene product(s) is included in any one of a variety of expression vectors for expressing the mevalonate pathway gene product(s). Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences.

[0083] Numerous suitable expression vectors are known to those of skill in the art, and many are commercially available. The following vectors are provided by way of example, for bacterial host cells: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, lambda-ZAP vectors (Stratagene); pTrc99a, pKK223-3, pDR540, and pRIT2T (Pharmacia). However, any other plasmid or other vector may be used so long as it is compatible with the host cell.

[0084] For generating a genetically modified host cell comprising one or more heterologous nucleic acids comprising nucleotide sequences encoding mevalonate pathway enzymes, a mevalonate pathway enzyme-encoding nucleotide sequence is inserted into an expression vector. The mevalonate pathway enzyme-encoding nucleotide sequence in the expression vector is operably linked to an appropriate expression control sequence(s) (e.g., a promoter) to direct synthesis of the encoded gene product. Similarly, for generating a genetically modified host cell from a parent host cell, an expression vector comprising nucleotide sequences encoding a mevalonate pathway enzyme will be used. The mevalonate pathway enzyme coding sequences are operably linked to appropriate expression control sequence(s) to direct synthesis of the encoded gene product. Depending on the host/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see e.g., Bitter et al. (1987) *Methods in Enzymology*, 153:516-544).

[0085] Suitable promoters for use in prokaryotic host cells include, but are not limited to, a bacteriophage T7 RNA polymerase promoter; a trp promoter; a lac operon promoter; a hybrid promoter, e.g., a lac/tac hybrid promoter, a tac/trc hybrid promoter, a trp/lac promoter, a T7/lac promoter; a trc promoter; a tac promoter, and the like; an araBAD promoter; a salicylate promoter; *in vivo* regulated promoters, such as an *ssaG* promoter or a related promoter (*see*, e.g., U.S. Patent Publication No. 20040131637), a *pagC* promoter (Pulkkinen and Miller, *J. Bacteriol.*, 1991: 173(1): 86-93; Alpuche-Aranda et al., PNAS, 1992; 89(21): 10079-83), a *nirB* promoter (Harborne et al. (1992) *Mol. Micro.* 6:2805-2813), and the like (*see*, e.g., Dunstan et al. (1999) *Infect. Immun.* 67:5133-5141; McKelvie et al. (2004) *Vaccine* 22:3243-3255; and Chatfield et al. (1992) *Biotechnol.* 10:888-892); a sigma70 promoter, e.g., a consensus sigma70 promoter (see, e.g., GenBank Accession Nos. AX798980, AX798961, and AX798183); a stationary phase promoter, e.g., a *dps* promoter, an *spv* promoter, and the like; a promoter derived from the pathogenicity island SPI-2 (*see*, e.g., WO96/17951); an actA promoter (*see*, e.g., Shetron-Rama et al. (2002) *Infect. Immun.* 70:1087-1096); an rpsM promoter (*see*, e.g., Valdivia and Falkow (1996). *Mol. Microbiol.* 22:367–378); a tet promoter (*see*, e.g., Hillen,W. and Wissmann,A. (1989) In Saenger,W. and Heinemann,U. (eds), *Topics in Molecular and Structural Biology, Protein–Nucleic Acid Interaction*. Macmillan, London, UK, Vol. 10, pp. 143–162); an SP6 promoter (*see*, e.g., Melton et al. (1984) *Nucl. Acids Res.* 12:7035-7056); and the like.

[0086] In addition, the expression vectors will in many embodiments contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as antibiotic resistance (e.g., tetracycline or ampicillin resistance) in prokaryotic host cells such as *E. coli*.

[0087] In some embodiments, a mevalonate pathway enzyme-encoding nucleotide sequence is operably linked to an inducible promoter. Similarly, where the genetically modified host cell is further genetically modified with one or more additional heterologous nucleic acid(s) comprising nucleotide sequences encoding enzymes other than mevalonate pathway enzymes, the nucleotide sequences can be operably linked to an inducible promoter. Inducible promoters are well known in the art. Suitable inducible promoters include, but are not limited to, the pL of bacteriophage λ; Plac; Ptrp; Ptac (Ptrp-lac hybrid promoter); an isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible promoter, e.g., a *lacZ* promoter; a tetracycline-inducible promoter; an arabinose inducible promoter, e.g., P_{BAD} (see, e.g., Guzman et al. (1995) *J. Bacteriol.* 177:4121-4130); a xylose-inducible promoter, e.g., Pxyl (see, e.g., Kim et al. (1996) *Gene* 181:71-76); a GAL1 promoter; a tryptophan promoter; a lac promoter; an alcohol-inducible promoter, e.g., a methanol-inducible promoter, an ethanol-inducible promoter; a raffinose-inducible promoter; a heat-inducible promoter, e.g., heat inducible lambda P_L promoter, a promoter controlled by a heat-sensitive repressor (e.g., CI857-repressed lambda-based expression vectors; *see*, e.g., Hoffmann et al. (1999) *FEMS Microbiol Lett.* 177(2):327-34); and the like.

[0088] In some embodiments, the nucleotide sequence encoding a mevalonate pathway enzyme is operably linked to a constitutive promoter. Suitable constitutive promoters for use in prokaryotic cells are known in the art and include, but are not limited to, a sigma70 promoter, e.g., a consensus sigma70 promoter.

[0089] Where a parent host cell has been genetically modified to produce two or more mevalonate pathway enzymes, nucleotide sequences encoding the two or more enzymes will in some embodiments each be contained on separate expression vectors. Where the host cell is genetically modified to express one or more mevalonate pathway enzymes, nucleotide sequences encoding the one or more mevalonate pathway enzymes will in some embodiments be contained in a single expression vector. Where nucleotide sequences encoding the one or more mevalonate pathway enzymes are contained in a single expression vector, in some embodiments, the nucleotide sequences will be operably linked to a common control element (e.g., a promoter), e.g., the common control element controls expression of all of the mevalonate pathway enzyme-encoding nucleotide sequences on the single expression vector.

[0090] Where nucleotide sequences encoding the mevalonate pathway enzyme(s) are contained in a single expression vector, in some embodiments, the nucleotide sequences will be operably linked to different control elements (e.g., a promoters), e.g., the different control elements control expression of each of the mevalonate pathway enzyme-encoding nucleotide sequences separately on a single expression vector.

[0091] Nucleotide sequences encoding MEV pathway gene products are known in the art, and any known MEV pathway gene product-encoding nucleotide sequence can used to generate a subject

genetically modified host cell. For example, nucleotide sequences encoding acetoacetyl-CoA thiolase, HMGS, HMGR, MK, PMK, MPD, and IDI are known in the art. The following are non-limiting examples of known nucleotide sequences encoding MEV pathway gene products, with GenBank Accession numbers and organism following each MEV pathway enzyme, in parentheses: acetoacetyl-CoA thiolase: (NC_000913 REGION: 2324131..2325315; *E. coli*), (D49362; *Paracoccus denitrificans*), and (L20428; *Saccharomyces cerevisiae*); HMGS: (NC_001145. complement 19061..20536; *Saccharomyces cerevisiae*), (X96617; *Saccharomyces cerevisiae*), (X83882; *Arabidopsis thaliana*), (AB037907; *Kitasatospora griseola*), and (BT007302; *Homo sapiens*); HMGR: (NM_206548; *Drosophila melanogaster*), (NM_204485; *Gallus gallus*), (AB015627; Streptomyces sp. KO-3988), (AF542543; *Nicotiana attenuata*), (AB037907; *Kitasatospora griseola*), (AX128213, providing the sequence encoding a truncated HMGR; *Saccharomyces cerevisiae*), and (NC_001145: complement (115734..118898; *Saccharomyces cerevisiae*)); MK: (L77688; *Arabidopsis thaliana*), and (X55875; *Saccharomyces cerevisiae*); PMK: (AF429385; *Hevea brasiliensis*), (NM_006556; *Homo sapiens*), (NC_001145. complement 712315..713670; *Saccharomyces cerevisiae*); MPD: (X97557; *Saccharomyces cerevisiae*), (AF290095; *Enterococcus faecium*), and (U49260; *Homo sapiens*); and IDI: (NC_000913, 3031087..3031635; *E. coli*), and (AF082326; *Haematococcus pluvialis*).

[0092] Also suitable for use are mevalonate pathway enzymes of gram positive bacteria, e.g., as described in Wilding et al. (2000) *J. Bacteriol.* 182:4319-4327. Various mevalonate pathway enzyme-encoding nucleotide sequences are known in the art; and any known mevalonate pathway enzyme-encoding nucleotide sequence, or a functional variant thereof, can be used. See, e.g., *Streptococcus pneumoniae* MK, MPD, and PMK, GenBank Accession No. AF290099; *Streptococcus pneumoniae* HMGS and HMGR, GenBank Accession No. AF290098; *Enterococcus faecium* MK, PMK, and MPD, GenBank Accession No. AF290095; *Enterococcus faecium* HMGS, acetyl-CoA acetyltransferases, and HMGR, GenBank Accession No. AF290094; *Enterococcus faecalis* MK, MPD, and PMK, GenBank Accession No. AF290093; *Enterococcus faecalis* HMGS, acetyl-CoA acetyltransferases, and HMGR, GenBank Accession No. AF290092; *Staphylococcus aureus* MK, MPD, and PMK, GenBank Accession No. AF290087; *Staphylococcus aureus* HMGS and HMGR, GenBank Accession No. AF290086; *Streptococcus pyogenes* MK, MPD, and PMK, GenBank Accession No. AF290097; and *Streptococcus pyogenes* HMGS and HMGR, GenBank Accession No. AF290096.

[0093] A non-limiting example of nucleotide sequences encoding aceoacetyl-CoA thiolase, HMGS, and HMGR is set forth in Figures 13A-C (SEQ ID NO:1) of U.S. Patent No. 7,183,089. A non-limiting example of nucleotide sequences encoding MK, PMK, MPD, and isopentenyl diphosphate isomerase (IDI) is set forth in Figures 16A-D of U.S. Patent No. 7,183,089. In some embodiments, the HMGR coding region is set forth in SEQ ID NO:13 of U.S. Patent No. 7,183,089, which encodes a truncated form of HMGR ("tHMGR") that lacks the transmembrane domain of wild-type HMGR. The transmembrane domain of HMGR contains the regulatory portions of the enzyme and has no catalytic activity.

[0094] The coding sequence of any known MEV pathway enzyme may be altered in various ways known in the art to generate targeted changes in the amino acid sequence of the encoded enzyme. The amino acid of a variant MEV pathway enzyme will usually be substantially similar to the amino acid sequence of any known MEV pathway enzyme, *i.e.* will differ by at least one amino acid, and may differ by at least two, at least 5, at least 10, or at least 20 amino acids, but typically not more than about fifty amino acids. The sequence changes may be substitutions, insertions or deletions. For example, as described below, the nucleotide sequence can be altered for the codon bias of a particular host cell.

DXP pathway enzymes

[0095] The DXP pathway comprises: 1-deoxy-D-xylulose-5-phosphate synthase (Dxs), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (IspC), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (IspD), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE), 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (IspG).

[0096] In some embodiments, a genetically modified host cell is one that has been genetically modified with one or more heterologous nucleic acids comprising nucleotide sequences encoding one or more DXP pathway enzymes.

[0097] Nucleotide sequences encoding DXP pathway enzymes are known in the art, and can be used in a subject method. Variants of any known nucleotide sequence encoding a DXP pathway enzyme can be used, where the encoded enzyme retains enzymatic activity. Variants of any known nucleotide sequence encoding a DXP pathway enzyme selected from 1-deoxy-D-xylulose-5-phosphate synthase (dxs); 1-deoxy-D-xylulose-5-phosphate reductoisomerase (IspC; dxr), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (IspD; YbgP), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE; YchB), 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF; YbgB), 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (IspG), and isopentenyl diphosphate isomerase can be used, where a variant differs in nucleotide sequence by one or more nucleotides from a reference sequence (e.g., a known sequence); and where a variant nucleotide sequence includes one or more nucleotide substitutions, insertions, truncations, or deletions, compared to a reference sequence, e.g., compared to a known sequence.

[0098] The coding sequence of any known DXP pathway enzyme may be altered in various ways known in the art to generate targeted changes in the amino acid sequence of the encoded enzyme. The amino acid of a variant DXP pathway enzyme will in some embodiments be substantially similar to the amino acid sequence of any known DXP pathway enzyme, *i.e.* will differ by at least one amino acid, and may differ by at least two, at least 5, at least 10, or at least 20 amino acids, but typically not more than about fifty amino acids. The sequence changes may be substitutions, insertions or deletions. For example, as described below, the nucleotide sequence can be altered for the codon bias of a particular host cell. In addition, one or more nucleotide sequence differences can be introduced that result in conservative amino acid changes in the encoded protein.

[0099] Nucleotide sequences encoding 1-deoxy-D-xylulose-5-phosphate synthase (dxs) are known in the art. See, e.g., GenBank Accession No. DQ768815 (*Yersinia pestis* dxs); GenBank Accession No. AF143812 (*Lycopersicon esculentum* dxs); GenBank Accession No. Y18874 (*Synechococcus* PCC6301 dxs); GenBank Accession No. AF035440; *E. coli* dxs); GenBank Accession No. AF282878 (*Pseudomonas aeruginosa* dxs); GenBank Accession No. NM_121176 (*Arabidopsis thaliana* dxs); and GenBank Accession No. AB026631 (*Streptomyces sp.* CL190 dxs). Swissprot accession No. O78328 (*Capsicum annum*). See also Figure 5 of U.S. Patent Publication No. 2003/0219798 for nucleotide sequences encoding dxs.

[00100] Nucleotide sequences encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase (IspC; dxr) are known in the art. See, e.g., GenBank Accession No. AF282879 (*Pseudomonas aeruginosa* dxr); GenBank Accession No. AY081453 (*Arabidopsis thaliana* dxr); and GenBank Accession No. AJ297566 (*Zea mays* dxr). See also Figure 31 of U.S. Patent Publication No. 2003/0219798 for nucleotide sequences encoding dxr.

[00101] Nucleotide sequences encoding 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (IspD; YbgP) are known in the art. See, e.g., GenBank Accession No. AF230737 (*Arabidopsis thaliana*); GenBank Accession No. CP000034.1 (nucleotides 2725605-2724895; *Shigella dysenteriae*); and GenBank Accession No. CP000036.1 (nucleotides 2780789 to 2781448; *Shigella boydii*). See also SEQ ID NO:5 of U.S. Patent No. 6,660,507 (*Methylomonas* IspD).

[00102] Nucleotide sequences encoding 4-diphosphocytidyl-2-C-methyl-D-erythritol (IspE; YchB) kinase are known in the art. See, e.g., GenBank Accession No. CP000036.1 (nucleotides 1839782-1840633; *Shigella boydii*); GenBank Accession No. AF288615 (*Arabidopsis thaliana*) and GenBank Accession No. CP000266.1 (nucleotides 1272480-1271629; *Shigella flexneri*). See also, SEQ ID NO:7 of U.S. Patent No. 6,660,507 (*Methylomonas 16a* IspE).

[00103] Nucleotide sequences encoding 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF; YbgB) are known in the art. See, e.g., GenBank Accession No. AE017220.1 (nucleotides 3025667-3025216; *Salmonella enterica* IspF); GenBank Accession No. NM_105070 (*Arabidopsis thaliana*); GenBank Accession No. AE014073.1 (nucleotides 2838621-283841; *Shigella flexneri*).

[00104] Nucleotide sequences encoding 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (IspG; GcpE) are known in the art. See, e.g., GenBank Accession No. CP000034.1 (nucleotides 2505082 to 2503964; *Shigella dysenteriae* IspG); GenBank Accession No. NM_180902 (*Arabidopsis thaliana*); GenBank Accession No. AE008814.1 (nucleotides 15609-14491; *Salmonella typhimurium* IsgG); GenBank Accession No. AE014613.1 (nucleotides 383225-384343; *Salmonella enterica* GcpE); GenBank Accession No. AE017220.1 (nucleotides 2678054-2676936; *Salmonella enterica* GcpE; and GenBank Accession No. BX95085.1 (nucleotides 3604460-3603539; *Erwinia carotova* GcpE).

[00105] IspH genes are known in the art. See, e.g., GenBank Accession No. AY168881 (*Arabidopsis thaliana*).

[00106] Nucleotide sequences encoding IPP isomerase are known in the art. See, e.g., (J05090; *Saccharomyces cerevisiae*); Wang and Ohnuma (2000) *Biochim. Biophys. Acta* 1529:33-48; GenBank Accession No. NM_121649 (*Arabidopsis thaliana*); U.S. Patent No. 6,645,747; SEQ ID NO:1 of WO 02/095011; and SEQ ID NO:50 of WO 02/083720.

[00107] Nucleotide sequences having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99%, or higher, nucleotide sequence identity to a known nucleotide sequence encoding a DXP pathway enzyme are also suitable for use, where the nucleotide sequence encodes a functional DXP pathway enzyme.

[00108] In some embodiments, the nucleic acid with which the host cell is genetically modified such that it produces IPP via a DXP pathway is an expression vector that includes a nucleic acid comprising a nucleotide sequence that encodes a DXP pathway enzyme(s). Suitable expression vectors include, but are not limited to, baculovirus vectors, bacteriophage vectors, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral vectors (e.g. viral vectors based on vaccinia virus, poliovirus, adenovirus, adeno-associated virus, SV40, herpes simplex virus, and the like), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as *E. coli* and yeast). Suitable vectors include chromosomal, nonchromosomal and synthetic DNA sequences.

[00109] Numerous suitable expression vectors are known to those of skill in the art, and many are commercially available. The following vectors are provided by way of example, for eukaryotic host cells: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, and pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used so long as it is compatible with the host cell.

[00110] Depending on the host/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see e.g., Bitter et al. (1987) *Methods in Enzymology*, 153:516-544).

[00111] In some embodiments, a nucleotide sequence encoding a DXP pathway enzyme is operably linked to a promoter. Non-limiting examples of suitable eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Suitable promoters for expression in yeast include, but are not limited to, CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, and TP1; and, e.g., AOX1 (e.g., for use in *Pichia*). Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector may also contain a ribosome binding site for translation initiation and a transcription terminator. The expression vector may also include appropriate sequences for amplifying expression.

[00112] Generally, an expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the *S. cerevisiae* TRP1 gene, etc.; and a promoter derived from a highly-expressed gene to direct transcription of the coding sequence. Such promoters

can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The promoter can be constitutive or inducible.

[00113] In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel, et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant, et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 31987, Acad. Press, N.Y., Vol. 153, pp.516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II. A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used (Cloning in Yeast, Ch. 3, R. Rothstein In: DNA Cloning Vol. 11, A Practical Approach, Ed. DM Glover, 1986, IRL Press, Wash., D.C.). Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

Prenyl transferases

[00114] In some embodiments, a host cell is genetically modified to include one or more nucleic acids comprising a nucleotide sequence(s) encoding one or more mevalonate pathway enzymes or one or more DXP pathway enzymes, as described above; and a nucleic acid comprising a nucleotide sequence that encodes a prenyl transferase.

[00115] Prenyltransferases constitute a broad group of enzymes catalyzing the consecutive condensation of IPP resulting in the formation of prenyl diphosphates of various chain lengths. Suitable prenyltransferases include enzymes that catalyze the condensation of IPP with allylic primer substrates to form isoprenoid compounds with from about 2 isoprene units to about 6000 isoprene units or more, e.g., 2 isoprene units (Geranyl Pyrophosphate synthase), 3 isoprene units (Farnesyl pyrophosphate synthase), 4 isoprene units (geranylgeranyl pyrophosphate synthase), 5 isoprene units, 6 isoprene units (hexadecylpyrophosphate synthase), 7 isoprene units, 8 isoprene units (phytoene synthase, octaprenyl pyrophosphate synthase), 9 isoprene units (nonaprenyl pyrophosphate synthase, 10 isoprene units (decaprenyl pyrophosphate synthase), from about 10 isoprene units to about 15 isoprene units, from about 15 isoprene units to about 20 isoprene units, from about 20 isoprene units to about 25 isoprene units, from about 25 isoprene units to about 30 isoprene units, from about 30 isoprene units to about 40 isoprene units, from about 40 isoprene units to about 50 isoprene units, from about 50 isoprene units to about 100 isoprene units, from about 100 isoprene units to about 250 isoprene units, from about 250 isoprene units to about 500 isoprene units, from about 500 isoprene units to about 1000 isoprene units, from about 1000 isoprene units to about 2000 isoprene units, from about 2000 isoprene units to about 3000 isoprene units, from about 3000 isoprene units to about 4000 isoprene units, from about 4000 isoprene units to about 5000 isoprene units, or from about 5000 isoprene units to about 6000 isoprene units or more.

[00116] Suitable prenyltransferases include, but are not limited to, an *E*-isoprenyl diphosphate synthase, including, but not limited to, geranyl diphosphate (GPP) synthase, farnesyl diphosphate (FPP) synthase, geranylgeranyl diphosphate (GGPP) synthase, hexaprenyl diphosphate (HexPP) synthase, heptaprenyl diphosphate (HepPP) synthase, octaprenyl (OPP) diphosphate synthase, solanesyl diphosphate (SPP) synthase, decaprenyl diphosphate (DPP) synthase, chicle synthase, and gutta-percha synthase; and a *Z*-isoprenyl diphosphate synthase, including, but not limited to, nonaprenyl diphosphate (NPP) synthase, undecaprenyl diphosphate (UPP) synthase, dehydrodolichyl diphosphate synthase, eicosaprenyl diphosphate synthase, natural rubber synthase, and other *Z*-isoprenyl diphosphate synthases.

[00117] The nucleotide sequences of a numerous prenyl transferases from a variety of species are known, and can be used or modified for use in generating a subject genetically modified host cell. Nucleotide sequences encoding prenyl transferases are known in the art. *See*, e.g., Human farnesyl pyrophosphate synthetase mRNA (GenBank Accession No. J05262; *Homo sapiens*); farnesyl diphosphate synthetase (FPP) gene (GenBank Accession No. J05091; *Saccharomyces cerevisiae*); isopentenyl diphosphate:dimethylallyl diphosphate isomerase gene (J05090; *Saccharomyces cerevisiae*); Wang and Ohnuma (2000) *Biochim. Biophys. Acta* 1529:33-48; U.S. Patent No. 6,645,747; *Arabidopsis thaliana* farnesyl pyrophosphate synthetase 2 (FPS2) / FPP synthetase 2 / farnesyl diphosphate synthase 2 (At4g17190) mRNA (GenBank Accession No. NM_202836); *Ginkgo biloba* geranylgeranyl diphosphate synthase (ggpps) mRNA (GenBank Accession No. AY371321); *Arabidopsis thaliana* geranylgeranyl pyrophosphate synthase (GGPS1) / GGPP synthetase / farnesyltranstransferase (At4g36810) mRNA (GenBank Accession No. NM_119845); *Synechococcus elongatus* gene for farnesyl, geranylgeranyl, geranylfarnesyl, hexaprenyl, heptaprenyl diphosphate synthase (SelF-HepPS) (GenBank Accession No. AB016095); etc.

[00118] A nucleotide sequence encoding a prenyltransferase can be operably linked to a promoter. Suitable promoters are as described above, and include, e.g., constitutive promoters and inducible promoters.

Terpene synthases

[00119] In some embodiments, a host cell is genetically modified to include one or more nucleic acids comprising a nucleotide sequence(s) encoding one or more mevalonate pathway enzymes or one or more DXP pathway enzymes, as described above; a nucleic acid comprising a nucleotide sequence that encodes a prenyl transferase; and a nucleic acid comprising a nucleotide sequence encoding a terpene synthase.

[00120] In some embodiments, the terpene synthase is one that modifies FPP to generate a sesquiterpene. In other embodiments, the terpene synthase is one that modifies GPP to generate a monoterpene. In other embodiments, the terpene synthase is one that modifies GGPP to generate a diterpene. The terpene synthase acts on a polyprenyl diphosphate substrate, modifying the polyprenyl diphosphate substrate by cyclizing, rearranging, or coupling the substrate, yielding an isoprenoid or isoprenoid precursor.

[00121] Suitable terpene synthases include, but are not limited to, an abietadiene synthase, an amorphadiene synthase, a camphene synthase, a carene synthase, an α-farnesene synthase, a β-farnesene synthase, a geraniol synthase, a germacrene synthase, a humulene synthase, a linalool synthase, a limonene synthase, a myrcene synthase, an ocimene synthase, a patchoulol synthase, an α-pinene synthase, a β-pinene synthase, a selinene synthase, a sabinine synthase, a γ-terpinene synthase, a terpinolene synthase, a valencene synthase, etc.

[00122] Nucleotide sequences encoding terpene synthases are known in the art, and any known terpene synthase-encoding nucleotide sequence can be used to genetically modify a host cell. For example, the following terpene synthase-encoding nucleotide sequences, followed by their GenBank accession numbers and the organisms in which they were identified, are known and can be used: (-)-germacrene D synthase mRNA (AY438099; *Populus balsamifera subsp. trichocarpa x Populus deltoids*); E,E-alpha-farnesene synthase mRNA (AY640154; *Cucumis sativus*); 1,8-cineole synthase mRNA (AY691947; *Arabidopsis thaliana*); terpene synthase 5 (TPS5) mRNA (AY518314; *Zea mays*); terpene synthase 4 (TPS4) mRNA (AY518312; *Zea mays*); myrcene/ocimene synthase (TPS10) (At2g24210) mRNA (NM_127982; *Arabidopsis* thaliana); geraniol synthase (GES) mRNA (AY362553; *Ocimum basilicum*); pinene synthase mRNA (AY237645; *Picea sitchensis*); myrcene synthase 1e20 mRNA (AY195609; *Antirrhinum majus*); (E)-β-ocimene synthase (0e23) mRNA (AY195607; *Antirrhinum majus*); E-β-ocimene synthase mRNA (AY151086; *Antirrhinum majus*); terpene synthase mRNA (AF497492; *Arabidopsis thaliana*); (-)-camphene synthase (AG6.5) mRNA (U87910; *Abies grandis*); (-)-4S-limonene synthase gene (e.g., genomic sequence) (AF326518; *Abies grandis*); delta-selinene synthase gene (AF326513; *Abies grandis*); amorpha-4,11-diene synthase mRNA (AJ251751; *Artemisia annua*); E-α-bisabolene synthase mRNA (AF006195; *Abies grandis*); gamma-humulene synthase mRNA (U92267; *Abies grandis*); δ-selinene synthase mRNA (U92266; *Abies grandis*); pinene synthase (AG3.18) mRNA (U87909; *Abies grandis*); myrcene synthase (AG2.2) mRNA (U87908; *Abies grandis*); etc.

[00123] A nucleotide sequence encoding a terpene synthase can be operably linked to a promoter. Suitable promoters are as described above, and include, e.g., constitutive promoters and inducible promoters.

[00124] In some embodiments, a nucleic acid comprising a nucleotide sequence encoding a mevalonate pathway enzyme, a DXP pathway enzyme, a prenyltransferase, a terpene synthase, or other enzyme mentioned above, is present as an extrachromosomal element in the host cell. In other embodiments, a nucleic acid comprising a nucleotide sequence encoding a mevalonate pathway enzyme, a DXP pathway enzyme, a prenyltransferase, a terpene synthase, or other enzyme mentioned above, is integrated into the host cell's genome.

Codon usage

[00125] In some embodiments, the nucleotide sequence encoding a mevalonate pathway enzyme, a DXP pathway enzyme, a prenyltransferase, a terpene synthase, or other enzyme mentioned above, is modified such that the nucleotide sequence reflects the codon preference for the particular host cell. As one non-limiting example, the nucleotide sequence can be modified for *E. coli* codon preference. See, e.g., Gouy and Gautier (1982) *Nucleic Acids Res.* 10(22):7055-7074; Eyre-Walker (1996) *Mol. Biol. Evol.* 13(6):864-872. See also Nakamura et al. (2000) *Nucleic Acids Res.* 28(1):292. As another example, the nucleotide sequence will in some embodiments be modified for yeast codon preference. See, e.g., Bennetzen and Hall (1982) *J. Biol. Chem.* 257(6): 3026-3031.

Additional genetic modifications

[00126] In some embodiments, a genetically modified host cell is one that is genetically modified to include one or more nucleic acids comprising a nucleotide sequence(s) that encode heterologous IPP biosynthetic pathway enzymes; and that is further genetically modified to achieve enhanced production of an isoprenoid or isoprenoid precursor, and/or that is further genetically modified such that an endogenous isoprenoid biosynthetic pathway gene (e.g., an endogenous DXP pathway gene; and endogenous mevalonate pathway gene) is functionally disabled. The term "functionally disabled," as used herein, refers to a genetic modification of a nucleic acid, which modification results in production of a gene product encoded by the nucleic acid that is produced at below normal levels, and/or is non-functional.

PRODUCTION OF AN ISOPRENOID OR ISOPRENOID PRECURSOR

[00127] As noted above, an isoprenoid-producing cell is cultured *in vitro* in a defined medium as described above. The isoprenoid-producing cell is cultured *in vitro* under suitable conditions and for such a time that IPP biosynthetic pathway enzymes are produced by the cell; and the IPP biosynthetic pathway enzymes catalyze the production of an isoprenoid or isoprenoid precursor. A subject method is useful for production of a variety of isoprenoid or isoprenoid precursor compounds.

[00128] The temperature at which isoprenoid-producing cell is cultured is generally from about 18°C to about 40°C, e.g., from about 18°C to about 20°C, from about 20°C to about 25°C, from about 25°C to about 30°C, from about 30°C to about 35°C, or from about 35°C to about 40°C (e.g., at about 37°C).

[00129] In some embodiments, a subject method provides for production of an isoprenoid or isoprenoid precursor in a recoverable amount of from about 1 mg/L to about 50 g/L, e.g., from about 1 mg/L to about 5 mg/L, from about 5 mg/L to about 10 mg/L, from about 10 mg/L to about 25 mg/L, from about 25 mg/L to about 50 mg/L, from about 50 mg/L to about 100 mg/L, from about 100 mg/L to about 250 mg/L, from about 250 mg/L to about 500 mg/L, from about 500 mg/L to about 1 g/L, from about 1 g/L to about 5 g/L, from about 5 g/L to about 10 g/L, from about 10 g/L to about 15 g/L, from about 15 g/L to about 20 g/L, from about 20 g/L to about 25 g/L, from about 25 g/L to about 30 g/L, from about 30 g/L to about 40 g/L, or from about 40 g/L to about 50 g/L. Isoprenoid and/or isoprenoid precursor production is readily determined using well-known methods, e.g., gas chromatography-mass

spectrometry, liquid chromatography-mass spectrometry, ion chromatography-mass spectrometry, pulsed amperometric detection, uv-vis spectrometry, and the like.

[00130] The amount of isoprenoid or isoprenoid precursor that is produced using a subject method can also be expressed as an amount per gram of dry cell weight. Thus, e.g., in some embodiments, a subject method provides for production of an isoprenoid or isoprenoid precursor in a recoverable amount of at least 1 mg isoprenoid compound per gram of dry cell weight (mg/g), at least 2 mg/g, at least 5 mg/g, at least 10 mg/g, at least 15 mg/g, at least 20 mg/g, at least 25 mg/g, at least 30 mg/g, at least 35 mg/g, at least 40 mg/g, at least 45 mg/g, at least 50 mg/g, or more than 50 mg/g.

[00131] Isoprenoid precursors that can be produced using the method of the invention include, but are not limited to, IPP, DMAPP, an intermediate in a mevalonate pathway (e.g., mevalonate), and an intermediate in a DXP pathway.

[00132] Isoprenoids that can be produced using a subject method include, e.g., C_5-C_{10} isoprenoids, C_5-C_{15} isoprenoids, C_5-C_{20} isoprenoids, C_{10}-C_{20} isoprenoids, C_{10}-C_{25} isoprenoids, C_{10}-C_{30} isoprenoids, C_{10}-C_{40} isoprenoids, and the like. Isoprenoids that can be produced using ta subject method include, but are not limited to, hemiterpenes, monoterpenes, diterpenes, triterpenes, and polyterpenes. For example, isoprenoids that can be produced using a subject method include, but are not limited to, monoterpenes, including but not limited to, limonene, citranellol, geraniol, menthol, perillyl alcohol, linalool, thujone; sesquiterpenes, including but not limited to, periplanone B, gingkolide B, amorphadiene, artemisinin, artemisinic acid, valencene, nootkatone, epi-cedrol, epi-aristolochene, farnesol, gossypol, sanonin, periplanone, santatol, and forskolin; diterpenes, including but not limited to, casbene, eleutherobin, paclitaxel, prostratin, and pseudopterosin; triterpenes, including but not limited to, arbrusideE, bruceantin, testosterone, progesterone, cortisone, digitoxin. Isoprenoids also include, but are not limited to, carotenoids such as lycopene, α- and β-carotene, α- and β-cryptoxanthin, bixin, zeaxanthin, astaxanthin, and lutein. Isoprenoids also include, but are not limited to, triterpenes, steroid compounds, and compounds that are composed of isoprenoids modified by other chemical groups, such as mixed terpene-alkaloids, and coenzyme Q-10.

[00133] In some embodiments, an isoprenoid that can be produced using a subject methodis selected from abietadiene, amorphadiene, carene, α-farnesene, β-farnesene, farnesol, geraniol, geranylgeraniol, isoprene, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, β-pinene, sabinene, γ-terpinene, terpinolene, and valencene.

[00134] In some embodiments, the isoprenoid or isoprenoid precursor is produced at a level that is at least 2-fold higher than the level of the isoprenoid or isoprenoid precursor that is produced when the isoprenoid-producing cell is cultured in minimal medium (e.g., minimal medium without the supplement(s)). The total production of an isoprenoid or isoprenoid precursor is increased, compared to the total production of the isoprenoid or isoprenoid precursor when the isoprenoid-producing cell is grown in minimal medium (e.g., compared to the total production of the isoprenoid or isoprenoid

precursor when the isoprenoid-producing cell is grown in MOPS minimal medium). The total production can be expressed as mg/L-OD, where the OD (e.g., OD_{600} is a measure of the number of cells. For example, the total production (in mg/L-optical density, or mg/L-OD) of an isoprenoid or isoprenoid precursor produced by an isoprenoid-producing cell that is cultured *in vitro* in a defined culture medium as described above, is at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at least about 3.5-fold, at least about 4-fold, at least about 4.5-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, or at least about 500-fold, or more, higher than the total production of the isoprenoid or isoprenoid compound when the isoprenoid-producing cell is cultured in a minimal medium (e.g., in MOPS minimal medium). For example, the total production of an isoprenoid or isoprenoid precursor produced by an isoprenoid-producing cell cultured *in vitro* in a defined medium, as described above, is from about 2-fold to about 2.5-fold, from about 2.5-fold to about 3-fold, from about 3-fold to about 3.5-fold, from about 3.5-fold to about 4-fold, from about 4-fold to about 4.5-fold, from about 4.5-fold to about 5-fold, from about 5-fold to about 7-fold, or from about 7-fold to about 10-fold, from about 10-fold to about 50-fold, from about 50-fold to about 100-fold, from about 100-fold to about 200-fold, from about 200-fold to about 300-fold, from about 300-fold to about 400-fold, or from about 400-fold to about 500-fold, or more, higher than the total production of the isoprenoid or isoprenoid compound when the cell is cultured in the minimal medium.

[00135] In some embodiments, a subject method of producing an isoprenoid or isoprenoid precursor compound comprises culturing an isoprenoid-producing cell, as described above; and further comprises recovering the isoprenoid or isoprenoid precursor compound. An isoprenoid or isoprenoid produced by the isoprenoid-producing cell can be recovered (e.g., isolated, purified) from a cell lysate, from a cell supernatant (e.g., from the culture medium), or both cell lysate and cell supernatant. Methods of recovering an isoprenoid or isoprenoid precursor compound from cell lysate and from cell supernatant (e.g., from cell culture medium) are known in the art. For example, the isoprenoid-producing cell can be sonicated, subjected to detergent lysis, or subjected to another method for releasing the contents of the cytosol. An isoprenoid or isoprenoid precursor compound can be recovered from the cell culture medium and/or a cell lysate using any of a variety of methods, including, but not limited to, high performance liquid chromatography (HPLC), size exclusion chromatography, and the like. In some embodiments, an isoprenoid or isoprenoid precursor compound is secreted from the isoprenoid-producing cell, and is captured in an organic solvent which overlays the cell culture medium; in these embodiments, the isoprenoid or isoprenoid precursor compound can be recovered from the organic solvent.

[00136] In some embodiments, isoprenoid-producing cells are cultured in a defined medium as described above, optionally supplemented with one or more additional agents, such as an inducer (e.g., where the isoprenoid-producing cell is a prokaryotic cell that is genetically modified with one or more

nucleic acids comprising nucleotide sequences encoding one or more mevalonate pathway enzymes, and where the nucleotide sequence is under the control of an inducible promoter, or where a nucleotide sequence encoding an enzyme that is not directly in the mevalonate pathway but that generates a precursor that feeds into the mevalonate pathway, or that modifies a product of the mevalonate pathway, is under the control of an inducible promoter, etc.); and the culture medium is overlaid with an organic solvent, e.g. dodecane, forming an organic layer. The isoprenoid compound produced by the isoprenoid-producing cell partitions into the organic layer, from which it can be purified. In some embodiments, where the isoprenoid-modifying enzyme-encoding nucleotide sequence is operably linked to an inducible promoter, an inducer is added to the culture medium; and, after a suitable time, the isoprenoid compound is isolated from the organic layer overlaid on the culture medium.

[00137] In some embodiments, the isoprenoid or isoprenoid precursor compound will be separated from other products which may be present in the organic layer. Separation of the isoprenoid compound from other products that may be present in the organic layer is readily achieved using, e.g., standard chromatographic techniques.

[00138] In some embodiments, the isoprenoid or isoprenoid precursor compound that is recovered is pure, e.g., at least about 40% pure, at least about 50% pure, at least about 60% pure, at least about 70% pure, at least about 80% pure, at least about 90% pure, at least about 95% pure, at least about 98%, or more than 98% pure, where "pure" in the context of an isoprenoid compound refers to an isoprenoid compound that is free from other isoprenoid or isoprenoid precursor compounds, macromolecules, contaminants, etc.

EXAMPLES

[00139] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1: Varying culture medium composition to increase isoprenoid production

MATERIALS AND METHODS

Media formulations

[00140] Luria broth with Millers modifications was used for growth on agar plates and overnight cultures. M9 glucose, supplemented with micronutrients, and Neidhardt's MOPS-buffered, defined media (Neidhardt et al. (1974) *J. Bacteriol.* 119:736) were used for the batch reactor and shake flask experiments. All chemicals were purchased from Sigma (St. Louis, MO) or Fischer Scientific (Pittsburgh, PA). During experiments to determine optimal media supplementation the base media formulation (referred to as "minimal-MOPS" medium hereafter) contained MOPS, tricine, 1% glucose, Fe_SO_4, NH_4Cl, K_2SO_4, $CaCl_2$, $MgCl_2$, NaCl, K_2HPO_4, micronutrients (ammonium molybdate, boric acid, cobalt chloride, cupric sulfate, manganese chloride, zinc sulfate), and antibiotics (Neidhardt et al. (1974) *supra*) [125]. The variants of this medium contained the following supplements at concentrations as per Neidhardt et al. (Neidhardt et al. (1974) *supra*): C1 Medium (serine, methionine, glycine, adenine, guanine), Shikimate Medium (aromatic amino acids, p-amino benzoic acid, p-hydroxy benzoic acid, 2,3 dihydroxybenzoic acid), C1+Shikimate Medium (contains all supplements found in the C1 and Shikimate media), 8E Medium (alanine, glutamine, glutamic acid, isoleucine, leucine, methionine, serine and valine (Zhang et al. (2006) *Biochimie* 88:1145)), MGL Medium (methionine, glycine and leucine), and Neidhardt's Defined Rich Medium (all amino acids and vitamin supplements).

Strain construction

[00141] *E. coli* DH1 was used as the cloning and expression host in this study (see Table 1 for strains used in this study). The amorpha-4-11-diene–producing strain used in this study contained a three plasmid system encoding the *E. coli* genes *atoB, idi, ispA;* the yeast genes *HMGS, HMGR, MK, PMK, MPD;* and a synthetic *ADS* as previously reported. Martin et al. (2003) *Nat. Biotech.* 21:796-802.

Table 1. Strains and plasmids used in this study.

Strain/ Plasmid	Genotype/Description
DH1	endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17(r_K^- m_K^+) λ^-
pMevT	pBad33 derivative containing the *atoB*, HMGS and tHMGR genes under control of P_{LAC}; *Cm*^{r}
pMBIS	pBBR1MCS-3 containing MK, PMK, MPD, idi, ispA under P_{LAC}; *Tc*^{R}
pADS	pTrc99A containing synthetic amorpha-4-11-diene synthase; *Ap*^{r}
pMevT-C159A	pMevT derivative containing *HMGS(C159A)*
pMBI*S*	pMBIS derivative containing catalytically inactive farnesyl diphosphate synthase and isopentenyl diphosphate isomerase
pADS-mut1	pADS derivative containing catalytically inactive amorpha-4-11-diene cyclase

[00142] Plasmids pMevT, pMBIS, and pADS are described in, e.g., e.g., U.S. Patent No. 7,183,089; U.S. Patent No. 7,172,886; U.S. Patent No. 7,192,751; U.S. Patent Publication Nos. 2006/007946,

2003/0148479, and 2004/0005678; and Martin et al. (2003) *Nat. Biotech.* 21:796-802. Nucleotide sequences of the plasmids are provided in U.S. Provisional Patent Application No. 60/802,266 and in U.S. Patent No. 7,183,089: pMevT (SEQ ID NO:3); pMBIS (SEQ ID NO:4); and pADS (SEQ ID NO:5). The strain DH1 is described in, e.g., Meselson Yuan (1968) *Nature* 217(134): p. 1110-4. Plasmids pMevT-C159A, pMBI*S*, and pADS-mut1 are described below.

[00143] Since transcriptomic and proteomic analysis is inherently relative, a control strain was designed such that the heterologous proteins would be expressed but key enzymes were catalytically inactive. This accounted for the protein expression artifact in the relative transcript profile in comparisons between the amorpha-4-11-diene producing strain and the control allowing detection of gene expression changes in response to biochemical flux through the exogenous pathway.

[00144] Following the work of Rokosz *et al* (*Arch Biochem Biophys* 312:1-13 (1994)), the catalytic cysteine of the *S. cerevisiae* HMGS active site in pBAD33MevT was replaced with an alanine by site-directed mutagenesis (QuickChange Site-directed mutagenesis kit, Stratagene) using primers mutAHS-F and mutAHS-R, thereby creating a full-length, inactive HMGS[124]. The yeast HMGS mutant with cysteine changed to alanine at amino acid position 159, named HMGS(C159A), was verified by DNA sequence of the entire operon. the plasmid pBad33MevT containing the HMGS(C159A) mutant was named pMevT(C159A), also referred to as pBad33MevT(C159A), (SEQ ID NO:11, U.S. Patent No. 7,183,089).

[00145] Since the farnesyl diphosphate synthase and isopentyl diphosphate isomerase expressed on the pMBIS plasmid were copies of endogenous genes, the plasmid encoded-copies were inactivated by point mutations, *ispA* (R96Q) (Song and Poulter (1994) Proc Natl Acad Sci USA 91(8): p. 3044-8) and *idi* (E116V) (Carrigan and Poulter (2003) J Am Chem Soc, 125(30): p. 9008-9; and Hahn et al. (1999) J Bacteriol, 1999. 181(15): p. 4499-504), to prevent any effect on endogenous metabolism due to enzymatic activity. The inactivated pMBIS plasmid was produced by point mutations in the *idi* and *ispA* genes. The IDIE116V mutant was generated from pBAD24-IDI using the standard QuikChange (Stratagene, La Jolla, CA) procedure with the idi-f and idi-R primers. IDIE116V was then amplified using the primers idi-f and idi-r and ligated into the Xma I site of pMevB. The IspAR96Q mutant was generated from pBAD24-IspA using the standard QuikChange (Stratagene, La Jolla, CA) procedure with the ispa-f and ispa-r primers. IspAR96Q was then amplified using the primers ispa-f and ispa-r and ligated into the SacII-SacI site of the pMB-IE116V plasmid. The final plasmid, pMB-IE116V-SR96Q, was verified by sequencing.

[00146] Inactivation of pMBIS was tested by monitoring amorpha-4-11-diene production. *E. coli* DH10B was co-transformed with pADS and pMevB, pMBIS, or pMB-IE116V-SR96Q. Fifty (50) mL of 2YT containing carbenicillin (50 μg/L) and tetracycline (5 μg/L) in a 250-mL baffled shake flask was inoculated with 1 mL of overnight LB culture of each of the freshly transformed strains. The cultures were grown at 37°C at 200 rpm to OD_{600} = 0.2-0.3 before inducing with IPTG (1 mM). At this time, mevalonate (final concentration of 5 mM) and dodecane (5 mL) were added to the culture. After

48 h of growth, the dodecane layer was sampled and analyzed by GC-MS. The resulting plasmid was designated pMBI*S*.

[00147] Finally, to prevent the synthetic amorpha-4-11-diene synthase from straining the FPP pool in the control strain, it was inactivated by converting three required aspartates in the first aspartate rich motif to alanines and cloned into the same pTrc99A plasmid used for pADS and named pADS-mut1. The inactive pathway strain (IAPS) control used in this study was created by transforming DH1 with pMevT-IAP, pMBI*S* and pADS-mut1.

Batch reactor growth and biomass collection

[00148] *E. coli* DH1 was transformed with plasmids encoding the amorpha-4-11-diene synthesis pathway (pMevT, pMBIS and pADS) or the inactive pathway control (pMevT-C159A, pMBI*S* and pADS-mut1) and grown on LB agar plates. Single colonies were chosen for 10-ml, overnight culture in Luria Broth with Millers modification. Overnight LB cultures were used to inoculate a second overnight culture in M9-glucose medium supplemented with micronutrients. The M9-glucose overnight cultures were then used as 1% inoculum for a 200-ml starter culture (M9-glucose). The 200-ml starter cultures were grown overnight and then used to inoculate a 10-L batch reactor (M9-glucose) to a starting OD_{600} of ~0.05. The batch reactor was operated with a 1 volume/initial volume house air supply, 700 RPM stirring, pH controlled with 5% NaOH at 6.4-6.9 pH, and an operating temperature of 30°C. The exhaust air was routed through a gas trap containing 200-ml of dodecane to capture any amorpha-4-11-diene the culture produced. The exhaust from the gas trap was then analyzed with an in-line mass selective detector for CO_2 concentration. All media were supplemented with 50 μg/ml carbenicillin, 5 μg/ml tetracycline, and 25-μg/ml chloramphenicol to maintain plasmids, and all M9-glucose media were supplemented with 1% glucose and micronutrients as per Neidhardt (Neidhardt et al. (1974) *supra*).

[00149] Batch reactor cultures were grown until to an OD_{600} of ~0.3 and sampled (T0) prior to induction with 50 mM IPTG. Biomass samples were taken every cell doubling for a total of three post-induction samples (T1-T3) after which the cultures were grown for an additional 13-15 hours and then sampled for amorpha-4-11-diene and mevalonate. Biomass samples collected for transcriptional analysis were centrifuged at 10,000 rpm for three minutes at room temperature, and the supernatant was discarded. The cell pellets were immediately transferred to a liquid nitrogen bath and frozen for storage at -80°C.

Transcript analysis sample preparation

[00150] RNA was extracted from the cell samples using the RNEasy Midi kit (QIAGEN) following the manufactures instructions. Using 40 μg aliquots of extracted RNA from each batch reactor time point pre-labeled cDNA was synthesized using random-primed reverse transcription reactions in a 40-μl volume containing 12.5-μg random primers (Invitrogen, Carlsbad, CA), 1x RT buffer (Invitrogen, Carlsbad, CA), 0.01 mM DTT (Invitrogen, Carlsbad, CA), 1 unit/μl Superase-In (Ambion, Austin, TX), 0.5 mM dATP/dCTP/dGTP (Invitrogen, Carlsbad, CA), 0.1 mM dTTP (Invitrogen, Carlsbad, CA), 0.4 mM amino-allyl-dUTP (Ambion, Austin, TX) and 10 units/μl Superscript II (Invitrogen, Carlsbad, CA)

following the enzyme manufacturer's instructions. The cDNA was base hydrolyzed in 100 mM NaOH/10 mM EDTA at 65°C for ten minutes and then neutralized using 7.0 pH HEPES at a final concentration of 500 mM. The Tris remaining in the cDNA suspension was removed by three buffer exchange spins using Micron YM-30 columns (Millipore) and eluted in a final volume of ~15 □l water. The cDNA was then labeled using either Alexa 555 or Alexa 647 (Invitrogen, Carslbad, CA) following the manufacturers protocol.

Microarray hybridization

[00151] Glass microarrays printed with 70-mer oligonucleotides (Operon) designed to probe every open reading frame (ORF) of *E. coli* MG1655 were hybridized in a TECAN hybridization station with ~6-10 μg of labeled cDNA per detection channel. The hybridization program included a pre-hybridization (5x SSC/0.2% SDS/1% BSA, 42°C, 60 minutes), a 17-hour hybridization (Ambion Hyb Solution #3, 41°C, medium agitation), two low stringency washes (1x SSC/0.2%SDS, 41°C, 2 minutes each), two high stringency washes (0.1x SSC/0.2%SDS, 25°C, 2 minutes each), and two final washes (0.1x SSC, 25°C, 2 minutes each). Following hybridization, the slides were scanned with an Axon 4500 with at 60% laser power and PMT gain adjusted to balance the total intensity histogram.

Transcriptional profile data analysis

[00152] The raw scans were globally normalized using Genepix software and then processed using SNOMAD's loess normalization to correct for any hybridization artifacts (Colantuoni et al., *SNOMAD (Standardization and NOrmalization of MicroArray Data): web-accessible gene expression data analysis.* Bioinformatics, 2002. 18(11): p. 1540-1). Local Z-scores generated by SNOMAD and the serial analysis for microarray (SAM) software (Tusher et al. (2001) Proc Natl Acad Sci U S A, 98(9): p. 5116-21) were used as guides to determine biologically significant expression changes. The biologically significant genes were then mined using hierarchal clustering (Cluster 3.0) to determine a base set of clusters in each data set (Eisen (1998) Proc Natl Acad Sci U S A 95(25): p. 14863-8). Once a base set of clusters was chosen, k-means clustering was also used to search the data set for temporal patterns in gene expression (Cluster 3.0).

Shake flask growth for media testing

[00153] In order to test the effects of various media components on amorpha-4-11-diene production the amorpha-4-11-diene producing strain (APS) was grown in 50-ml shake flask cultures using variations of Neidhart's MOPS-buffered minimal medium listed above. *E. coli* DH1 was transformed with pMevT, pMBIS and pADS and plated onto minimal agar plates. Single colonies were chosen and grown in a 10-ml overnight culture in minimal medium supplemented with 1% glucose media. This overnight culture was used to inoculate 50-ml shake flasks containing the various media formulations and a 20% v/v dodecane overlay in order to capture the volatile amorpha-4-11-diene. The cultures were grown to ~0.3 OD_{600} and induced with 50 mM IPTG and then sampled approximately every 6-12 hours for 48-76 hours for amorpha-4-11-diene production.

Amorpha-4,11-diene production assays

[00154] Amorpha-4,11-diene production levels from *E. coli* strains expressing the full amorpha-4-11-diene production pathway as well as the inactivated production pathway were assayed under a variety of conditions. The amorpha-4-11-diene concentration in the dodecane capture fluid was assayed at multiple time points by diluting 10-μL of the dodecane overlay into 990-μL of ethyl acetate spiked with 5 μg/mL trans,trans-caryophyllene (both Sigma) as an internal standard.

[00155] Dodecane/ethyl acetate extracts were analyzed on a Hewlett-Packard 6890 gas chromatograph/mass spectrometer (GC/MS). A 1-μL sample was separated on the GC using a DB-5 column (Agilent Technologies, Inc., Palo Alto, Calif.) and helium carrier gas at 1 L/min. The oven cycle for each sample was 80°C for two minutes, increasing temperature at 30°C/min to a temperature of 160°C, increasing temperature at 3°C/min to 170°C, increasing temperature at 50°C/min to 300°C, and a hold at 300°C for two minutes. The resolved samples were analyzed by a Hewlett-Packard model 5973 mass selective detector that monitored ions 189 and 204 m/z. A standard curve for amorpha-4-11-diene was determined, based on a pure standard. The amorpha-4,11-diene concentration is based on the relative abundance of 189 and 204 m/z ions to the abundance of the total ions in the mass spectra.

GC-MS quantification of mevalonate

[00156] Mevalonate (mevalonic acid) concentration in cultures of engineered *E. coli* was determined by GC-MS analysis. *E. coli* culture (560 μL) was mixed with 140 μL of 500 mM HCl in a glass GC vial to convert mevalonate from mevalonic acid to mevalonic acid lactone. Ethyl acetate (700 μL), spiked with 500 μg/ml (-)-trans-caryophyllene as an internal standard, was added to each vial, and then the samples were shaken at maximum speed on a Fisher Vortex Genie 2 mixer (Fisher Scientific) for 3-5 minutes. The ethyl acetate extract of acidified culture was diluted 1:100 with fresh ethyl acetate in a clean GC vial before analysis.

[00157] Diluted ethyl acetate extracts were analyzed using an Agilent Technologies 6890 gas chromatograph with an Agilent Technologies model 5973 mass selective detector (GC-MS) operating in electron impact mode. The GC column used was an Agilent Technologies DB-5ms (30 m x 250 μm x 0.25 μm). Helium was used as the carrier gas at a constant flow of 1 ml/min, and 1-μL splitless injections were preformed. The injection port was maintained at 250°C, the MS source temperature was maintained at 230°C, and the MS quad temperature was held constant at 150°C. The oven cycle for each sample and the ions monitored were modified from published methods (Woollen (2001) J Chromatogr B Biomed Sci Appl, 760(1): p. 179-84). The column temperature profile was 70°C for 2 minutes; ramped at 15°C/min to 185°C; ramped at 30°C/min to 300°C; and held at 300°C for 3 minutes. The selected ions monitored were m/z 71 and 58 for mevalonic acid lactone, and m/z 189 and 204 for (-)-trans-caryophyllene. Retention time, mass spectrum and concentration of extracted mevalonic acid lactone were confirmed using commercial DL-mevalonic acid lactone (Sigma).

Intracellular metabolite extraction and analysis

[00158] The concentrations of intracellular acyl-CoAs and adenylate pool constituents were determined by LC-MS analysis of trichloroacetic acid (TCA) culture extracts taken during the exponential phase of growth. To simultaneously and rapidly quench cellular metabolism, isolate *E. coli* cells from the growth medium and extract metabolites, cells were centrifuged through a layer of silicone oil into a denser solution of TCA by method similar to that of Shimazu et al (Shimazu (2004) Anal Biochem, 328(1): p. 51-9). Using 15-mL Falcon tubes (Fischer Scientific), 2 mL silicone oil (AR200 from Fluka) was layered over 0.5 mL 10% trichloroacetic acid (Fluka) in Deuterium Oxide (Sigma). The TCA layer was spiked with 10 μM crotonyl-CoA as an internal standard. Tubes were stored on ice until time of sampling. To each tube, 10 mL of cell culture was carefully added above the silicone oil layer. Tubes were then quickly centrifuged at 4°C for 3 min at 10,000×g. By centrifugation, the cells traverse the silicone layer, lyse in the TCA layer and quench metabolism. The spent medium was carefully removed by aspiration and the TCA extract layer was transferred to a 2-mL centrifuge tube using a small gauge needle and syringe. To neutralize the TCA, 1 mL of ice cold 0.5 M tri-n-octylamine in 1,1,2-trichloro-1,2,2-trifluoroethane (both Sigma) was added, tubes were vortexed for 1 minute and then centrifuged at max speed for 2 minutes to separate the layers. The aqueous layer was removed for analysis by LC-MS.

[00159] The neutralized TCA extract was analyzed using a Hewlett-Packard 1100 series LC-MS using electrospray ionization. A 50-μL sample was separated on a C-18 reverse phase HPLC column (250 x 2.1 mm Inertsil 3-um ODS-3 by Varian) using a two solvent gradient system adapted from J.J. Dalluge et al (Dalluge (2002) Anal Bioanal Chem, 374(5): p. 835-40). Solvent A was 100 mM ammonium acetate buffer at pH 6, and Solvent B was 70% Solvent A and 30% acetonitrile. The HPLC column was equilibrated each run with 8% Solvent B (92% Solvent A) for 12 minutes. Using a 0.25 mL/min flow rate and linear gradients as indicated, the elutant program was: 8% Solvent B at 0 min to 50% Solvent B at 5 min, gradient increase to 100% Solvent B at 13 min, isocratic at 100% Solvent B until 19 min, gradient returning to 8% Solvent B at 26 min. The resolved metabolite samples were analyzed by electrospray ionization mass selective detector (ESI-MS) operated in positive mode. The following ESI-MS parameters were used: drying gas, 12 L/min; nebulizer pressure, 60 psig; drying gas temperature, 300°C; capillary voltage, 2500 V. Selected ions corresponding to the protonated molecular ion of each metabolite were monitored: adenosine 5′-triphosphate (ATP) – m/z 508, adenosine 5′-diphosphate (ADP) – m/z 428, adenosine 5′-monophosphate (AMP) – m/z 348, coenzyme A – m/z 768, acetyl-CoA – m/z 810, propionyl-CoA – m/z 824, crotonyl-CoA – m/z 836, acetoacetyl-CoA – m/z 852, malonyl-CoA – m/z 854, succinyl-CoA – m/z 868, methylmalonyl-CoA – m/z 868, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) - m/z 912. Retention times, mass spectra and concentrations of extracted metabolites were confirmed using commercial standards (Sigma).

RESULTS

[00160] Initially, a systems biology approach was taken in order to develop an understanding of how expression of the amorpha-4-11-diene producing pathway affected the heterologous host. *E. coli* DH1 was transformed with either the amorpha-4-11-diene producing pathway (Figure 4), or the inactivated pathway and grown in 10-L batch reactors until an OD^{600} of ~0.3. At that point biomass and growth medium samples were taken for later analysis (designated pre-induction, T0), and expression of the heterologous pathway was induced with 50 mM IPTG. The OD_{600} and CO_2 off-gas were monitored, and biomass and growth medium samples were taken every cell doubling for a total of three post-induction time points (designated T1, T2 and T3 respectively). Growth medium samples were analyzed for glucose, ammonium, and acetate while biomass samples were analyzed for acyl-CoAs, mRNA and protein profiles.

[00161] The production of the olefinic sesquiterpene, amorpha-4-11-diene, by the engineered mevalonate pathway imposed a metabolic burden on the heterologous host due to a) heterologous protein expression and b) biochemical flux through the exogenous pathway. Using proteomic, transcriptomic, and metabolomic profiling and various versions of the amorpha-4-11-diene pathway, we examined the burden associated with amorpha-4-11-diene production. Since point mutations in genes encoding key enzymes of the engineered pathway genes prevented any biochemical flux from acetyl-CoA to amorpha-4-11-diene, the impact on the *E. coli* host due solely to the expression of heterologous pathway proteins was evaluated by comparing post-induction to pre-induction profiles in the inactivated pathway strain (designated IAPS). Using a similar comparison strategy, the combined burdens of heterologous protein expression and active flux through the engineered pathway were profiled in the amorpha-4-11-diene–producing strain (designated APS) by comparing post-induction biomass samples with pre-induction samples. Finally, the specific burden on the heterologous host associated solely with active biochemical flux through the amorpha-4-11-diene production pathway was profiled using direct comparison of biomass from each strain at each point over a time course.

[00162] There were 863, 866 and 933 mRNA transcripts observed in the APS, IAPS and APS vs IAPS profiles, respectively, that had a biologically significant expression change in at least one time point. Additionally, there were 713, 655 and 745 proteins detected in the APS, IAPS and APS vs IAPS profiles respectively. The interpretation of this data revealed that the expression of the heterologous proteins, as well as biochemical flux through the amorpha-4-11-diene pathway, significantly impacted amino acid anabolism.

Amino Acid Metabolism

[00163] In the medium used for the batch growth of the APS and IAPS the cells are forced to synthesize all their amino acids from glucose and ammonia, which places a significant metabolic burden on the organism. After induction of expression from the plasmids in the APS and IAPS, there is significant alteration of genes involved in amino acid metabolism. By combing our knowledge of what intracellular signals modulate transcription of amino acid biosynthetic genes with the measurement of

their expression profiles we can gain insight into the metabolic state of the amorpha-4-11-diene-producing strain.

[00164] The expression profiles reveal that the amorpha-4-11-diene producing strain (APS) is experiencing a high serine turn over which is straining the single carbon metabolism. This strain is reflected in the time course expression profile of the LRP regulon as well as the genes in the methionine, serine, glycine and histidine biosynthetic pathways.

Single-carbon metabolism

[00165] The transcript and protein profiles of genes involved in the biosynthesis of serine, glycine and methionine are consistent with a significant metabolic burden imposed on single carbon metabolism by heterologous protein expression. Single carbon units are used, via carrier molecules, in a wide variety of metabolic functions, including the biosynthesis of purines, histidine, thymine, pantothenate, methionine, tRNA modification, chemotaxis, and nucleic or protein methylation. During growth in minimal medium, the methyl groups utilized in C1 metabolism are ultimately derived from serine, and both the mRNA and gene product (3-phosphoglycerate dehydrogenase) of *serA* were up-regulated in the APS at T3 (Table 2). Transcription of *serA* was up-regulated in the inactive-pathway strain (IAPS) at T2-T3 by 2-3-fold as well, though the enzyme level remained constant over the entire time course in this strain. When the two strains were compared directly by microarray and proteomic analysis, the expression of *serA* was 4-fold higher in the APS at T3 while the protein levels were equivalent.

[00166] In Table 2, transcript expression ratios of stress regulon genes in the *E. coli* DP10 pBAD33MevT time course (relative to pre-induction), the *E. coli* DP10 pBAD33MevT vs. *E. coli* DP10 pBAD33MevT-C159A time course (relative to the inactive pathway control) and the *E. coli* DP10 pBAD33MevT-C159A time course (relative to pre-induction). Both fold-changes and local Z-scores (in parenthesis) are shown.

Table 2

MevT			MevT vs MevT-C159A			MevT-C159A		
Oxidative Stress								
	1 hr	3 hr		1 hr	3 hr		1 hr	3 hr
dps	2.9 (3.1)	4.1 (4.0)	*katE*	ND	3.8 (3.2)	*dps*	1.4 (1.1)	2.1 (2.3)
sufA	2.1 (3.1)	3.6 (4.0)	*katG*	2.6 (2.8)	3.8 (3.1)	*soxS*	1.4 (1.2)	2.4 (1.9)
poxB	1.8 (2.6)	2.2 (3.8)	*dps*	2.2 (3.1)	3.2 (12.1)	*sodB*	1.6 (1.5)	2.2 (1.5)
ahpC	1.9 (2.5)	2.6 (2.0)	*sufA*	1.6 (2.1)	2.4 (4.7)			
ynhC	1.7 (3.2)	3.1 (3.5)	*sufB*	1.6 (2.2)	2.4 (6.5)			
sufB	1.4 (2.4)	3.2 (3.3)	*sufC*	1.7 (2.2)	2.2 (4.0)			
katE	1.2 (2.6)	3.0 (3.6)	*sufD*	1.9 (2.2)	2.1 (3.7)			
sufD	1.8 (2.9)	2.9 (3.7)						
sufC	2.1 (2.3)	2.4 (3.2)						
Osmotic Stress								
	1 hr	3 hr		1 hr	3 hr			
osmC	3.3 (4.2)	5.5 (6.0)	*otsB*	1.3 (4.2)	5.3 (4.9)			
osmY	2.9 (3.8)	6.6 (6.3)	*otsA*	1.2 (2.5)	5.3 (4.5)			
osmE	2.4 (3.8)	3.0 (4.5)	*osmC*	3.2 (4.1)	4.6 (3.5)			
otsA	1.8 (2.4)	3.9 (5.2)	*osmY*	2.3 (4.6)	3.6 (5.8)			
betA	1.5 (2.0)	2.8 (4.1)	*betA*	1.3 (2.7)	3.2 (3.7)			
otsB	1.9 (2.4)	2.9 (4.1)	*osmB*	0.9 (2.8)	3.2 (3.0)			
betB	1.7 (2.1)	2.5 (3.5)	*betB*	1.6 (2.7)	2.4 (2.8)			
proP	1.6 (1.6)	2.1 (2.8)	*osmE*	1.3 (2.2)	2.1 (3.3)			
			proP	1.4 (2.3)	1.9 (3.3)			
			betT	0.8 (2.0)	1.5 (2.7)			
			kdpA	1.2 (1.8)	1.9 (2.8)			
Heat Shock								
	1 hr	3 hr		1 hr	3 hr		1 hr	3 hr
clpB	8.4 (5.5)	2.9 (3.2)	*clpB*	2.5 (6.8)	0.4 (-2.3)	*ibpB*	5.3 (9.2)	37.9 (6.1)
ibpB	6.6 (6.6)	2.5 (3.3)	*ibpB*	2.5 (5.8)	0.5 (-4.3)	*clpB*	9.4 (6.8)	22.5 (7.9)
ibpA	5.6 (5.2)	3.7 (4.2)	*ibpA*	1.7 (2.3)	0.3 (-4.6)	*ibpA*	5.7 (6.9)	42.1 (6.2)
dnaK	5.1 (6.0)	2.6 (2.0)	*grpE*	0.8 (1.1)	0.4 (-3.3)	*grpE*	5.3 (4.3)	5.0 (5.9)
groS	3.1 (4.6)	2.8 (2.1)	*hchA*	2.4 (3.5)	ND	*dnaK*	6.4 (5.3)	6.0 (6.8)
grpE	3.0 (3.1)	1.3 (0.9)	*pphA*	2.2 (4.0)	ND	*htpG*	3.4 (3.3)	3.3 (4.4)
hslV	2.9 (3.1)	2.3 (2.5)	*dnaK*	2.0 (5.6)	0.7 (-2.1)	*groS*	4.1 (1.7)	4.4 (5.4)
htpG	2.8 (3.6)	1.4 (1.4)	*htpG*	2.0 (3.2)	0.7 (-2.0)	*hslU*	3.2 (2.5)	2.7 (4.1)
groL	2.3 (2.7)	1.7 (1.2)	*groS*	1.1 (1.4)	0.5 (-2.9)	*groL*	3.4 (1.8)	3.8 (4.1)
clpA	2.2 (2.4)	1.4 (1.2)	*groL*	0.8 (-1.0)	0.3 (-3.1)	*hslV*	2.7 (1.8)	2.1 (3.5)
dnaJ	2.1 (2.2)	1.6 (1.5)				*dnaJ*	2.2 (2.2)	2.6 (2.8)
hslU	2.0 (2.3)	1.4 (1.1)				*clpA*	1.7 (2.8)	2.8 (1.9)
lon	1.9 (2.0)	1.9 (2.0)				*htpX*	1.8 (2.5)	2.5 (2.2)
						lon	2.2 (1.8)	2.1 (2.6)
						gapA	1.9 (1.1)	2.5 (2.7)
						clpP	1.5 (1.9)	2.1 (1.6)

[00167] Serine is subsequently converted into glycine by serine hydroxymethyltransferase (SHMT) in a reaction that transfers a methyl group to the C1 carrier molecule, tetrahydrofolate (THF). Figure 3. This reaction provides all of the estimated 902 μmol/g-biomass of single carbon units required during growth in minimal medium, and as such the expression level of the gene encoding SHMT, *glyA,* is an important indicator of the state of single carbon metabolism. The expression of *glyA* was up-regulated 2.8-fold at T3 in the APS (Table 2). Expression of this gene was also up-regulated 2.8-fold in the IAPS control though this was observed at T2, after which expression of this gene returned to pre-induction

levels at T3. When the two strains were compared directly, *glyA* expression was significantly higher at all time points in the APS relative to the control strain.

[00168] Another important indication of the single carbon limitation in the APS and IAPS cultures is the expression profiles of the methionine biosynthetic genes. Methionine is the least utilized amino acid for protein synthesis in *E. coli,* but it is the key precursor to the primary methyl-carrier cofactor in *E. coli*, S-adenosyl-methionine (SAM). SAM acts as a transcriptional co-repressor, along with MetJ, of all the methionine biosynthetic genes except for *metH.* The expression of expression of *glyA* is coordinated with methionine biosynthesis through the action of another transcriptional regulator, MetR. Thus, methionine biosynthesis plays a central role in single carbon metabolism and the transcriptional profile of the *met* genes provides a biomarker for the state of single carbon metabolism during growth in minimal media. The expression of all methionine biosynthetic genes except for *metH* were strongly up-regulated in the APS at T3, matching the profile observed for *glyA.* Expression of the *met* genes also matched *glyA* expression in the IAPS, with an up-regulation at T2 followed by a down-regulation at T3. When the two strains are compared directly, the combined pattern is seen in the methionine biosynthetic genes: expression is higher at T2 in the control strain and higher at T3 in the amorpha-4-11-diene-producing strain.

[00169] As previously mentioned, the biosynthesis of methionine consumes a single carbon unit in the form of methyl-tetrahydrofolate. Tetrahydrofolate is an important carrier of methyl groups and is synthesized from chorismate. It has been recently reported that one gene in this pathway , *folE,* is transcriptionally repressed by the MetJ-SAM complex (Marincs (2006) Biochem J, 396(2): p. 227-34), and indeed expression of this gene is co-regulated with *glyA* and the *met* genes in both strains (Table 2). Taken together this data confirms that there are significant regulatory changes occurring in both strains in response to a burden on single carbon metabolism imposed by heterologous protein expression. While the protein profiles are fairly equivalent, the transcriptional response suggests a more severe burden in the APS that occurs later in the growth cycle.

The heterologous pathway affects the LRP regulon

[00170] The leucine-responsive regulatory protein (Lrp) plays an important role in coordinating amino acid metabolism with the nutritional quality of the growth medium by regulating the transcription of several genes in *E. coli.* While there were no significant differences observed in the Lrp levels in either strain over the time course (Table 2), there were changes observed in the expression of leucine biosynthetic genes which likely had a significant impact on both strain's metabolism. Lrp's affinity for certain promoters is modulated by the intracellular leucine concentration, and the activity of this transcriptional regulator is reported to be 4-10 fold higher in minimal media (Calvo and Matthews (1994) Microbiol Rev, 58(3): p. 466-90). Following IPTG induction of the heterologous amorpha-4-11-diene pathway, a strong down-regulation of the leucine biosynthetic pathway was observed in both strains. Transcription of the *leu* operon was strongly down-regulated in the APS by the first post-induction time point (T1) and then expression increased at T2 and T3, though overall *leu* transcript

levels remained lower relative to pre-induction during the entire time course. In the IAPS, expression of the *leu* operon was down-regulated as well over the entire time course, though the initial response to IPTG induction was not as severe. The enzyme levels, relative to pre-induction, mirror the transcriptional response in each strain (Table 2). When transcript profiles are compared directly by microarray analysis, expression of the *leu* genes was equivalent in the two strains prior to IPTG induction, but then were down-regulated in the APS more significantly at T1 and T2. Since transcription of the leucine biosynthetic genes is inhibited by the availability of leucine-charged tRNA, this data suggests leucine availability in both strains increased after IPTG induction of heterologous gene expression.

[00171] Since Lrp is a transcriptional regulator, any significant changes in leucine availability would be observed as modulation of gene expression in the Lrp regulon in the microarray data. Indeed, significant transcriptional changes were observed in the Lrp regulon for both strains though determining whether changes in leucine biosynthesis contributed to this response was complicated by the fact that most transcriptional units in this regulon are controlled by additional regulatory signals and the effect of leucine on LRP affinity varies for each promoter. There were a few transcriptional units in the LRP regulon that were observed in all three microarray comparison methodologies that have been reported to be differentially regulated by Lrp-leucine (as compared to Lrp in its unbound form) and which are not under the control of other transcriptional regulators in the growth conditions used in this study.

[00172] One such transcriptional unit is *livKHMGF*, which encodes a branched-chain amino acid transporter and is repressed by Lrp but only in the presence of excess intracellular leucine (Haney (1992) J Bacteriol, 174(1): p. 108-15). The time course expression profile of this transcriptional unit exhibited a post-induction up-regulation at T2 in the APS followed by a significant down-regulation at T3 while the IAPS control had a minor down-regulation at T2. Expression of the *liv* operon was observed to increase in the APS relative to the IAPS control until T2, after which expression was strongly down-regulated at T3. These data suggest that leucine bound to Lrp increases post-induction, but only at T3. Another operon, the dipeptide transporter encoded by the *oppABCDF*, is repressed by Lrp alone but activated by Lrp bound to leucine (Andrews and Short (1986) J Bacteriol, 165(2): p. 434-42). There was a transient down-regulation of the *opp* genes observed in the APS, which reached a minimum at T2, after which expression returned to pre-induction levels at T3. In the IAPS, expression of *oppABCDF* remained stable in the first two post-induction samples but was up-regulated by T3. The strain-to-strain comparison of the *opp* expression profile indicates that expression of these genes was significantly higher in the APS prior to IPTG induction. Following induction, *opp* expression was equivalent in both strains for T1 and T2, but then increased in the APS at T3. Again, this data is consistent with increased leucine availability, but only at the final time point and with a stronger effect in the APS.

Histidine biosynthesis

[00173] The production of histidine starts with ATP and 5-phosphoribosyl-a-1-pyrophosphate (PRPP) and requires ten biochemical reactions utilizing eight enzymes and 41 molecules of ATP. Histidine biosynthesis is also a consumer of single-carbon units because it starts with an adenine molecule (in the form of ATP), and thus requires two single-carbon units, one of which is recycled to the purine pool as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR).

[00174] The *his* operon is regulated by the alarmone ppGpp, where increasing concentrations of the nucleotide lead to higher operon expression. The second main regulatory feature is transcription attenuation in the leader section of the *his* operon, which coordinates expression based on the amount of $His\text{-}tRNA^{His}$. The attenuator regulation of this operon is not as sensitive as would be theoretically possible — even when 77-88% of the tRNA's are charged there is still significant basal readthrough. Charged tRNA levels need to drop to <12% for there to be a significant increase in operon transcription due to attenuator regulation.

[00175] The *his* operon's full length transcript has an *in vivo* half life of ~3 minutes while a secondary transcript of the 5 distal genes (*hisBHAFI*) is much more stable and has a ~15 minute half life in the cell. The processing of the primary transcript into several secondary transcripts is modulated by the concentrations of various pathway intermediates. Inhibition of the first enzymatic step in the pathway leads to an increase in the primary transcript levels, while inhibitors that block steps down stream of AICAR lead to increases in both the primary transcript and the stable secondary transcript. The addition of an inhibitor that lowers the over all formylation level in the cell causes a decrease in primary transcript and an increase in the stable secondary transcript (Alifano (1994) Gene, 146(1): p. 15-21). Because AICAR must be formylated in order to return to the purine pool, the organism must coordinate the generation of AICAR by histidine biosynthesis with the overall formylating capacity of the cell.

[00176] There was a strong up-regulation of the primary transcript in the APS as indicated by the time profile of the *hisGDC* genes. There was also an up-regulation of *hisB* over the time course after an initial drop at time one with the time three expression level reaching the pre-induction level. This time course profile is consistent with an increase in primary transcript expression due to low histidine levels. The gene expression time course of the histidine biosynthetic genes in the IAPS showed the exact opposite profile with a strong down-regulation by T3. Since histidine requires single carbon units the low levels of charged histidine tRNA's in the APS could reflect an overall strain on the C1 metabolism as discussed above.

The chorismate pathway

[00177] Serine and single carbon units are utilized by several biochemical pathways branching from chorismate. The biosynthesis of tryptophan and enterobactin directly consume one and three molecules of serine, respectively, while quinone biosynthesis utilizes SAM in three sequential methylation

reactions. Many of the genes in the highly branched chorismate pathway are expressed constitutively, but several are transcriptionally regulated in response to aromatic amino acid and iron levels.

[00178] The biosynthesis of the tryptophan is transcriptionally regulated at the terminal pathway branching from chorismate by both a repressor protein and transcriptional attenuation. There was an up-regulation of tryptophan biosynthetic genes observed in the APS transcriptional profile at the third cell doubling post-induction. Expression of the *trpEDCBA* operon was approximately 2-4-fold higher in the APS at T3 relative to pre-induction and to the IAPS control, though no change was observed in the protein products of these genes (Table 2). One of the three DAHP synthase isozymes that catalyzes the first committed step of chorismate biosynthesis, which is encoded by *aroH*, is tryptophan dependent and was up-regulated ~2-fold at T3 in the APS along with the *trp* operon genes. The transcriptional unit containing *aroF* (encoding DAHP(Tyr) synthase) and *tyrA*, which is regulated in response to intracellular tyrosine levels, was up-regulated in the APS 2-4-fold over the entire time course relative to the IAPS control. The opposite trend in the tryptophan biosynthetic genes was observed in the IAPS, with a down-regulation at the third cell doubling that was matched by some of the protein products.

[00179] To scavenge iron from the environment *E. coli* releases the catecholate siderophore enterobactin into its growth medium to bind Fe(III). Enterobactin is synthesized from the shikimate pathway intermediate chorismate via a pathway consisting of six enzymes encoded in three operons (*entABCE*, *entD* and *entF*). These operons were up-regulated in both the IAPS and the APS, though the post-induction response was far stronger in the APS. Transcription of the *ent* operon is controlled by the ferric uptake regulation protein (Fur); thus, an up-regulation of this pathway suggests a more significant iron limitation in the APS than in the IAPS. Since the Fur regulon was observed to be up-regulated in both strains relative to pre-induction levels the strain-to-strain comparison provided insight into the relative activation of this regulon.

[00180] The time course expression profile of the Fur regulon observed in the strain-to-strain comparison clearly shows a strong shift in the relative activation following IPTG induction of the heterologous proteins. Expression of the *ent* biosynthetic genes is higher in the IAPS at T0-T1, but the relative expression is higher in the APS at T2-T3. The iron-sulfur cluster repair genes encoded by the *sufABCSDE* operon, the succinate dehydrogenase/2-ketoglutarate dehydrogenase complex encoded by *sdhABCDE-sucABCD,* and portions of a ribonucleoside-diphosphate reductase complex encoded by *nrdHEIF* have all been reported to be Fur regulated, and each exhibited a temporal pattern consistent with de-repression of this regulon in the APS. Thus, active biochemical flux through the amorpha-4-11-diene production pathway activates the Fur regulon.

The Heat Shock Regulon

[00181] As a colonizer of the mammalian gut, *E. coli* has an optimal growth temperature around 37°C but can grow in a fairly wide range of temperatures (at least 25°C to 42°C). The heat shock regulon is actually the cell's response to misfolded protein in the cytoplasm or in the periplasm and can be induced by a wide range of conditions including growth at high temperature (~ 42°C or higher),

exposure to solvents, protein over expression, viral infection and alterations in C1 metabolism. While this regulon is most often identified as a stress regulon, this is really a misnomer since this group of proteins is required for growth at all temperatures. The genes in this regulon consist mostly of chaperones and proteases and, as such, are critical for the normal functioning of *E. coli*'s translational machinery.

[00182] There was surprisingly little response in the heat shock regulon of either the APS or the IAPS following induction. A strong heat shock response is expected during high level expression of heterologous genes from an inducible promoter and encoded on a plasmid. The expression vectors used in this study had low copy number (higher for the pADS) and weak promoters, and so it would appear that the protein folding machinery in the cell was not significantly stressed by the expression of the heterologous proteins.

[00183] There was one interesting exception to this in the APS for the small heat shock proteins encoded by *ibpA* and *ibpB*. The proteins encoded by these genes are associated with the management of inclusion bodies and function along with *clpB* as chaperones shuttling protein to and from an aggregated state. The *ibpA* and *ibpB* genes were six- and seven-fold up-regulated respectively in the APS by the third cell doubling. This was the most significant difference in heat shock regulon expression when one compares the APS to the IAPS. While the expression of these genes is classically associated with the formation of inclusion bodies, the fact the IAPS expressing the same heterologous proteins did not significantly up-regulate these genes suggests something else caused this effect. There have been a few studies linking an altered heat shock response to changes in single carton metabolism. One study has shown that when *E. coli* is starved for single carbon units by blocking the production of tetrahydrofolate (THF) there is a strong up-regulation of *ibpA* and *ibpB*. The study found that several other members of the HSR (*dnaK, dnaJ, groE(mopA), groL (mopB), clpB)* were up-regulated as well but this was an extreme case of chemical blockage of all THF production. In the APS, where there appears to be a strain on single carbon production (not a complete cessation), the induction if *ibpA* and *ibpB* may be part of the cell's adaptation.

Metabolite analysis of the amorpha-4-1-diene strain

[00184] Glucose and nitrogen uptake were higher in the amorpha-4-11-diene producing strain (APS) in the first two cell-doublings following IPTG induction of heterologous protein expression. Additionally, the APS accumulated acetyl-CoA in the post-induction samples and excreted more acetate over the entire time course than the IAPS. Both strains up-regulated expression of the acetate-evolving *ackA-pta* pathway 2-3-fold at later time points, though by an equal amount, since the strain-to-strain transcriptomic comparisons show no differential expression for these genes. Since both strains grew in a condition of carbon excess, the higher glucose uptake, acetate excretion and CO_2 evolution in the APS suggest there was higher glycolytic flux in this strain compared to the IAPS.

[00185] There was no significant accumulation of HMG-CoA observed, while acetoacetyl-CoA and mevalonate concentrations increased over time. Malonyl-CoA is an important biomarker of a growth

inhibition associated with HMG-CoA accumulation (Pitera et al. (2007) *Metab Eng* 9(2): 193-207), but there was no significant accumulation of this acyl-CoA observed in the APS, indicating that the enzymatic activity in the engineered pathway was balanced, and HMG-CoA levels remained below growth-inhibiting concentrations. Mevalonate accumulation to approximately 400 nM/OD_{600} was observed at later time points in the APS, indicating the rate of biochemical flux through the later stages of the amorpha-4-11-diene pathway limited production. Indeed, the only heterologous proteins that were not detected by proteomic analysis were mevalonate kinase and mevalonate phosphokinase, which implies these proteins were not highly expressed and the conversion of mevalonate into prenyl phosphates may limit overall production titer.

Media supplementation improves amorpha-4-11-diene titer

[00186] The APS transcriptional and proteomic profiles suggested a possible iron and single carbon unit limitation was associated with amorpha-4-11-diene production in *E. coli*. We measured amorpha-4-11-diene production in several different media to determine if simple supplementation of iron or certain amino acids could improve titer. The APS was grown in M9-glucose medium with and without additional $FeSO_4$; there was no significant difference in amorpha-4-11-diene production or growth (data not shown).

[00187] Next, the effects of various amino acid supplementation strategies on amorpha-4-11-diene production were tested. The minimal medium specific production titer was 45 $mg/L\text{-}OD_{600}$; supplementation of methionine, glycine and leucine (MGL media) reduced both growth and sesquiterpene production (Figure 5). Specific amorpha-4-11-diene production (mg/L-OD) and total amorpha-4-11-diene production (mg/L) were measured in various media formulations 48-64 hours post-induction in three biological replicates. MOPS-minimal medium supplemented with the aromatic amino acids as well as precursors to tetrahydrofolate, quinone and enterobactin (shikimate media) increased specific production to 64 mg/L-OD. The MOPS-minimal, MGL and shikimate media all had a low total production titer, most likely because they did not support growth to as high a final cell density as the MOPS-defined rich, 8-AA or C1 media. The MOPS-defined rich growth medium, which contains all supplements, provided both the high final titer (402 mg/L) and specific production (69 mg/L-OD) that would be expected. The 8-AA medium has been reported to increase recombinant protein production by supplementing eight amino acids (alanine, glutamine, glutamic acid, isoleucine, leucine, methionine, serine and valine). The performance of this growth medium matched MOPS-defined rich medium very closely, both in final cell density and amorpha-4-11-diene production. The C1 medium, which is MOPS minimal medium supplemented with serine, glycine, methionine, adenine, and guanine, provided the highest final production titer of 499 mg/L and specific production at 92 mg/L-OD. This represented a nearly 5-fold improvement in total amorpha-4-11-diene production and a 2-fold improvement in specific production.

[00188] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

What is claimed is:

1. A method of making an isoprenoid compound, the method comprising culturing in a defined culture medium *in vitro* a plurality of cells that are capable of making the isoprenoid compound, wherein the defined culture medium comprises:

a) serine; and

b) a subset of amino acids consisting of zero to eight amino acids selected from glycine, alanine, glutamine, glutamic acid, isoleucine, leucine, valine, and methionine, wherein the defined culture medium does not comprise one or more amino acids that are not part of the subset,

wherein the cells are genetically modified with one or more nucleic acids comprising nucleotide sequences encoding a heterologous mevalonate pathway enzyme or a heterologous 1-deoxy-D-xylulose 5-diphosphate (DXP) pathway enzyme, and

wherein said culturing provides for production of the isoprenoid compound in a recoverable amount.

2. The method of claim 1, wherein the subset is one amino acid.

3. The method of claim 2, wherein the one amino acid is methionine.

4. The method of claim 2, wherein the one amino acid is glycine.

5. The method of claim 1, wherein the subset is two amino acids, and wherein the two amino acids are glycine and methionine.

6. The method of claim 1, wherein the subset is seven amino acids, and wherein the seven amino acids are alanine, glutamine, glutamic acid, isoleucine, leucine, methionine, and valine.

7. The method of claim 1, wherein the defined culture medium does not additionally comprise an amino acid other than serine and the subset.

8. The method of claim 1, wherein the culture medium further comprises a purine.

9. The method of claim 8, wherein the purine is adenine.

10. The method of claim 8, wherein the purine is guanine.

11. The method of claim 8, wherein the purine is both adenine and guanine.

12. The method of claim 1, wherein the defined culture medium comprises a minimal medium that is supplemented with serine and the subset.

13. The method of claim 1, wherein the cells are prokaryotic cells.

14. The method of claim 1, wherein the cells are *Escherichia coli*.

15. The method of claim 1, wherein the cells are eukaryotic cells.

16. The method of claim 1, wherein the cells are yeast cells.

17. The method of claim 1, wherein the cells are *Saccharomyces cerevisiae*.

18. The method of claim 1, wherein the isoprenoid compound is made at least in part via the mevalonate pathway.

19. The method of claim 1, wherein the isoprenoid compound is made at least in part via the DXP pathway.

20. The method of claim 1, wherein the cells are further genetically modified with a nucleic acid comprising a nucleotide sequence encoding a heterologous isopentenyl pyrophosphate isomerase.

21. The method of claim 1, wherein the cells are further genetically modified with a nucleic acid comprising a nucleotide sequence encoding a heterologous prenyltransferase

22. The method of claim 21, wherein the prenyltransferase is selected from a geranyl pyrophosphate synthase, a farnesyl pyrophosphate synthase, and a geranylgeranyl pyrophosphate synthase.

23. The method of claim 1, wherein the cells are further genetically modified with a nucleic acid comprising a nucleotide sequence encoding a heterologous terpene synthase.

24. The method of claim 1, wherein the one or more nucleic acids is present extrachromosomally in the cells.

25. The method of claim 1, wherein the one or more nucleic acids is integrated into the genome of the cells.

26. The method of claim 1, wherein the isoprenoid compound is a C_5C_{20} isoprenoid compound.

27. The method of claim 1, wherein the isoprenoid compound is selected from abietadiene, amorphadiene, carene, α-farnesene, β-farnesene, farnesol, geraniol, geranylgeraniol, isoprene, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, β-pinene, sabinene, γ-terpinene, terpinolene, and valencene.

28. The method of claim 1, wherein the isoprenoid or isoprenoid precursor is produced in a recoverable amount of at least 350 mg/L.

29. The method of claim 1, wherein the isoprenoid or isoprenoid precursor is produced in a recoverable amount of at least 400 mg/L.

30. The method of claim 1, wherein the isoprenoid or isoprenoid precursor is produced in a recoverable amount of at least 500 mg/L.

31. The method of claim 1, wherein the isoprenoid or isoprenoid precursor is produced in a recoverable amount of at least 1 g/L.

32. The method of claim 1, wherein the isoprenoid or isoprenoid precursor is produced in a recoverable amount of at least 10 g/L.

33. The method of claim 1, wherein the isoprenoid or isoprenoid precursor is produced in a recoverable amount of at least 50 g/L.

34. A method of making an isoprenoid compound, the method comprising culturing in a defined medium *in vitro* a plurality of cells that are capable of making the isoprenoid compound, wherein the defined culture medium comprises:

a) serine;

b) methionine;

c) glycine; and

d) a purine,

wherein the medium does not include any amino acid other than serine, methionine, and glycine,

wherein the cells are genetically modified with one or more nucleic acids comprising nucleotide sequences encoding a heterologous mevalonate pathway enzyme or a heterologous 1-deoxy-D-xylulose 5-diphosphate (DXP) pathway enzyme, and

wherein said culturing provides for production of the isoprenoid compound in a recoverable amount.

35. The method of claim 34, wherein the isoprenoid is a C_5-C_{20} isoprenoid.

36. The method of claim 34, wherein the one or more nucleic acids comprise nucleotide sequences encoding a heterologous mevalonate kinase, a heterologous hydroxymethylglutaryl-CoA (HMG-CoA) reductase, or both a heterologous mevalonate kinase and a heterologous HMG-CoA reductase.

37. The method of claim 34, wherein the cells are *Escherichia coli* and wherein the one or more nucleic acids comprise nucleotide sequences encoding one or more heterologous mevalonate pathway enzymes.

38. The method of claim 34, wherein the purine comprises both guanine and adenine.

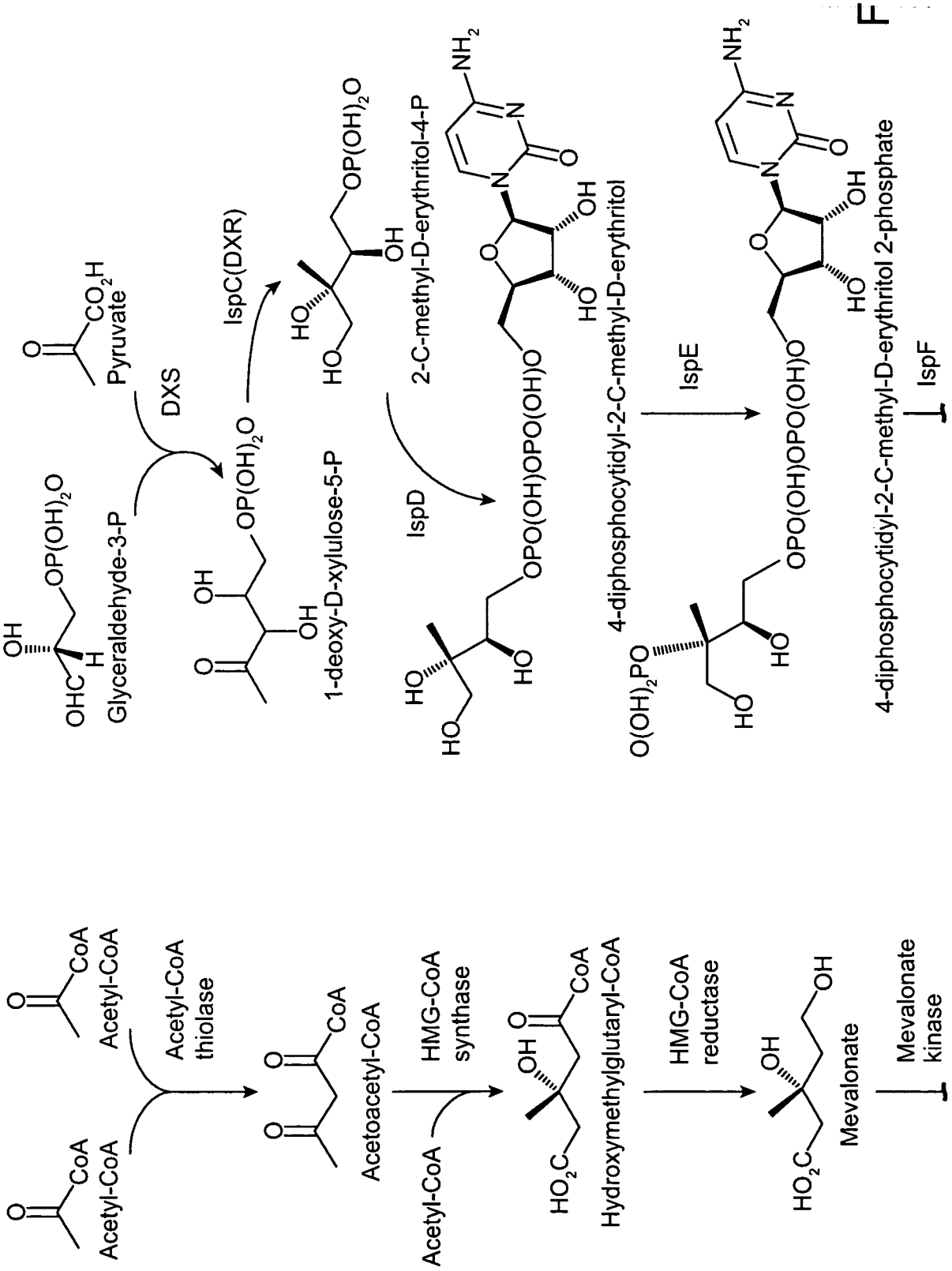

Acetyl-CoA
Acetyl-CoA
Acetyl-CoA thiolase
Acetoacetyl-CoA
Acetyl-CoA
HMG-CoA synthase
Hydroxymethylglutaryl-CoA
HMG-CoA reductase
Mevalonate
Mevalonate kinase
Glyceraldehyde-3-P
Pyruvate
DXS
1-deoxy-D-xylulose-5-P
IspC(DXR)
2-C-methyl-D-erythritol-4-P
IspD
4-diphosphocytidyl-2-C-methyl-D-erythritol
IspE
4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate
IspF

FIG. 1

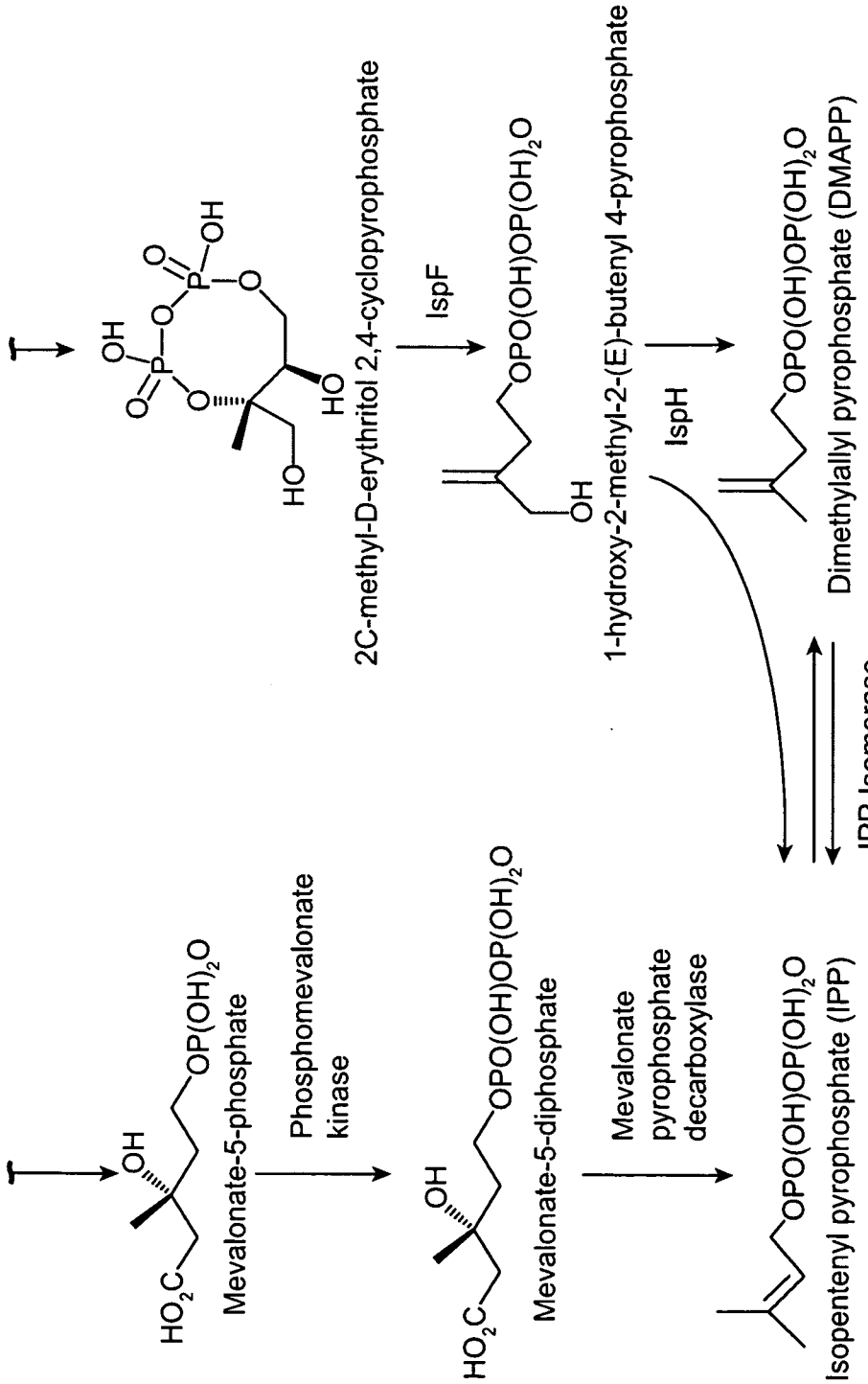

Mevalonate-5-phosphate
Phosphomevalonate kinase
Mevalonate-5-diphosphate
Mevalonate pyrophosphate decarboxylase
Isopentenyl pyrophosphate (IPP)
IPP Isomerase
Dimethylallyl pyrophosphate (DMAPP)
IspH
1-hydroxy-2-methyl-2-(E)-butenyl 4-pyrophosphate
IspF
2C-methyl-D-erythritol 2,4-cyclopyrophosphate

FIG. 1 (Cont.)

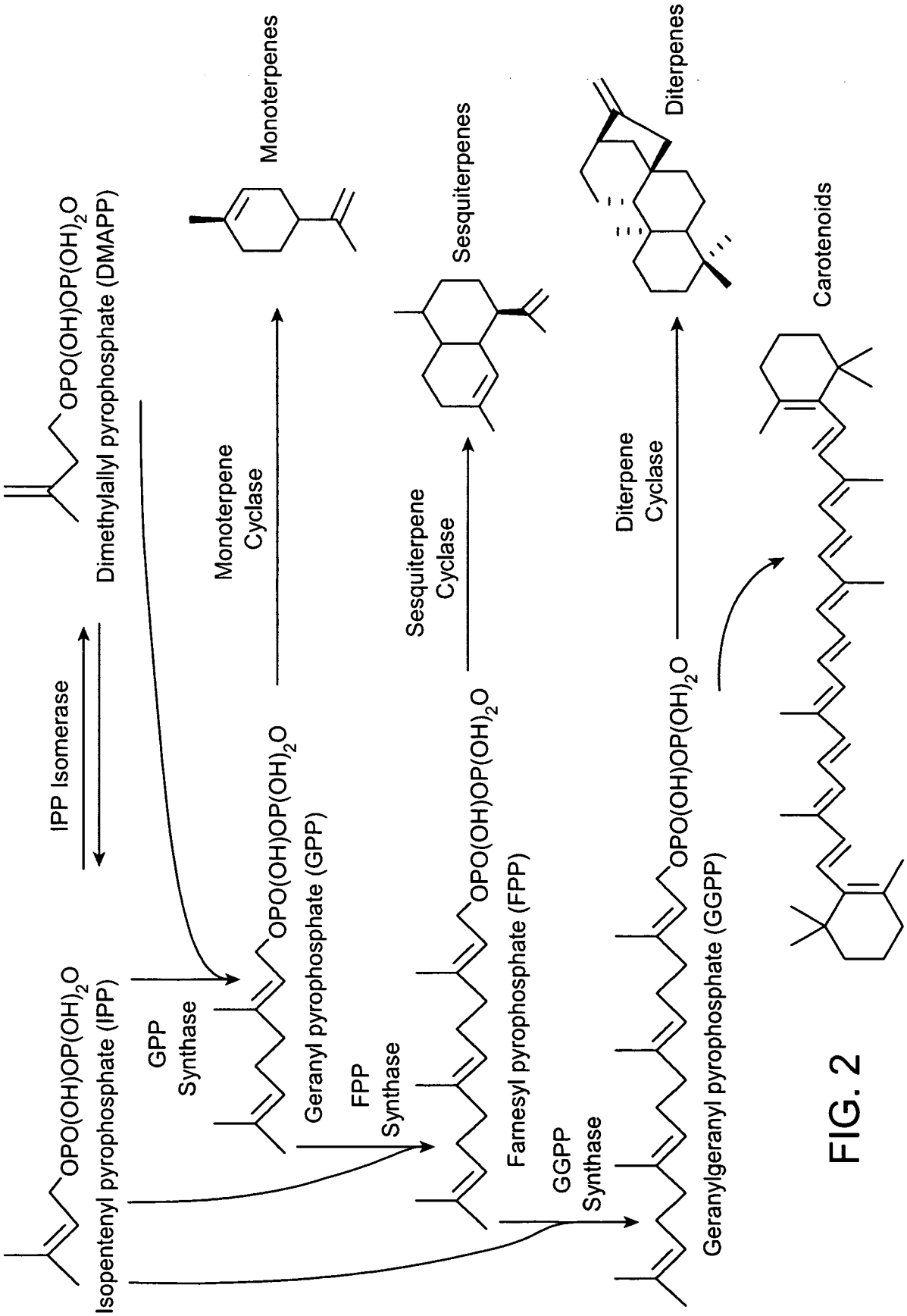

OPO(OH)OP(OH)2O
Isopentenyl pyrophosphate (IPP)
IPP Isomerase
OPO(OH)OP(OH)2O
Dimethylallyl pyrophosphate (DMAPP)
GPP Synthase
OPO(OH)OP(OH)2O
Geranyl pyrophosphate (GPP)
Monoterpene Cyclase
Monoterpenes
FPP Synthase
OPO(OH)OP(OH)2O
Farnesyl pyrophosphate (FPP)
Sesquiterpene Cyclase
Sesquiterpenes
GGPP Synthase
OPO(OH)OP(OH)2O
Geranylgeranyl pyrophosphate (GGPP)
Diterpene Cyclase
Diterpenes
Carotenoids

FIG. 2

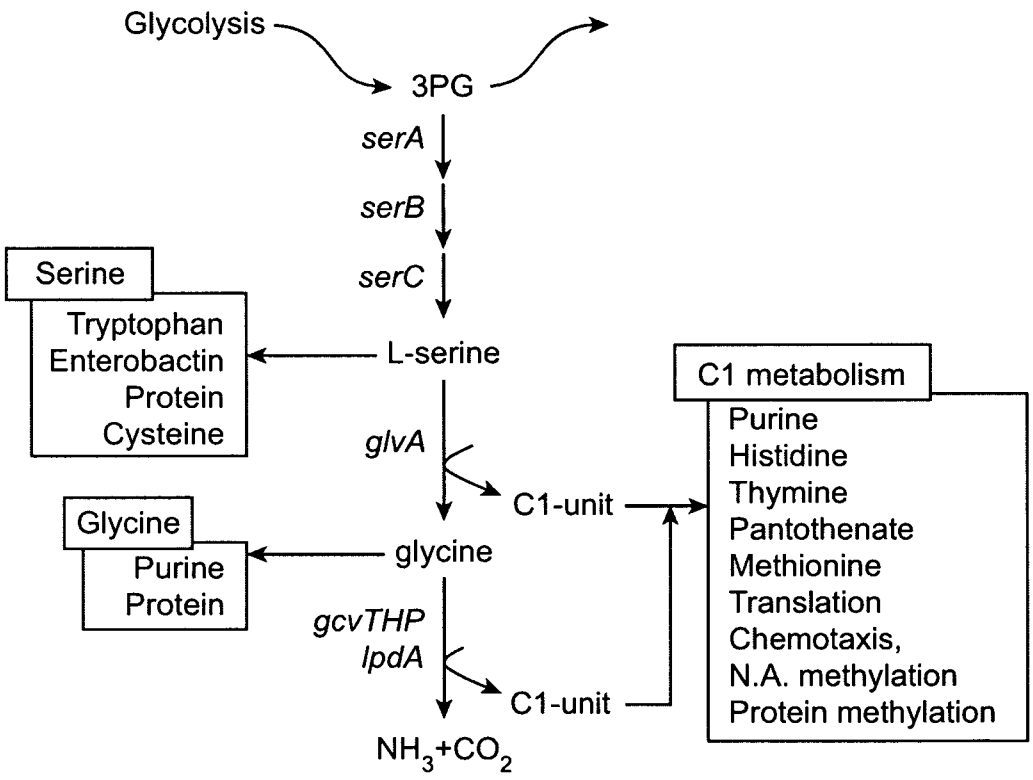

Glycolysis
3PG
serA
serB
serC
Serine
Tryptophan
Enterobactin
Protein
Cysteine
L-serine
C1 metabolism
Purine
Histidine
Thymine
Pantothenate
Methionine
Translation
Chemotaxis,
N.A. methylation
Protein methylation
glvA
C1-unit
Glycine
Purine
Protein
glycine
gcvTHP
lpdA
C1-unit
NH3+CO2

FIG. 3

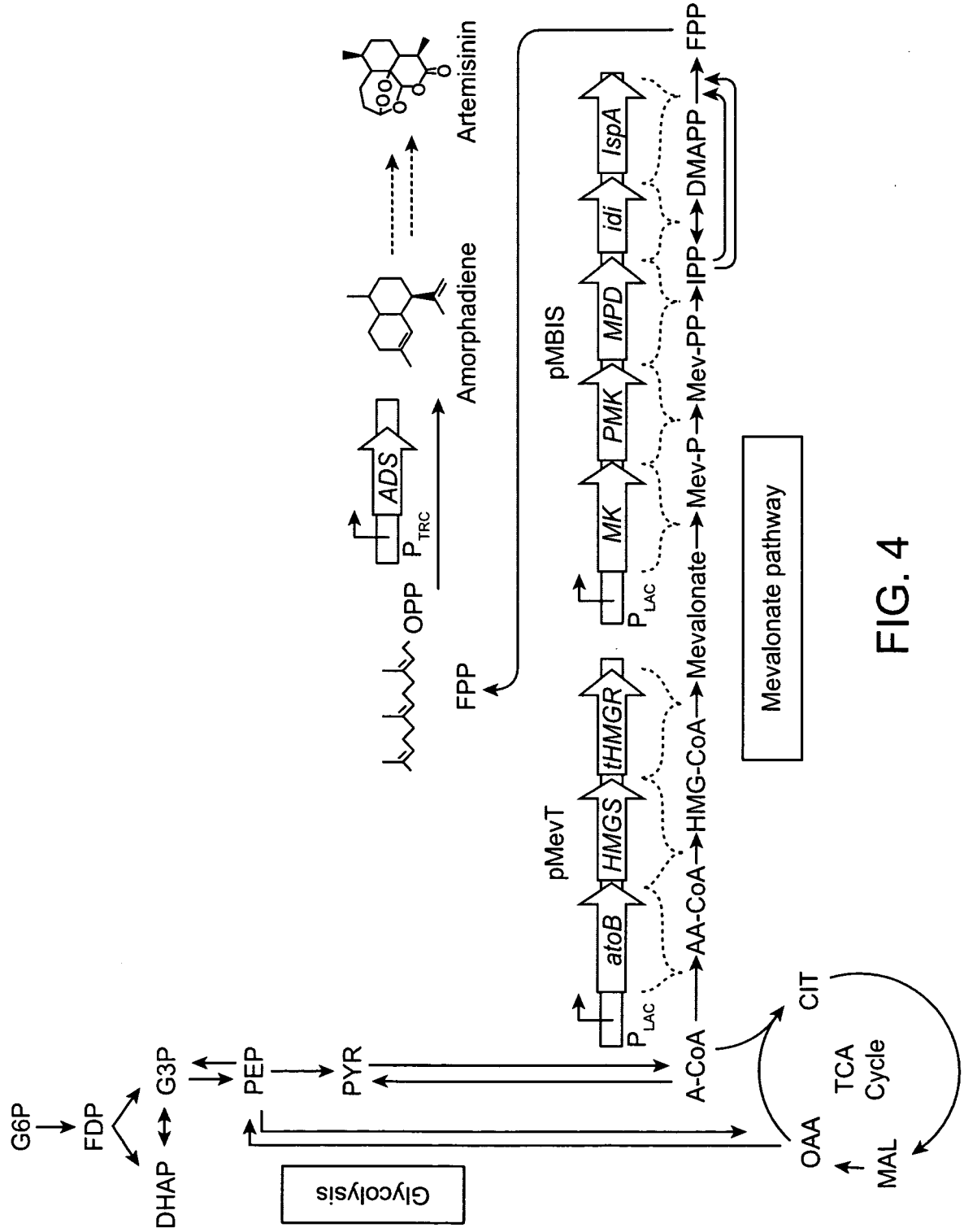

G6P
FDP
DHAP
G3P
PEP
PYR
Glycolysis
A-CoA
CIT
TCA Cycle
OAA
MAL
pMevT
pMBIS
P LAC
atoB
HMGS
tHMGR
AA-CoA
HMG-CoA
Mevalonate
P LAC
MK
PMK
MPD
idi
IspA
Mev-P
Mev-PP
IPP
DMAPP
FPP
Mevalonate pathway
FPP
OPP
P TRC
ADS
Amorphadiene
Artemisinin

FIG. 4

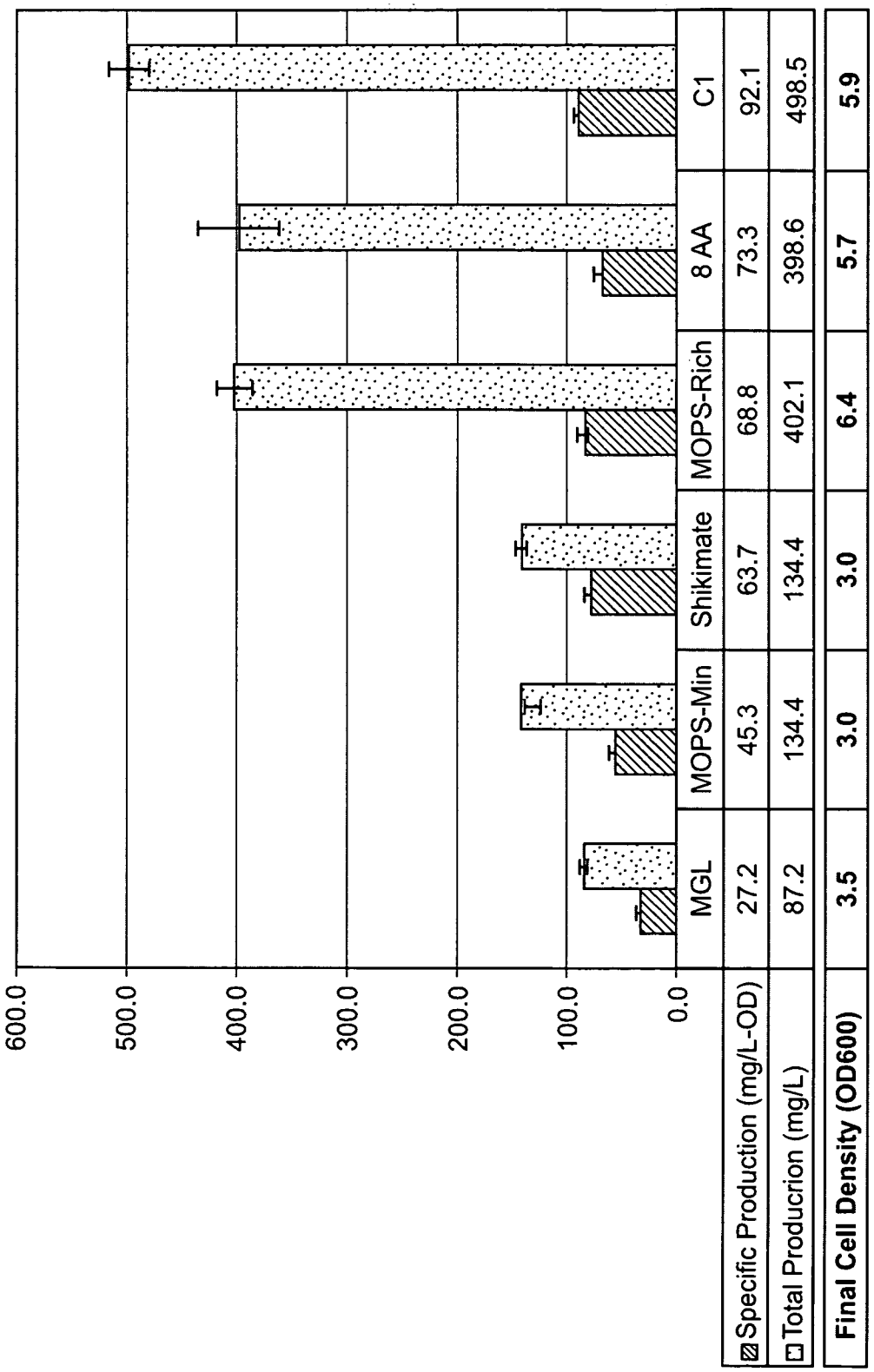

600.0
500.0
400.0
300.0
200.0
100.0
0.0
MGL
MOPS-Min
Shikimate
MOPS-Rich
8 AA
C1
Specific Production (mg/L-OD)
27.2
45.3
63.7
68.8
73.3
92.1
Total Producrion (mg/L)
87.2
134.4
134.4
402.1
398.6
498.5
Final Cell Density (OD600)
3.5
3.0
3.0
6.4
5.7
5.9

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/07990

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 5/00 (2008.04)
USPC - 435/166
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12P 5/00 (2008.04)
USPC: 435/166

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
435/166; 435/167; 435/170; 435/171;
435/69.1; 800/278

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DialogPRO b 2,6,144,155,315,440,344,345,348,349,351,371,652,654; Freepatentsonline
Search Terms: isoprenoid, mevalonate pathway, mevalonate pathway, DOXP pathway, prenyl transferase, terpene transferase, isopentenyl pyrophosphate isomerase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 6660507 B2 (Cheng) 9 December 2003 (09.12.2003), Cheng Col. 3, lines 37-44, Col. 4, lines 57-58, Col. 5 lines 41-43, Col. 18, lines 55-63, lines 63-67, Col. 6, lines 24-27, Col. 19, lines 6-14, Col. 21, lines 6-10, table 3.	1-19, 21-22, 24-35, 37 and 38 ---------- 20, 23, and 36
Y	US 6989257 B2 (Berry) 24 January 2006 (24.01.2006), Col. 20, lines 54-65.	20, 36
Y	WO/2002/064764 A2 (Aharoni) 22 August 2002 (22.08.2002), page 10, lines 5-6, page 14, lines 19-20.	23
A	US 7208298 B2 (Miyake) 24 April 2007 (24.04.2007), Col. 1, lines 16-18, Col 2, lines 42-67; Col. 12, lines 66-67.	1-38

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 September 2008 (25.09.2008)	02 OCT 2008
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2007)