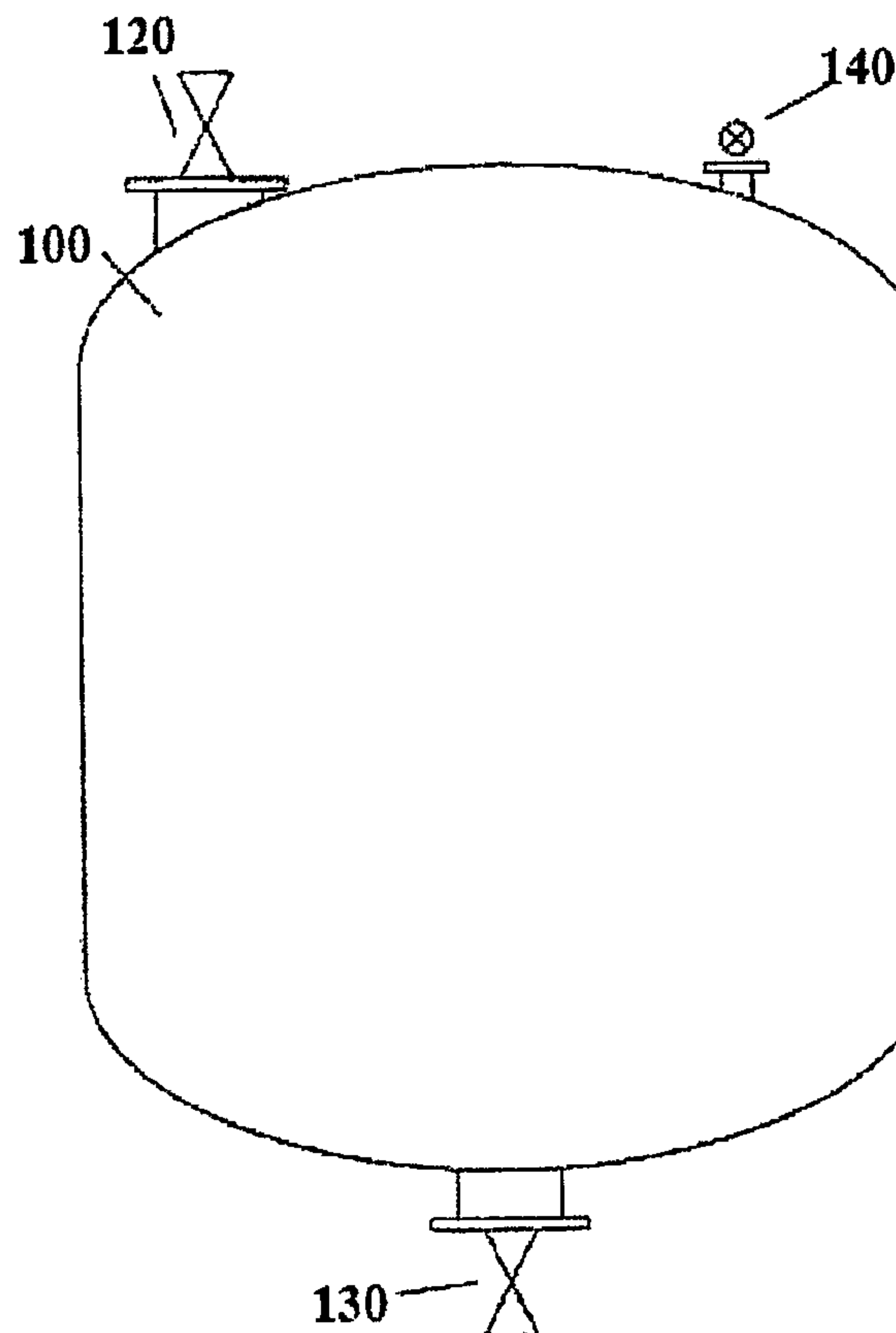




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(54) Title: APPARATUS FOR MAKING BIO-ORGANIC COMPOUNDS



(57) Abrégé/Abstract:

A system and method for producing bio-organic compounds may include a vessel, a first phase comprising an aqueous medium including host cells capable of producing a bio-organic compound, where the bio-organic compound comprises a second phase in contact with the aqueous medium.

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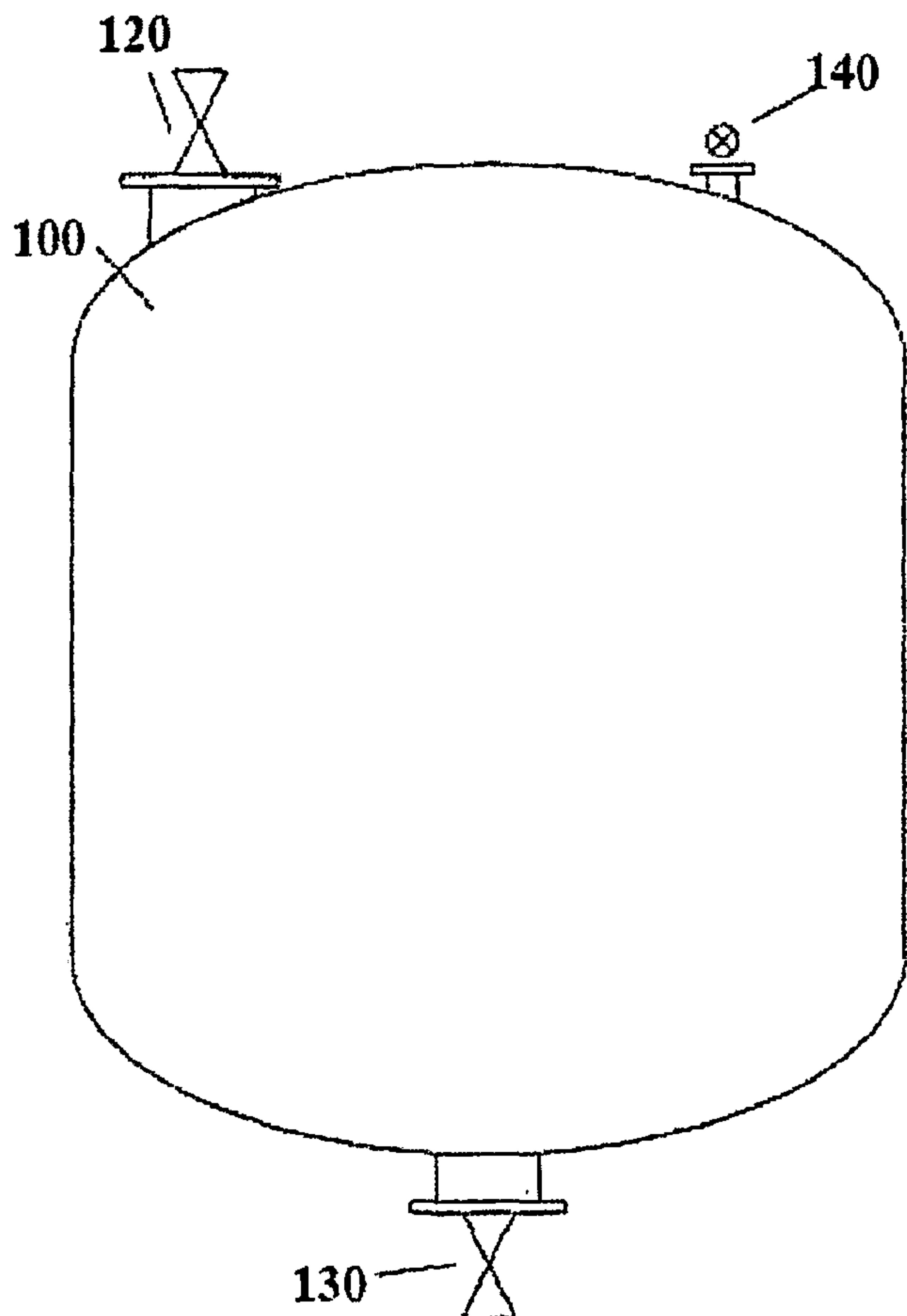
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(54) Title: APPARATUS FOR MAKING BIO-ORGANIC COMPOUNDS



(57) Abstract: A system and method for producing bio-organic compounds may include a vessel, a first phase comprising an aqueous medium including host cells capable of producing a bio-organic compound, where the bio-organic compound comprises a second phase in contact with the aqueous medium.

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DEMANDES OU BREVETS VOLUMINEUX

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APPARATUS FOR MAKING BIO-ORGANIC COMPOUNDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional application nos. 60/808,989 filed May 26, 2006 entitled MICROORGANISMS FOR PRODUCTION OF ISOPRENOIDS; US 60/808,666 filed May 26, 2006 entitled BIOFUELS AND METHODS FOR PRODUCTION; U.S. 60/870,592 filed December 12, 2006 entitled PRODUCTION OF ISOPRENOIDS; and U.S. 60/922,782, filed April 10, 2007 entitled APPARATUS FOR MAKING BIO-ORGANIC COMPOUNDS the contents of all of which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

[0002] Traditionally, bio-organic compounds of interest have been manufactured by extraction from natural sources such as plants, microbes, and animals. However, extraction yields are usually very low as most bio-organic compounds accumulate in nature in small amounts. Given that these quantities are far less than is for many commercial applications, there remains a need for systems and procedures that produce bio-organic compounds on an industrial scale.

[0003] The present invention addresses this need. Provided are various industrial-scale systems for making bio-organic compounds using host cells. These bio-organic compounds have at least five carbon atoms and can be a carbohydrate such as a mono- or poly-alcohol, ester, ether, aldehyde, ketone, or a hydrocarbon such as an alkane, alkene, or alkyne. The bio-organic compound can be linear or cyclic and can be saturated or unsaturated.

SUMMARY OF THE INVENTION

[0004] The present invention provides various bio-organic compound production systems. In one aspect, a bio-organic compound production system is provided which comprises:

- a. at least one vessel having a capacity of at least 100 liters;
 - b. an aqueous medium, within the vessel, comprising a first phase;
 - c. a plurality of host cells, within the aqueous medium, capable of making, producing or synthesizing at least one bio-organic compound;
- and,

- d. a liquid organic second phase, comprising the at least one bio-organic compound, in contact with the first phase.

[0005] In another aspect, a method of producing at least one bio-organic compound is provided. The method comprises:

- a. culturing in an aqueous medium a plurality of host cells that make, produce or synthesize the at least one bio-organic compound wherein the aqueous medium comprises a first phase;
- b. forming an organic second phase comprising the bio-organic compound in contact with the first phase;
- c. separating at least a portion of the organic second phase from the first phase; and,
- d. isolating the at least one bio-organic compound from the organic second phase.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] Figure 1 is a vessel having a capacity of at least 100 liters for use in the present invention.

[0007] Figure 2 is another vessel embodiment.

[0008] Figure 3 is a schematic representation of the mevalonate ("MEV") pathway for the production of isopentenyl diphosphate ("IPP").

[0009] Figure 4 is a schematic representation of the DXP pathway for the production of IPP and dimethylallyl pyrophosphate ("DMAPP"). Dxs is 1-deoxy-D-xylulose-5-phosphate synthase; Dxr is 1-deoxy-D-xylulose-5-phosphate reductoisomerase (also known as IspC); IspD is 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; IspE is 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; IspF is 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG is 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (IspG); and ispH is isopentenyl/dimethylallyl diphosphate synthase.

[0010] Figure 5 is a schematic representation of the conversion of IPP and DMAPP to geranyl pyrophosphate ("GPP"), farnesyl pyrophosphate ("FPP"), and geranylgeranyl pyrophosphate ("GGPP").

[0011] Figure 6 shows a map of expression plasmid pMBIS-gpps.

[0012] Figures 7 shows a map of expression plasmid Pam00408

[0013] Figure 8 shows a map of expression plasmid pAM424.

[0014] Figure 9 shows a map of expression plasmids pTrc99A-ADS, pTrc99A-FSA, pTrc99A-LLS, pTrc99A-LMS, pTrc99A-GTS, pTrc99A-APS, pTrc99A-BPS, pTrc99A-PHS, pTrc99A-TS, pTrc99A-CS, pTrc99A-SS, and pAM373.

[0015] Figure 10 are schematics for the construction of plasmids pAM489-pAM498.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0016] 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. Reference is made here to a number of terms that shall be defined to have the following meanings:

[0017] “Bio-organic compound” refers to an organic compound having at least five carbon atoms that can be made by a host cell by taking a carbohydrate carbon source and converting the carbohydrate carbon source into the desired product.

[0018] “Deoxyxylulose 5-phosphate pathway” or “DXP pathway” is used herein to refer to the pathway that converts glyceraldehyde-3-phosphate and pyruvate to IPP and DMAPP. The DXP pathway is illustrated schematically in Figure 4.

[0019] “Endogenous” refers to a substance or process that can occur naturally, *e.g.*, in a non-recombinant host cell.

[0020] “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 (that is, not naturally found in) a given host cell; (b) the nucleic acid comprises a nucleotide sequence that is naturally found in (that is, is “endogenous to”) a given host cell, but the nucleotide sequence is produced in an unnatural (for example, greater than expected or greater than naturally found) amount in the cell; (c) the nucleic acid comprises a nucleotide sequence that differs in sequence from an endogenous nucleotide sequence, but the nucleotide sequence encodes the same protein (having the same or substantially the same amino acid sequence) and is produced in an unnatural (for example, greater than expected or greater than naturally found) amount in the cell; or (d) the nucleic acid comprises two or more nucleotide sequences that are not found in the same relationship to each other in nature (for example, the nucleic acid is recombinant).

[0021] “Host cell” and “microorganism” are used interchangeably herein to refer to any archae, bacterial, or eukaryotic living cell into which a heterologous nucleic acid can be or has been inserted. The term also relates to the progeny of the original cell,

which may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to natural, accidental, or deliberate mutation.

[0022] “Isoprenoid” and “isoprenoid compound” are used interchangeably herein and refer to a compound derivable from isopentenyl diphosphate.

[0023] “Isolate” and “isolating” when referred to a bio-organic compound is the enrichment of the amount of the bio-organic compound in a composition. Consequently, the amount of the bio-organic compound in a composition after the bio-organic compound has been isolated or subject to an isolating step is greater than the amount present in the composition prior to such step.

[0024] “Mevalonate pathway” or “MEV pathway” is used herein to refer to the biosynthetic pathway that converts acetyl-CoA to IPP. The MEV pathway is illustrated schematically in Figure 3.

[0025] “Naturally occurring” as applied to a nucleic acid, an enzyme, a cell, or an organism, refers to a nucleic acid, enzyme, cell, or organism that is found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism that can be isolated from a source in nature and that has not been intentionally modified by a human in the laboratory is naturally occurring.

[0026] “Optional” or “optionally” means that the subsequently described feature or structure may or may not be present, or that the subsequently described event or circumstance may or may not occur, and that the description includes instances where a particular feature or structure is present and instances where the feature or structure is absent, or instances where the event or circumstance occurs and instances where the event or circumstance does not occur.

[0027] “Pyrophosphate” is used interchangeably herein with “diphosphate”.

[0028] As used herein, a composition that is a “substantially pure” compound is substantially free of one or more other compounds, *i.e.*, the composition contains greater than 80 vol.%, greater than 90 vol.%, greater than 95 vol.%, greater than 96 vol.%, greater than 97 vol.%, greater than 98 vol.%, greater than 99 vol.%, greater than 99.5 vol.%, greater than 99.6 vol.%, greater than 99.7 vol.%, greater than 99.8 vol.%, greater than 99.9 vol.% of the compound; or less than 20 vol.%, less than 10 vol.%, less than 5 vol.%, less than 4 vol.%, less than 3 vol.%, less than 2 vol.%, less than 1 vol.%, less than 0.5 vol.%, less than 0.1 vol.%, or less than 0.01 vol.% of the one or more other compounds, based on the total volume of the composition.

[0029] In the following description, all numbers disclosed herein are approximate values, regardless whether the word "about" or "approximate" is used in connection therewith. They may vary by 1 percent, 2 percent, 5 percent, or, sometimes, 10 to 20 percent. Whenever a numerical range with a lower limit, RL and an upper limit, RU, is disclosed, any number falling within the range is specifically disclosed. In particular, the following numbers within the range are specifically disclosed: $R=RL+k*(RU-RL)$, wherein k is a variable ranging from 1 percent to 100 percent with a 1 percent increment, i.e., k is 1 percent, 2 percent, 3 percent, 4 percent, 5 percent, ..., 50 percent, 51 percent, 52 percent, ..., 95 percent, 96 percent, 97 percent, 98 percent, 99 percent, or 100 percent. Moreover, any numerical range defined by two R numbers as defined in the above is also specifically disclosed.

[0030] In addition to the definitions above, certain compounds described herein have one or more double bonds that can exist as either the Z or E isomer. The invention in certain embodiments encompasses these compounds as individual isomers in a substantially pure form as well as mixtures of various isomers, e.g., racemic mixtures of stereoisomer.

Apparatus for Making Bio-organic Compounds

[0031] The present invention provides various production systems for making bio-organic compounds. In some embodiments, the bio-organic compounds may be produced using batch, continuous, fed-batch or semi-continuous fermentation processes.

[0032] Batch fermentation may be a closed system where the composition of the media is fixed at the beginning of the fermentation and not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the media is inoculated with the desired organism or organisms and fermentation is permitted to occur adding nothing to the system. In some embodiments, however, "batch" fermentation is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system may change constantly up to the time the fermentation is stopped. Within batch cultures, cells may moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of production of end product or intermediate.

[0033] A variation on the standard batch system is the fed-batch system. Fed-batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that additional carbon source or substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt

to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO₂.

[0034] Continuous fermentation is an open system where a defined fermentation media is added continuously to one or more bioreactors which may be in series and an equal amount of conditioned media is removed simultaneously from the system for additional processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth. Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to media being drawn off must be balanced against the cell growth rate in the fermentation.

[0035] Accordingly, in some embodiments of the invention, a bio-organic production system is provided which comprises:

- a. at least one vessel having a capacity of at least 100 liters;
- b. an aqueous medium, within the at least one vessel, comprising a first phase;
- c. a plurality of host cells, within the aqueous medium, capable of making, producing or synthesizing at least one bio-organic compound; and,
- d. a liquid organic second phase comprising the at least one bio-organic compound in contact with the first phase.

[0036] A suitable vessel for use in the present invention can be any vessel for holding the host cells and aqueous medium for fermentation. For example, the vessel can be a tank for a reactor or fermenter or it can be a part of a centrifuge that can separate heavier materials from lighter materials in subsequent processing steps. Alternatively, one or a plurality of vessels may be used in a continuous or semi-continuous process.

[0037] A general illustrative example of a suitable vessel 100 is shown in Figure 1. The vessel 100 includes: an inlet port 120 for the addition of host cells, fermentation media, and other compounds, nutrients or compositions to assist, regulate or improve fermentation of

the host cells, production of the bio-organic compound or compounds, and performance of additional production steps into the vessel; an outlet port 130 for removing the materials during or at the end of the fermentation process, and a gas outlet 140 for venting off exhaust gases such as carbon dioxide produced during or after the fermentation process. Vessel 100 may be completely filled with host cells, fermentation media and other materials so that there is no space for gas at the top of the vessel. Alternatively, vessel 100 can be partially filled thus leaving void space occupied by a gas. The amount, pressure and composition of the gas in the void space may be controlled to optimize or maximize growth of the host cells and production of the bio-organic compound or bio-organic compounds. For example, during fermentation of aerobic host cells, the gas typically may comprise air or other oxygen-containing gas at various pressures above, at or below atmospheric pressure, for example for microaerophilic and anaerobic host cells the oxygen concentration of the gas may be controlled within a range lower than atmospheric concentration while still above zero while during fermentation for anaerobic host cells, the gas typically has little to no oxygen and can completely comprise mostly or completely of nitrogen or other suitable gas.

[0038] In a closed system, inlet port 120, outlet port 130 and gas outlet 140 of vessel 100 shown in Figure 1 may be closed or under positive pressure during the fermentation process. Alternatively, particularly when using aerobic host cells, vessel 100 can be used as an open system whereby one or more of the ports and outlet are opened to the atmosphere providing a system for gas/liquid mass transfer (air or oxygen in and carbon dioxide out). If desired, gas outlet 140 may function both as a gas outlet and as a gas inlet where oxygen or air or other gas may be introduced into the system. In some embodiments, vessel 100 includes separate gas inlets and separate gas outlets. In such open systems, additional hardware may be included on the vessel for preventing contamination or infiltration of other organisms or other materials into the vessel during the fermentation.

[0039] Another vessel embodiment is illustrated in Figure 2. In addition to inlet port 220, outlet port 230, gas inlet 235, and gas outlet 240 similar to the vessel in Figure 1, the vessel 200 of Figure 2 includes an agitator 250 for mixing. In some embodiments, agitator 250 may comprise a motor-driven shaft 252 which may include a shaft seal 251 and is connected to one or more impellers 254. Agitator 250 may be typically attached to the top or bottom of the vessel 200. Optionally, each impeller 254 may be terminated with one or more paddles 256. Impellers 254 may be any suitable shape and may be selected specifically to control amount of mixing, growth rate of the host cells, production rate of the bio-organic compound, shear rate and oxygen or other gas transfer rates. Additionally, one or more

baffles 258 can be added to the vessel 200 to further improve mixing. In another embodiment, agitation may be supplied in the form of a recycle line with a pump that draws material from one portion of the vessel such as the bottom and reintroduces the material into the vessel at another portion of the vessel such as the top. Agitation within the vessel of the host cells and the fermentation medium aids in ensuring that the host cells are exposed to adequate nutrients to enable them to grow and produce the bio-organic compounds.

[0040] If the fermentation process is an aerobic process, oxygen or air can be bubbled through a sparger 260 for improved gas/liquid mass transfer. The sparger 260 may include one or more gas outlets (not shown) that are submerged within the fermentation media, preferably at or near the bottom of the vessel. In some embodiments, the sparger 260 may be a sparging ring having multiple gas outlets arranged in a generally circular or round configuration. Alternatively, for shear sensitive organisms or to reduce foaming, passive aeration of the vessel may be provided, such as use of various aeration screens, membranes, fibers or other passive aeration devices or by removing a portion of the media from the vessel, oxygenating it and returning it to the vessel.

[0041] If temperature control is desired, then a heater or heat exchanger may be used to heat or cool the fermentation reaction. In one embodiment, the temperature may be controlled using a heating/cooling jacket 270 surrounding and/or attached to at least a portion of vessel 200 that may be connected to a heat exchanger (not shown) that circulates temperature controlled heat exchange fluid through jacket 270. Alternatively, a heater, or heat exchanger may be immersed in the fermentation medium. Illustrative examples of this type of heater or heat exchanger include an electric immersion heater, an immersed coiled or linear tube heat exchanger carrying a heat-exchange fluid such as heated water or oil, and one or more spargers that inject a heated stream such as air and/or water into the fermentation medium. Alternatively or additionally, a heater or heat exchanger can be attached to the outside of the vessel. Such heaters and heat exchangers include electrical heat tape on outside sidewalls of the vessel and heated or jacketed recycle lines attached to the vessel.

[0042] Vessel 200 can include additional inlet and outlet ports. In some embodiments, the additional inlet and outlet ports may be located on the top, sides or bottom of the vessel 200. In some embodiments, the additional inlet ports include feed lines for the addition of oxygen or other gases, nutrients, foam and pH control agents during the fermentation reaction. Any of the inlet and outlet ports may include sterilization mechanisms for multiple uses including in-process use, and multiple connection or reconnection during the fermentation process.

[0043] In addition, one or more probe ports 280 and/or sampling valves 290 can be positioned at various places on vessel 200 to help monitor critical parameters such as concentrations of various products and metabolites, pH, liquid level, pressure, foam, dissolved oxygen concentration, temperature, agitation rate, power, voltage, valve positions and cell density during the fermentation process.

[0044] It should be understood that the vessels in Figures 1 and 2 are for illustrative purposes and that many different vessel configurations for the fermentation process may be used, for example, according to the type of host cell, the bio-organic compound or compounds produced, the production volume, the type of fermentation process, the type of downstream processing, the separation process and other considerations.

[0045] A vessel such as that shown in Figure 2 is suitable for use in batch fermentation processes. If a continuous or semi-continuous fermentation process is desired (as opposed to a batch fermentation process) where materials are constantly added to or withdrawn from the vessel, the vessel typically includes additional inlet and outlet ports which may be located on the top, bottom or on the sides of the vessel. These additional inlet and outlet ports facilitate the flow of materials in and out of the vessel. In some embodiments, one or more vessels continuously receive host cells, fermentation medium, and optional additives while continuously discharging host cells, byproducts, and/or bio-organic compounds from the vessels. In these embodiments, the discharge from one vessel may be used as the feedstock to another vessel that optionally also receives fresh host cells, fermentation medium, nutrients, and/or other additives. A single vessel or a series of vessels together can be configured to provide the desired average residence time for the host cells. A portion of the discharge from one of the down-stream vessels can be returned to one or more upstream vessels to recycle the discharge to an earlier stage of processing, or other materials from processing steps further downstream can be reintroduced into the vessels.

[0046] The vessels used in some embodiments of the present invention include additional hardware that may be attached to the vessel to facilitate processing. Such hardware may include additional hardware for facilitating clean-in-place and sterilize-in-place processing. In some embodiments, one, some or each of the ports, outlets, inlets, valves and all of the hardware inside the vessel may be sterilized in place. In some embodiments, the sterilization may occur using steam sterilization. For example, any of the ports, outlets or sampling valves may include or have attached to them additional hardware that provides for steam supply to and condensate return from the port outlet or valve such that it may be steam sterilized prior to use or reuse.

[0047] The vessel or vessels may have a capacity of at least 100 liters. In some embodiments, the vessel has a capacity of from 100 to 3,000,000 liters such as at least 1000 liters, at least 5,000 liters, at least 10,000 liters, vessel at least 25,000 liters, at least 50,000 liters, at least 75,000 liters, at least 100,000 liters, at least 250,000 liters, at least 500,000 liters or at least 1,000,000 liters.

[0048] The vessel or vessels may include or have attached to them sensors and probes for measuring various parameters such as pressure, pH, dissolved oxygen concentration, temperature, gas flow rates, liquid flow rates, liquid level, valve positions, foaming, agitation, power, voltage and any other parameters useful in controlling or optimizing the growth of the host cells and the production of the bio-organic compound or compounds. The sensors and probes may feed information to one or more automated systems for controlling and recording the various parameters measured and for adjusting any of the various parameters by controlling air flowrates, power, heating or cooling to control vessel temperature, stirring rpms, pumps, sterilization or clean in place of the vessel or any of the inlet, outlet, addition, sampling valves or other ports, outlet flow control or any other relevant mechanism for controlling a parameter or parameters of the fermentation. Such adjustments may occur using any known control mechanism, such as for example, control or actuation of various valves, pumps or motors and may use proportional, proportional-integral or proportional -integral-derivative control systems.

[0049] The automated system or systems may additionally be controlled and monitored by a central control system, which may be a local or plant wide control system and may control production of just one bio-organic compound production process or multiple bio-organic compound production processes. The automated system or systems and central control system may comprise any suitable software, firmware and/or hardware, which may be proprietary or off the shelf or a combination thereof and may communicate using any suitable communication system. Non-limiting examples of such communication systems include hardwired systems that may be digital or analog, and may include direct connection or be in the form of a network such as a LAN or a WAN or ethernet. In addition, in some embodiments the communication system may be wireless and may be proprietary, BLUETOOTH, ultra wide band, 802.11 a,b,g or n or ZigBee, including TDMA, FDMA, OFDM, and CDMA and may operate in any suitable frequency band such as 2.4 GHz or 5.8 GHz.

[0050] Any of the vessels used in the production of the bio-organic compounds may include additional hardware, such as additional agitators, additional inlet ports, outlet ports,

sampling ports, additional heating/cooling equipment, such as additional heating coils, additional aeration equipment such as additional spargers, additional sensors and probes, additional cleaning or sterilization equipment to facilitate processing or any other parameter of the fermentation.

[0051] In some embodiments of the invention, an isoprenoid production system is provided which comprises:

- a. at least one vessel having a capacity of at least 100 liters;
- b. an aqueous medium, within the at least one vessel, comprising a first phase;
- c. a plurality of host cells, within the aqueous medium, capable of making, producing or synthesizing one or more isoprenoid compounds; and,
- d. a liquid organic second phase comprising the one or more isoprenoid compounds in contact with the first phase.

[0052] In some embodiments, the isoprenoid compound or compounds is a C₅ isoprenoid. These compounds are derived from one isoprene unit and are also called hemiterpenes. An illustrative example of a hemiterpene is isoprene. In other embodiments, the isoprenoid compound or compounds is a C₁₀ isoprenoid. These compounds are derived from two isoprene units and are also called monoterpenes. An illustrative example of a monoterpene is myrcene. In other embodiments, the isoprenoid compound or compounds is a C₁₅ isoprenoid. These compounds are derived from three isoprene units and are also called sesquiterpenes. An illustrative example of a sesquiterpene is patchoulol (which is also known as patchouli alcohol). In other embodiments, the isoprenoid compound or compounds is a C₂₀ isoprenoid. These compounds are derived from four isoprene units and also called diterpenes. An illustrative example of a diterpene is taxadiene. In yet other examples, the isoprenoid compound or compounds is a C₂₀₊ isoprenoid. These compounds are derived from more than four isoprene units and include: triterpenes (C₃₀ isoprenoid compounds derived from 6 isoprene units) such as squalene; tetraterpenes (C₄₀ isoprenoid compounds derived from 8 isoprenoids) such as β-carotene; and polyterpenes (C₄₀₊ isoprenoid compounds derived from more than 8 isoprene units) such as polyisoprene. In some embodiments, the isoprenoid compound or compounds may be any combination of two or more isoprenoid compounds.

[0053] In another aspect of the present invention, a method for producing at least one bio-organic compound is provided which comprises:

- a. culturing in an aqueous medium a plurality of host cells that produce, make or synthesize at least one bio-organic compound wherein the aqueous medium comprises a first phase;
- b. forming a liquid organic second phase comprising the at least one bio-organic compound in contact with the first phase;
- c. separating at least a portion of the second phase from the first phase; and,
- d. isolating the at least one bio-organic compound from the second phase.

[0054] The isoprenoid production system may include one or more additional processing components including: 1) one or more separation systems for separating the at least one bio-organic compound from the aqueous media and the organic second phase; 2) one or more reactors for biologically or chemically altering the at least one bio-organic compound such as by addition, substitution, hydrogenation, alkylation, hydroxylation, condensation, halogenation or any other suitable reaction; 2) one or more blending vessels or systems for blending the at least one bio-organic compound with one or more additional components; 3) and one or more additional purification or separation systems for further purifying the bio-organic composition or the at least one bio-organic compound.

[0055] The second phase may comprise the at least one bio-organic compound. The bio-organic compound can form a portion, most, or substantially all of the second phase. In certain embodiments, the bio-organic compound forms 1% to 99%, such as 5% to 95%, 10% to 90%, 20% to 80%, 25% to 75%, 35% to 65%, or 40% to 50% of the second phase. In certain embodiments, the second phase consists essentially of the bio-organic compound.

[0056] In some embodiments, the plurality of host cells includes more than one type of host cell, such as more than one species or strain of host cells, for example 2-5 species or strains of host cells, for example 2, 3, 4 or 5 species or strains of host cells. In some embodiments the plurality of host cells may produce more than one bio-organic compound, such as 2-5 bio-organic compounds, for example 2, 3, 4, or 5 bio-organic compounds.

[0057] The bio-organic compound or compounds may be isolated from the first phase and/or second phase using any suitable separation method. In some embodiments, the bio-organic compound is isolated from the second phase such that it is substantially pure.

[0058] In some embodiments, the organic second phase occurs spontaneously as a result of chemical and molecular interactions such as differences in solubility, or hydrophobicity, density, concentration or any other spontaneous phase separation mechanism. In other embodiments, separation of the first and second phases is induced in a

separation vessel or vessels or system that may be the same or a different vessel or vessels or processing system as the fermentation vessel or vessels. In some embodiments, phase separation is induced by centrifugation such as continuous or batch centrifugation. In other embodiments, phase separation is induced by the introduction of a deemulsifier or a nucleating agent into the fermentation reaction. A deemulsifier prevents or limits the amount of the bio-organic compound or compounds that emulsify with the aqueous phase.

Illustrative examples of deemulsifiers include flocculants and coagulants. A nucleating agent facilitates the aggregation of smaller droplets of the bio-organic compound to coalesce and eventually form a separate phase. If sufficient amounts of a nucleating agent are used, the nucleating agent itself forms an organic second phase into which the bio-organic compound migrates. Illustrative examples of nucleating agents include droplets of the bio-organic compound or compounds itself and organic solvents such as dodecane, isopropyl myristate, and methyl oleate. Some embodiments may include a combination of one or more of the above phase separation materials and methods.

[0059] Once phase separation occurs, the separate phases can be individually drawn from the separation vessel. Any amount of the second phase can be separated from the first phase, *e.g.* all, a portion, 1% to 100% such as 5% to 95%, 10% to 90%, 20% to 80%, 25% to 75%, 35% to 65%, or 40% to 50% of the second phase may be separated from the first phase. If the organic second phase is less dense than the aqueous first phase, then one or more taps can be provided or placed on the separation vessel near the interface between the two phases (preferably within the organic second phase) to decant the organic second phase before removing the denser aqueous phase. Alternatively, the aqueous first phase can be removed from the separation vessel using an outlet near the bottom of the separation vessel until the organic second phase appears. At which point, the organic second phase can be transferred into a separate location for further processing or storage. Both of the aqueous first and organic second phases can flow out of the separation vessel under the force of gravity, gas pressure or through the use of a pump or pumps or a combination thereof.

[0060] If the organic second phase is denser than the aqueous first phase, then one or more taps can be provided or placed on the separation vessel near the interface between the two phases (preferably within the organic second phase) to decant the aqueous first phase before removing the denser organic second phase. Alternatively the organic second phase may be removed from the separation vessel using an outlet near the bottom of the separation vessel.

[0061] For a continuous process in which the aqueous first phase is denser than the organic second phase, a separation vessel with one or more taps can contain a specified volume of the fermentation medium and host cells, and the continually-produced organic second phase may be decanted through the taps to storage or further processing. If the organic second phase is denser than the aqueous first phase, the organic second phase can be removed continuously from the bottom of the separation vessel at a rate that prevents complete depletion of the organic second phase from the separation vessel to avoid drawing from the aqueous first phase.

[0062] In some embodiments, the bio-organic compound may be isolated from the organic second phase using adsorption, a process in which molecules move from a bulk liquid onto the surface of adsorbents. Illustrative examples of adsorbents include activated carbon; aluminas; aluminosilicates such as zeolites; clays such as fuller's earth; molecular sieves; organic polymers such as polystyrene and resins; and silicas such silica gel. Depending on the adsorbent used, the adsorbent may be used to capture the desired bio-organic product or unwanted byproducts. Isolation by adsorption may be performed using a batch, continuous or semi-continuous process.

[0063] In other embodiments, the bio-organic compound may be isolated from the organic second phase using distillation, a method of separating substances based on differences in their volatilities. In batch distillation, an entire batch of liquid is initially charged to a vessel and then heated or reduced in pressure within the vessel. Vapor is thereby continuously generated and may be condensed to form a liquid distillate which is collected. In continuous equilibrium distillation, a continuously flowing liquid feed is heated or reduced in pressure so as to cause partial vaporization of the mixture and separate recovery of liquid and vapor components. The liquid and vapor disengage while flowing through a distillation column, and the products emerge as vapor and liquid streams. When the vapor and liquid approach phase equilibrium, this is called a flashing process. If desired, the vapor product can be condensed to form a liquid distillate.

[0064] In other embodiments, the bio-organic compound or compounds are isolated from the organic second phase using gas-liquid extraction. This process is also known as stripping and is the transfer of a component dissolved in a liquid stream into a vapor stream in a more concentrated form. Temperature and pressure can be optimized for the transfer of the desired bio-organic compound. Illustrative examples of vapor streams include air and steam. Typically, the liquid stream flows down a column while the vapor stream is bubbled up (flowing countercurrently to the liquid stream).

[0065] In other embodiments, the bio-organic compound is isolated from the organic second phase using liquid-liquid extraction. Also known as solvent extraction, liquid-liquid extraction is the transfer of a substance from one liquid phase into another immiscible liquid phase.

[0066] In a batch liquid-liquid extraction system, the feed liquid (the organic second phase) is mixed with a second immiscible liquid phase in a suitable vessel. The mixture is then permitted to settle into layers and separate into extract and raffinate and the lighter layer can be decanted from the vessel. The desired bio-organic compound or compounds can be in the extract or raffinate depending on the product and solvent used.

[0067] In a continuous liquid-liquid extraction system, differences in density, vapor pressure at a given temperature, or boiling points are used to separate the desired bio-organic product from the feed liquid (the organic phase). Such systems can use mixer/settler tanks, towers or columns, centrifuges and combinations thereof to effect separation.

[0068] In other embodiments, the bio-organic compound is isolated from the organic second and /or the aqueous first phase using ultrafiltration, a pressure-driven membrane process used to separate solution components on the basis of molecular size and shape.

Under an applied pressure difference across an ultrafiltration membrane, solvent and small solute species pass through the membrane and are collected as permeate while larger solute species are retained by the membrane and recovered as a concentrated retentate.

Ultrafiltration involves solutes whose molecular dimensions are ten or more times larger than those of the solvent and are usually below ½ micron in size. The solutes or the materials to be separated usually have molecular weights greater than 500 amu, such as macromolecules, colloidal dispersions, and emulsions. A non-limiting example of an ultrafiltration system is a tangential flow ultrafiltration system.

[0069] In some embodiments, the host cells are capable of producing from about 10 to about 50 grams, more than about 15 grams, more than about 20 grams, more than about 25 grams or more than about 30 grams of bio-organic compound per liter of fermentation medium.

[0070] In some embodiments, the host cells are capable of producing from about 50 to about 1500 milligrams, such as more than about 100 milligrams, more than about 150 milligrams, more than about 200 milligrams, more than about 250 milligrams, more than about 500 milligrams, more than about 750 milligrams or more than about 1000 milligrams of bio-organic compound per gram of dry cell weight.

Fuel Composition Production System

[0071] In some embodiments, the invention comprises a fuel composition production system comprising:

- a. at least one vessel having a capacity of at least 100 liters;
- b. an aqueous medium, within the vessel, comprising a first phase;
- c. a plurality of host cells, within the aqueous medium, capable of making, producing or synthesizing at least one bio-organic compound; and,
- d. a liquid organic second phase comprising the at least one bio-organic compound in contact with the first phase.

The fuel composition production system may include one or more additional processing components including: 1) one or more separation systems for separating the at least one bio-organic compound from the aqueous media and the organic second phase; 2) one or more reactors for biologically or chemically altering the at least one bio-organic compound such as by addition, substitution, hydrogenation, alkylation, hydroxylation, condensation, halogenation or any other suitable reaction; 2) one or more blending vessels or systems for blending the at least one bio-organic compound with one or more additional fuel components such as a petroleum-based fuel, a fuel additive or a combination thereof; and, 3) one or more additional purification or separation systems for further purifying the fuel composition or the at least one bio-organic compound.

[0072] In some embodiments, the fuel additive is selected from the group consisting of oxygenates, antioxidants, environmental protectants, thermal stability improvers, cetane improvers, stabilizers, cold flow improvers, combustion improvers, anti-foams, anti-haze additives, corrosion inhibitors, lubricity improvers, icing inhibitors, injector cleanliness additives, smoke suppressants, drag reducing additives, metal deactivators, dispersants, detergents, deemulsifiers, dyes, markers, static dissipaters, biocides and combinations thereof.

[0073] In some embodiments, the fuel composition production system comprises:

- a) one or more batch, fed-batch or continuous flow fermentation systems comprising:
 - i) at least one vessel having a capacity of at least 100 liters;
 - ii) an aqueous medium, within the at least one vessel, comprising a first phase;

- iii) a plurality of host cells, within the aqueous medium, capable of making, producing or synthesizing at least one bio-organic compound; and,
- iv) a liquid organic second phase comprising the at least one bio-organic compound in contact with the first phase;
- b) one or more first phase separation systems whereby the first phase and the second organic phase or one or more components of the second organic phase are separated;
- c) optionally one or more second phase separation systems whereby the at least one bio-organic compound is separated from the second organic phase;
- d) optionally one or more reactors or vessels wherein the at least one bio-organic compound is chemically or biologically modified;
- e) optionally one or more purification systems whereby the bio-organic compound or the modified bio-organic compound is purified or further purified;
- f) optionally one or more blending vessels or systems for blending the at least one bio-organic compound with one or more additional fuel components; and
- g) optionally one or more further purification systems whereby the blend of the at least one bio-organic compound and the one or more additional fuel components is purified or further purified.

[0074] In some embodiments, the one or more first phase separation systems comprises one or more systems, vessels or other phase separation components detailed herein configured specifically to separate the first phase from the second organic phase. In some embodiments the one or more second phase separation systems includes one or more systems, vessels or phase separation components detailed herein configured specifically to separate the bio-organic compound or compounds from the second organic phase.

[0075] In some embodiments, the one or more reactors wherein the at least one bio-organic compound is chemically or biologically modified comprises the same or different vessel or vessels used for the fermentation or the separation systems. Alternatively, the one or more reactors may comprises one or more different vessels, which may include additional hardware, sensors, ports, probes, and/or control systems suitable for the specific reaction or reactions or other modifications to the bio-organic compound or compounds that are performed therein. The reactors may be batch, fed batch or continuous reactors.

[0076] In some embodiments, the bio-organic compounds or modified bio-organic compounds or the fuel compositions may be purified or further purified using one or more purification systems. The purification systems may comprise any suitable purification system including any system that may remove unwanted compounds from the bio-organic compound or compounds or that may separate the unwanted compounds from the bio-organic compounds. In some embodiments, the purification system may comprise one or more systems, vessels or phase separation components detailed herein that may be specifically configured to achieve the desired purity of the bio-organic compound or compounds. In some embodiments, the purification may be accomplished using one or more separation systems in series to achieve the desired purity. In some embodiments, the separation systems may be configured differently from each other in order to achieve the purity in stepwise fashion.

[0077] In some embodiments, the purification will be performed to achieve specifications or requirements of federal, state or local laws, rules or regulations for the bio-organic compounds or for fuel compositions. In some embodiments, the purification can improve the functionality of the bio-organic compounds or fuel compositions beyond the requirements of federal or state laws, rules or regulations. In some embodiments, the federal state or local laws, rules or regulations may pertain to environmental emissions, fuel performance, tax incentives, and other economic incentives. In some embodiments, the purification may reduce the environmental impact of, carbon footprint of, fuel efficiency obtained from, reliability obtained from, energy available from, or long term economic cost of the bio-organic compounds or fuel compositions.

[0078] In some embodiments the fuel composition system includes one or more blending vessels or systems for blending the at least one bio-organic compound with one or more additional fuel components. The blending vessel or blending system may be any suitable vessel or system. The blending vessel may include any or all of the inlets, outlets, ports, sensors, probes, agitators and other hardware identified for the bio-organic compound production vessel. The blending vessel may blend one or more fuel components with the bio-organic compound or compounds. For example, 2-5 fuel components, such as 3 or 4 fuel components. The blending system may be batch, continuous or fed batch.

[0079] In some embodiments, the invention comprises a method of making a fuel composition comprising:

- a. culturing in an aqueous medium a plurality of host cells that produce, make or synthesize at least one bio-organic compound wherein the aqueous medium comprises a first phase;
- b. forming a liquid organic second phase comprising the at least one bio-organic compound in contact with the first phase;
- c. separating at least a portion of the second phase from the first phase;
- d. isolating the at least one bio-organic compound from the second phase;
- e. optionally chemically or biologically modifying the at least one bio-organic compound;
- f. optionally purifying the bio-organic compound or the modified bio-organic compound;
- g. optionally blending the at least one bio-organic compound with one or more additional fuel components; and
- g) optionally purifying the blend of the one or more bio-organic compounds and the one or more additional fuel components.

[0080] In some embodiments, the fuel composition comprises a biofuel composition. In some embodiments, the biofuel further comprises at least one bio-organic compound and a petroleum-based fuel, a fuel additive or a combination thereof. In further embodiments, the petroleum-based fuel is a gasoline, jet fuel, kerosene, diesel fuel or a combination thereof.

[0081] In some embodiments, the bio-organic compound production system or the fuel composition production system may be built or created by retrofitting an ethanol production facility.

[0082] In some embodiments, the fuel composition production systems may comprise one or more automated control systems. The automated control systems may be the same or different from the control systems for the bio-organic production system and may comprise various sensors, probes and other equipment for measuring and controlling the various process parameters associated with each system within the fuel composition system and each step or the fuel composition production methods. The automated system or systems may additionally be controlled and monitored by a central control system, which may be a local or plant wide control system and may control production of just one bio-organic compound production process or multiple bio-organic compound production processes. The automated system or systems and central control system may comprise any suitable software, firmware and/or hardware, which may be proprietary or off the shelf or a combination thereof and may communicate using any suitable communication system. Non-limiting examples of such

communication systems include hardwired systems that may be digital or analog, and may include direct connection or be in the form of a network such as a LAN or a WAN or ethernet. In addition, in some embodiments the communication system may be wireless and may be proprietary, BLUETOOTH, ultra wide band, 802.11 a,b,g or n or ZigBee, including TDMA, FDMA, OFDM, and CDMA and may operate in any suitable frequency band such as 2.4 GHz or 5.8 GHz.

Host Cells

[0083] Any suitable host cell can be used in the practice of the present invention. In some embodiments, the host cell is a genetically modified host microorganism in which nucleic acid molecules have been inserted, deleted or modified (*i.e.*, mutated; e.g., by insertion, deletion, substitution, and/or inversion of nucleotides), to either produce the desired bio-organic compound, or effect an increased yield of the desired bio-organic compound.

[0084] Illustrative examples of suitable host cells include any archae, bacterial, or eukaryotic cell. Examples of archae cells include, but are not limited to those belonging to the genera: *Aeropyrum*, *Archaeoglobus*, *Halobacterium*, *Methanococcus*, *Methanobacterium*, *Pyrococcus*, *Sulfolobus*, and *Thermoplasma*. Illustrative examples of archae species include but are not limited to: *Aeropyrum pernix*, *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*.

[0085] Examples of bacterial cells include, but are not limited to those belonging to the genera: *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azobacter*, *Bacillus*, *Brevibacterium*, *Chromatium*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Mesorhizobium*, *Methylobacterium*, *Microbacterium*, *Phormidium*, *Pseudomonas*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Rhodococcus*, *Salmonella*, *Scenedesmun*, *Serratia*, *Shigella*, *Staphylococcus*, *Streptomyces*, *Synnecoccus*, and *Zymomonas*.

[0086] Illustrative examples of bacterial species include but are not limited to: *Bacillus subtilis*, *Bacillus amyloliquefacines*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum*, *Clostridium beigerinckii*, *Enterobacter sakazakii*, *Escherichia coli*, *Lactococcus lactis*, *Mesorhizobium loti*, *Pseudomonas aeruginosa*, *Pseudomonas mevalonii*, *Pseudomonas pudica*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Salmonella enterica*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus aureus*, and the like.

[0087] In general, if a bacterial host cell is used, a non-pathogenic strain is preferred. Illustrative examples of species with non-pathogenic strains include but are not limited to: *Bacillus subtilis*, *Escherichia coli*, *Lactibacillus acidophilus*, *Lactobacillus helveticus*, *Pseudomonas aeruginosa*, *Pseudomonas mevalonii*, *Pseudomonas putida*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and the like.

[0088] Examples of eukaryotic cells include but are not limited to fungal cells. Examples of fungal cells include, but are not limited to those belonging to the genera: *Aspergillus*, *Candida*, *Chrysosporium*, *Cryptococcus*, *Fusarium*, *Kluyveromyces*, *Neotyphodium*, *Neurospora*, *Penicillium*, *Pichia*, *Saccharomyces*, *Trichoderma* and *Xanthophyllomyces* (formerly *Phaffia*).

[0089] Illustrative examples of eukaryotic species include but are not limited to: *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Candida albicans*, *Chrysosporium lucknowense*, *Fusarium graminearum*, *Fusarium venenatum*, *Kluyveromyces lactis*, *Neurospora crassa*, *Pichia angusta*, *Pichia finlandica*, *Pichia kodamae*, *Pichia membranaefaciens*, *Pichia methanolica*, *Pichia opuntiae*, *Pichia pastoris*, *Pichia pijperi*, *Pichia quercuum*, *Pichia salictaria*, *Pichia thermotolerans*, *Pichia trehalophila*, *Pichia stipitis*, *Streptomyces ambofaciens*, *Streptomyces aureofaciens*, *Streptomyces aureus*, *Saccaromyces bayanus*, *Saccaromyces boulardi*, *Saccharomyces cerevisiae*, *Streptomyces fungicidicus*, *Streptomyces griseochromogenes*, *Streptomyces griseus*, *Streptomyces lividans*, *Streptomyces olivogriseus*, *Streptomyces rameus*, *Streptomyces tanashiensis*, *Streptomyces vinaceus*, *Trichoderma reesei* and *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*).

[0090] In general, if a eukaryotic cell is used, a non-pathogenic strain is preferred. Illustrative examples of species with non-pathogenic strains include but are not limited to: *Fusarium graminearum*, *Fusarium venenatum*, *Pichia pastoris*, *Saccaromyces boulardi*, and *Saccaromyces cerevisiae*.

[0091] In some embodiments, the host cells of the present invention have been designated by the Food and Drug Administration as GRAS or Generally Regarded As Safe. Illustrative examples of such strains include: *Bacillus subtilis*, *Lactibacillus acidophilus*, *Lactobacillus helveticus*, and *Saccharomyces cerevisiae*.

Engineering Pathways to make Bio-Organic Compounds

[0092] An illustrative example of a class of bio-organic compounds is isoprenoids. They comprise a diverse family of over 40,000 individual products, many of which are vital to living organisms. Isoprenoids serve to maintain cellular fluidity, electron transport, and

other metabolic functions. In addition to their usefulness in making fuel compositions, a vast number of natural and synthetic isoprenoids are useful as pharmaceuticals, cosmetics, perfumes, pigments and colorants, fungicides, antiseptics, nutraceuticals, and fine chemical intermediates.

[0093] Isoprenoid compounds are made in nature through two different metabolic pathways which converge at IPP and its isomer, DMAPP. In general, eukaryotes other than plants use the MEV isoprenoid pathway exclusively to convert acetyl-CoA to IPP, which is subsequently isomerized to DMAPP. Prokaryotes, with some exceptions, use the mevalonate-independent or DXP pathway to produce IPP and DMAPP separately through a branch point. In general, plants use both the MEV and DXP pathways for IPP synthesis. The methods described herein for engineering the MEV and DXP pathways to make the desired isoprenoid compound can be readily adapted to similarly engineer other pathways to make other bio-organic compounds.

MEV Pathway

[0094] A schematic representation of the MEV pathway is described in Figure 3. In general, the pathway comprises six steps.

[0095] In the first step, two molecules of acetyl-coenzyme A are enzymatically combined to form acetoacetyl-CoA. An enzyme known to catalyze this step is, for example, acetyl-CoA thiolase (also known as acetyl-CoA acetyltransferase). Illustrative examples of nucleotide sequences include but are not limited to the following GenBank accession numbers and the organism from which the sequences derived: (NC_000913 REGION: 2324131..2325315; *Escherichia coli*), (D49362; *Paracoccus denitrificans*), and (L20428; *Saccharomyces cerevisiae*).

[0096] In the second step of the MEV pathway, acetoacetyl-CoA is enzymatically condensed with another molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). An enzyme known to catalyze this step is, for example, HMG-CoA synthase. Illustrative examples of nucleotide sequences include but are not limited to: (NC_001145. complement 19061..20536; *Saccharomyces cerevisiae*), (X96617; *Saccharomyces cerevisiae*), (X83882; *Arabidopsis thaliana*), (AB037907; *Kitasatospora griseola*), (BT007302; *Homo sapiens*), and (NC_002758, Locus tag SAV2546, GeneID 1122571; *Staphylococcus aureus*).

[0097] In the third step, HMG-CoA is enzymatically converted to mevalonate. An enzyme known to catalyze this step is, for example, HMG-CoA reductase. Illustrative examples of nucleotide sequences include but are not limited to: (NM_206548; *Drosophila*

melanogaster), (NC_002758, Locus tag SAV2545, GeneID 1122570; *Staphylococcus aureus*), (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*).

[0098] In the fourth step, mevalonate is enzymatically phosphorylated to form mevalonate 5-phosphate. An enzyme known to catalyze this step is, for example, mevalonate kinase. Illustrative examples of nucleotide sequences include but are not limited to: (L77688; *Arabidopsis thaliana*), and (X55875; *Saccharomyces cerevisiae*).

[0099] In the fifth step, a second phosphate group is enzymatically added to mevalonate 5-phosphate to form mevalonate 5-pyrophosphate. An enzyme known to catalyze this step is, for example, phosphomevalonate kinase. Illustrative examples of nucleotide sequences include but are not limited to: (AF429385; *Hevea brasiliensis*), (NM_006556; *Homo sapiens*), and (NC_001145: complement 712315..713670; *Saccharomyces cerevisiae*).

[00100] In the sixth step, mevalonate 5-pyrophosphate is enzymatically converted into IPP. An enzyme known to catalyze this step is, for example, mevalonate pyrophosphate decarboxylase. Illustrative examples of nucleotide sequences include but are not limited to: (X97557; *Saccharomyces cerevisiae*), (AF290095; *Enterococcus faecium*), and (U49260; *Homo sapiens*).

[00101] If IPP is to be converted to DMAPP, then a seventh step is required. An enzyme known to catalyze this step is, for example, IPP isomerase. Illustrative examples of nucleotide sequences include but are not limited to: (NC_000913, 3031087..3031635; *Escherichia coli*), and (AF082326; *Haematococcus pluvialis*). If the conversion to DMAPP is required, an increased expression of IPP isomerase ensures that the conversion of IPP into DMAPP does not represent a rate-limiting step in the overall pathway.

DXP Pathway

[00102] A schematic representation of the DXP pathway is described in Figure 4. In general, the DXP pathway comprises seven steps. In the first step, pyruvate is condensed with D-glyceraldehyde 3-phosphate to make 1-deoxy-D-xylulose-5-phosphate. An enzyme known to catalyze this step is, for example, 1-deoxy-D-xylulose-5-phosphate synthase. Illustrative examples of nucleotide sequences include but are not limited to: (AF035440; *Escherichia coli*), (NC_002947, locus tag PP0527; *Pseudomonas putida* KT2440), (CP000026, locus tag SPA2301; *Salmonella enterica Paratyphi*, see ATCC 9150),

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(NC_007493, locus tag RSP_0254; *Rhodobacter sphaeroides* 2.4.1), (NC_005296, locus tag RPA0952; *Rhodopseudomonas palustris* CGA009), (NC_004556, locus tag PD1293; *Xylella fastidiosa Temecula1*), and (NC_003076, locus tag AT5G11380; *Arabidopsis thaliana*).

[00103] In the second step, 1-deoxy-D-xylulose-5-phosphate is converted to 2C-methyl-D-erythritol-4-phosphate. An enzyme known to catalyze this step is, for example, 1-deoxy-D-xylulose-5-phosphate reductoisomerase. Illustrative examples of nucleotide sequences include but are not limited to: (AB013300; *Escherichia coli*), (AF148852; *Arabidopsis thaliana*), (NC_002947, locus tag PP1597; *Pseudomonas putida* KT2440), (AL939124, locus tag SCO5694; *Streptomyces coelicolor* A3(2)), (NC_007493, locus tag RSP_2709; *Rhodobacter sphaeroides* 2.4.1), and (NC_007492, locus tag Pfl_1107; *Pseudomonas fluorescens* PfO-1).

[00104] In the third step, 2C-methyl-D-erythritol-4-phosphate is converted to 4-diphosphocytidyl-2C-methyl-D-erythritol. An enzyme known to catalyze this step is, for example, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase. Illustrative examples of nucleotide sequences include but are not limited to: (AF230736; *Escherichia coli*), (NC_007493, locus_tag RSP_2835; *Rhodobacter sphaeroides* 2.4.1), (NC_003071, locus_tag AT2G02500; *Arabidopsis thaliana*), and (NC_002947, locus_tag PP1614; *Pseudomonas putida* KT2440).

[00105] In the fourth step, 4-diphosphocytidyl-2C-methyl-D-erythritol is converted to 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate. An enzyme known to catalyze this step is, for example, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase. Illustrative examples of nucleotide sequences include but are not limited to: (AF216300; *Escherichia coli*) and (NC_007493, locus_tag RSP_1779; *Rhodobacter sphaeroides* 2.4.1).

[00106] In the fifth step, 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate is converted to 2C-methyl-D-erythritol 2, 4-cyclodiphosphate. An enzyme known to catalyze this step is, for example, 2C-methyl-D-erythritol 2, 4-cyclodiphosphate synthase. Illustrative examples of nucleotide sequences include but are not limited to: (AF230738; *Escherichia coli*), (NC_007493, locus_tag RSP_6071; *Rhodobacter sphaeroides* 2.4.1), and (NC_002947, locus_tag PP1618; *Pseudomonas putida* KT2440).

[00107] In the sixth step, 2C-methyl-D-erythritol 2, 4-cyclodiphosphate is converted to 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate. An enzyme known to catalyze this step is, for example, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase. Illustrative examples of nucleotide sequences include but are not limited to: (AY033515; *Escherichia*

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coli), (NC_002947, locus_tag PP0853; *Pseudomonas putida* KT2440), and (NC_007493, locus_tag RSP_2982; *Rhodobacter sphaeroides* 2.4.1).

[00108] In the seventh step, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate is converted into either IPP or its isomer, DMAPP. An enzyme known to catalyze this step is, for example, isopentyl/dimethylallyl diphosphate synthase. Illustrative examples of nucleotide sequences include but are not limited to: (AY062212; *Escherichia coli*) and (NC_002947, locus_tag PP0606; *Pseudomonas putida* KT2440).

[00109] In some embodiments, "cross talk" (or interference) between the host cell's own metabolic processes and those processes involved with the production of IPP as provided by the present invention are minimized or eliminated entirely. For example, cross talk is minimized or eliminated entirely when the host microorganism relies exclusively on the DXP pathway for synthesizing IPP, and a MEV pathway is introduced to provide additional IPP. Such host organisms would not be equipped to alter the expression of the MEV pathway enzymes or process the intermediates associated with the MEV pathway. Organisms that rely exclusively or predominately on the DXP pathway include, for example, *Escherichia coli*.

[00110] In some embodiments, the host cell produces IPP via the MEV pathway, either exclusively or in combination with the DXP pathway. In other embodiments, a host's DXP pathway is functionally disabled so that the host cell produces IPP exclusively through a heterologously introduced MEV pathway. The DXP pathway can be functionally disabled by disabling gene expression or inactivating the function of one or more of the naturally occurring DXP pathway enzymes.

[00111] In other embodiments, the host cell produces IPP via the DXP pathway, either exclusively or in combination with the MEV pathway. In other embodiments, a host's MEV pathway is functionally disabled so that the host cell produces IPP exclusively through a heterologously introduced DXP pathway. The MEV pathway can be functionally disabled by disabling gene expression or inactivating the function of one or more of the naturally occurring MEV pathway enzymes.

C₅ Compounds

[00112] Exemplary C₅ bio-organic compounds are hemiterpenes which are generally derived from IPP or DMAPP. An illustrative example of a hemiterpene is isoprene.

Isoprene

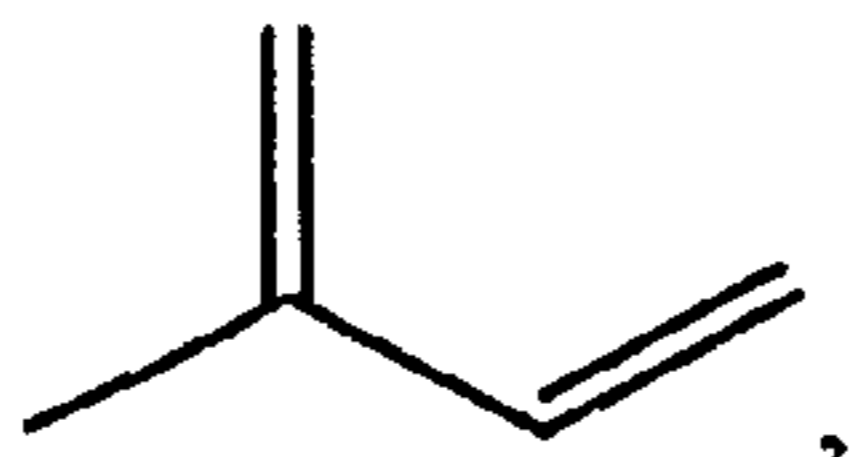
[00113] Isoprene, whose structure is

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is found in many plants. Isoprene is made from IPP by isoprene synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to: (AB198190; *Populus alba*) and (AJ294819; *Populus alba x Populus tremula*).

***C*₁₀ Compounds**

[00114] Exemplary *C*₁₀ bio-organic compounds are monoterpenes which are generally derived from geranyl pyrophosphate (GPP) which in turn is made by the condensation of IPP with DMAPP. An enzyme known to catalyze this step is, for example, geranyl pyrophosphate synthase.

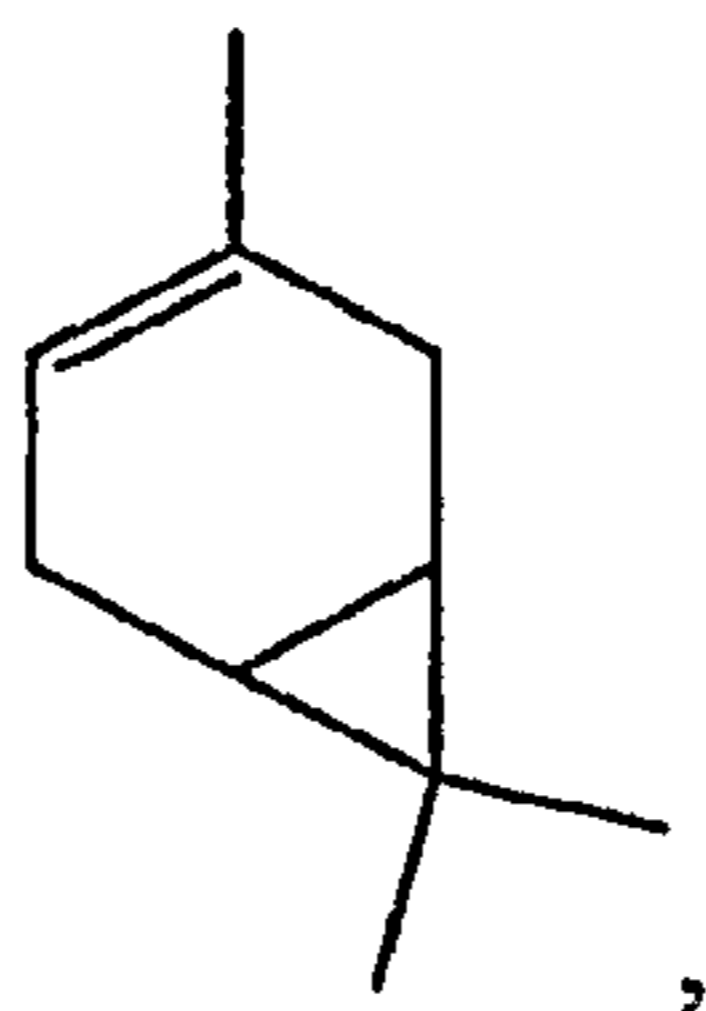
[00115] Figure 5 shows schematically how IPP and DMAPP can produce GPP, which can be further processed to a monoterpene.

[00116] Illustrative examples of nucleotide sequences for geranyl pyrophosphate synthase include but are not limited to: (AF513111; *Abies grandis*), (AF513112; *Abies grandis*), (AF513113; *Abies grandis*), (AY534686; *Antirrhinum majus*), (AY534687; *Antirrhinum majus*), (Y17376; *Arabidopsis thaliana*), (AE016877, Locus AP11092; *Bacillus cereus*; ATCC 14579), (AJ243739; *Citrus sinensis*), (AY534745; *Clarkia breweri*), (AY953508; *Ips pini*), (DQ286930; *Lycopersicon esculentum*), (AF182828; *Mentha x piperita*), (AF182827; *Mentha x piperita*), (MPI249453; *Mentha x piperita*), (PZE431697, Locus CAD24425; *Paracoccus zeaxanthinifaciens*), (AY866498; *Picrorhiza kurrooa*), (AY351862; *Vitis vinifera*), and (AF203881, Locus AAF12843; *Zymomonas mobilis*).

[00117] GPP is then subsequently converted to a variety of *C*₁₀ compounds. Illustrative examples of *C*₁₀ compounds include but are not limited to:

Carene

[00118] Carene, whose structure is



is found in the resin of many trees, particularly pine trees. Carene is made from GPP from carene synthase. Illustrative examples of suitable nucleotide sequences include but are not

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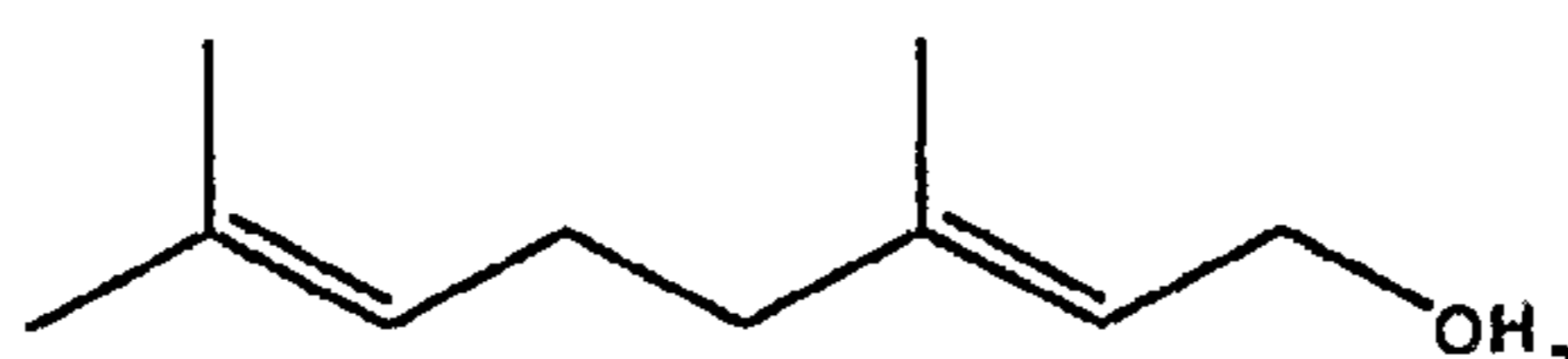
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limited to: (AF461460, REGION 43..1926; *Picea abies*) and (AF527416, REGION: 78..1871; *Salvia stenophylla*).

Geraniol

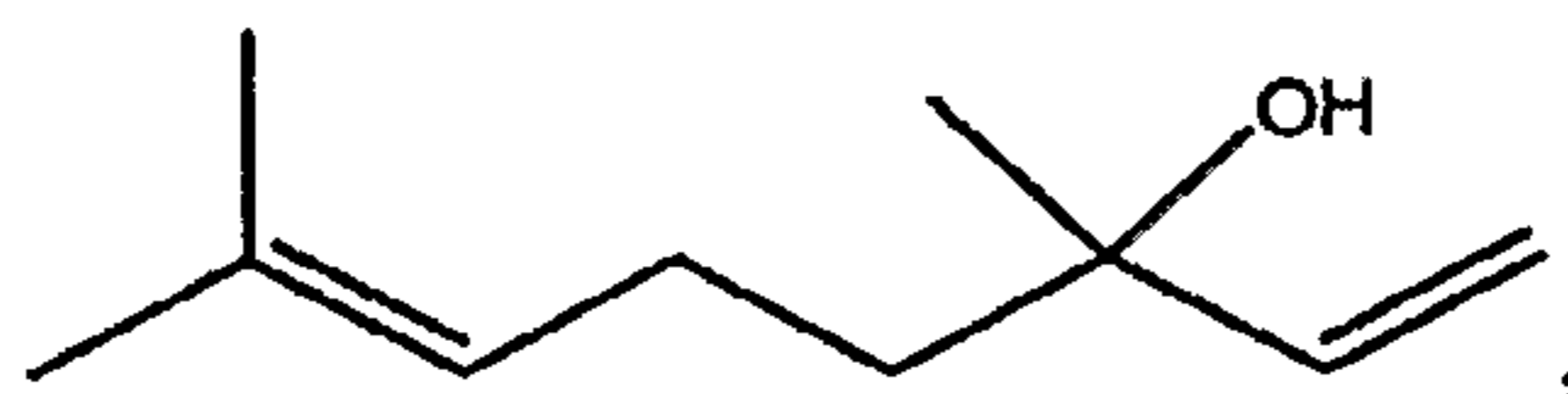
[00119] Geraniol (also known as rhodnol), whose structure is



is the main component of oil-of-rose and palmarosa oil. It also occurs in geranium, lemon, and citronella. Geraniol is made from GPP by geraniol synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to: (AJ457070; *Cinnamomum tenuipilum*), (AY362553; *Ocimum basilicum*), (DQ234300; *Perilla frutescens* strain 1864), (DQ234299; *Perilla citriodora* strain 1861), (DQ234298; *Perilla citriodora* strain 4935), and (DQ088667; *Perilla citriodora*)

[00120] Linalool

[00121] Linalool, whose structure is



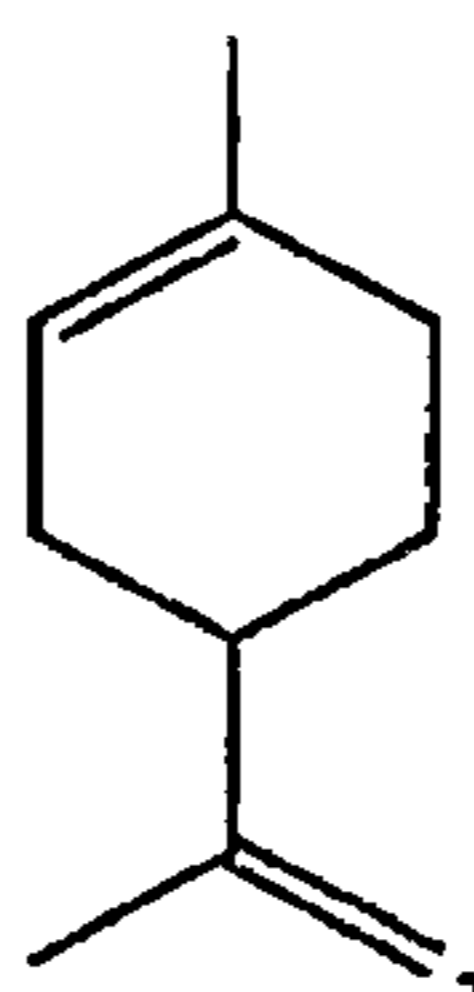
is found in many flowers and spice plants such as coriander seeds. Linalool is made from GPP by linalool synthase. Illustrative examples of a suitable nucleotide sequence include but are not limited to: (AF497485; *Arabidopsis thaliana*), (AC002294, Locus AAB71482; *Arabidopsis thaliana*), (AY059757; *Arabidopsis thaliana*), (NM_104793; *Arabidopsis thaliana*), (AF154124; *Artemisia annua*), (AF067603; *Clarkia breweri*), (AF067602; *Clarkia concinna*), (AF067601; *Clarkia breweri*), (U58314; *Clarkia breweri*), (AY840091; *Lycopersicon esculentum*), (DQ263741; *Lavandula angustifolia*), (AY083653; *Mentha citrate*), (AY693647; *Ocimum basilicum*), (XM_463918; *Oryza sativa*), (AP004078, Locus BAD07605; *Oryza sativa*), (XM_463918, Locus XP_463918; *Oryza sativa*), (AY917193; *Perilla citriodora*), (AF271259; *Perilla frutescens*), (AY473623; *Picea abies*), (DQ195274; *Picea sitchensis*), and (AF444798; *Perilla frutescens* var. *crispa* cultivar No. 79).

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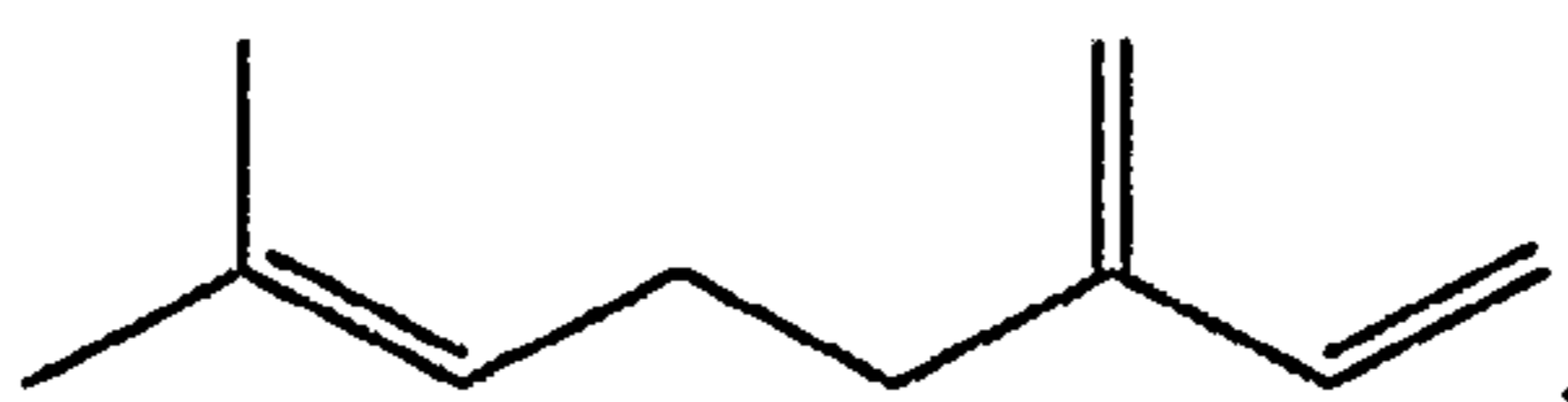
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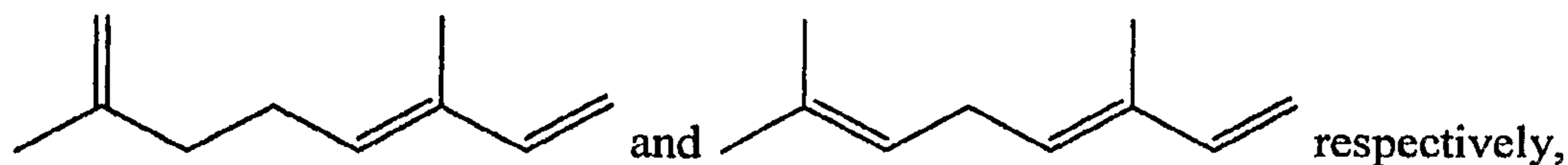
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Limonene**[00122]** Limonene, whose structure is

is found in the rind of citrus fruits and peppermint. Limonene is made from GPP by limonene synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to: (+)-limonene synthases (AF514287, REGION: 47..1867; *Citrus limon*) and (AY055214, REGION: 48..1889; *Agastache rugosa*) and (-)-limonene synthases (DQ195275, REGION: 1..1905; *Picea sitchensis*), (AF006193, REGION: 73..1986; *Abies grandis*), and (MHC4SLSP, REGION: 29..1828; *Mentha spicata*).

Myrcene**[00123]** Myrcene, whose structure is

is found in the essential oil in many plants including bay, verbena, and myrcia from which it gets its name. Myrcene is made from GPP by myrcene synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to: (U87908; *Abies grandis*), (AY195609; *Antirrhinum majus*), (AY195608; *Antirrhinum majus*), (NM_127982; *Arabidopsis thaliana* TPS10), (NM_113485; *Arabidopsis thaliana* ATTPS-CIN), (NM_113483; *Arabidopsis thaliana* ATTPS-CIN), (AF271259; *Perilla frutescens*), (AY473626; *Picea abies*), (AF369919; *Picea abies*), and (AJ304839; *Quercus ilex*).

Ocimene**[00124]** α - and β -Ocimene, whose structures are

are found in a variety of plants and fruits including *Ocimum basilicum* and is made from GPP by ocimene synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to: (AY195607; *Antirrhinum majus*), (AY195609; *Antirrhinum majus*), (AY195608; *Antirrhinum majus*), (AK221024; *Arabidopsis thaliana*), (NM_113485; *Arabidopsis thaliana* ATTPS-CIN), (NM_113483; *Arabidopsis thaliana* ATTPS-CIN),

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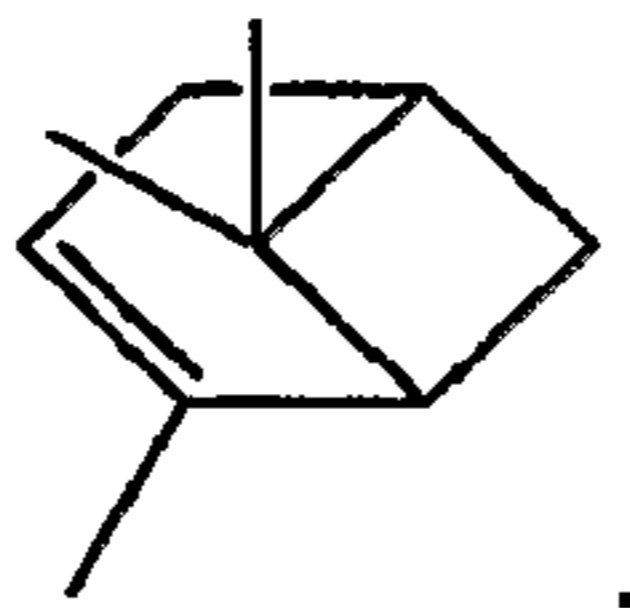
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(NM_117775; *Arabidopsis thaliana* ATTPS03), (NM_001036574; *Arabidopsis thaliana* ATTPS03), (NM_127982; *Arabidopsis thaliana* TPS10), (AB110642; *Citrus unshiu* CitMTSL4), and (AY575970; *Lotus corniculatus* var. *japonicus*).

α -Pinene

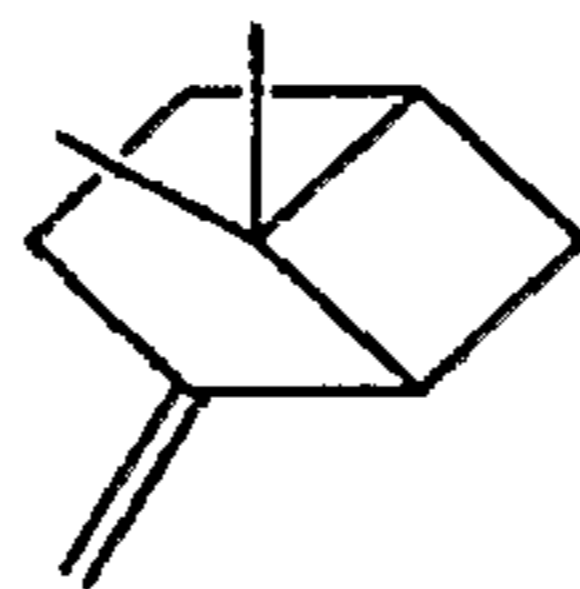
[00125] α -Pinene, whose structure is



is found in pine trees and eucalyptus. α -Pinene is made from GPP by α -pinene synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to: (+) α -pinene synthase (AF543530, REGION: 1..1887; *Pinus taeda*), (-) α -pinene synthase (AF543527, REGION: 32..1921; *Pinus taeda*), and (+)/(-) α -pinene synthase (AGU87909, REGION: 6111892; *Abies grandis*).

β -Pinene

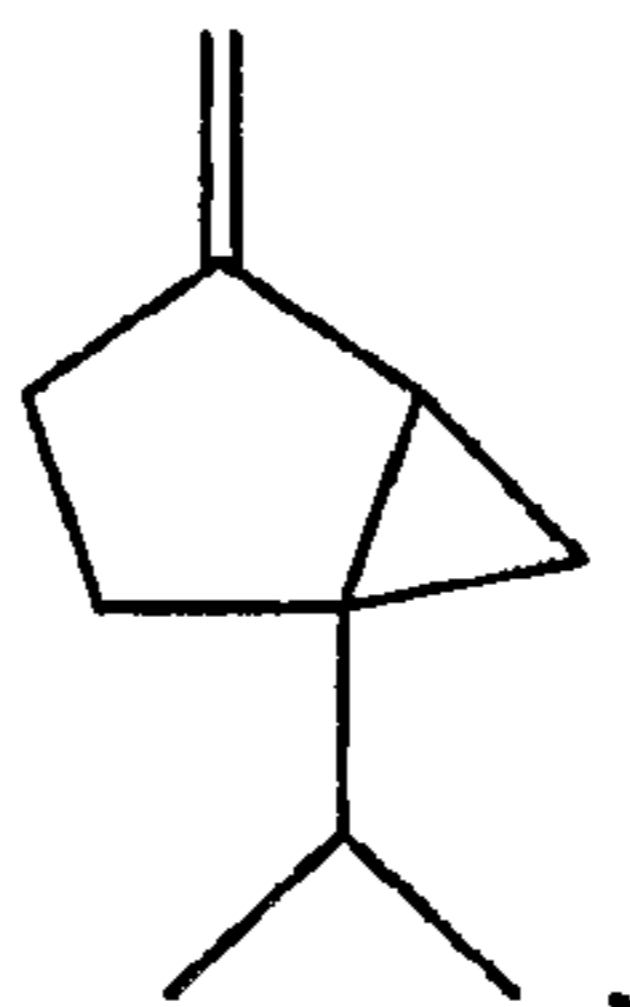
[00126] β -Pinene, whose structure is



is found in pine trees, rosemary, parsley, dill, basil, and rose. β -Pinene is made from GPP by β -pinene synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to: (-) β -pinene synthases (AF276072, REGION: 1..1749; *Artemisia annua*) and (AF514288, REGION: 26..1834; *Citrus limon*).

Sabinene

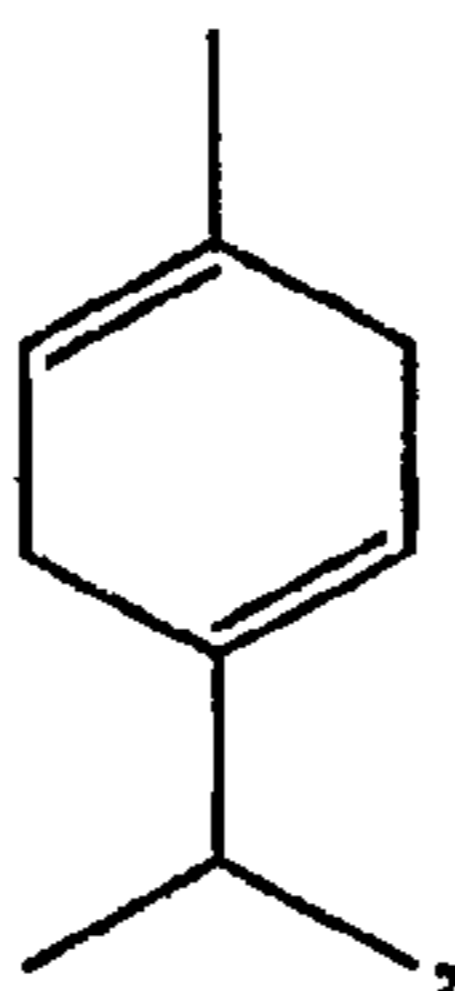
[00127] Sabinene, whose structure is



is found in black pepper, carrot seed, sage, and tea trees. Sabinene is made from GPP by sabinene synthase. An illustrative example of a suitable nucleotide sequence includes but is not limited to AF051901, REGION: 26..1798 from *Salvia officinalis*.

γ -Terpinene

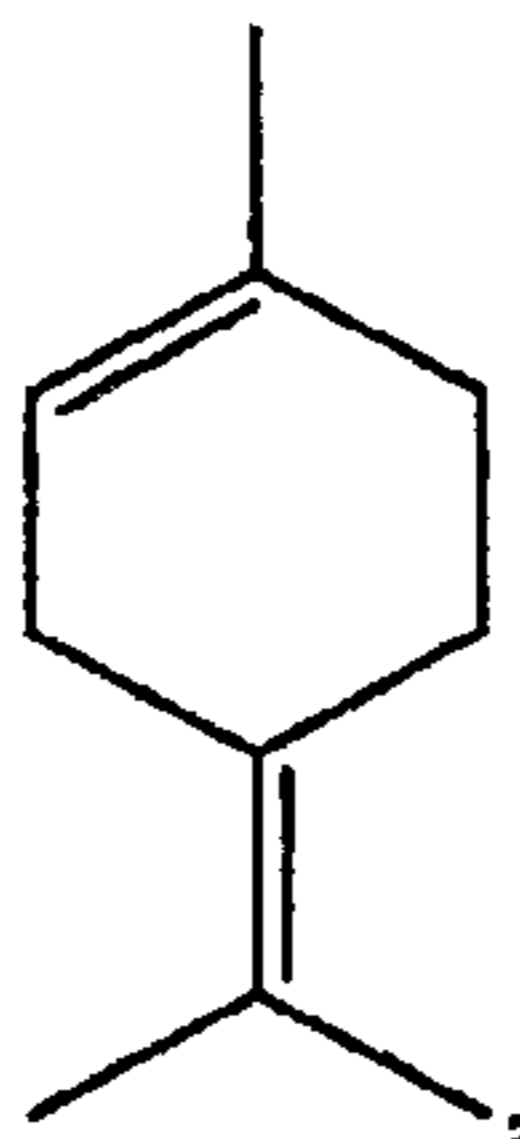
[00128] γ -Terpinene, whose structure is



is a constituent of the essential oil from citrus fruits. Biochemically, γ -terpinene is made from GPP by a γ -terpinene synthase. Illustrative examples of suitable nucleotide sequences include: (AF514286, REGION: 30..1832 from *Citrus limon*) and (AB110640, REGION 1..1803 from *Citrus unshiu*).

Terpinolene

[00129] Terpinolene, whose structure is



is found in black currant, cypress, guava, lychee, papaya, pine, and tea. Terpinolene is made from GPP by terpinolene synthase. Illustrative examples of a suitable nucleotide sequence include but is not limited to: (AY693650 from *Oscimum basilicum*) and (AY906866, REGION: 10..1887 from *Pseudotsuga menziesii*).

*C*₁₅ Compounds

[00130] Exemplary *C*₁₅ bio-organic compounds are sesquiterpenes which are generally derive from farnesyl pyrophosphate (FPP) which in turn is made by the condensation of two molecules of IPP with one molecule of DMAPP. An enzyme known to catalyze this step is, for example, farnesyl pyrophosphate synthase.

[00131] Figure 5 also shows schematically how IPP and DMAPP can be combined to produce FPP, which can be further processed to a sesquiterpene.

[00132] Illustrative examples of nucleotide sequences for farnesyl pyrophosphate synthase include but are not limited to: (ATU80605; *Arabidopsis thaliana*), (ATHFPS2R; *Arabidopsis thaliana*), (AAU36376; *Artemisia annua*), (AF461050; *Bos taurus*), (D00694;

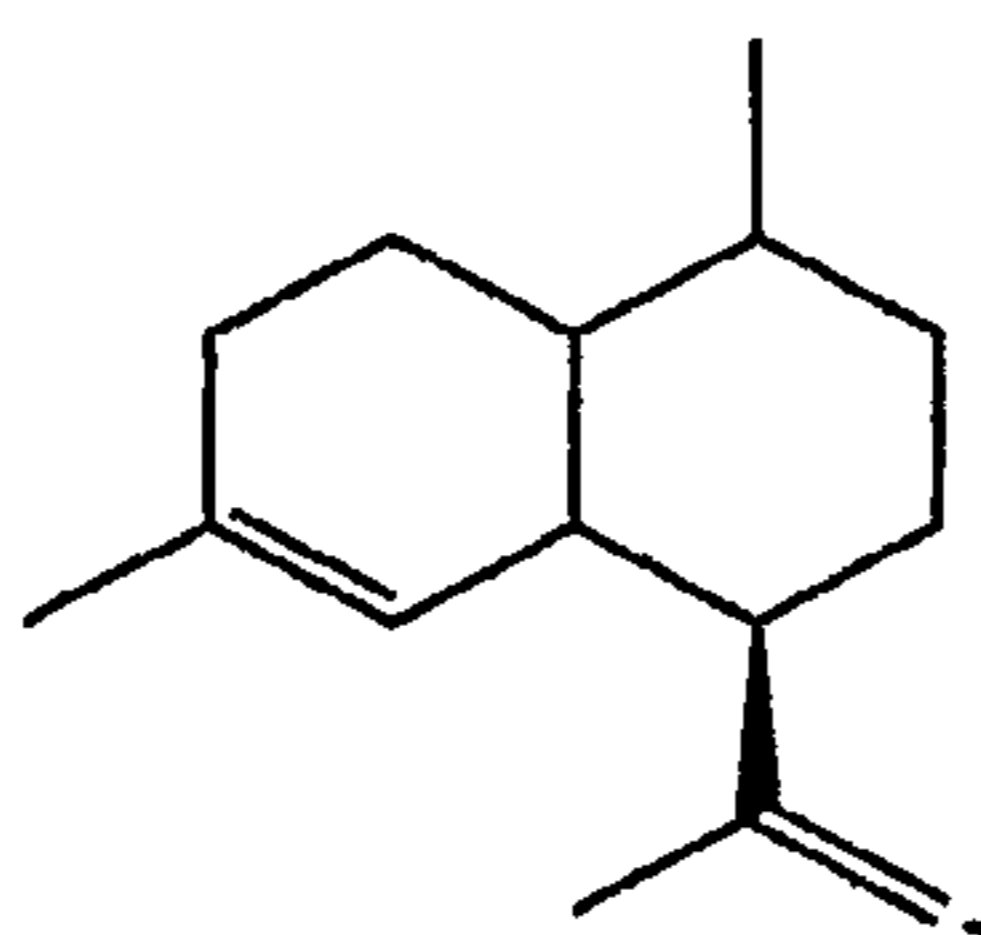
Escherichia coli K-12), (AE009951, Locus AAL95523; *Fusobacterium nucleatum subsp. nucleatum* ATCC 25586), (GFFPPSGEN; *Gibberella fujikuroi*), (CP000009, Locus AAW60034; *Gluconobacter oxydans* 621H), (AF019892; *Helianthus annuus*), (HUMFAPS; *Homo sapiens*), (KLPFPSQCR; *Kluyveromyces lactis*), (LAU15777; *Lupinus albus*), (LAU20771; *Lupinus albus*), (AF309508; *Mus musculus*), (NCFPPSGEN; *Neurospora crassa*), (PAFPS1; *Parthenium argentatum*), (PAFPS2; *Parthenium argentatum*), (RATFAPS; *Rattus norvegicus*), (YSCFPP; *Saccharomyces cerevisiae*), (D89104; *Schizosaccharomyces pombe*), (CP000003, Locus AAT87386; *Streptococcus pyogenes*), (CP000017, Locus AAZ51849; *Streptococcus pyogenes*), (NC_008022, Locus YP_598856; *Streptococcus pyogenes* MGAS10270), (NC_008023, Locus YP_600845; *Streptococcus pyogenes* MGAS2096), (NC_008024, Locus YP_602832; *Streptococcus pyogenes* MGAS10750), and (MZEFP; *Zea mays*).

[00133] Alternatively, FPP can also be made by adding IPP to GPP. Illustrative examples of nucleotide sequences encoding for an enzyme capable of this reaction include but are not limited to: (AE000657, Locus AAC06913; *Aquifex aeolicus* VF5), (NM_202836; *Arabidopsis thaliana*), (D84432, Locus BAA12575; *Bacillus subtilis*), (U12678, Locus AAC28894; *Bradyrhizobium japonicum* USDA 110), (BACFDPS; *Geobacillus stearothermophilus*), (NC_002940, Locus NP_873754; *Haemophilus ducreyi* 35000HP), (L42023, Locus AAC23087; *Haemophilus influenzae* Rd KW20), (J05262; *Homo sapiens*), (YP_395294; *Lactobacillus sakei subsp. sakei* 23K), (NC_005823, Locus YP_000273; *Leptospira interrogans serovar Copenhageni str. Fiocruz* L1-130), (AB003187; *Micrococcus luteus*), (NC_002946, Locus YP_208768; *Neisseria gonorrhoeae* FA 1090), (U00090, Locus AAB91752; *Rhizobium sp.* NGR234), (J05091; *Saccharomyces cerevisiae*), (CP000031, Locus AAV93568; *Silicibacter pomeroyi* DSS-3), (AE008481, Locus AAK99890; *Streptococcus pneumoniae* R6), and (NC_004556, Locus NP_779706; *Xylella fastidiosa* Temecula1).

[00134] FPP is then subsequently converted to a variety of C₁₅ compounds. Illustrative examples of C₁₅ compounds include but are not limited to:

Amorphadiene

[00135] Amorphadiene, whose structure is

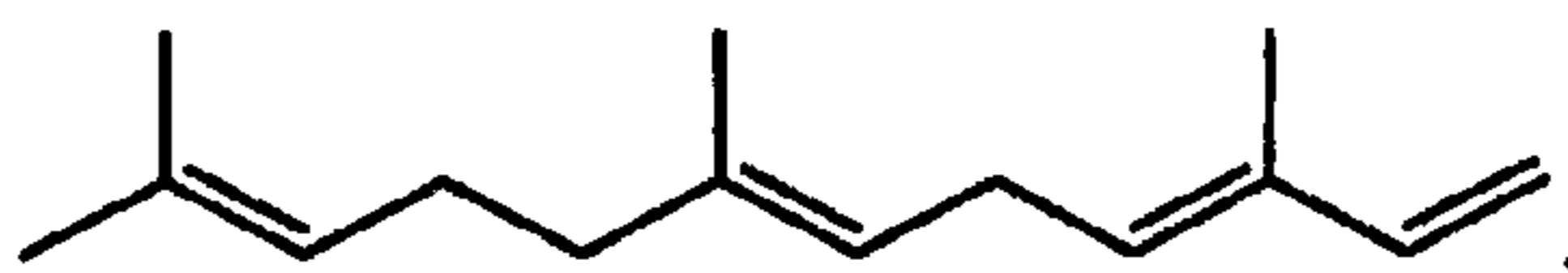


is a precursor to artemisinin which is made by *Artemisia annua*. Amorphadiene is made from FPP by amorphadiene synthase. An illustrative example of a suitable nucleotide sequence is SEQ ID NO. 37 of U.S. Patent Publication No. 2004/0005678.

[00136] Figure 5 shows schematically how IPP and DMAPP can be combined to produce FPP, which can then be further processed to produce amorphadiene.

 α -Farnesene

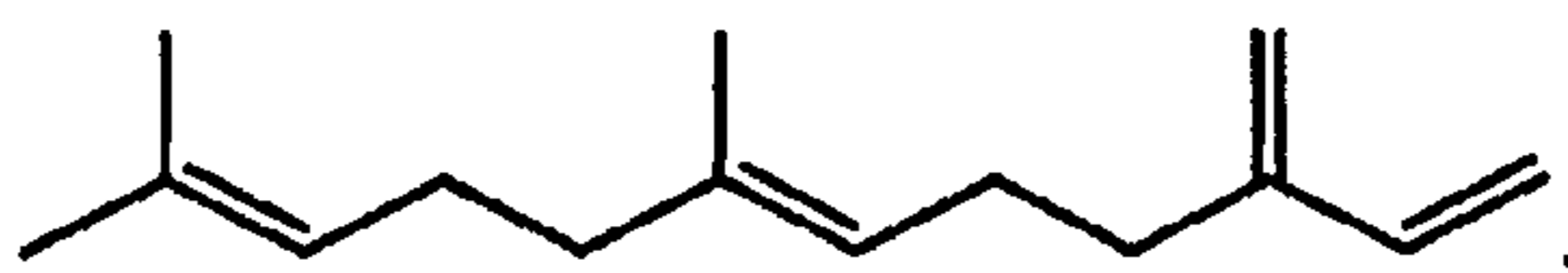
[00137] α -Farnesene, whose structure is



is found in various biological sources including but not limited to the Dufour's gland in ants and in the coating of apple and pear peels. α -Farnesene is made from FPP by α -farnesene synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to DQ309034 from *Pyrus communis cultivar d'Anjou* (pear; gene name AFS1) and AY182241 from *Malus domestica* (apple; gene AFS1). Pechouus *et al.*, *Planta* **219**(1):84-94 (2004).

 β -Farnesene

[00138] β -Farnesene, whose structure is



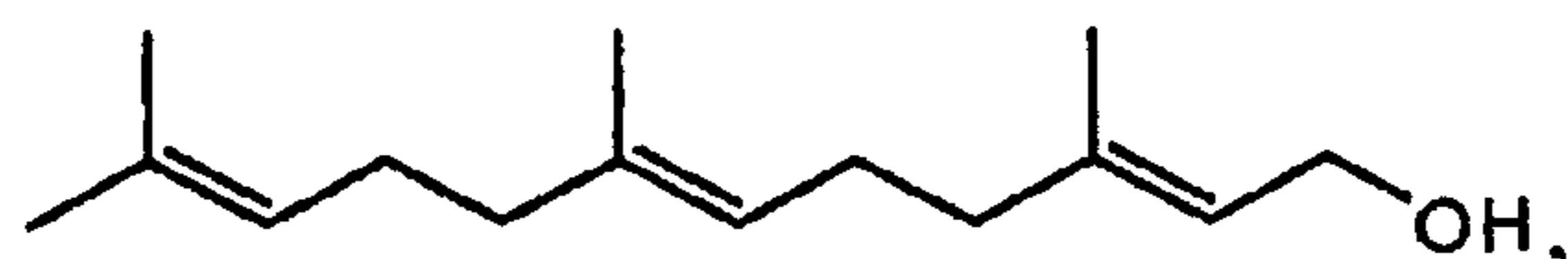
is found in various biological sources including but not limited to aphids and essential oils such as from peppermint. In some plants such as wild potato, β -farnesene is synthesized as a natural insect repellent. β -Farnesene is made from FPP by β -farnesene synthase. Illustrative examples of suitable nucleotide sequences include but is not limited to GenBank accession number AF024615 from *Mentha x piperita* (peppermint; gene Tspa11), and AY835398 from *Artemisia annua*. Picaud *et al.*, *Phytochemistry* **66**(9): 961-967 (2005).

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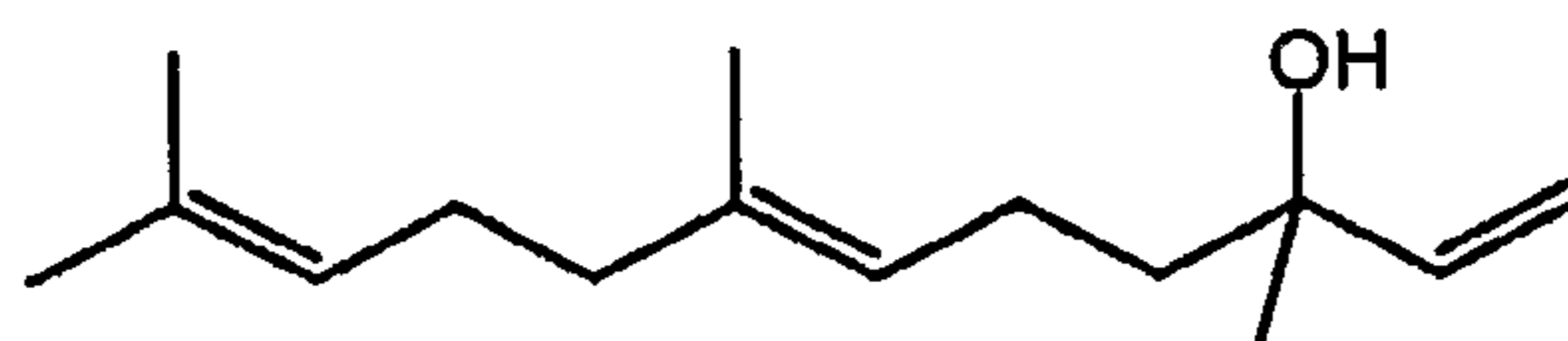
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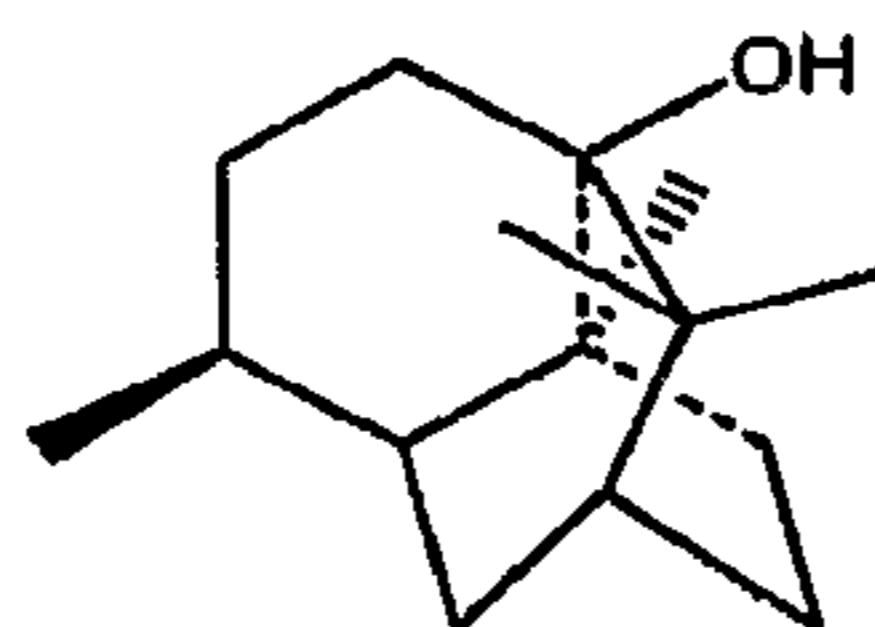
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Farnesol**[00139]** Farnesol, whose structure is

is found in various biological sources including insects and essential oils such as from citronella, neroli, cyclamen, lemon grass, tuberose, and rose. Farnesol is made from FPP by a hydroxylase such as farnesol synthase. Illustrative examples of suitable nucleotide sequences include but are not limited to GenBank accession number AF529266 from *Zea mays* and YDR481C from *Saccharomyces cerevisiae* (gene Pho8). Song, L., *Applied Biochemistry and Biotechnology* 128:149-158 (2006).

Nerolidol**[00140]** Nerolidol, whose structure is

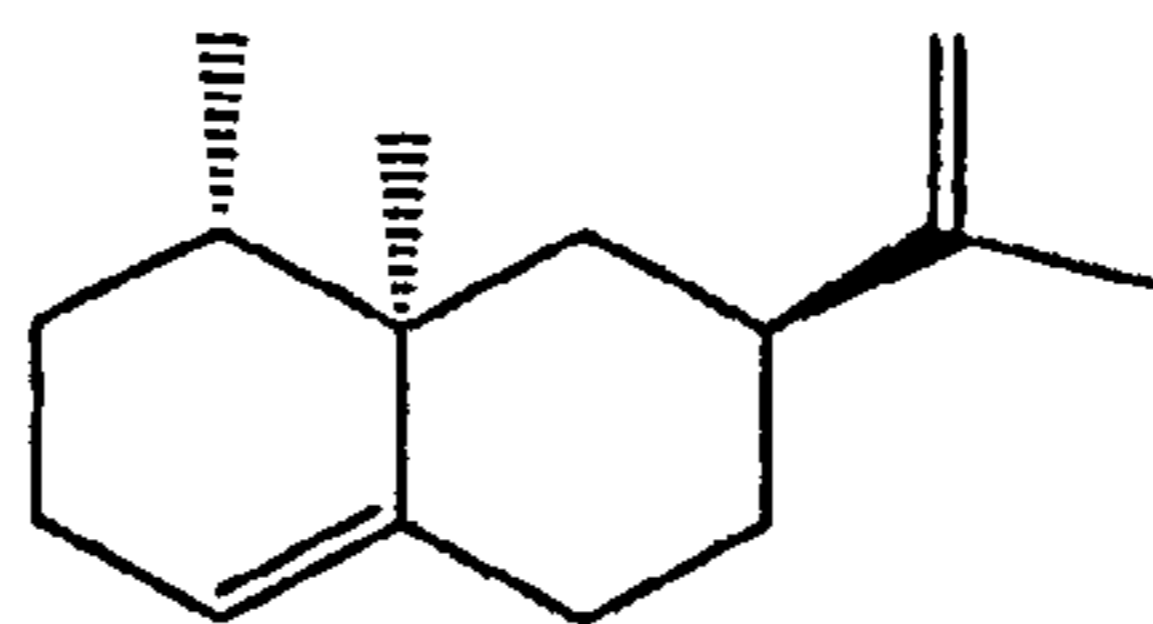
is also known as peruvial, and is found in various biological sources including as essential oils such as from neroli, ginger, jasmine, lavender, tea tree, and lemon grass. Nerolidol is made from FPP by a hydroxylase such as nerolidol synthase. An illustrative example of a suitable nucleotide sequence includes but is not limited to AF529266 from *Zea mays* (maize; gene tps1).

Patchoulol**[00141]** Patchoulol, whose structure is

is also known as patchouli alcohol and is a constituent of the essential oil of *Pogostemon patchouli*. Patchouliol is made from FPP by patchouliol synthase. An illustrative example of a suitable nucleotide sequence includes but is not limited to AY508730 REGION: 1..1659 from *Pogostemon cablin*.

Valencene

[00142] Valencene, whose structure is



is one of the main chemical components of the smell and flavour of oranges and is found in orange peels. Valencene is made from FPP by nootkatone synthase. Illustrative examples of a suitable nucleotide sequence includes but is not limited to AF441124 REGION: 1..1647 from *Citrus sinensis* and AY917195 REGION: 1..1653 from *Perilla frutescens*.

C₂₀ Compounds

[00143] Exemplary C₂₀ bio-organic compounds are diterpenes which are generally derived from geranylgeraniol pyrophosphate (GGPP) which in turn is made by the condensation of three molecules of IPP with one molecule of DMAPP. An enzyme known to catalyze this step is, for example, geranylgeranyl pyrophosphate synthase.

[00144] Figure 5 also shows schematically how IPP and DMAPP can be combined to produce GGPP, which can be further processed to a diterpene, or can be further processed to produce a carotenoid.

[00145] Illustrative examples of nucleotide sequences for geranylgeranyl pyrophosphate synthase include but are not limited to: (ATHGERPYRS; *Arabidopsis thaliana*), (BT005328; *Arabidopsis thaliana*), (NM_119845; *Arabidopsis thaliana*), (NZ_AAJM01000380, Locus ZP_00743052; *Bacillus thuringiensis serovar israelensis*, ATCC 35646 sq1563), (CRGGPPS; *Catharanthus roseus*), (NZ_AABF02000074, Locus ZP_00144509; *Fusobacterium nucleatum subsp. vincentii*, ATCC 49256), (GFGGPPSGN; *Gibberella fujikuroi*), (AY371321; *Ginkgo biloba*), (AB055496; *Hevea brasiliensis*), (AB017971; *Homo sapiens*), (MCI276129; *Mucor circinelloides f. lusitanicus*), (AB016044; *Mus musculus*), (AABX01000298, Locus NCU01427; *Neurospora crassa*), (NCU20940; *Neurospora crassa*), (NZ_AAKL01000008, Locus ZP_00943566; *Ralstonia solanacearum* UW551), (AB118238; *Rattus norvegicus*), (SCU31632; *Saccharomyces cerevisiae*), (AB016095; *Synechococcus elongates*), (SAGGPPS; *Sinapis alba*), (SSOGDS; *Sulfolobus acidocaldarius*), (NC_007759, Locus YP_461832; *Syntrophus aciditrophicus* SB), and (NC_006840, Locus YP_204095; *Vibrio fischeri* ES114).

[00146] Alternatively, GGPP can also be made by adding IPP to FPP. Illustrative examples of nucleotide sequences encoding an enzyme capable of this reaction include but

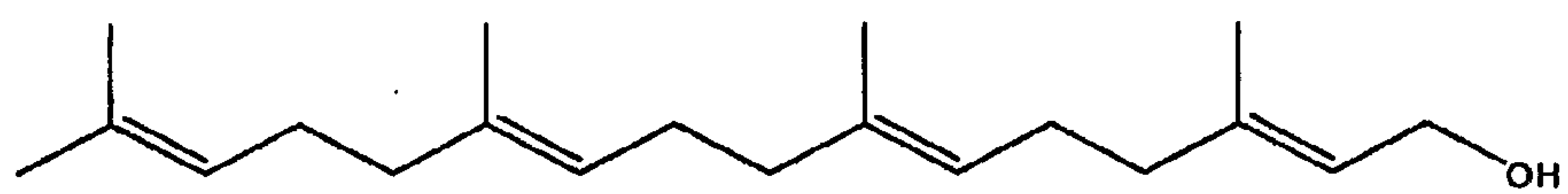
are not limited to: (NM_112315; *Arabidopsis thaliana*), (ERWCRTE; *Pantoea agglomerans*), (D90087, Locus BAA14124; *Pantoea ananatis*), (X52291, Locus CAA36538; *Rhodobacter capsulatus*), (AF195122, Locus AAF24294; *Rhodobacter sphaeroides*), and (NC_004350, Locus NP_721015; *Streptococcus mutans* UA159).

[00147] GGPP is then subsequently converted to a variety of C₂₀ isoprenoids.

Illustrative examples of C₂₀ compounds include but are not limited to:

Geranylgeraniol

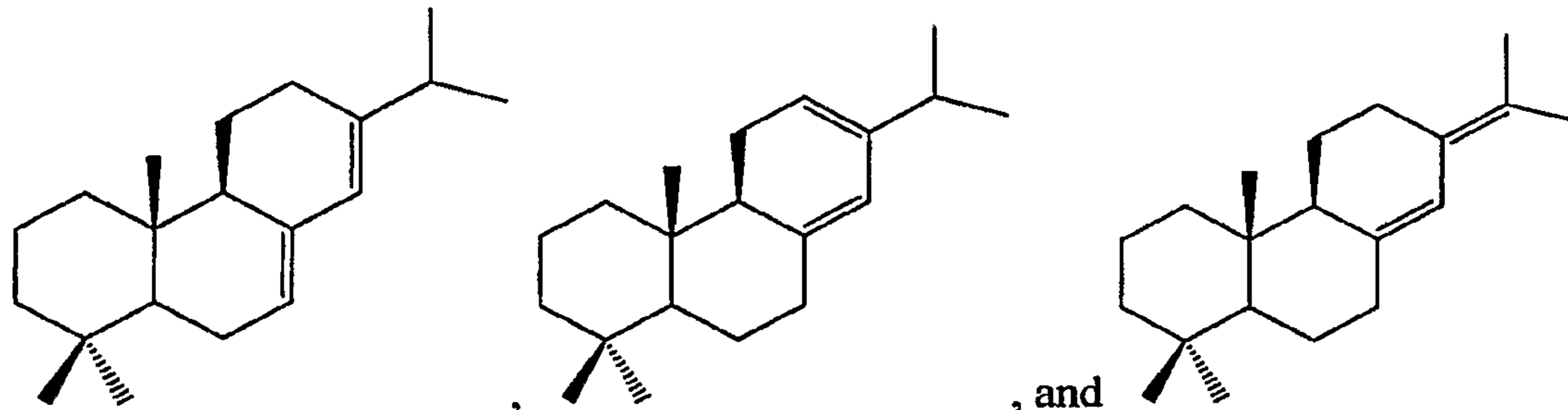
[00148] Geranylgeraniol, whose structure is



is a constituent of wood oil from *Cedrela toona* and of linseed oil. Geranylgeraniol can be made by e.g., adding to the expression constructs a phosphatase gene after the gene for a GGPP synthase.

Abietadiene

[00149] Abietadiene encompasses the following isomers:



and is found in trees such as *Abies grandis*. Abietadiene is made by abietadiene synthase.

An illustrative example of a suitable nucleotide sequence includes but are not limited to:

(U50768; *Abies grandis*) and (AY473621; *Picea abies*).

C₂₀₊ Compounds

[00150] C₂₀₊ bio-organic compounds are also within the scope of the present invention.

Illustrative examples of such compounds include sesterterpenes (C₂₅ compound made from five isoprene units), triterpenes (C₃₀ compounds made from six isoprene units), and tetraterpenes (C₄₀ compound made from eight isoprene units). These compounds are made by using similar methods described herein and substituting or adding nucleotide sequences for the appropriate synthase(s).

Engineering Pathways

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[00151] Although for illustrative purposes, the invention has been described with reference to engineering the MEV and/or DXP pathways, these methods can be adapted to similarly engineer suitable pathways to make non-isoprenoid bio-organic compounds. These pathways are typically engineered using recombinant DNA technology by expression of suitable heterologous sequences encoding one or more enzymes.

[00152] The subject nucleotide acids can be expressed by a single or multiple vectors. The nucleic acids can be arranged in a single operon, or in separate operons that are placed in one or multiple vectors. Where desired, two expression vectors can be employed, each of which contains one or more heterologous sequences operably linked in a single operon. While the choice of single or multiple vectors and the use of single or multiple operons may depend on the size of the heterologous sequences and the capacity of the vectors, it will largely depend on the overall yield of a given bio-organic compound that the vector is able to provide when expressed in a selected host cell. In some instances, two-operon expression system provides a higher yield of the bio-organic compound. The subject vectors can stay replicable episomally, or as an integral part of the host cell genome. Typically, the latter is preferred for a sustained propagation of the host cell.

[00153] In certain host cells, the subject nucleic acids may be controlled by one or more operons. In some instances, a two or three operon system provides a higher yield of a bio-organic compound over a single operon system.

[00154] Where desired, the subject nucleic acid sequences can be modified to reflect the codon preference of a selected host cell to effect a higher expression of such sequences in a host cell. For example, the subject nucleotide sequences will in some embodiments be modified for yeast codon preference. See, e.g., Bennetzen and Hall (1982) *J. Biol. Chem.* 257(6): 3026-3031. As another non-limiting example, the nucleotide sequences will in other embodiments 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. Codon usage tables for many organisms are available, which can be used as a reference in designing sequences of the present invention. The use of prevalent codons of a given host microorganism generally increases the likelihood of translation, and hence the expression level of the desired sequences.

[00155] Preparation of the subject nucleic acids can be carried out by a variety of routine recombinant techniques and synthetic procedures. Briefly, the subject nucleic acids can be prepared genomic DNA fragments, cDNAs, and RNAs, all of which can be extracted

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directly from a cell or recombinantly produced by various amplification processes including but not limited to PCR and rt-PCR.

[00156] Direct chemical synthesis of nucleic acids typically involves sequential addition of 3'-blocked and 5'-blocked nucleotide monomers to the terminal 5'-hydroxyl group of a growing nucleotide polymer chain, wherein each addition is effected by nucleophilic attack of the terminal 5'-hydroxyl group of the growing chain on the 3'-position of the added monomer, which is typically a phosphorus derivative, such as a phosphotriester, phosphoramidite, or the like. Such methodology is known to those of ordinary skill in the art and is described in the pertinent texts and literature (for example, Matteucci et al. (1980) Tet. Lett. 521:719; U.S. Pat. No. 4,500,707 to Caruthers et al.; and U.S. Pat. Nos. 5,436,327 and 5,700,637 to Southern et al.).

[00157] The level of transcription of a nucleic acid in a host microorganism can be increased in a number of ways. For example, this can be achieved by increasing the copy number of the nucleotide sequence encoding the enzyme (e.g., by using a higher copy number expression vector comprising a nucleotide sequence encoding the enzyme, or by introducing additional copies of a nucleotide sequence encoding the enzyme into the genome of the host microorganism, for example, by recA-mediated recombination, use of "suicide" vectors, recombination using lambda phage recombinase, and/or insertion via a transposon or transposable element). In addition, it can be carried out by changing the order of the coding regions on the polycistronic mRNA of an operon or breaking up an operon into individual genes, each with its own control elements, or increasing the strength of the promoter (transcription initiation or transcription control sequence) to which the enzyme coding region is operably linked (for example, using a consensus arabinose- or lactose-inducible promoter in an *Escherichia coli* host microorganism in place of a modified lactose-inducible promoter, such as the one found in pBluescript and the pBBR1MCS plasmids), or using an inducible promoter and inducing the inducible-promoter by adding a chemical to a growth medium. The level of translation of a nucleotide sequence in a host microorganism can be increased in a number of ways, including, but not limited to, increasing the stability of the mRNA, modifying the sequence of the ribosome binding site, modifying the distance or sequence between the ribosome binding site and the start codon of the enzyme coding sequence, modifying the entire intercistronic region located "upstream of" or adjacent to the 5' side of the start codon of the enzyme coding region, stabilizing the 3'-end of the mRNA transcript using hairpins and specialized sequences, modifying the codon usage of enzyme, altering expression of rare codon tRNAs used in the biosynthesis of the enzyme, and/or increasing the

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stability of the enzyme, as, for example, via mutation of its coding sequence. Determination of preferred codons and rare codon tRNAs can be based on a sequence analysis of genes derived from the host microorganism.

[00158] The subject vector can be constructed to yield a desired level of copy numbers of the encoded enzyme. In some embodiments, the subject vectors yield at least 10, between 10 to 20, between 20-50, between 50-100, or even higher than 100 copies of the desired enzyme. Low copy number plasmids generally provide fewer than about 20 plasmid copies per cell; medium copy number plasmids generally provide from about 20 plasmid copies per cell to about 50 plasmid copies per cell, or from about 20 plasmid copies per cell to about 80 plasmid copies per cell; and high copy number plasmids generally provide from about 80 plasmid copies per cell to about 200 plasmid copies per cell, or more.

[00159] Suitable low copy expression vectors for *Escherichia coli* include, but are not limited to, pACYC184, pBeloBac11, pBR332, pBAD33, pBBR1MCS and its derivatives, pSC101, SuperCos (cosmid), and pWE15 (cosmid). Suitable medium copy expression vectors for *Escherichia coli* include, but are not limited to pTrc99A, pBAD24, and vectors containing a ColE1 origin of replication and its derivatives. Suitable high copy number expression vectors for *Escherichia coli* include, but are not limited to, pUC, pBluescript, pGEM, and pTZ vectors. Suitable low-copy (centromeric) expression vectors for yeast include, but are not limited to, pRS415 and pRS416 (Sikorski & Hieter (1989) Genetics 122:19-27). Suitable high-copy 2 micron expression vectors in yeast include, but are not limited to, pRS425 and pRS426 (Christainson et al. (1992) Gene 110:119-122). Alternative 2 micron expression vectors include non-selectable variants of the 2 micron vector (Bruschi & Ludwig (1988) Curr. Genet. 15:83-90) or intact 2 micron plasmids bearing an expression cassette (as exemplified in U.S. Pat. Appl. 20050084972) or 2 micron plasmids bearing a defective selection marker such as LEU2d (Erhanrt et al. (1983) J. Bacteriol. 156 (2): 625-635) or URA3d (Okkels (1996) Annals of the New York Academy of Sciences 782(1): 202-207).

[00160] Regulatory elements include, for example, promoters and operators can also be engineered to increase the metabolic flux of the engineered pathways by increasing the expression of one or more genes that play a significant role in determining the overall yield of the bio-organic compound produced. A promoter is a sequence of nucleotides that initiates and controls the transcription of a nucleic acid sequence by an RNA polymerase enzyme. An operator is a sequence of nucleotides adjacent to the promoter that functions to control transcription of the desired nucleic acid sequence. The operator contains a protein-binding

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domain where a specific repressor protein can bind. In the absence of a suitable repressor protein, transcription initiates through the promoter. In the presence of a suitable repressor protein, the repressor protein binds to the operator and thereby inhibits transcription from the promoter.

[00161] In some embodiments of the present invention, promoters used in expression vectors are inducible. In other embodiments, the promoters used in expression vectors are constitutive. In some embodiments, one or more nucleic acid sequences are operably linked to an inducible promoter, and one or more other nucleic acid sequences are operably linked to a constitutive promoter.

[00162] Non-limiting examples of suitable promoters for use in prokaryotic host cells include a bacteriophage T7 RNA polymerase promoter; a *trp* promoter; a *lac* operon promoter; a hybrid promoter, for example, 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; *in vivo* regulated promoters, such as an *ssaG* promoter or a related promoter (*see*, for example, U.S. Patent Publication No. 20040131637), a *pagC* promoter (Pulkkinen and Miller, *J. Bacteriol.* (1991) 173(1):86-93; Alpuche-Aranda et al. (1992) *Proc. Natl. Acad. Sci. U S A.* 89(21):10079-83), a *nirB* promoter (Harborne et al. (1992) *Mol. Micro.* 6:2805-2813), and the like (*see*, for example, 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, for example, a consensus sigma70 promoter (*see*, for example, GenBank Accession Nos. AX798980, AX798961, and AX798183); a stationary phase promoter, for example, a *dps* promoter, an *spv* promoter, and the like; a promoter derived from the pathogenicity island SPI-2 (*see*, for example, WO96/17951); an *actA* promoter (*see*, for example, Shetron-Rama et al. (2002) *Infect. Immun.* 70:1087-1096); an *rpsM* promoter (*see*, for example, Valdivia and Falkow (1996) *Mol. Microbiol.* 22:367-378); a *tet* promoter (*see*, for example, Hillen et al. (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*, for example, Melton et al. (1984) *Nucl. Acids Res.* 12:7035-7056); and the like.

[00163] In some embodiments, the total activity of a heterologous enzyme that plays a larger role in the overall yield of a bio-organic compound relative to other enzymes in the respective pathways is increased by expressing the enzyme from a strong promoter. Suitable strong promoters for *Escherichia coli* include, but are not limited to *Trc*, *Tac*, *T5*, *T7*, and *P_{Lambda}*. In another embodiment of the present invention, the total activity of one or more

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engineered pathway enzymes in a host is increased by expressing the enzyme from a strong promoter on a high copy number plasmid. Suitable examples, for *Escherichia coli* include, but are not limited to using Trc, Tac, T5, T7, and P_{Lambda} promoters with pBAD24, pBAD18, pGEM, pBluescript, pUC, and pTZ vectors.

[00164] Non-limiting examples of suitable promoters for use in eukaryotic host cells include, but are not limited to, a CMV immediate early promoter, an HSV thymidine kinase promoter, an early or late SV40 promoter, LTRs from retroviruses, and a mouse metallothionein-I promoter.

[00165] Non-limiting examples of suitable constitutive promoters for use in prokaryotic host cells include a sigma70 promoter (for example, a consensus sigma70 promoter). Non-limiting examples of suitable inducible promoters for use in bacterial host cells include the pL of bacteriophage λ ; Plac; P_{trp}; P_{tac} (P_{trp}-lac hybrid promoter); an isopropyl-beta-D44 thiogalactopyranoside (IPTG)-inducible promoter, for example, a *lacZ* promoter; a tetracycline inducible promoter; an arabinose inducible promoter, for example, PBAD (see, for example, Guzman et al. (1995) *J. Bacteriol.* 177:4121-4130); a xylose-inducible promoter, for example, P_{xyl} (see, for example, Kim et al. (1996) *Gene* 181:71-76); a GAL1 promoter; a tryptophan promoter; a lac promoter; an alcohol-inducible promoter, for example, a methanol-inducible promoter, an ethanol-inducible promoter; a raffinose-inducible promoter; a heat-inducible promoter, for example, heat inducible lambda PL promoter; a promoter controlled by a heat-sensitive repressor (for example, CI857-repressed lambda-based expression vectors; see, for example, Hoffmann et al. (1999) *FEMS Microbiol Lett.* 177(2):327-34); and the like.

[00166] Non-limiting examples of suitable constitutive promoters for use in yeast host cells include an ADH1, an ADH2, a PGK, or a LEU2 promoter. Non-limiting examples of suitable inducible promoters for use in yeast host cells include, but are not limited to, a divergent galactose-inducible promoter such as a GAL 1 or a GAL 10 promoter (West et al. (1984) *Mol. Cell. Biol.* 4(11):2467-2478), or a CUP1 promoter. Where desired, the subject vector comprises a promoter that is stronger than a native *E. Coli* Lac promoter.

[00167] Non-limiting examples of operators for use in bacterial host cells include a lactose promoter operator (LacI repressor protein changes conformation when contacted with lactose, thereby preventing the LacI repressor protein from binding to the operator), a tryptophan promoter operator (when complexed with tryptophan, TrpR repressor protein has a conformation that binds the operator; in the absence of tryptophan, the TrpR repressor

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protein has a conformation that does not bind to the operator), and a tac promoter operator (see, for example, deBoer et al. (1983) Proc. Natl. Acad. Sci. U.S.A. 80:21-25.).

[00168] The genes in the expression vector typically will also encode a ribosome binding site to direct translation (that is, synthesis) of any encoded mRNA gene product. For suitable ribosome binding sites for use in *Escherichia coli*, see Shine et al. (1975) Nature 254:34, and Steitz, in Biological Regulation and Development: Gene Expression (ed. R. F. Goldberger), vol. 1, p. 349, 1979, Plenum Publishing, N.Y. Insertion of the ribosome binding site encoding nucleotide sequence 5'-AAAACA-3' upstream of a coding sequence facilitates efficient translation in a yeast host microorganism (Looman et al. (1993) Nuc. Ac. Res. 21:4268-4271; Yun et. al. (1996) Mol. Microbiol. 19:1225-1239).

[00169] Other regulatory elements that may be used in an expression vector include transcription enhancer elements and transcription terminators. See, for example, Bitter et al. (1987) Methods in Enzymology, 153:516-544.

[00170] An expression vector may be suitable for use in particular types of host microorganisms and not others. One of ordinary skill in the art, however, can readily determine through routine experimentation whether a particular expression vector is suited for a given host microorganism. For example, the expression vector can be introduced into the host organism, which is then monitored for viability and expression of any genes contained in the vector.

[00171] The expression vector may also contain one or more selectable marker genes that, upon expression, confer one or more phenotypic traits useful for selecting or otherwise identifying host cells that carry the expression vector. Non-limiting examples of suitable selectable markers for eukaryotic cells include dihydrofolate reductase and neomycin resistance. Non-limiting examples of suitable selectable markers for prokaryotic cells include tetracycline, ampicillin, chloramphenicol, carbenicillin, and kanamycin resistance.

[00172] For production of a bio-organic product at an industrial scale, it may be impractical or too costly to use a selectable marker that requires the addition of an antibiotic to the fermentation media. Accordingly, some embodiments of the present invention employ host cells that do not require the use of an antibiotic resistance conferring selectable marker to ensure plasmid (expression vector) maintenance. In these embodiments of the present invention, the expression vector contains a plasmid maintenance system such as the 60-kb IncP (RK2) plasmid, optionally together with the RK2 plasmid replication and/or segregation system, to effect plasmid retention in the absence of antibiotic selection (see, for example, Sia et al. (1995) J. Bacteriol. 177:2789-97; Pansegrau et al. (1994) J. Mol. Biol. 239:623-63). A

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suitable plasmid maintenance system for this purpose is encoded by the *parDE* operon of RK2, which codes for a stable toxin and an unstable antitoxin. The antitoxin can inhibit the lethal action of the toxin by direct protein-protein interaction. Cells that lose the expression vector that harbors the *parDE* operon are quickly deprived of the unstable antitoxin, resulting in the stable toxin then causing cell death. The RK2 plasmid replication system is encoded by the *trfA* gene, which codes for a DNA replication protein. The RK2 plasmid segregation system is encoded by the *parCBA* operon, which codes for proteins that function to resolve plasmid multimers that may arise from DNA replication.

[00173] The subject vectors can be introduced into a host cell stably or transiently by variety of established techniques. For example, one method involves a calcium chloride treatment wherein the expression vector is introduced via a calcium precipitate. Other salts, for example calcium phosphate, may also be used following a similar procedure. In addition, electroporation (that is, the application of current to increase the permeability of cells to nucleic acids) may be used. Other transformation methods include microinjection, DEAE dextran mediated transformation, and heat shock in the presence of lithium acetate. Lipid complexes, liposomes, and dendrimers may also be employed to transfect the host microorganism.

[00174] Upon transformation, a variety of methods can be practiced to identify the host cells into which the subject vectors have been introduced. One exemplary selection method involves subculturing individual cells to form individual colonies, followed by testing for expression of the desired gene product. Another method entails selecting transformed host cells based upon phenotypic traits conferred through the expression of selectable marker genes contained within the expression vector. Those of ordinary skill can identify genetically modified host cells using these or other methods available in the art.

[00175] The introduction of various pathway sequences of the invention into a host cell can be confirmed by methods such as PCR, Southern blot or Northern blot hybridization. For example, nucleic acids can be prepared from the resultant host cells, and the specific sequences of interest can be amplified by PCR using primers specific for the sequences of interest. The amplified product is subjected to agarose gel electrophoresis, polyacrylamide gel electrophoresis or capillary electrophoresis, followed by staining with ethidium bromide, SYBR Green solution or the like, or detection of DNA with a UV detection. Alternatively, nucleic acid probes specific for the sequences of interest can be employed in a hybridization reaction. The expression of a specific gene sequence can be ascertained by detecting the corresponding mRNA via reverses-transcription coupled PCR, Northern blot hybridization, or

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by immunoassays using antibodies reactive with the encoded gene product. Exemplary immunoassays include but are not limited to ELISA, radioimmunoassays, and sandwich immunoassays.

[00176] The yield of a bio-organic compound via one or more metabolic pathways disclosed herein can be augmented by inhibiting reactions that divert intermediates from productive steps towards formation of the bio-organic product. Inhibition of the unproductive reactions can be achieved by reducing the expression and/or activity of enzymes involved in one or more unproductive reactions. Such reactions include side reactions of the TCA cycle that lead to fatty acid biosynthesis, alanine biosynthesis, the aspartate superpathway, gluconeogenesis, heme biosynthesis, and/or glutamate biosynthesis, at a level that affects the overall yield of the bio-organic compound.

[00177] A variety of methods are available for knocking out or knocking down a gene of interest. For example, a reduced gene expression may be accomplished by deletion, mutation, and/or gene rearrangement. It can also be carried out with the use of antisense RNA, siRNA, miRNA, ribozymes, triple stranded DNA, and transcription and/or translation inhibitors. In addition, transposons can be employed to disrupt gene expression, for example, by inserting it between the promoter and the coding region, or between two adjacent genes to inactivate one or both genes.

[00178] The amount of microorganism per liter of fermentation, or the density of microorganism, can be measured by measuring the weight of microorganism isolated from a given volume of the fermentation medium. A common measure is the dry weight of cells per liter of fermentation medium. Another method which can be used to monitor the fermentation while it is progressing is by a measurement of the optical density of the medium. A common method is to measure the optical density at a wavelength of 600 nm, referred to the OD₆₀₀, or the OD. The OD can be correlated to a the density of a specific type of organism within a specific medium, but the specific relationship between OD and amount of microorganism per volume will not generally be applicable across all types of organisms in all types of media. A calibration curve can be created by measuring the OD and the dry cell weight over a range of cell densities. In some cases, these correlations can be used in different fermentation of the same or similar microorganisms in the same or similar media.

EXAMPLES

[00179] The practice of the present invention can employ, unless otherwise indicated, conventional techniques of the biosynthetic industry and the like, which are within the skill of

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the art. To the extent such techniques are not described fully herein, one can find ample reference to them in the scientific literature.

[00180] In the following examples, efforts have been made to ensure accuracy with respect to numbers used (for example, amounts, temperature, and so on), but variation and deviation can be accommodated, and in the event a clerical error in the numbers reported herein exists, one of ordinary skill in the arts to which this invention pertains can deduce the correct amount in view of the remaining disclosure herein. Unless indicated otherwise, temperature is reported in degrees Celsius, and pressure is at or near atmospheric pressure at sea level. All reagents, unless otherwise indicated, were obtained commercially. The following examples are intended for illustrative purposes only and do not limit in any way the scope of the present invention.

[00181] Example 1

[00182] This example describes methods for making expression plasmids that encode enzymes of the MEV pathway from *Saccharomyces cerevisiae* organized in operons.

[00183] Expression plasmid pMevT was generated by inserting the MevT operon (SEQ ID NO: 1) into the pBAD33 vector. The MevT operon encodes the set of MEV pathway enzymes that together transform the ubiquitous precursor acetyl-CoA to (R)-mevalonate, namely acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase. The MevT operon was generated by PCR amplifying from *Escherichia coli* genomic DNA the coding sequence of the *atoB* gene (GenBank accession number NC_000913 REGION: 2324131..2325315) (encodes an acetoacetyl-CoA thiolase), from *Saccharomyces cerevisiae* genomic DNA the coding sequence of the *ERG13* gene (GenBank accession number X96617, REGION: 220..1695) (encodes a HMG-CoA synthase), and from *Saccharomyces cerevisiae* genomic DNA a segment of the coding region of the *HMG1* gene (GenBank accession number M22002, REGION: 1660..3165) (encodes a truncated HMG-CoA reductase (tHMGR)). The upstream PCR primer used for the amplification of the *HMG1* gene fragment included an artificial start codon. The amplified fragments were spliced together using overlap extensions (SOEing), during which process ribosome binding sites were introduced after the *atoB* and the *ERG13* coding sequences. After the addition of 3' A overhangs, the MevT operon was ligated into the TA cloning vector pCR4 (Invitrogen, Carlsbad, CA), and sequenced to ensure accuracy. The MevT operon was subsequently ligated into the *XmaI PstI* restriction enzyme site of vector pBAD33 (Guzman et al. (1995) *J. Bacteriol.* 177(14): 4121-4130). To place the operon under the control of the P_{Lac} promoter,

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the *araC-P_{BAD}NsiI-XmaI* fragment of pBAD33 was replaced with the *NsiI-XmaI* fragment of pBBR1MCS, yielding expression plasmid pMevT (see U.S. Patent Number 7,192,751).

[00184] Expression plasmid pAM36-MevT66 was generated by inserting the MevT66 operon into the pAM36 vector. Vector pAM36 was generated by inserting an oligonucleotide cassette containing *AscI-SfiI-AsiSI-XhoI-PacI-FsII-PmeI* restriction enzyme sites into the pACYC184 vector (GenBank accession number XO6403), and by removing the *tet* resistance gene in pACYC184. The MevT66 operon was synthetically generated using the nucleotide sequence SEQ ID NO: 1 as a template, which comprises the *atoB* gene from *Escherichia coli* (GenBank accession number NC_000913 REGION: 2324131..2325315), the *ERG13* gene from *Saccharomyces cerevisiae* (GenBank accession number X96617, REGION: 220..1695), and a truncated version of the *HMG1* gene from *Saccharomyces cerevisiae* (GenBank accession number M22002, REGION: 1777..3285), all three sequences being codon-optimized for expression in *Escherichia coli*. The synthetically generated MevT66 operon was flanked by a 5' *EcoRI* restriction enzyme site and a 3' *Hind III* restriction enzyme site, and could thus be cloned into compatible restriction enzyme sites of a cloning vector such as a standard pUC or pACYC origin vector. From this construct, the MevT66 operon was PCR amplified with flanking *SfiI* and *AsiSI* restriction enzyme sites, the amplified DNA fragment was digested to completion using *SfiI* and *AsiSI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 4.2 kb DNA fragment was gel extracted using a Qiagen gel purification kit (Valencia, CA), and the isolated DNA fragment was ligated into the *SfiI AsiSI* restriction enzyme site of the pAM36 vector, yielding expression plasmid pAM36-MevT66.

[00185] Expression plasmid pAM25 was generated by inserting the MevT66 operon into the pAM29 vector. Vector pAM29 was created by assembling the p15A origin of replication and *kan* resistance gene from pZS24-MCS1 (Lutz and Bujard (1997) *Nucl Acids Res.* 25:1203-1210) with an oligonucleotide-generated *lacUV5* promoter. The DNA synthesis construct comprising the MevT66 operon (see above) was digested to completion using *EcoRI* and *Hind III* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the 4.2 kb DNA fragment was gel extracted, and the isolated DNA fragment was ligated into the *EcoRI HindIII* restriction enzyme site of pAM29, yielding expression plasmid pAM25.

[00186] Expression plasmid pMevB-Cm was generated by inserting the MevB operon into the pBBR1MCS-1 vector. The MevB operon encodes the set of enzymes that together convert (R)-mevalonate to IPP, namely mevalonate kinase, phosphomevalonate kinase, and

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mevalonate pyrophosphate carboxylase. The MevB operon was generated by PCR amplifying from *Saccharomyces cerevisiae* genomic DNA the coding sequences of the *ERG12* gene (GenBank accession number X55875, REGION: 580..1911) (encodes a mevalonate kinase), the *ERG8* gene (GenBank accession number Z49939, REGION: 3363..4718) (encodes a phosphomevalonate kinase), and the *MVD1* gene (GenBank accession number X97557, REGION: 544..1734) (encodes a mevalonate pyrophosphate carboxylase), and by splicing the PCR fragments together using overlap extensions (SOEing). By choosing appropriate primer sequences, the stop codons of *ERG12* and *ERG8* were changed from TAA to TAG during amplification to introduce ribosome binding sites. After the addition of 3' A overhangs, the MevB operon was ligated into the TA cloning vector pCR4 (Invitrogen, Carlsbad, CA). The MevB operon was excised by digesting the cloning construct to completion using *PstI* restriction enzyme, resolving the reaction mixture by gel electrophoresis, gel extracting the 4.2 kb DNA fragment, and ligating the isolated DNA fragment into the *PstI* restriction enzyme site of vector pBBR1MCS-1 (Kovach *et al.*, *Gene* 166(1): 175-176 (1995)), yielding expression plasmid pMevB-Cm.

[00187] Expression plasmid pMBI was generated by inserting the MBI operon into the pBBR1MCS-3 vector. The MBI operon encodes the same enzymes as the MevB operon, as well as an isopentenyl pyrophosphatase isomerase that catalyzes the conversion of IPP to DMAPP. The MBI operon was generated by PCR amplifying from *Escherichia coli* genomic DNA the coding sequence of the *idi* gene (GenBank accession number AF119715) using primers that contained an *XmaI* restriction enzyme site at their 5' ends, digesting the amplified DNA fragment to completion using *XmaI* restriction enzyme, resolving the reaction mixture by gel electrophoresis, gel extracting the 0.5 kb fragment, and ligating the isolated DNA fragment into the *XmaI* restriction enzyme site of expression plasmid pMevB-Cm, thereby placing *idi* at the 3' end of the MevB operon. The MBI operon was subcloned into the *Sall* and *SacI* restriction enzyme sites of vector pBBR1MCS-3 (Kovach *et al.*, *Gene* 166(1): 175-176 (1995)), yielding expression plasmid pMBI (see U.S. Patent Number 7,192,751).

[00188] Expression plasmid pMBIS was generated by inserting the *ispA* gene into pMBI. The *ispA* gene encodes a farnesyl pyrophosphate synthase that catalyzes the conversion of IPP and DMAPP to FPP. The coding sequence of the *ispA* gene (GenBank accession number D00694, REGION: 484..1383) was PCR amplified from *Escherichia coli* genomic DNA using a forward primer with a *SacII* restriction enzyme site and a reverse primer with a *SacI* restriction enzyme site. The amplified PCR product was digested to completion with *SacII* and *SacI* restriction enzymes, the reaction mixture was resolved by gel

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electrophoresis, and the 0.9 kb DNA fragment was gel extracted. The isolated DNA fragment was ligated into the *SacII SacI* restriction enzyme site of pMBI, thereby placing the *ispA* gene 3' of *idi* and the *MevB* operon, and yielding expression plasmid pMBIS (see U.S. Patent Number 7,192,751).

[00189] Expression plasmid pMBIS-gpps was derived from expression plasmid pMBIS by replacing the *ispA* coding sequence with a nucleotide sequence encoding a geranyl diphosphate synthase ("gpps"). A DNA fragment comprising a nucleotide sequence encoding the geranyl diphosphate synthase was generated synthetically using the coding sequence of the *gpps* gene of *Arabidopsis thaliana* (GenBank accession number Y17376, REGION: 52..1320), codon-optimized for expression in *Escherichia coli*, as a template. The nucleotide sequence was flanked by a leader *SacII* restriction enzyme site and a terminal *SacI* restriction enzyme site, and can be cloned into compatible restriction enzyme sites of a cloning vector such as a standard pUC or pACYC origin vector. The synthetically generated geranyl diphosphate synthase sequence was isolated by digesting the DNA synthesis construct to completion using *SacII* and *SacI* restriction enzymes, resolving the reaction mixture by gel electrophoresis, gel extracting the approximately 1.3 kb DNA fragment, and ligating the isolated DNA fragment into the *SacII SacI* restriction enzyme site of expression plasmid pMBIS, yielding expression plasmid pMBIS-gpps (see Figure 6 for a plasmid map).

[00190] Expression plasmid pAM45 was generated by inserting the MBIS operon into pAM36-MevT66 and adding *lacUV5* promoters in front of the two operons. The MBIS operon was PCR amplified from pMBIS using primers comprising a 5' *XhoI* restriction enzyme site and a 3' *PacI* restriction enzyme site. The amplified PCR product was digested to completion using *XhoI* and *PacI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the 5.4 kb DNA fragment was gel extracted, and the isolated DNA fragment was ligated into the *XhoI PacI* restriction enzyme site of pAM36-MevT66, yielding plasmid pAM43. A DNA fragment comprising a nucleotide sequence encoding the *lacUV5* promoter was synthesized from oligonucleotides and sub-cloned into the *AscI SfiI* and *AsiSI XhoI* restriction enzyme sites of pAM43, yielding expression plasmid pAM45.

[00191] Example 2

[00192] This example describes methods for making expression vectors encoding enzymes of the MEV pathway from *Staphylococcus aureus* organized in operons.

[00193] Expression plasmid pAM41 was derived from expression plasmid pAM25 by replacing the coding sequence of the *HMG1* gene, which encodes the *Saccharomyces cerevisiae* HMG-CoA reductase, with the coding sequence of the *mvaA* gene, which encodes

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the *Staphylococcus aureus* HMG-CoA reductase (GenBank accession number BA000017, REGION: 2688925..2687648). The coding sequence of the *mvaA* gene was PCR amplified from *Staphylococcus aureus* subsp. aureus (ATCC 70069) genomic DNA using primers 4-49 *mvaA* *SpeI* (SEQ ID NO: 2) and 4-49 *mvaAR* *XbaI* (SEQ ID NO: 3), the amplified DNA fragment was digested to completion using *SpeI* restriction enzyme, the reaction mixture was resolved by gel electrophoresis, and the approximately 1.3 kb DNA fragment was gel extracted. The *HMG1* coding sequence was removed from pAM25 by digesting the plasmid to completion using *HindIII* restriction enzyme. The terminal overhangs of the resulting linear DNA fragment were blunted using T4 DNA polymerase. The DNA fragment was then partially digested using *SpeI* restriction enzyme, the reaction mixture was resolved by gel electrophoresis, and the 4.8 kb DNA fragment was gel extracted. The isolated DNA fragment was ligated with the *SpeI*-digested *mvaA* PCR product, yielding expression plasmid pAM41. The nucleotide sequence of the *atoB(opt):ERG13(opt):mvaA* operon contained in pAM41 is SEQ ID NO: 41. ERG13 is also known as HMGS or HMG-CoA synthase.

[00194] Expression plasmid pAM52 was derived from expression plasmid pAM41 by replacing the coding sequence of the *ERG13* gene, which encodes the *Saccharomyces cerevisiae* HMG-CoA synthase, with the coding sequence of the *mvaS* gene, which encodes the *Staphylococcus aureus* HMG-CoA synthase (GenBank accession number BA000017, REGION: 2689180..2690346). The coding sequence of the *mvaS* gene was PCR amplified from *Staphylococcus aureus* subsp. aureus (ATCC 70069) genomic DNA using primers HMGS 5' Sa *mvaS*-S (SEQ ID NO: 4) and HMGS 3' Sa *mvaS*-AS (SEQ ID NO: 5), and the amplified DNA fragment was used as a PCR primer to replace the coding sequence of the *HMG1* gene in pAM41 according to the method of Geiser *et al.* (*BioTechniques* 31:88-92 (2001)), yielding expression plasmid pAM52. The nucleotide sequence of the *atoB(opt):mvaS:mvaA* operon contained in pAM52 is SEQ ID NO: 42.

[00195] Expression plasmid pAM97 was derived from expression plasmid pAM45 by replacing the *MevT66* operon with the (*atoB(opt):mvaS:mvaA*) operon of expression plasmid pAM52. Expression plasmid pAM45 was digested to completion using *AsiSI* and *SfiI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, and the 8.3 kb DNA fragment lacking the *MevT66* operon was gel extracted. The (*atoB(opt):mvaS:mvaA*) operon of pAM52 was PCR amplified using primers 19-25 *atoB* *SfiI*-S (SEQ ID NO: 6) and 19-25 *mvaA*-*AsiSI*-AS (SEQ ID NO: 7), the PCR product was digested to completion using *SfiI* and *AsiSI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the 3.7 kb DNA fragment was gel extracted, and the isolated DNA fragment was ligated into

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the *AsiSI SfiI* restriction enzyme site of expression plasmid pAM45, yielding expression plasmid pAM97.

[00196] Expression plasmid pAM97-MBI was derived from expression plasmid pAM97 and pAM45 by replacing the MBIS operon of pAM97 with the MBI operon of pAM45. The MBI operon was PCR amplified from pAM45 using primers 9-70C (SEQ ID NO: 8) and 26-39B (SEQ ID NO: 9), the reaction mixture was resolved by gel electrophoresis, the 4.5 kb DNA fragment was gel extracted, and the isolated DNA fragment was digested to completion using *SacI* and *XhoI* restriction enzymes. Expression plasmid pAM97 was digested to completion using *SacI* and *XhoI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the 7.6 kb fragment was gel extracted, and the isolated DNA fragment was ligated with the MBI operon PCR product, yielding expression plasmid pAM97-MBI.

[00197] Expression plasmid pAM97-MevB was derived from expression plasmid pAM97 and pAM45 by replacing the MBIS operon of pAM97 with the MevB operon of pAM45. The MevB operon was PCR amplified from pAM45 using primers 9-70C (SEQ ID NO: 8) and 26-39A (SEQ ID NO: 10), the reaction mixture was resolved by gel electrophoresis, the 3.9 kb DNA fragment was gel extracted, and the isolated DNA fragment was digested to completion using *SacI* and *XhoI* restriction enzymes. Expression plasmid pAM97 was digested to completion using *SacI* and *XhoI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the 7.6 kb fragment was gel extracted, and the isolated DNA fragment was ligated with the MevB operon PCR product, yielding expression plasmid pAM97-MevB.

[00198] Expression plasmid pAM128 was generated by inserting the (*atoB(opt):mvaS:mvaA*) and MBIS operons of expression plasmid pAM97 into a vector that comprises the RK2 plasmid replication, segregation, and maintenance system, which obviates the continuous need for antibiotic selection of host cell transformants. The RK2 plasmid was digested to completion using *PstI* restriction enzyme, the reaction mixture was resolved by gel electrophoresis, the approximately 6.3 kb DNA fragment containing the entire *par* locus was gel extracted, and the isolated DNA fragment was subcloned into the *PstI* restriction enzyme site of the mini RK2 replicon pRR10 (Roberts et al. (1990) *J Bacteriol.* 172(11): 6204-6216), yielding vector pAM132. Expression plasmid pAM97 was digested to completion using *AscI* and *SacI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 9.4 kb DNA fragment was gel extracted, and the isolated

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DNA fragment was ligated into the *MluI SacI* restriction enzyme site of pAM132, yielding expression plasmid pAM128.

[00199] Example 3

[00200] This example describes methods for making expression vectors that encode enzymes of the MEV pathway from *Enterococcus faecalis* organized in operons.

[00201] Plasmid pAM16 was generated by inserting the coding sequence of the *mvaE* gene of *Enterococcus faecalis* (GenBank accession number AF290092 REGION: 1479..3890) (encodes an acetyl-CoA acetyltransferase/HMG-CoA reductase (HMGR)) into the pBlueScripII-KS(+) vector. The coding sequence of the *mvaE* gene was PCR amplified from *Enterococcus faecalis* genomic DNA (ATCC 700802) using 5' phosphorylated primers 4-40 mvaEF BamHI (SEQ ID NO: 11) and 4-40 mvaERHindIII (SEQ ID NO: 12). (Note that primer 4-40 mvaEF BamHI changes the start codon of the *mvaE* gene from TTG to ATG in the amplified PCR product.) The resulting PCR product was ligated into the *SmaI* restriction enzyme site of pBlueScripII-KS(+) (Stratagene, La Jolla, CA), yielding expression plasmid pAM16.

[00202] Plasmid pAM18 was generated by inserting the coding sequence of the *mvaS* gene of *Enterococcus faecalis* (GenBank accession number AF290092 REGION: 142..1293) (encodes a HMG-CoA synthase (HMGS)) into the pBlueScripII-KS(+) vector. The coding sequence of the *mvaS* gene was PCR amplified from *Enterococcus faecalis* genomic DNA (ATCC 700802) using 5' phosphorylated primers 4-40 mvaSF BglII (SEQ ID NO: 13) and 4-39 mvaSR BamHI (SEQ ID NO: 14), and the PCR product was ligated into the *SmaI* restriction enzyme site of pBlueScripII-KS(+) (Stratagene, La Jolla, CA), yielding expression plasmid pAM18.

[00203] Expression plasmid pAM22 was generated by inserting the coding sequence of the *mvaE* gene of expression plasmid pAM16 into the pZE21- $P_{L-lacO1}$ vector. Vector pZE21- $P_{L-lacO1}$ is a derivative of vector pZE21-MCS-1 in which the tet promoter was replaced with the $P_{L-lacO1}$ promoter (Lutz and Bujard (1997) *Nucl Acids Res.* 25:1203-1210). Expression plasmid pAM16 was digested to completion using *BamHI* and *HindIII* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 2.4 kb DNA fragment containing the *mvaE* coding sequence was gel extracted, and the isolated DNA fragment was inserted into the *BamHI HindIII* restriction enzyme site of pZE21- $P_{L-lacO1}$, yielding expression plasmid pAM22.

[00204] Expression plasmid pAM33 was generated by inserting the coding sequence of the *mvaS* gene of expression plasmid pAM18 into expression plasmid pAM22. Expression

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plasmid pAM18 was digested to completion using *BglII* and *BamHI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 1.2 kb DNA fragment containing the coding sequence of the *mvaS* gene was gel extracted, and the isolated DNA fragment was inserted into the *BamHI* site of expression plasmid pAM22, yielding expression plasmid pAM33.

[00205] Expression plasmid pAM34 was generated by inserting the *mvaS-mvaE* operon of expression plasmid pAM33 into vector pAM29. The *mvaS-mvaE* operon was isolated by partially digesting pAM33 using *EcoRI* restriction enzyme, digesting the resulting linear DNA fragment using *MluI* restriction enzyme, resolving the reaction mixture by gel electrophoresis, and gel extracting the approximately 3.6 kb DNA fragment. The vector backbone of pAM29 was obtained by digesting to completion expression vector pAM25 using *MluI* and *EcoRI* restriction enzymes, resolving the reaction mixture by gel electrophoresis, and gel extracting the approximately 2.1 kb DNA fragment. The two isolated DNA fragments were ligated, yielding expression plasmid pAM34.

[00206] Example 4

[00207] This example describes methods for making expression plasmids that encode enzymes of the DXP pathway from *Escherichia coli* organized in operons.

[00208] Expression plasmid pAM408 was generated by inserting genes encoding enzymes of the "top" DXP pathway into the pAM29 vector. Enzymes of the "top" DXP pathway include 1-deoxy-D-xylulose-5-phosphate synthase (encoded by the *dxs* gene of *Escherichia coli*), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (encoded by the *dxr* gene of *Escherichia coli*), 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (encoded by the *ispD* gene of *Escherichia coli*), and 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (encoded by the *ispE* gene of *Escherichia coli*), which together transform pyruvate and D-glyceraldehyde-3-phosphate to 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate. DNA fragments comprising nucleotide sequences that encode enzymes of the "top" DXP pathway were generated by PCR amplifying the coding sequences of the *dxs* (GenBank accession number U00096 REGION: 437539..439401), *dxr* (GenBank accession number U00096 REGION: 193521..194717), *ispD* (GenBank accession number U00096 REGION: 2869803..2870512), and *ispE* (GenBank accession number U00096 REGION: 1261249..1262100) genes from *Escherichia coli* strain DH1 (ATCC #33849) with added optimal Shine Dalgarno sequences and 5' and 3' restriction enzyme sites using the PCR primers shown in SEQ ID NOS: 15-18. The PCR products were resolved by gel electrophoresis, gel extracted using a Qiagen (Valencia, CA) gel purification kit, digested to

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completion using appropriate restriction enzymes (*XhoI* and *KpnI* for the PCR product comprising the *dxs* gene; *KpnI* and *Apal* for the PCR product comprising the *dxr* gene; *Apal* and *NdeI* for the PCR product comprising the *ispD* gene; *NdeI* and *MluI* for the PCR product comprising the *ispE* gene), and purified using a Qiagen (Valencia, CA) PCR purification kit. Roughly equimolar amounts of each PCR product were then added to a ligation reaction to assemble the individual genes into an operon. From this ligation reaction, 1 μ l of reaction mixture was used to PCR amplify 2 separate gene cassettes, namely the *dxs-dxr* and the *ispD-ispE* gene cassettes. The *dxs-dxr* gene cassette was PCR amplified using primers 67-1A-C (SEQ ID NO: 15) and 67-1D-C (SEQ ID NO: 18), and the *ispD-ispE* gene cassette was PCR amplified using primers 67-1E-C (SEQ ID NO: 19) and 67-1H-C (SEQ ID NO: 22). The two PCR products were resolved by gel electrophoresis, and gel extracted. The PCR product comprising the *dxs-dxr* gene cassette was digested to completion using *XhoI* and *Apal* restriction enzymes, and the PCR product comprising the *ispD-ispE* gene cassette was digested to completion using *Apal* and *MluI* restriction enzymes, and the two PCR products were purified. Vector pAM29 was digested to completion using *Sall* and *MluI* restriction enzymes, and the two digested PCR products containing the "top" DXP pathway operon were ligated into the *Sall MluI* restriction enzyme site of the pAM29 vector, yielding expression plasmid pAM408 (see Figure 7 for a plasmid map).

[00209] Expression plasmid pAM409 was generated by inserting genes encoding enzymes of the "bottom" DXP pathway into the pAM369 vector. Enzymes of the "bottom" DXP pathway include 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (encoded by the *ispF* gene of *Escherichia coli*), 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (encoded by the *ispG* gene of *Escherichia coli*), and isopentenyl/dimethylallyl diphosphate synthase (encoded by the *ispH* gene of *Escherichia coli*), which together transform 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate to IPP and DMAPP. IPP is also converted to DMAPP through the activity of isopentyl diphosphate isomerase (encoded by the *idi* gene of *Escherichia coli*). DMAPP can be further converted to FPP through the activity of farnesyl diphosphate synthase (encoded by the *ispA* gene of *Escherichia coli*). An operon encoding enzymes of the "bottom" DXP pathway as well as an isopentyl diphosphate isomerase and a farnesyl diphosphate synthase was generated by PCR amplifying the *ispF* (GenBank accession number U00096 REGION: 2869323..2869802), *ispG* (GenBank accession number U00096 REGION: 2638708..2639826), *ispH* (GenBank accession number U00096 REGION: 26277..27227), *idi* (GenBank accession number AF119715), and *ispA* (GenBank accession number D00694 REGION: 484..1383) genes from

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Escherichia coli strain DH1 (ATCC #33849) with added optimal Shine Dalgarno sequences and 5' and 3' restriction enzyme sites using the appropriate PCR primers. The PCR products were resolved by gel electrophoresis, gel extracted, digested with the appropriate restriction enzymes (*Bam*HI and *Apa*I for the PCR product comprising the *ispF* gene; *Kpn*I and *Apa*I for the PCR product comprising the *ispG* gene; *Sal*I and *Kpn*I for the PCR product comprising the *ispH* gene; *Sal*I and *Hind*III for the PCR product comprising the *idi* gene; *Hind*III and *Nco*I for the PCR product comprising the *ispA* gene), and purified. Roughly equimolar amounts of each PCR product were then added to a ligation reaction to assemble the individual genes into an operon. From this ligation reaction, 1 µl of reaction mixture was used to PCR amplify 2 separate gene cassettes, namely the *ispF-ispG* and the *ispH-idi-ispA* gene cassettes. The *ispF-ispG* gene cassette was PCR amplified using primers 67-2A-C (SEQ ID NO: 23) and 67-2D-C (SEQ ID NO: 26), and the *ispH-idi-ispA* gene cassette was PCR amplified using primers 67-2E-C (SEQ ID NO: 27) and 67-2J-C (SEQ ID NO: 32). The two PCR products were resolved by gel electrophoresis, and gel extracted. The PCR product comprising the *ispF-ispG* gene cassette was digested to completion using *Bam*HI and *Kpn*I restriction enzymes, and the PCR product comprising the *ispH-idi-ispA* gene cassette was digested to completion using *Kpn*I and *Nco*I restriction enzymes, and the two PCR products were purified. Vector pAM369 was created by assembling the p15A origin of replication from pAM29 and beta-lactamase gene for ampicillin resistance from pZE12-luc (Lutz and Bujard (1997) *Nucl Acids Res.* 25:1203-1210) with an oligonucleotide-generated lacUV5 promoter. Vector pAM369 was digested to completion using *Bam*HI and *Nco*I restriction enzymes, and the 2 isolated PCR products containing the "bottom" DXP pathway operon were ligated into the *Bam*HI *Nco*I restriction enzyme site of the pAM369 vector, yielding expression plasmid pAM409.

[00210] Expression plasmid pAM424, a derivative of expression plasmid pAM409 containing the broad-host range RK2 origin of replication, was generated by transferring the lacUV5 promoter and the *ispFGH-idi-ispA* operon of pAM409 to the pAM257 vector. Vector pAM257 was generated as follows: the RK2 *par* locus was PCR-amplified from RK2 plasmid DNA (Meyer et al. (1975) *Science* 190:1226-1228) using primers 9-156A (SEQ ID NO: 33) and 9-156B (SEQ ID NO: 34), the 2.6 kb PCR product was digested to completion using *Aat*II and *Xho*I restriction enzymes, and the DNA fragment was ligated into a plasmid containing the p15 origin of replication and the chloramphenicol resistance gene from vector pZA31-luc (Lutz and Bujard (1997) *Nucl Acids Res.* 25:1203-1210), yielding plasmid pAM37-*par*; pAM37-*par* was digested to completion using restriction enzymes *Sac*I and

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HindIII, the reaction mixture was resolved by gel electrophoresis, the DNA fragment comprising the RK2 *par* locus and the chloramphenicol resistance gene was gel extracted, and the isolated DNA fragment was ligated into the *SacI HindIII* site of the mini-RK2 replicon pRR10 (Roberts et al. (1990) *J Bacteriol.* 172:6204–6216), yielding vector pAM133; pAM133 was digested to completion using *BglII* and *HindIII* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 6.4 kb DNA fragment lacking the ampicillin resistance gene and *oriT* conjugative origin was gel extracted, and the isolated DNA fragment was ligated with a synthetically generated DNA fragment comprising a multiple cloning site that contained *PciI* and *XhoI* restriction enzyme sites, yielding vector pAM257. Expression plasmid pAM409 was digested to completion using *XhoI* and *PciI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, and the approximately 4.4 kb DNA fragment was gel extracted. Vector pAM257 was digested to completion using restriction enzymes *XhoI* and *PciI*, and the isolated DNA fragment containing the *lacUV5* promoter and *ispFGH-idi-ispA* operon was ligated into the *XhoI PciI* restriction enzyme site of the pAM257 vector, yielding expression plasmid pAM424 (see Figure 8 for a plasmid map).

[00211] Example 5

[00212] This example describes methods for making expression plasmids that encode enzymes that convert FPP or GPP.

[00213] Expression plasmid pTrc99A-ADS was generated by inserting a nucleotide sequence encoding an amorpha-4,11-diene synthase (“ADS”) into vector pTrc99A. The amorpha-4,11-diene synthase sequence was generated synthetically, so that upon translation the amino acid sequence would be identical to that described by Merke et al. (2000) *Ach. Biochem. Biophys.* 381:173-180, so that the nucleotide sequence encoding the amorpha-4,11-diene synthase was optimized for expression in *Escherichia coli*, and so that the nucleotide sequence was flanked by a 5' *NcoI* and a 3' *XmaI* restriction enzyme site (see U.S. Patent Number 7,192,751). The nucleotide sequence was digested to completion using *NcoI* and *XmaI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 1.6 kb DNA fragment was gel-extracted, and the isolated DNA fragment was inserted into the *NcoI XmaI* restriction enzyme site of the pTrc99A vector (Amman et al. (1985) *Gene* 40:183-190), yielding expression plasmid pTrc99A-ADS (see Figure 9 for a plasmid map).

[00214] Expression plasmid pAM113 is a chloramphenicol-resistant derivative of pTrc99A-ADS. It was generated by PCR amplifying the chloramphenicol resistance gene

from vector pZA31-luc (Lutz and Bujard (1997) *Nucl Acids Res.* 25:1203-1210) using 5'-phosphorylated primers 19-137 cml-pAM37-AS (SEQ ID NO: 35) and 19-137 cml-pAM37-S (SEQ ID NO: 36), and inserting the 920 bp PCR product into the *FspI* restriction enzyme site of expression plasmid pTrc99A-ADS, yielding expression plasmid pAM113.

[00215] Expression plasmid pC9 was generated by inserting a genomic DNA fragment of *Bacillus subtilis* 6051 comprising the coding sequence of the *nudF* gene and upstream genomic sequences (GenBank accession number Z99116 REGION: 49364..48548) into vector pTrc99A (Amann et al. (1988) *Gene* 69:301-315). Expression plasmid pNudF-H was generated by inserting the coding sequence of the *Bacillus subtilis* 6051 *nudF* gene (GenBank accession number Z99116 REGION: 49105..48548) into vector pTrc99A. Expression plasmid pyhfR was generated by inserting the coding sequence of the *Bacillus subtilis* 6051 *yhfR* gene (GenBank accession number Z99109 REGION: 97583..97002) into vector pTrc99A.

[00216] Expression plasmid pAM373 was generated by inserting a nucleotide sequence encoding the β -farnesene synthase ("FSB") of *Artemisia annua* (GenBank accession number AY835398), codon-optimized for expression in *Escherichia coli*, into the pTrc99A vector. The nucleotide sequence encoding the β -farnesene synthase was generated synthetically, and was amplified by PCR from its DNA synthesis construct using the appropriate primers. To create a leader *NcoI* restriction enzyme site in the PCR product comprising the β -farnesene synthase coding sequence, the codon encoding the second amino acid in the original polypeptide sequence (TCG coding for serine) was replaced by a codon encoding aspartic acid (GAC) in the 5' PCR primer (SEQ ID NO: 37). The resulting PCR product was partially digested using *NcoI* restriction enzyme, and digested to completion using *SacI* restriction enzyme, the reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the β -farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the *NcoI SacI* restriction enzyme site of the pTrc99A vector, yielding expression plasmid pAM373 (see Figure 9 for a plasmid map).

[00217] Expression plasmids pTrc99A-FSA, pTrc99A-GTS, pTrc99A-PS, pTrc99A-TS were generated by inserting a DNA fragment comprising a nucleotide sequence encoding an α -farnesene synthase ("FSA"), a γ -terpinene synthase ("GTS"), an α -pinene synthase ("APS"), or a terpinolene synthase ("TS") into the pTrc99A vector. The DNA fragment insert was generated synthetically, using as a template for example the coding sequence of the α -farnesene synthase gene of *Picea abies* (GenBank accession number AY473627, REGION:

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24..1766), the coding sequence of the β -farnesene synthase gene of *Artemisia annua* (GenBank accession number AY835398), the coding sequence of the γ -terpinene synthase gene of *Citrus limon* (GenBank accession number AF514286 REGION: 30..1832), the coding sequence of the α -pinene synthase gene of *Abies grandis* (GenBank accession number U87909, REGION: 6..1892) or of *Pinus taeda* (GenBank accession number AF543530 REGION: 1..1887), or the coding sequence of the terpinolene synthase gene of *Ocimum basilicum* (GenBank accession number AY693650) or of *Pseudotsuga menziesii* (GenBank accession number AY906866 REGION:10..1887) or of *Abies grandis* (GenBank accession number AF139206), all nucleotide sequences being codon-optimized for expression in *Escherichia coli*. The DNA fragments for FSA was amplified by PCR from its DNA synthesis construct using the primer sequences SEQ ID NO: 39 and SEQ ID NO: 40. The resulting PCR product was digested to completion using *NcoI* and *SacI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the α -farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the *NcoI SacI* restriction enzyme site of the pTrc99A vector, yielding expression plasmid pTrc99A-FSA (see Figure 9 for a plasmid map). The DNA fragments for GTS, APS, and TS were designed to be flanked by a leader *XmaI* restriction enzyme site and a terminal *XbaI* restriction enzyme site, and were cloned into compatible restriction enzyme sites of a cloning vector such as a standard pUC or pACYC origin vector, from which they could be liberated again by digesting to completion the DNA synthesis construct using *XbaI* and *XmaI* restriction enzymes, resolving the reaction mixture by gel electrophoresis, and gel extracting the 1.7 to 1.9 terpene synthase encoding DNA fragment. The isolated DNA fragments were ligated into the *XmaI XbaI* restriction enzyme site of vector pTrc99A (Amman *et al.*, *Gene* 40:183-190 (1985)), yielding plasmids pTrc99A-GTS, pTrc99A-APS, or pTrc99A-TS (see Figure 9 for plasmid maps).

[00218] Expression plasmids pRS425-FSA and pRS425-FSB were generated by inserting a nucleotide sequence encoding an α -farnesene synthase ("FSA") or a β -farnesene synthase ("FSB"), respectively, into the pRS425-Gal1 vector (Mumberg *et. al.* (1994) *Nucl. Acids. Res.* 22(25): 5767-5768). The nucleotide sequence inserts were generated synthetically, using as a template for example the coding sequence of the α -farnesene synthase gene of *Picea abies* (GenBank accession number AY473627, REGION: 24..1766) or of the β -farnesene synthase gene of *Artemisia annua* (GenBank accession number AY835398), codon-optimized for expression in *Saccharomyces cerevisiae*. The synthetically generated nucleotide sequence was flanked by a 5' *BamHI* site and a 3' *XhoI* site, and could

thus be cloned into compatible restriction enzyme sites of a cloning vector such as a standard pUC or pACYC origin vector. The synthetically generated nucleotide sequence was isolated by digesting to completion the DNA synthesis construct using *BamHI* and *XhoI* restriction enzymes. The reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the α -farnesene synthase or β -farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the *BamHI XhoI* restriction enzyme site of the pRS425-Gall vector, yielding expression plasmid pRS425-FSA or pRS425-FSB, respectively.

[00219] Expression plasmids pTrc99A-LLS, pTrc99A-LMS, pTrc99A-BPS, pTrc99A-PHS, pTrc99A-CS, and pTrc99A-SS are generated by inserting a nucleotide sequence encoding a linalool synthase ("LLS"), limonene synthase ("LMS"), β -pinene synthase ("BPS"), β -phellandrene ("PHS"), carene synthase ("CS"), or sabinine synthase ("SS") into the pTrc99A vector. The nucleotide sequence inserts are generated synthetically, using as a template for example the coding sequence of the linalool synthase gene of *Artemisia annua* (GenBank accession number AF154124, REGION: 13..1764), the coding sequence of the limonene synthase gene of *Abies grandis* (GenBank accession number AF006193 REGION: 73..1986), the coding sequence of the β -pinene synthase of *Artemisia annua* (GenBank accession number AF276072 REGION: 1..1749), the coding sequence of the β -phellandrene synthase gene of *Abies grandis* (GenBank accession number AF139205 REGION: 34..1926), the coding sequence of the carene synthase gene of *Salvia stenophylla* (GenBank accession number AF527416 REGION: 78..1871), or the coding sequence of the sabinene synthase gene of *Salvia officinalis* (GenBank accession number AF051901 REGION: 26..1798). The nucleotide sequences encoding the β -pinene, sabinine, and β -phellandrene synthases are flanked by a leader *XmaI* restriction enzyme site and a terminal *XbaI* restriction enzyme site, the nucleotide sequences encoding the linalool and carene synthases are flanked by a leader *NcoI* restriction enzyme site and a terminal *XmaI* restriction enzyme site, and the nucleotide sequence encoding the limonene synthase is flanked by a leader *NcoI* restriction enzyme site and a terminal *PstI* restriction enzyme site. The DNA synthesis constructs are digested to completion using *XmaI* and *XbaI* (for the β -pinene, sabinine, and β -phellandrene synthase constructs), *NcoI* and *XmaI* restriction enzymes (for the linalool and carene synthase constructs), or *XbaI* and *PstI* restriction enzymes (for the limonene synthase construct). The reaction mixtures are resolved by gel electrophoresis, the approximately 1.7 to 1.9 kb DNA fragments are gel extracted, and the isolated DNA fragments are ligated into the *XmaI XbaI* restriction enzyme site (for the β -pinene, sabinine, and β -phellandrene synthase inserts), the

NcoI XmaI restriction enzyme site (for the linalool and carene synthase inserts), or the *XbaI PstI* restriction enzyme site (for the limonene synthase insert) of the pTrc99A vector, yielding expression plasmids pTrc99A-LLS, pTrc99A-LMS, pTrc99A-BPS, pTrc99A-PHS, pTrc99A-CS, and pTrc99A-SS (see Figure 9 for plasmid maps).

[00220] Example 6

[00221] This example describes the generation of *Escherichia coli* host strains useful in the invention.

[00222] As detailed in Table 1, the host strains were created by transforming chemically competent *Escherichia coli* parent cells with one or more expression plasmids of Example 1 through 5.

Table 1. *E. coli* host strains

Host Strain	E.coli Parent Strain	Expression Plasmids	Antibiotic Selection
B32	DH1	pMevT	100 ug/mL
B292	B	pMBIS	carbenicillin
B210	DP	pTrc99A-ADS	5 ug/mL tetracycline 34 ug/mL chloramphenicol
B153	DH1	pAM97	100 ug/mL
B282	DP	pTrc99A-ADS	carbenicillin 34 ug/mL chloramphenicol
B255	DH1	pAM128	100 ug/mL
B256	DP	pAM113	carbenicillin 34 ug/mL chloramphenicol
B86	DH1	pAM52 pMBIS pTrc99A-ADS	50 ug/mL kanamycin 100 ug/mL carbenicillin
B61	DH1	pAM25 pBBR1MCS-3 pTrc99A	5 ug/mL tetracycline
B62		pAM34 pBBR1MCS-3 pTrc99A	
B003	DH10B	pTrc99A-ADS	100 µg/ml carbenicillin
B617		pAM408 pTrc99A-ADS	100 ug/mL carbenicillin 50 ug/mL kanamycin
B618		pAM424 pTrc99A-ADS	100 ug/mL carbenicillin 35 µg/ml chloramphenicol

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Host Strain	E.coli Parent Strain	Expression Plasmids	Antibiotic Selection
B619		pAM408 pAM424 pTrc99A-ADS	100 µg/ml carbenicillin 50 µg/ml kanamycin 35 µg/ml chloramphenicol
B650	DH10B	pAM373	100 µg/ml carbenicillin
B651		pAM408 pAM373	100 µg/ml carbenicillin 50 µg/ml kanamycin
B652		pAM424 pAM373	100 µg/ml carbenicillin 35 µg/ml chloramphenicol
B653		pAM408 pAM424 pAM373	100 µg/ml carbenicillin 50 µg/ml kanamycin 35 µg/ml chloramphenicol
B286	DH1	pAM97-MevB pC9	100 ug/mL carbenicillin
B287		pAM97-MevB pnudF-H	34 ug/mL chloramphenicol.
B288		pAM97-MevB pyhfR	
B291		pAM97-MBI pyhfR	
B592	DH1	pMevT pMBIS pTrc99A-FSA	100 ug/mL carbenicillin 34 ug/mL chloramphenicol
B552		pMevT pMBIS pAM373	5 ug/mL tetracycline
Example 21 host cell (production of GTS, APS, TS)		pMevT pMBIS-gpps pTrc99A-GTS or -APS or -TS	
Example 21 host cell (production of LLS, LMS, BPS, PHS, CS, SS)		pMevT pMBIS-gpps pTrc99A-LLS or -LMS or -BPS or -PHS or -CS or - SS	100 ug/mL carbenicillin 34 ug/mL chloramphenicol 5 ug/mL tetracycline

[00223] Host cell transformants were selected on Luria Bertoni (LB) agar containing antibiotics as detailed in Table 1. Single colonies were transferred from LB agar to culture tubes containing 5 mL of LB liquid medium and antibiotics. B003, B617, B618, B619, B650,

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B651, B652, and B653 host cell transformants were incubated at 30°C on a rotary shaker at 250 rpm for 30 hours. All other host cell transformants were incubated at 37°C on a rotary shaker at 250 rpm until growth reached stationary phase. The cells were adapted to minimal media by passaging them through 4 to 5 successive rounds of M9-MOPS media containing 0.8% glucose and antibiotics (see Table 2 for the composition of the M9-MOPS medium). The cells were stored at -80°C in cryo-vials in 1 mL stock aliquots made up of 400 uL sterile 50% glycerol and 600 uL liquid culture.

Table 2 – Composition of M9-MOPS Culture Medium

Component	Quantity (per L)
Na ₂ HPO ₄ 7H ₂ O	12.8 g
KH ₂ PO ₄	3 g
NaCl	0.5 g
NH ₄ Cl	1 g
MgSO ₄	2 mmol
CaCl ₂	0.1 mmol
Thiamine	0.1 ug
MOPS buffer pH 7.4	100 mmol
(NH ₃) ₆ Mo ₇ O ₂₄ 4H ₂ O	3.7 ug
H ₃ BO ₄	25 ug
CoCl ₂	7.1 ug
CuSO ₄	2.4 ug
MnCl ₂	16 ug
ZnSO ₄	2.9 ug
FeSO ₄	0.28 mg

[00224] Example 7

[00225] This example demonstrates expression plasmid stability in the absence of antibiotics in an *Escherichia coli* host strain that harbors an expression plasmid comprising the RK2 plasmid replication, segregation, and maintenance system.

[00226] A seed culture of host strain B255 was established by adding a stock aliquot of the strain to a 125 mL flask containing 40 mL M9-MOPS, 2% glucose, 0.5% yeast extract, and antibiotics as detailed in Table 1, and by growing the culture overnight.

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[00227] The seed culture was used to inoculate at an initial OD₆₀₀ of approximately 0.05, two 250 mL flasks each containing 40 mL M9-MOPS medium, 2% glucose, and 0.5% yeast extract. Culture #1 also contained 100 ug/mL carbenicillin and 34 ug/mL chloramphenicol. Culture #2 did not receive any antibiotics. Both cultures were incubated at 37°C on a rotary shaker at 250 rpm until they reached an OD₆₀₀ of approximately 0.2, at which point the production of amorpho-4,11-diene in the host cells was induced by adding 40 uL of 1M IPTG to the culture medium. At the time of induction, the cultures were overlain with 8 mL of an organic overlay to capture the amorpho-4,11-diene. Samples were taken periodically for a total of 72 hours. Production of amorpho-4,11-diene by the host strain in the 2 cultures was confirmed by GC/MS as described in Example 10.

[00228] To assess plasmid stability in the two cell cultures, a sample of each culture was removed at 72 hours and streaked onto a LB agar plate (no antibiotics). After overnight incubation at 37°C, 50 individual colonies derived from each culture were replica-plated onto a LB agar-plus-antibiotics (34 ug/mL chloramphenicol, 100 ug/mL carbenicillin) plate and a LB agar-minus-antibiotics (no antibiotic) plate. After another overnight incubation at 37°C, the LB agar-plus-antibiotics and the LB agar-minus-antibiotics plate were each found to contain approximately 50 colonies, indicating that plasmid retention both in the presence and in the absence of antibiotics in the culture medium had been approximately 100%.

[00229] Example 8

[00230] This example demonstrates increased specific activity and stability of the *Enterococcus faecalis* HMGR compared to the *Saccharomyces cerevisiae* tHMGR in an *Escherichia coli* host strain.

[00231] Seed cultures of host strains B61 and B62 were established by adding a stock aliquot of each strain to 125 mL flasks containing 20 mL M9-MOPS medium, 0.8% glucose, and antibiotics as detailed in Table 5, and by growing the cultures to saturation. The seed cultures were diluted 1:100 into 140 mL of fresh medium in a 500 mL flask, and grown again to an OD₅₅₀ of approximately 0.1, at which point production of amorpho-4,11-diene was induced by adding 140 uL 1 M IPTG to each culture. At 4, 12, 20, 28, 36, and 49 hours post-induction, samples were removed from each culture, and cells were pelleted by centrifugation. The cell pellets were snap frozen on dry ice, and then stored at -80°C.

[00232] To conduct enzyme assays, cell pellets were thawed on ice, and then lysed using Bugbuster (Novagen, Madison, WI) containing protease inhibitor mix #3 (Calbiochem, San Diego, CA), benzonase (20 uL per 5 mL bugbuster; Novagen, Madison, WI), and

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lysozyme (30 ug/mL). Enzyme activity of the *Saccharomyces cerevisiae* tHMGR was assayed in 50 mM Tris HCl (pH7.5), 0.2 mM NADPH (Sigma, St. Louis, MO), and 0.3 mM DL-3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) sodium salt (Sigma, St. Louis, MO). The assay was started by adding cell lysate, and the disappearance of NADPH was monitored by absorbance at 340nm. To account for non-specific disappearance of NADPH, results obtained in a control assay lacking HMG-CoA were subtracted from results obtained in test samples. Enzyme activity of the *Enterococcus faecalis* HMGR was measured similarly except that the assay buffer contained 100 mM potassium phosphate buffer (pH6.5), 0.4 mM NADPH, 1.0 mM EDTA, and 100 mM KCl.

[00233] Protein assays were done by the method of Bradford ((1976) *Anal Biochem.* 72:248-254). Specific activities were calculated as Δ nmol NADPH/min/mg protein.

[00234] Example 9

[00235] This example describes the calibration of OD₆₀₀ with dry cell weight ("DCW").

[00236] To obtain the relationship between DCW and OD₆₀₀, a representative strain, B32, was grown in high cell density processes similar to those described in Examples 10-12. Samples were taken throughout the runs, and the OD₆₀₀ and DCW were measured for each sample. To determine the DCW, the cells were pelleted and the supernatant discarded. The cell pellet was washed once with water, and was then dried in an oven at 80°C for at least 3 days. The tubes containing cell pellets were weighed, the weight of the tube was subtracted from the measured weights, and the remaining weight was divided by the initial volume of each sample (0.0015 L) to obtain the DCW.

[00237] Example 10

[00238] This example demonstrates increased production of amorpho-4,11-diene in *Escherichia coli* host strains expressing the *Staphylococcus aureus* HMGR and HMGS compared to host strains expressing the *Saccharomyces cerevisiae* tHMGR and HMGS.

[00239] Seed cultures of host strains B32, B153, B210, B282, B292, B86, B255, and B256 were established by adding a stock aliquot of each strain to separate 125 mL flasks containing 25 mL M9-MOPS medium, 0.8% glucose, and antibiotics as detailed in Table 1, and by growing the cultures overnight.

[00240] The seed cultures were used to inoculate at an initial OD₆₀₀ of approximately 0.05 separate 250 mL flasks containing 40 mL M9-MOPS medium, 2% glucose, and antibiotics. The cultures were incubated at 30°C on a rotary shaker at 250 rpm until they reached an OD₆₀₀ of approximately 0.2, at which point the production of amorpho-4,11-diene

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in the host cells was induced by adding 40 uL of 1M IPTG to the culture medium. The cultures were overlain with 8mL of an organic overlay (e.g., dodecane, methyl oleate or isopropyl myristate). Samples of the organic overlay layer and the broth were taken once a day for 72 hours. Broth samples were used to measure the OD₆₀₀. Amorpha-4,11-diene concentration was measured by transferring 5 uL of the organic overlay layer to a clean glass vial containing 500 uL ethyl acetate spiked with beta- or trans-caryophyllene as an internal standard.

[00241] The organic overlay/ethyl acetate samples were analyzed on a Hewlett-Packard 6890 gas chromatograph/mass spectrometer (GC/MS) by scanning only for two ions, the molecular ion (204 m/z) and the 189 m/z ion, as described in Martin et al. (2001) *Biotechnol. Bioeng.* 75:497-503. To expedite run times, the temperature program and column matrix was modified to achieve optimal peak resolution and the shortest overall runtime. A 1 uL sample was separated on the GC using a DB-XLB column (available from Agilent Technologies, Inc., Palo Alto, CA) and helium carrier gas. The temperature program for the analysis was as follows: 100°C for 0.75 minutes, increasing temperature at 60°C/minute to a temperature of 300°C, and a hold at 300°C for 0.5 minutes. The resolved samples were analyzed by a Hewlett-Packard model 5973 mass-selective detector that monitored ions 189 and 204 m/z. Previous mass spectra demonstrated that the amorpha-4,11-diene synthase product was amorpha-4,11-diene, and that amorpha-4,11-diene had a retention time of 3.7 minutes using this GC protocol. Beta- or trans-caryophyllene was used as an internal standard for quantitation. Amorpha-4,11-diene titer was calculated using the ratio of internal standard to amorpha-4,11-diene peak areas based upon a quantitative calibration curve of purified amorpha-4,11-diene (0.63-10 mg/L of KJF17-109-3) in caryophyllene-spiked ethyl acetate.

[00242] Example 11

[00243] This example demonstrates increased production of amorpha-4,11-diene by an *Escherichia coli* host strain grown at suboptimal temperature.

[00244] A seed culture of host strain B32 was established by adding 0.5 mL of a stock aliquot of the strain to a 250 mL flask containing 50 mL M9-MOPS medium and antibiotics as detailed in Table 1, and by growing the culture overnight at 37°C on a rotary shaker at 250 rpm.

[00245] The seed culture was used to inoculate at an initial OD₆₀₀ of approximately 0.05 four 250 mL flasks, each containing 40 mL fermentor batch medium (see Table 6 for medium composition), 100 mM MOPS buffer pH7.1, and antibiotics. The cultures were

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incubated on a rotary shaker at 250 rpm at either 30°C or 37°C until they reached an OD₆₀₀ of 0.18 to 0.22, at which point the production of amorpho-4,11-diene in the host cells was induced by adding 40 uL of 1M IPTG to the culture medium. At the time of induction, the cultures were overlain with 8mL of an organic overlay to capture the amorpho-4,11-diene. Samples were taken once a day, and analyzed as described in Example 10.

[00246] Example 12

[00247] This example demonstrates increased production of amorpho-4,11-diene by an *Escherichia coli* host strain grown under restricted carbon source conditions.

[00248] A seed culture of host strain B32 for fermentation runs 050608-1 and 050629-1 was established by adding 0.25 uL of a stock aliquot of the strain to a 250 mL flask containing 50 mL M9-MOPS medium and antibiotics as detailed in Table 1, and by incubating the culture at 37°C on a rotary shaker at 250 rpm until it reached an OD₆₀₀ of 1 to 2.

[00249] A seed culture of host strain B32 for fermentation run 060403-3 was established by adding a stock aliquot of the strain to a 250 mL flask containing 50 mL M9-MOPS medium and antibiotics as detailed in Table 1, and by incubating the culture overnight at 37°C on a rotary shaker at 250 rpm. The seed culture was used to inoculate at an initial OD₆₀₀ of approximately 1 a 250 mL flask containing 40 mL M9-MOPS medium and antibiotics, and the culture was again incubated at 37°C on a rotary shaker at 250 rpm until it reached an OD₆₀₀ of 3 to 5.

[00250] For all fermentation processes, the KH₂PO₄, K₂HPO₄ 3H₂O, EDTA, citric acid, and (NH₄)₂SO₄ were heat sterilized in the bioreactor (2L Applikon Bioconsole ADI 1025s with ADI 1010 controllers, Applikon Biotechnology, Foster City, CA). The remaining media components were filter sterilized as stock solutions and injected through the headplate. Table 3 shows the final media composition for fermentation runs 050608-1 and 050629-1. Table 4 shows the final media composition for fermentation run 060403-3. The starting volume for run 050608-1 was 0.8 L, the starting volume for 050629-1 was 1.2 L and the starting volume for 060403-3 was 1 L. All runs were inoculated by injecting 50 mL of the seed culture through the headplate.

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050608-1 and 050629-1

TABLE 3 – Composition of Fermentation Medium of Fermentation Runs
050608-1 and 050629-1

Component	Batch Medium (per L)	Feed Solution (per L)
Glucose	5 g	590-650 g
KH ₂ PO ₄	4.2 g	-
K ₂ HPO ₄ 3H ₂ O	15.7 g	-
Citric acid	1.7 g	-
(NH ₄) ₂ SO ₄	2 g	-
MgSO ₄ 7H ₂ O	1.2 g	12 g
EDTA	8.4 mg	13 g
CoCl ₂ 6H ₂ O	0.25 mg	0.4 mg
MnCl ₂ 4H ₂ O	1.5 mg	2.35 mg
CuCl ₂ 2H ₂ O	0.15 mg	0.25 mg
H ₃ BO ₄	0.3 mg	0.5 mg
Na ₂ MoO ₄ 2H ₂ O	0.25 mg	0.4 mg
Zn(CH ₃ COO) ₂ 2H ₂ O	1.3 mg	1.6 mg
Fe(III)citrate hydrate	10.0 mg	4.0 mg
Thiamine HCl	4.5 mg	-
Carbenicillin	100 ug	100 ug
Tetracycline	5 ug	5 ug
Chloramphenicol	34 ug	34 ug

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Table 4 – Composition of Fermentation Medium of Fermentation Run 060403-3

Component	Batch medium (per L)	Feed solution (per L)
Glucose	15 g	650 g
KH ₂ PO ₄	4.2 g	-
K ₂ HPO ₄ 3H ₂ O	15.7 g	-
Citric acid	1.7 g	-
(NH ₄) ₂ SO ₄	2 g	-
MgSO ₄ 7H ₂ O	1.2 g	12 g
EDTA	8.4 mg	13 mg
CoCl ₂ 6H ₂ O	2.5 mg	4 mg
MnCl ₂ 4H ₂ O	15 mg	23.5 mg
CuCl ₂ 2H ₂ O	1.5 mg	2.5 mg
H ₃ BO ₄	3 mg	5 mg
Na ₂ MoO ₄ 2H ₂ O	2.5 mg	4 mg
Zn(CH ₃ COO) ₂ 2H ₂ O	13 mg	16 mg
Fe(III)citrate hydrate	100 mg	40 mg
Thiamine HCl	4.5 mg	-
Carbenicillin	100 ug	100 ug
Tetracycline	5 ug	5 ug
Chloramphenicol	34 ug	34 ug

[00251] For fermentation run 050608-1 (excess carbon), the feed was initiated at induction, and feed rates were adjusted manually. For fermentation run 050629-1 (carbon-restricted), the feed was delivered to the fermentor according to the protocol shown in Table 5. For fermentation run 060403-3 (lowest carbon), the feed was started automatically when the initial glucose bolus (15 g) was exhausted and the dissolved oxygen spiked. Up to a maximum of 27.6 g/hr, the rate of the feed was calculated according to the following equation:

$$m_s(t) = S(t_0) \mu e^{\mu(t-t_0)}$$

$$\mu = 0.12$$

$$S(t_0) = 15g$$

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wherein t_0 is the time at which the initial glucose was depleted. Upon reaching the maximum rate, the glucose feed was restricted to a rate of 9.5 g/hr, and held constant at this rate for the remainder of the run.

Table 5 – Feed Protocol for Fermentation Run 050629-1

Run Time (hours)	Glucose Feed Rate (g/hr)
0	0
7	0.37
10	0.74
12	1.11
14	1.48
16	2.22
18	2.96
20	3.69
22	4.80
24	5.91
31	7.39
33	5.54
47	3.69

[00252] Runs 050608-1 and 050629-1 were carried out at 37°C. Airflow in the bioreactor was set at 1-2 L/min; pH was maintained at 7 using ammonium hydroxide and/or sodium hydroxide; initial agitation was 500-600 rpm; foam was controlled with antifoam B (Sigma-Aldich, St. Louis, MO); the dissolved oxygen levels were maintained above 30% using an agitation cascade. After 5-6 hours of cultivation, production of amorpha-4,11-diene by the host cells was induced by adding 0.8 mL of 1 M IPTG to run 050608-1 and 1.2 mL IPTG to run 050629-1. Upon induction, the culture temperature was reduced to 30°C.

[00253] Run 060403-3 was carried out at 30°C. Airflow in the bioreactor was set at 1-2 L/min; pH was maintained at 7 using ammonia hydroxide. Dissolved oxygen was maintained above 30% by an agitation cascade and oxygen enrichment. At an OD₆₀₀ of approximately 28 (19 hours after inoculation), production of amorpha-4,11-diene by the host cells was induced by adding 1 mL 1 M IPTG.

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[00254] Amorpha-4,11-diene was captured and extracted according to two different protocols. For runs 050608-1 and 050629-1, volatile amorpha-4,11-diene present in the off-gas was captured by venting the off-gas through a gas-washer containing 200 mL heptanol. The heptanol was then diluted into ethyl acetate until the amorpha-4,11-diene concentration in the sample was between 0.63 mg/L and 20 mg/L. For run 060403-3, amorpha-4,11-diene was captured in the bioreactor by adding 200 mL of an organic overlay to the fermentor at the time of induction. Product concentration was measured by combining 25 uL broth plus organic overlay with 975 uL acetonitrile, shaking the sample at maximum speed on a Fisher Vortex Genie 2™ mixer (Scientific Industries, Inc., Bohemia, NY) for at least 3 minutes, removing cells from the sample by centrifugation, and diluting the acetonitrile solution into ethyl acetate until the amorpha-4,11-diene concentration in the sample was between 0.63 and 20 mg/L. The ethyl acetate samples were analyzed by GC/MS as described in Example 10.

[00255] Example 13

[00256] This example demonstrates increased amorpha-4,11-diene production by an *Escherichia coli* host strain grown under restricted carbon source conditions and at suboptimal temperature.

[00257] A seed culture of host strain B153 was established by adding a stock aliquot of the strain to a 250 mL flask containing 50 mL M9-MOPS medium and antibiotics as detailed in Table 1, and growing the culture at 37°C on a rotary shaker at 250 rpm to an OD₆₀₀ of 3.5 to 4.5.

[00258] 2 L bioreactors (Biocontroller ADI 1010 with Bioconsole ADI 1025, Applikon Biotechnology, Foster City, CA) were set up and run in the same way as described in Example 12 for run 060403-3, except that strain and induction time were varied.

[00259] Production of amorpha-4,11-diene in the host cells was induced by adding 1 mL of 1 M IPTG to the culture medium. Amorpha-4,11-diene was captured and extracted according to two different protocols. In one method, volatile amorpha-4,11-diene present in the off-gas was captured by venting the off-gas through a gas-washer containing 200 mL heptanol. The heptanol was then diluted into ethyl acetate until the amorpha-4,11-diene concentration in the sample was between 0.63 and 20 mg/L. In another, amorpha-4,11-diene was captured by adding 200 mL of an organic overlay to the fermentor at the time of induction.

[00260] Amorpha-4,11-diene was extracted from the culture medium by combining 25 uL broth with 975 uL acetonitrile, shaking the sample at maximum speed on a Fisher Vortex

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Genie 2™ mixer (Scientific Industries, Inc., Bohemia, NY) for at least 3 minutes, removing cells from the sample by centrifugation, and diluting the acetonitrile solution into ethyl acetate until the amorpho-4,11-diene concentration in the sample was between 0.63 and 20 mg/L. The ethyl acetate samples were analyzed by GC/MS as described in Example 10.

[00261] Example 14

[00262] This example demonstrates increased amorpho-4,11-diene production by an *Escherichia coli* host strain grown under restricted carbon and nitrogen source conditions and at suboptimal temperature.

[00263] A seed culture of host strain B86 was established by adding a stock aliquot of the strain to a 250 mL flask containing 50 mL M9-MOPS medium and antibiotics as detailed in Table 1. The culture was grown overnight at 37°C on a rotary shaker at 250 rpm, sub-cultured the following morning into the same medium at an OD₆₀₀ of approximately 1, and grown again at 37°C and 250 rpm to an OD₆₀₀ of 3 to 5.

[00264] Four 2 L bioreactors (Biocontroller ADI 1010 with Bioconsole ADI 1025, Applikon Biotechnology, Foster City, CA) were set up and run in the same way as described in Example 12 for run 060403-3, except that the nitrogen restricted runs did not contain ammonia sulfate in the feed.

[00265] An exponential glucose feed with a 6 hour doubling time was initiated automatically when the initial glucose bolus (15 g) was exhausted and the dissolved oxygen spiked. Up to a maximum of 30.4 g/hr, the rate of the feed was calculated according to the following equation:

$$m_s(t) = S_0 \mu e^{\mu(t-t_0)}$$

$$\mu = 0.12 \text{ min}^{-1}$$

$$S_0 = 15 \text{ g}$$

wherein μ is the specific growth rate, and t_0 is the time at which the initial glucose bolus was depleted. Upon reaching the maximum rate, the glucose feed was reduced to a rate of 11.4 g/hr, and held constant at this rate for the remainder of the run. In fermentation runs 060710-4, 060724-5, and 060619-5 (carbon- and nitrogen-restricted), the glucose feed was further reduced when ammonia restriction lead to glucose accumulation in the medium.

[00266] Fermentation was carried out at the reduced temperature of 30°C. Airflow in the bioreactor was set at 1 vvm; initial agitation was at 700 rpm; foam was controlled with antifoam B (Sigma-Aldich, St. Louis, MO); and dissolved oxygen tension was controlled at 40% using an agitation cascade (700-1,200 rpm) and oxygen enrichment. In fermentation run

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060327-3 (carbon-restricted), the pH was maintained at 7 using 20% NH₄OH; in fermentation runs 060710-4, 060724-5, and 060619-5 (carbon- and nitrogen-restricted), pH was maintained at 7 initially using 20% NH₄OH, and starting at 72 hours using a 50/50 mixture of 2.5 N NaOH and 10 N NH₄OH, to further restrict the amount of ammonia going into the fermentor.

[00267] Production of amorpha-4,11-diene in the host cells was induced at an OD₆₀₀ of approximately 30 by adding 1 mL of 1 M IPTG to the culture medium.

[00268] Amorpha-4,11-diene was captured by overlaying the medium with 10% (v/v) of an organic overlay. Amorpha-4,11-diene was then extracted by combining 25 uL of broth with 975 uL methanol, shaking the sample at maximum speed on a Fisher Vortex Genie 2™ mixer (Scientific Industries, Inc., Bohemia, N.Y.) for at least 15 minutes, removing cells from the sample by centrifugation, and adding 10 uL of the methanol solution to 990 uL ethyl acetate containing 10 uL/L trans-caryophyllene.

[00269] Samples were analyzed by GC/MS as described in Example 10.

[00270] Example 15

[00271] This example describes the production of amorpha-4,11-diene via the DXP pathway in an *Escherichia coli* host strain.

[00272] Seed cultures of host strains B003, B617, B618, and B619 were established by adding a stock aliquot of each strain to separate 125 mL flasks containing 25 mL M9-MOPS and antibiotics as detailed in Table 1, and by growing the cultures overnight.

[00273] The seed cultures were used to inoculate at an initial OD₆₀₀ of approximately 0.05, separate 250 mL flasks containing 40 mL M9-MOPS medium, 45 ug/mL thiamine, micronutrients, 1.00E-5 mol/L FeSO₄, 0.1 M MOPS, 0.5% yeast extract, 20 g/L of D-glucose, and antibiotics. Cultures were incubated at 30°C in a humidified incubating shaker at 250 rpm until they reached an OD₆₀₀ of 0.2 to 0.3, at which point the production of amorpha-4,11-diene in the host cells was induced by adding 40 uL of 1M IPTG to the culture medium.

[00274] At the time of induction, the cultures were overlain with 8 mL of an organic overlay to capture the amorpha-4,11-diene. Samples were taken at various time points, and amorpha-4,11-diene was extracted and analyzed by GC/MS as described in Example 10. Experiments were performed using 2 independent clones of each host strain, and results were averaged. Deviation between samples was found to be less than 10%.

[00275] Example 16

[00276] This example describes the production of 3-methyl-but-3-en-1-ol and 3-methyl-but-2-en-1-ol in *Escherichia coli* host strains.

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[00277] Seed cultures of host strains B286, B287, B288, and B291 were established by streaking out a stock aliquot of each strain on LB agar containing antibiotics as detailed in Table 1. Three independent colonies were picked for each strain, and each colony was inoculated into 7 mL of LB media containing antibiotics. The cultures were grown overnight at 37°C on a rotary shaker at 250 rpm until late exponential phase. The cultures were then inoculated at an OD₆₀₀ of approximately 0.05, into a 250 mL flask containing 40 ml of M9-MOPS, 2% glucose, 0.5% yeast extract, and antibiotics. The cultures were grown overnight at 37°C on a rotary shaker at 250 rpm until they reached an OD₆₀₀ of approximately 0.2, at which point they were induced by adding 40 uL of 1 M IPTG. The cultures were grown for 72 hours at 30°C on a rotary shaker at 250 rpm. One to two times per day, the OD₆₀₀ of each culture was measured, and a 700 uL sample was removed. To extract the 3-methyl-but-3-en-1-ol and 3-methyl-but-2-en-1-ol from the culture broth, 600 uL of ethyl acetate was added to 300 uL of each removed sample. The sample was then vortexed for 15 minutes, and 400 uL of the upper ethyl acetate phase was transferred to a clean glass vial for analysis.

[00278] The samples were analyzed on a Hewlett-Packard 6890 gas chromatograph/mass spectrometer (GC/MS). A 1 uL sample was separated on the GC using a DB-5 column (Agilent Technologies, Inc., Palo Alto, CA) and helium carrier gas. The temperature program for the analysis was as follows: 60°C for 3 minutes, increasing temperature at 60°C/minute to a temperature of 300°C, and a hold at 300°C for 2 minutes. The total run time was 9 minutes. The resolved samples were analyzed by a Hewlett-Packard model 5973 mass selective detector. Previous mass spectra demonstrated that 3-methyl-3-buten-1-ol and 3-methyl-2-buten-1-ol have a retention time of 2.067 minutes using this GC protocol. To focus detection on 3-methyl-but-3-en-1-ol and 3-methyl-but-2-en-1-ol, a selective-ion-monitoring method was employed that monitors only ions 56 and 68 in 3-methyl-but-3-en-1-ol and 3-methyl-but-2-en-1-ol.

[00279] Example 17

[00280] This example describes the production of amorpha-4,11-diene by a *Saccharomyces cerevisiae* host strain.

[00281] The generation of host strain EPY224 is described in Ro *et al.* (*Nature* 440: 940-943; 2006) and in PCT Patent Publication WO2007/005604. Host strain EPY224 was cured of expression plasmid pRS425ADS by growth in YPD medium (Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, 2005 ed., ISBN 0-87969-728-8), plating for single colonies on YPD agar, and then patching single colonies onto CSM-Met His agar and CSM-Met Leu agar. Clones that grew on CSM-Met His agar but not on CSM-

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Met Leu agar were cured (i.e., had lost the plasmid pRS425ADS). One such clone was designated EPY300. EPY300 was transformed with expression plasmid pRS425-ADS-LEU2d, a plasmid identical to pRS425-ADS except that instead of LEU2 it contains a LEU2d selection marker (Erhart and Hollenberg (1983) *J. Bacteriol.* 156: 625-635) yielding host strain Y185.

[00282] Y185 host cell transformants were selected on synthetic defined media, containing 2% glucose and all amino acids except histidine, leucine, and methionine (CSM-glucose; MP Biomedicals, Solon, OH). The host strain EPY300 is auxotrophic for leucine biosynthesis (*leu2*), but expression plasmid pRS425-ADS-LEU2d in Y185 restores leucine prototrophy (*LEU2*). Single colonies were patched onto selective medium (CSM-glucose-histidine, leucine, methionine), and grown for 2 days. The cells were scraped from the plate and transferred to 1 mL of 25% (v/v) glycerol in a cryotube. The suspension was mixed, and then stored at -80°C.

[00283] Seed flasks of host strain Y185 were established by adding a stock aliquot of the strain to a 125 mL flask containing 25 mL of CSM-glucose lacking leucine and methionine, and by growing the cultures overnight. The cultures were used to inoculate at an initial OD₆₀₀ of approximately 0.05 a 250 mL baffled flask containing 40 mL of synthetic defined media lacking leucine, and containing 0.2% glucose, 1.8% galactose, and 1 mM methionine. The culture was incubated at 30°C on a rotary shaker at 200 rpm. Because the presence of glucose in the media prevents induction of the *GALI* promoter by galactose, amorpho-4,11-diene production was not induced until the cells had used up the glucose in the media and had switched to using galactose as their main carbon source. At the time of inoculation, the cultures were overlain with 8 mL of an organic overlay to capture the amorpho-4,11-diene. Samples were taken at 72 hours by transferring 5 uL of the organic solvent layer to a clean glass vial containing 500 uL ethyl acetate containing a known concentration of beta- or trans-caryophyllene as an internal standard.

[00284] The organic overlay/ethyl acetate samples were analyzed on a Hewlett-Packard 6890 gas chromatograph/mass spectrometer (GC/MS) as described in Example 10.

[00285] After 72 hours of growth, 3 yeast cultures were found to produce 60.68, 54.48, and 59.25 mg/L amorpho-4,11-diene.

[00286] Example 19

[00287] This example describes the production of amorpho-4,11-diene in an *Saccharomyces cerevisiae* host strain where the host strain includes a native mevalonate

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pathway as well as a heterologous mevalonate pathway that is under control of a heterologous regulatory control.

[00288] Yeast strains CEN.PK2-1C (Y002) (MATA; ura3-52; trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2) and CEN.PK2-1D (Y003) (MAT α ; ura3-52; trp1-289; leu2-3,112; his3 Δ 1; MAL2-8C; SUC2) (J. P. van Dijken *et al.*, *Enzyme Microb Technol* **26**, 706 (Jun 1, 2000) were cultivated in either standard rich medium (YPD) or in defined synthetic medium (. D. Rose, F. Winston, P. Heiter, *Methods in yeast genetics: a laboratory course manual*. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1990) lacking appropriate nutrients allowing for selection of integrative transformants, plasmid retention, and meiotic progeny.

[00289] DNA-mediated transformations into *S. cerevisiae* were conducted using the lithium acetate procedure as described by R. H. Schiestl, R. D. Gietz, *Curr Genet* **16**, 339 (Dec, 1989). All gene disruptions and replacements were confirmed by phenotypic analysis, colony polymerase chain reaction ("PCR") and sequencing of amplified genomic DNA. Plasmids pAM489-pAM498 were constructed using the pCR 2.1 (Invitrogen, Carlsbad CA) and are schematically described by Figure 7A-C and Table 6. The HISMX marker sequences are described in M. S. Longtine *et al.*, *Yeast* **14**, 953 (Jul, 1998). Propagation of plasmid DNA was performed in *Escherichia coli* strain DH5 α .

Table 6

Strain	5'HR	Gene #1	Crick Promoter	Watson Promoter	Gene #2	Genetic Marker	3'HR
pAM489	TRP1	tHMGR	GAL1	GAL10	ERG20	TRP1	TRP1
pAM490	TRP1	tHMGR	CUP1	CUP1	ERG20	TRP1	TRP1
pAN491	URA3	tHMGR	GAL1	GAL10	ERG13	URA3	URA3
pAM492	URA3	IDI1	CUP1	CUP1	tHMGR	URA3	URA3
pAM493	ADE1	tHMGR	GAL1	GAL10	IDI1	ADE1	URA3
pAM494	ADE1	tHMGR	CUP1	CUP1	IDI1	ADE1	ADE1
pAM495	HIS3	ERG12	GAL1	GAL10	ERG10	HISMx	HIS3
pAM496	HIS3	ERG12	CUP1	CUP1	ERG10	HISMx	HIS3
pAM497	LEU2	ERG19	GAL1	GAL1	ERG8	HISMx	LEU2
pAM498	LEU2	ERG19	CUP1	CUP1	ERG8	HISMx	LEU2

[00290] *S. cerevisiae* strains Y002 and Y003 were prepared for introduction of inducible mevalonate pathway genes by the following. The *ERG9* promoter was replaced with the *S. cerevisiae* *MET3* promoter by PCR amplification of the KanMX-PMET3 region from pAM328 (SEQ ID NO: 43) using primers 50-56-pw100-G (SEQ ID NO: 44) and 50-56-pw101-G (SEQ ID NO: 45) containing 45 basepairs of homology to the native *ERG9* promoter. 10 μ g of the resulting PCR product was transformed into exponentially growing

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Y002 and Y003 strains using 40% w/w polyethelene glycol 3350 (Sigma-Aldrich St Louis, MO), 100 mM lithium acetate (Sigma), 10 μ g Salmon Sperm DNA (Invitrogen) and incubation at 30°C for 30 minutes followed by a 42°C heat shock for 30 minutes (as described by Schiestl & Gietz, *Curr. Genet.* 16: 339 (1989)). Positive recombinants were identified by their ability to grow on rich medium containing 0.5 μ g/ml Geneticin (Invitrogen Co, Carlsbad, CA) and confirmed by diagnostic PCR. The resultant clones were given the designation Y93 (MAT A) and Y94 (MAT alpha). Next, the *ADE1* open reading frame was replaced with the *Candida glabrata LEU2* gene (*CgLEU2*). The 3.5KB *CgLEU2* genomic locus was amplified from *C. glabrata* genomic DNA (ATCC, Manassas, VA) using primers 61-67-CPK066-G (SEQ ID NO: 46) and 61-67-CPK067-G (SEQ ID NO: 47) containing 50 basepairs of flanking homology to the *ADE1* open reading frame (ORF). 10 μ g of the resulting PCR product was transformed into exponentially growing Y93 and Y94 as described above. *ade1*- strains were selected for growth in the absence of leucine supplementation and confirmed by diagnostic PCR. The resultant clones were given the designation Y176 (MAT A) and Y177 (MAT alpha).

[00291] To generate *S. cerevisiae* strain Y188, 2 μ g's of plasmid DNA from pAM491 (SEQ ID NO: 48) and pAM495 (SEQ ID NO:49), respectively, were digested overnight with PmeI (New England Biolabs, Beverly, MA) and introduced into exponentially growing Y176 as described above. Positive recombinants were selected for by growth on medium lacking uracil and histidine. Integration into the correct genomic locus was confirmed by diagnostic PCR.

[00292] To generate *S. cerevisiae* strain Y189, 2 μ g's of plasmid DNA from pAM489 (SEQ ID NO: 50) and pAM497 (SEQ ID NO: 51), respectively, were digested overnight with PmeI and introduced into exponentially growing Y177 as described above. Positive recombinants were selected for by growth on medium lacking tryptophan and histidine. Integration into the correct genomic locus was confirmed by diagnostic PCR.

[00293] Approximately 1 X 10⁷ cells from Y188 and Y189 were mixed on a YPD medium plate for 6 hours at room temperature to allow for mating. The mixed cell culture was then plated to medium lacking histidine, uracil and tryptophan to select for growth of diploid cells. 2 μ g of plasmid DNA from pAM493 (SEQ ID NO: 52) was digested overnight with PmeI and introduced into exponentially growing diploid cells as described above. Positive recombinants were selected for by growth on medium lacking adenine. Integration

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into the correct genomic locus was confirmed by diagnostic PCR. The resultant strain was given the designation Y238.

[00294] To generate haploid strains containing the full complement of introduced genes, Y238 was sporulated in 2% potassium acetate and 0.02% raffinose liquid medium. Approximately 200 genetic tetrads (tetrads are four-spored meiotic products) were isolated using a Singer Instruments MSM300 series micromanipulator (Singer Instrument Co, LTD. Somerset, UK). Independent genetic isolates containing the appropriate complement of introduced genetic material were identified by their ability to grow in the absence of adenine, histidine, uracil, and tryptophan. Integration of all introduced DNA was confirmed by diagnostic PCR. The resultant strains were given the designation Y210 (MAT A) and Y211 (MAT alpha).

[00295] 2 μ g of plasmid DNA from pAM426 (SEQ ID NO:53), containing *S. cerevisiae* condon optimized Amorphadeine Synthase (ADS) expressed from the *S. cerevisiae* *GALI* promoter, was introduced into exponentially growing Y210 and Y211 as described above. *S. cerevisiae* strains that contained the pAM426 plasmid were selected for by their ability to grow in the absence of leucine supplementation. The resultant strains were given the designation Y225 (MAT A) and Y227 (MAT alpha).

[00296] 2 μ g of plasmid DNA from pAM322 (SEQ ID NO: 54), containing *S. cerevisiae* condon optimized Amorphadeine Synthase (ADS) and cytochrome P450 monooxygenase (AMO) expressed from the *S. cerevisiae* *GALI* and the cytochrome P450 oxidoreductase (CPR) expressed from the *S. cerevisiae* *GAL10* promoter, was introduced into exponentially growing Y210 and Y211 as described above. *S. cerevisiae* strains that contained the pAM322 plasmid were selected for by their ability to grow in the absence of leucine supplementation. The resultant strains were given the designation Y222 (MAT A) and Y224 (MAT alpha).

[00297] Example 19

[00298] This example describes the production of α -farnesene or β -farnesene in *Escherichia coli* host strains.

[00299] Seed cultures of host strains B552 and B592 were established by adding a stock aliquot of each strain to a 125 mL flask containing 25 mL M9-MOPS, 0.8% glucose, 0.5% yeast extract, and antibiotics as detailed in Table 1, and by growing the cultures overnight.

[00300] The seed cultures were used to inoculate at an initial OD₆₀₀ of approximately 0.05, 250 mL flasks containing 40 mL M9-MOPS, 2% glucose, 0.5% yeast extract, and

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antibiotics. Cultures were incubated at 30°C on a rotary shaker at 250 rpm until they reached an OD₆₀₀ of approximately 0.2, at which point the production of α -farnesene or β -farnesene in the host cells was induced by adding 40 μ L of 1 M IPTG. At the time of induction, the cultures were overlain with 8 mL of an organic overlay to capture the α -farnesene. Samples were taken every 24 hours up to 120 hours (total of 5 time points) by transferring 2 μ L to 10 μ L of the organic overlay layer to a clean glass vial containing 1 mL ethyl acetate spiked with trans-caryophyllene as an internal standard. In addition, 1 mL aliquots of the cultures were spun down, cell pellets were resuspended in 250 μ L sterile water, and the cell suspensions were transferred to a glass vial containing 1 mL ethyl acetate spiked with trans-caryophyllene as an internal standard. In addition, 0.5 mL aliquots of the whole culture broth were added to a glass vials containing 1 mL ethyl acetate spiked with trans-caryophyllene as an internal standard. The whole culture broth samples were extracted in the ethyl acetate by vortexing the glass vials for 10 minutes, after which 600 μ L of the ethyl acetate extraction was transferred to a clean glass vial.

[00301] The organic overlay/ethyl acetate samples and the ethyl acetate-extracted whole culture broth samples were analyzed on an Agilent 6890N gas chromatograph equipped with an Agilent 5975 mass spectrometer (GC/MS) in full scan mode (50-500 m/z). To expedite run times, the temperature program and column matrix was modified to achieve optimal peak resolution and the shortest overall runtime. A 1 μ L sample was separated using a HP-5MS column (Agilent Technologies, Inc., Palo Alto, CA) and helium carrier gas. The temperature program for the analysis was as follows: 150°C hold for 3 minutes, increasing temperature at 25°C/minute to a temperature of 200°C, increasing temperature at 60°C/minute to a temperature of 300°C, and a hold at 300°C for 1 minute. Previous mass spectra demonstrated that the β -farnesene synthase product was β -farnesene, and that β -farnesene had a retention time of 4.33 minutes using this GC protocol. Farnesene titers were calculated by comparing generated peak areas against a quantitative calibration curve of purified β -farnesene (Sigma-Aldrich Chemical Company, St. Louis, MO) in trans-caryophyllene-spiked ethyl acetate.

[00302] Host strain B592 produced approximately 400 mg/L of α -farnesene at 120 hours (averaged over 3 independent clones), and had a maximal specific productivity of approximately 46 mg/L/OD₆₀₀. Host strain B552 produced approximately 1.1 g/L of β -farnesene at 120 hours (averaged over 3 independent clones), and had a maximal specific productivity of approximately 96 mg/L/OD₆₀₀ (1 representative clone).

[00303] Example 20

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[00304] This example describes the production of β -farnesene via the DXP pathway in an *Escherichia coli* host strain.

[00305] Seed cultures of host strains B650, B651, B652, and B653 were established by adding a stock aliquot of each strain to separate 125 mL flasks containing 25 mL M9-MOPS and antibiotics as detailed in Table 1, and by growing the cultures overnight.

[00306] The seed cultures were used to inoculate at an initial OD₆₀₀ of approximately 0.05 separate 250 mL flasks containing 40 mL M9-MOPS minimal medium, 45 ug/mL thiamine, micronutrients, 1.00E-5 mol/L FeSO₄, 0.1 M MOPS, 0.5% yeast extract, 20 g/L of D-glucose, and antibiotics. The cultures were incubated at 30°C in a humidified incubating shaker at 250 rpm until they reached an OD₆₀₀ of 0.2 to 0.3, at which point the production of β -farnesene in the host cells was induced by adding 40 uL of 1 M IPTG to the culture medium. At the time of induction, the cultures were overlain with 8 mL of an organic overlay to capture the β -farnesene. Samples were taken at various time points by transferring 100 uL samples of the upper organic overlay layer to a clean tube. The tube was centrifuged to separate out any remaining cells or media, and 10 uL of the organic overlay samples were transferred into 500 uL ethyl acetate spiked with beta- or trans-caryophyllene as an internal standard in clean glass GC vials. The mixtures were vortexed for 30 seconds, and then analyzed as described in Example 18. *Escherichia coli* host strain B653 produced approximately 7 mg/g DCW β -farnesene.

[00307] Example 21

[00308] This example describes the production of α -farnesene or β -farnesene in a *Saccharomyces cerevisiae* host strain.

[00309] Strain EPY300 was generated by removing the expression plasmid from *Saccharomyces cerevisiae* strain EPY224 (Ro *et al.* (2006) *Nature* 440: 940-943; PCT Patent Publication WO2007/005604) by culturing in rich medium. Strain EPY300 was then transformed with expression plasmids pRS425-FSA or pR425-FSB, yielding host strains Y166 and Y164, respectively.

[00310] Host cell transformants were selected on synthetic defined media, containing 2% glucose and all amino acids except leucine (SM-glu). The host strain EPY300 was auxotrophic for leucine biosynthesis (*leu2*), but expression plasmid pRS425-FSA or pRS425-FSB restores leucine prototrophy (*LEU2*). Single colonies were transferred to culture vials containing 5 mL of liquid SM-glu lacking leucine. The cultures were incubated by shaking at 30°C until growth reaches stationary phase. The cells were stored at -80°C in cryo-vials in 1 mL frozen aliquots made up of 400 μ L 50% glycerol and 600 μ L liquid culture.

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[00311] Seed cultures were established by adding a stock aliquot to a 125 mL flask containing 25 mL SM-glu lacking leucine, and growing the cultures overnight. The seed cultures were used to inoculate at an initial OD₆₀₀ of approximately 0.05 250 mL baffled flasks containing 40 mL of synthetic defined media lacking leucine, 0.2% glucose, and 1.8% galactose. Cultures were incubated at 30°C on a rotary shaker at 200 rpm. Because the presence of glucose in the media prevents induction of the *Gall* promoter by galactose, farnesene production was not induced until the cells use up the glucose in the media and switch to using galactose as their main carbon source. The cultures are overlain with 8 mL methyl oleate or isopropyl myristate. Samples were taken once every 24 hours by transferring 2-10 uL of the organic solvent layer to a clean glass vial containing 500 uL ethyl acetate containing a known concentration of beta- or trans-caryophyllene as an internal standard. In addition, 0.5 mL aliquots of the whole culture broth were added to a glass vials containing 1 mL ethyl acetate spiked with trans-caryophyllene as an internal standard. The whole culture broth samples were extracted in the ethyl acetate by vortexing the glass vials for 10 minutes, after which 600 uL of the ethyl acetate extraction was transferred to a clean glass vial.

[00312] Host strain Y166 produced approximately 9.8 mg/L of α -farnesene at 120 hours (averaged over 3 independent clones), and had a maximal specific productivity of approximately 3 mg/L/OD₆₀₀ (1 representative clone). Host strain Y164 produced approximately 56 mg/L of β -farnesene at 120 hours (averaged over 3 independent clones), and had a maximal specific productivity of approximately 20 mg/L/OD₆₀₀ (1 representative clone).

[00313] Example 22

[00314] This example describes the production of γ -terpinene, α -pinene, and terpinolene in *Escherichia coli* host strains.

[00315] Seed cultures of host strains for production of γ -terpinene (*E. coli* DH1-T1r [pMevT, pMevB-Gpps, pAM445]), α -pinene (*E. coli* DH1-T1r [pMevT, pMevB-Gpps, pAM443 or pAM442]) or terpinolene (*E. coli* DH1-T1r [pMevT, pMevB-Gpps, pAM444]) were established by adding a stock aliquot of each strain to separate 125 mL flasks containing 25 mL M9-MOPS, 2% glucose, 0.5% yeast extract, and antibiotics as detailed in Table 1, and by growing the cultures overnight to late exponential phase.

[00316] The seed cultures were used to inoculate at an initial OD₆₀₀ of approximately 0.05, 250 mL flasks containing 40 mL M9-MOPS, 2% glucose, 0.5% yeast extract, and antibiotics. At time of inoculation, the cultures were also overlain with 4 mL hexadecane.

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Cultures were incubated at 30°C on a rotary shaker at 200 - 250 rpm until they reached an OD₆₀₀ of approximately 0.2, at which point the production of the compound of interest in the host cells in the host cells was induced by adding 40 uL of 1 M IPTG. Samples were taken once per day for 96 hours by transferring 200 uL of the hexadecane layer to a 0.6 mL microfuge tube. For analysis, the hexadecane overlay was diluted 1:1 or 1:10 with ethyl acetate spiked with trans-caryophyllene as an internal standard in a 1.8 mL GC vial. In addition, 1 mL aliquots of the cultures were spun down, cell pellets were resuspended in 250 uL sterile water, and the cell suspensions were transferred to a glass vial containing 1 mL ethyl acetate spiked with trans-caryophyllene as an internal standard. The cell pellets were extracted in the ethyl acetate by vortexing the glass vials for 15 minutes, after which 500 uL of the ethyl acetate extraction was transferred to a clean glass vial.

[00317] The hexadecane/ethyl acetate samples and the ethyl acetate-extracted cell pellet samples were analyzed on an Agilent 6890N gas chromatograph equipped with an Agilent 5975 mass spectrometer (GC/MS) in full scan mode (50-500 m/z). To expedite run times, the temperature program and column matrix was modified to achieve optimal peak resolution and the shortest overall runtime. A 1 µL sample was split (a split ratio between 1:2 and 1:50 was selected based on sample concentration) and then separated using a HP-5MS column (Agilent Technologies, Inc., Palo Alto, CA) and helium carrier gas. The temperature program for the analysis was as follows: 75°C hold for 3 minutes, increasing temperature at 20°C/minute to a temperature of 115°C, increasing temperature at 60°C/minute to a temperature of 300°C, and a hold at 300°C for 0.5 minute. The various products, γ-terpinene, α-pinene, and terpinolene were observed at 5.4, 4.1, 5.4, and 5.9 minutes, respectively. Titters were calculated by comparing generated peak areas against a quantitative calibration curve of purified standards in trans-caryophyllene-spiked ethyl acetate.

[00318] Example 23

[00319] This example describes the production of linalool, limonene, β-pinene, β-phellandrene, carene, or sabinine in *Escherichia coli* host strains.

[00320] Seed cultures are established by adding a stock aliquot of each strain to separate 125 mL flasks containing 25 mL M9-MOPS, 0.5% yeast extract, 2% glucose, and antibiotics as detailed in Table 1, and by growing the cultures overnight.

[00321] The seed cultures are used to inoculate at an initial OD₆₀₀ of approximately 0.05, 250 mL baffled flasks containing 40 mL M9-MOPS, 0.5% yeast extract, 2% glucose, and antibiotics. Cultures are incubated at 30°C on a rotary shaker at 250 rpm until they reach

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an OD₆₀₀ of approximately 0.2, at which point the production of the compound of interest in the host cells is induced by adding 40 ul of 1 M IPTG to the culture medium. The compound of interest is separated from the culture medium through solvent-solvent extraction, or by settling and decantation if the titer of the compound of interest is large enough to saturate the media and to form a second phase.

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DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS**What is claimed is:**

1. A bio-organic compound production system comprising:
 - a. a vessel having a capacity of at least 100 liters;
 - b. an aqueous medium, within the vessel, forming a first phase;
 - c. a plurality of host cells, within the aqueous medium, capable of making at least one bio-organic compound; and,
 - d. a liquid organic second phase, comprising the at least one bio-organic compound, in contact with the first phase.
2. The system as in claim 1 wherein the at least one bio-organic compound is a C₅ bio-organic compound.
3. The system as in claim 1 wherein the at least one bio-organic compound is a C₁₀ bio-organic compound.
4. The system as in claim 1 wherein the at least one bio-organic compound is a C₁₅ bio-organic compound.
5. The system as in claim 1 wherein the at least one bio-organic compound is a C₂₀ bio-organic compound.
6. The system as in claim 1 wherein the at least one bio-organic compound is a C₂₀₊ bio-organic compound.
7. The system as in claim 1 wherein the at least one bio-organic compound is an isoprenoid compound.
8. The system as in claim 1 wherein the vessel has a capacity of at least 1000 liters.
9. The system as in claim 1 wherein the vessel has a capacity of at least 10,000 liters.

10. The system as in claim 1 wherein the vessel has a capacity of at least 50,000 liters.
11. The system as in claim 1 wherein the vessel has a capacity of at least 100,000 liters.
12. The system as in claim 1 wherein the vessel has a capacity of at least 500,000 liters.
13. The system as in claim 1 wherein the vessel has a capacity of at least 1,000,000 liters.
14. The system as in claim 1 wherein the organic second phase comprises the bio-organic compound.
15. The system as in claim 1 wherein the organic second phase comprises at least 90% bio-organic compound.
16. A system for making C₅-C₂₀ isoprenoid compounds comprising:
 - a. a vessel having a capacity of at least 100 liters;
 - b. an aqueous medium, within the vessel, comprising a first phase;
 - c. a plurality of host cells, within the aqueous medium, capable of making at least one isoprenoid; and,
 - d. a liquid organic second phase, comprising the at least one isoprenoid, in contact with the first phase.
17. The system of claim 16 wherein the isoprenoid compound is a hemiterpene.
18. The system of claim 16 wherein the isoprenoid compound is a monoterpene.
19. The system of claim 16 wherein the isoprenoid compound is a sesquiterpene.
20. The system of claim 16 wherein the isoprenoid compound is a diterpene.
21. A method for producing a bio-organic compound comprising:
 - a. culturing in an aqueous medium a plurality of host cells that produce at least one bio-organic compound wherein the aqueous medium and host cells comprise a first phase;
 - b. forming a liquid organic second phase comprising the at least one bio-organic compound in contact with the second phase;
 - c. separating at least a portion of the second phase from the first phase; and,

- d. isolating the at least one bio-organic compound from the second phase.
22. The method of claim 21 wherein the organic second phase is formed by induction.
23. The method of claim 21 wherein the organic second phase is separated by decanting the second phase from the first phase.
24. The system as in claim 21 wherein the at least one bio-organic compound is a C₅ bio-organic compound.
25. The system as in claim 21 wherein the at least one bio-organic compound is a C₁₀ bio-organic compound.
26. The system as in claim 21 wherein the at least one bio-organic compound is a C₁₅ bio-organic compound.
27. The system as in claim 21 wherein the at least one bio-organic compound is a C₂₀ bio-organic compound.
28. The system as in claim 21 wherein the at least one bio-organic compound is a C₂₀₊ bio-organic compound.
29. The method of claim 21 wherein the isolation step comprises adsorption.
30. The method of claim 21 where the isolation step comprises distillation.
31. The method of claim 21 wherein the isolation step comprises gas-liquid extraction.
32. The method of claim 21 wherein the isolation step comprises liquid-liquid extraction.
33. The method of claim 21 wherein the isolation step comprises ultrafiltration.
34. The method of claim 21 wherein the at least one bio-organic compound is an isoprenoid compound.

35. A method for producing a C₅-C₂₀ isoprenoid compound comprising:
- a. culturing in an aqueous medium a plurality of host cells that produce at least one C₅-C₂₀ isoprenoid compound in a vessel having a capacity of at least 100 liters;
 - b. forming an organic phase comprising the at least one C₅-C₂₀ isoprenoid compound;
 - c. separating at least a portion of the organic phase from the aqueous medium; and,
 - d. isolating the at least one C₅-C₂₀ isoprenoid compound from the organic phase.
36. The method of claim 35 wherein the at least one isoprenoid compound is a hemiterpene.
37. The method of claim 35 wherein the at least one isoprenoid compound is a monoterpene.
38. The method of claim 35 wherein the at least one isoprenoid compound is a sesquiterpene.
39. The method of claim 35 wherein the at least one isoprenoid compound is a diterpene.
40. The method of claim 35 wherein the organic phase is formed by induction.
41. The method of claim 35 wherein the organic phase is separated by decanting the organic phase from the aqueous phase.
42. The method of claim 35 wherein the isolation step comprises adsorption.
43. The method of claim 35 wherein the isolation step comprises distillation.
44. The method of claim 35 wherein the isolation step comprises gas-liquid extraction.
45. The method of claim 35 wherein the isolation step comprises liquid-liquid extraction.
46. The method of claim 35 wherein the isolation step comprises ultrafiltration.

47. A fuel composition production system comprising:
- a. one or more fermentation systems comprising:
 - i) at least one vessel having a capacity of at least 100 liters;
 - ii) an aqueous medium, within the at least one vessel, comprising a first phase;
 - iii) a plurality of host cells, within the aqueous medium, capable of making, producing or synthesizing at least one bio-organic compound; and,
 - iv) a liquid organic second phase comprising the at least one bio-organic compound in contact with the first phase;
 - b. one or more first phase separation systems whereby the first phase and the second organic phase or one or more components of the second organic phase are separated;
 - c. optionally one or more second phase separation systems whereby the at least one bio-organic compound is separated from the second organic phase;
 - d. optionally one or more reactors or vessels wherein the at least one bio-organic compound is chemically or biologically modified;
 - e. optionally one or more purification systems whereby the bio-organic compound or the modified bio-organic compound is purified or further purified;
 - f. optionally one or more blending vessels or systems for blending the at least one bio-organic compound with one or more additional fuel components; and
 - g. optionally one or more further purification systems whereby the blend of the at least one bio-organic compound and the one or more additional fuel components is purified or further purified.
48. A method of making a fuel composition comprising:
- a. culturing in an aqueous medium a plurality of host cells that produce, make or synthesize at least one bio-organic compound wherein the aqueous medium comprises a first phase;
 - b. forming a liquid organic second phase comprising the at least one bio-organic compound in contact with the first phase;
 - c. separating at least a portion of the second phase from the first phase;
 - d. isolating the at least one bio-organic compound from the second phase;

- e. optionally chemically or biologically modifying the at least one bio-organic compound;
- f. optionally purifying the bio-organic compound or the modified bio-organic compound;
- g. optionally blending the at least one bio-organic compound with one or more additional fuel components; and
- h. optionally purifying the blend of the one or more bio-organic compounds and the one or more additional fuel components.

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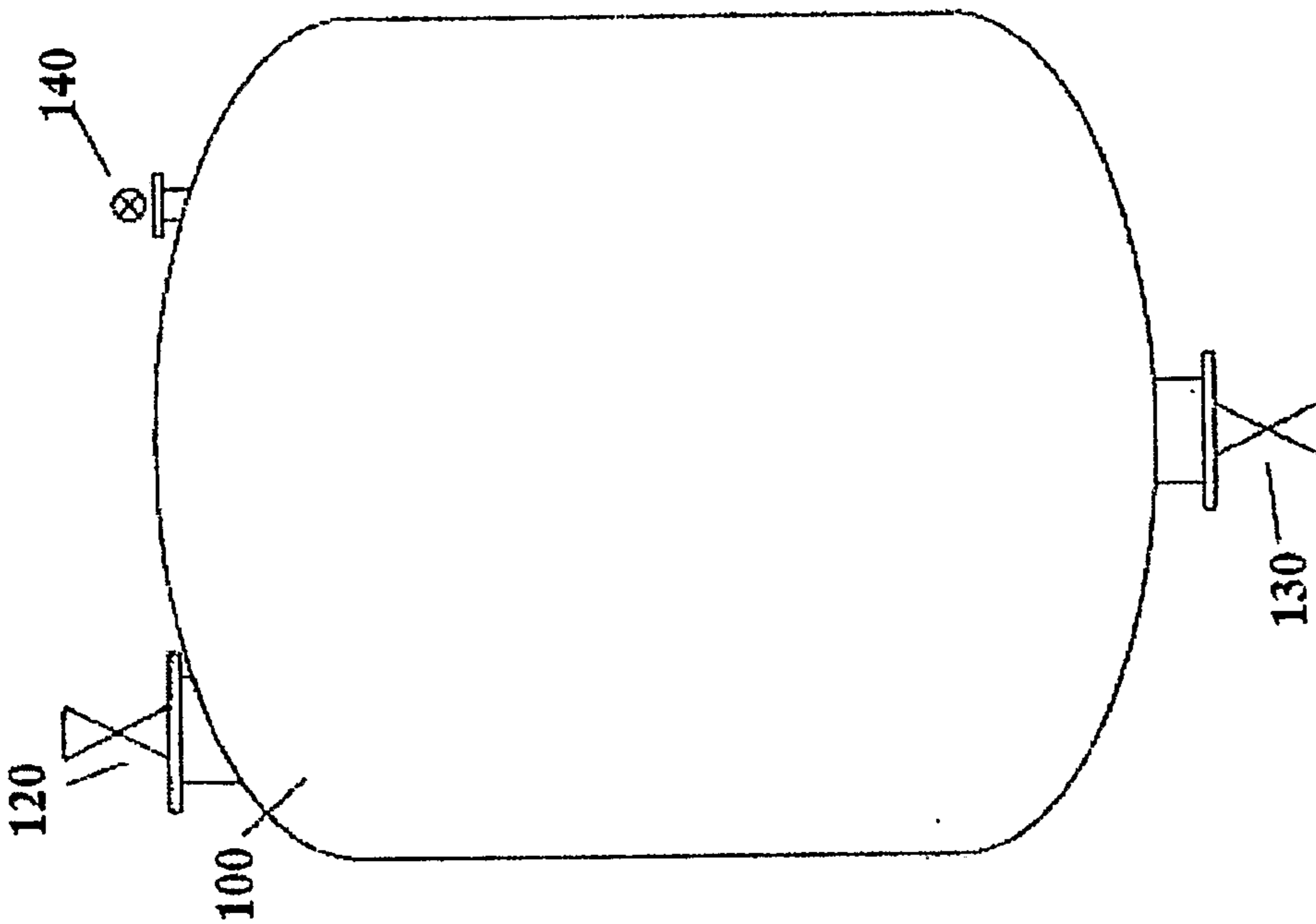


Figure 1

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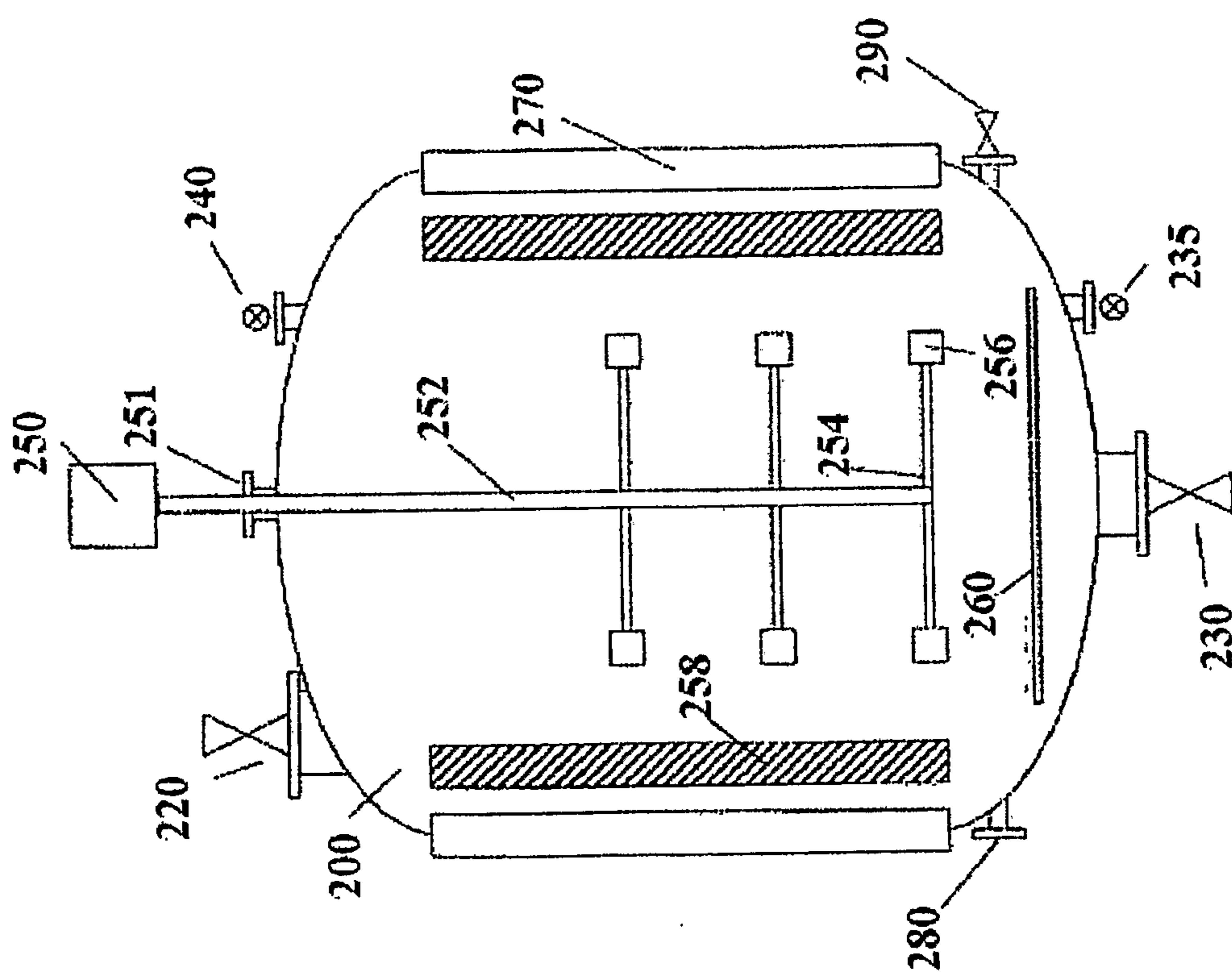


FIGURE 2

Figure 3
Mevalonate pathway

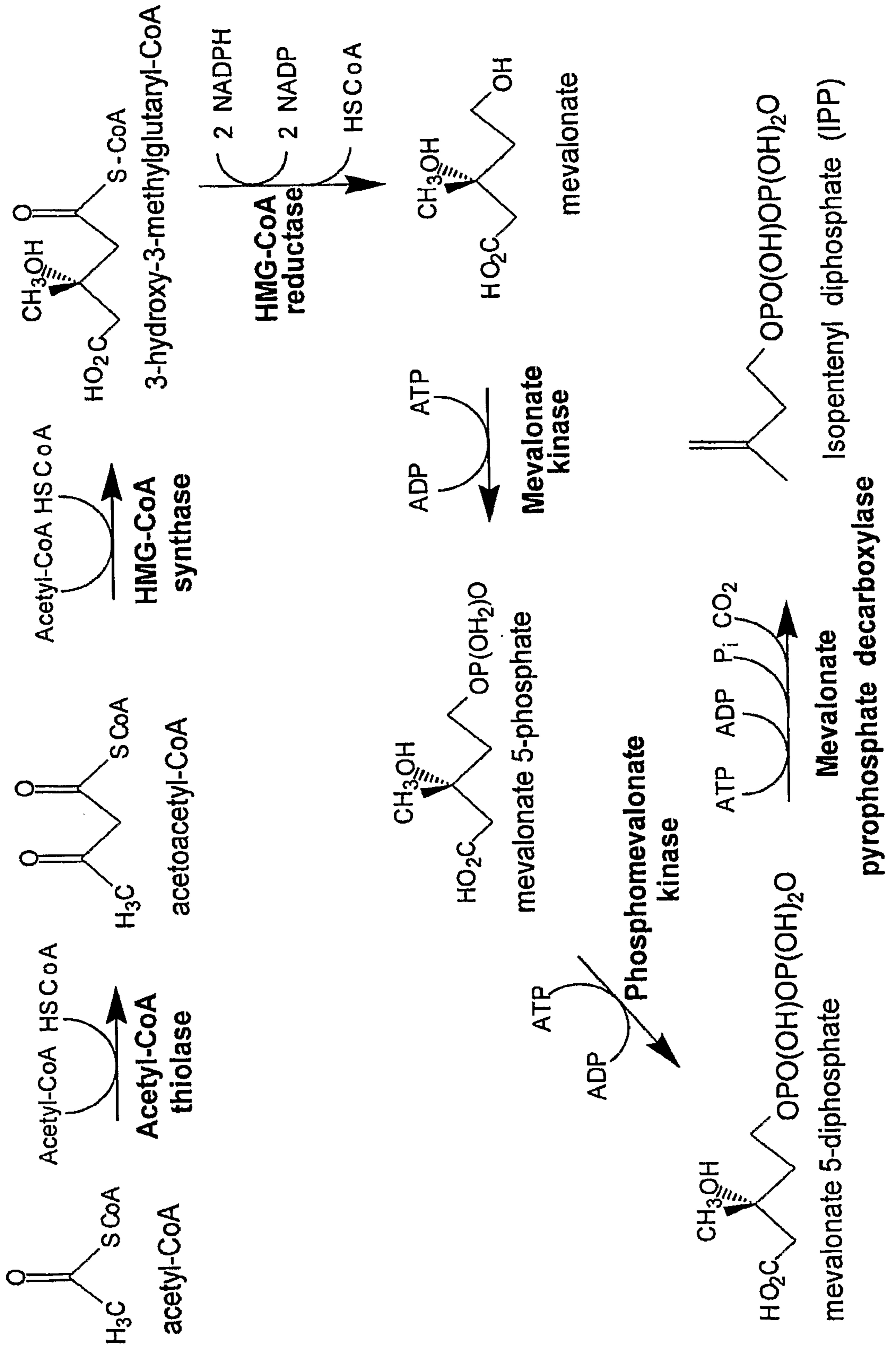
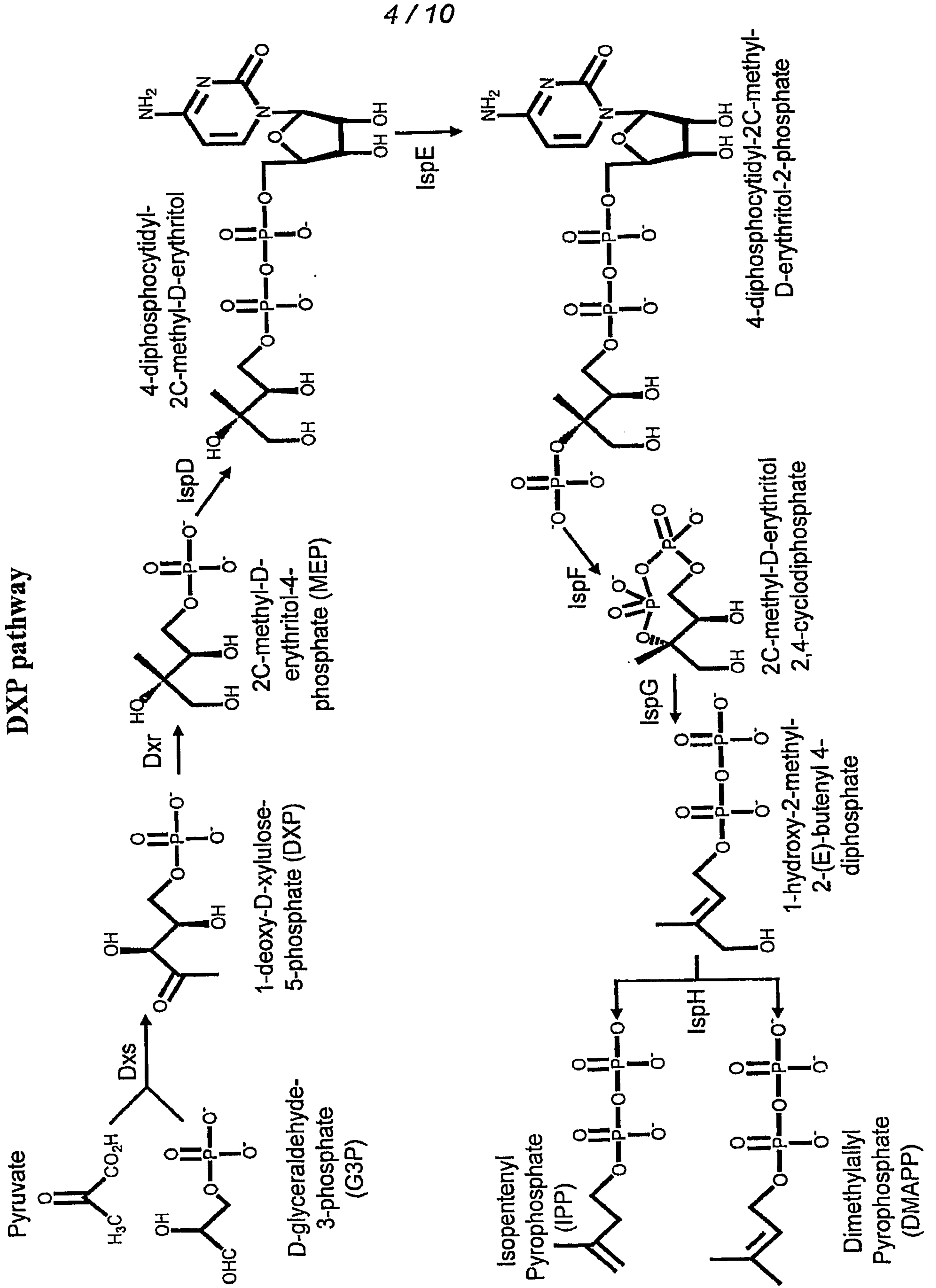


Figure 4
DXP pathway



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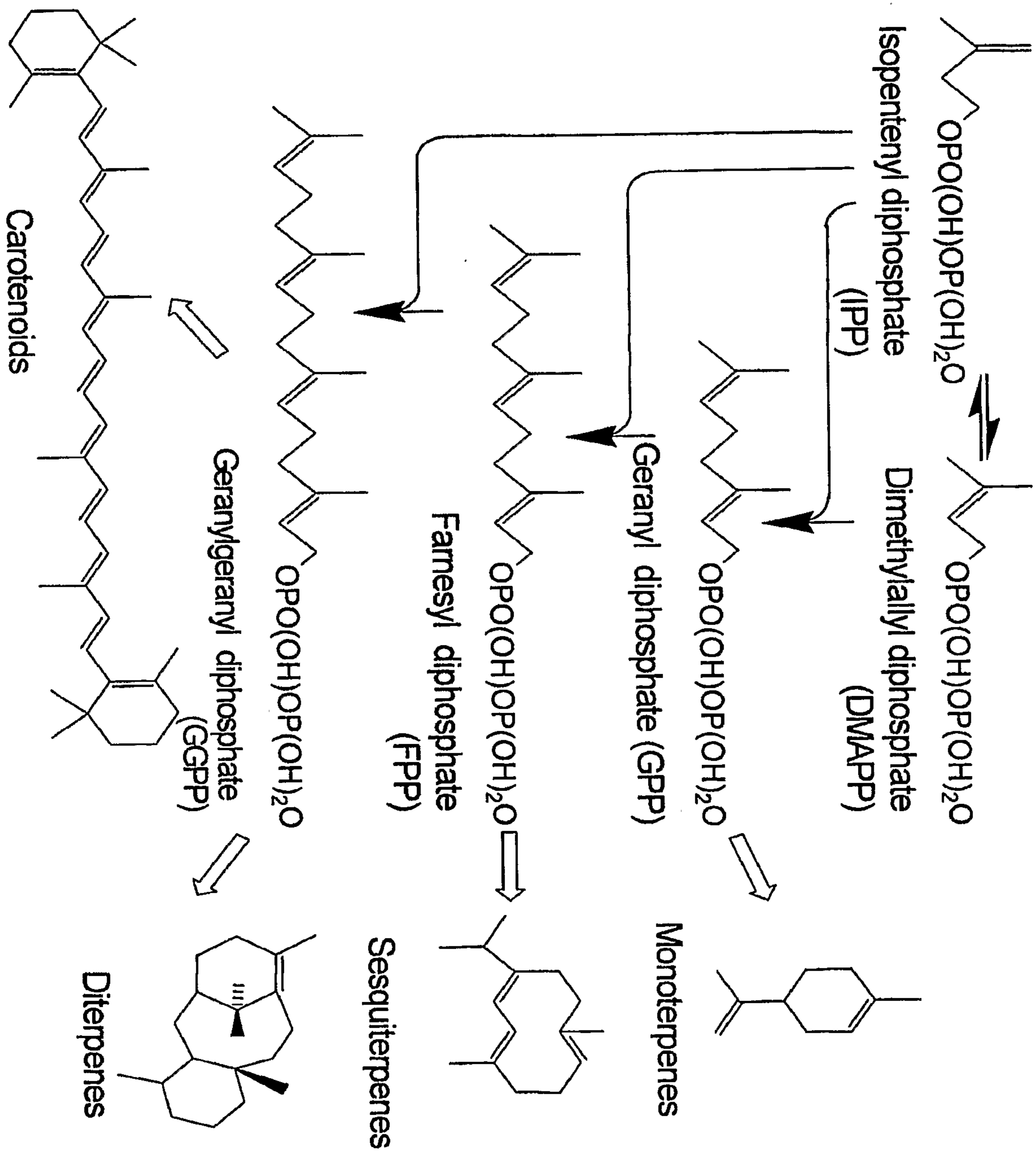
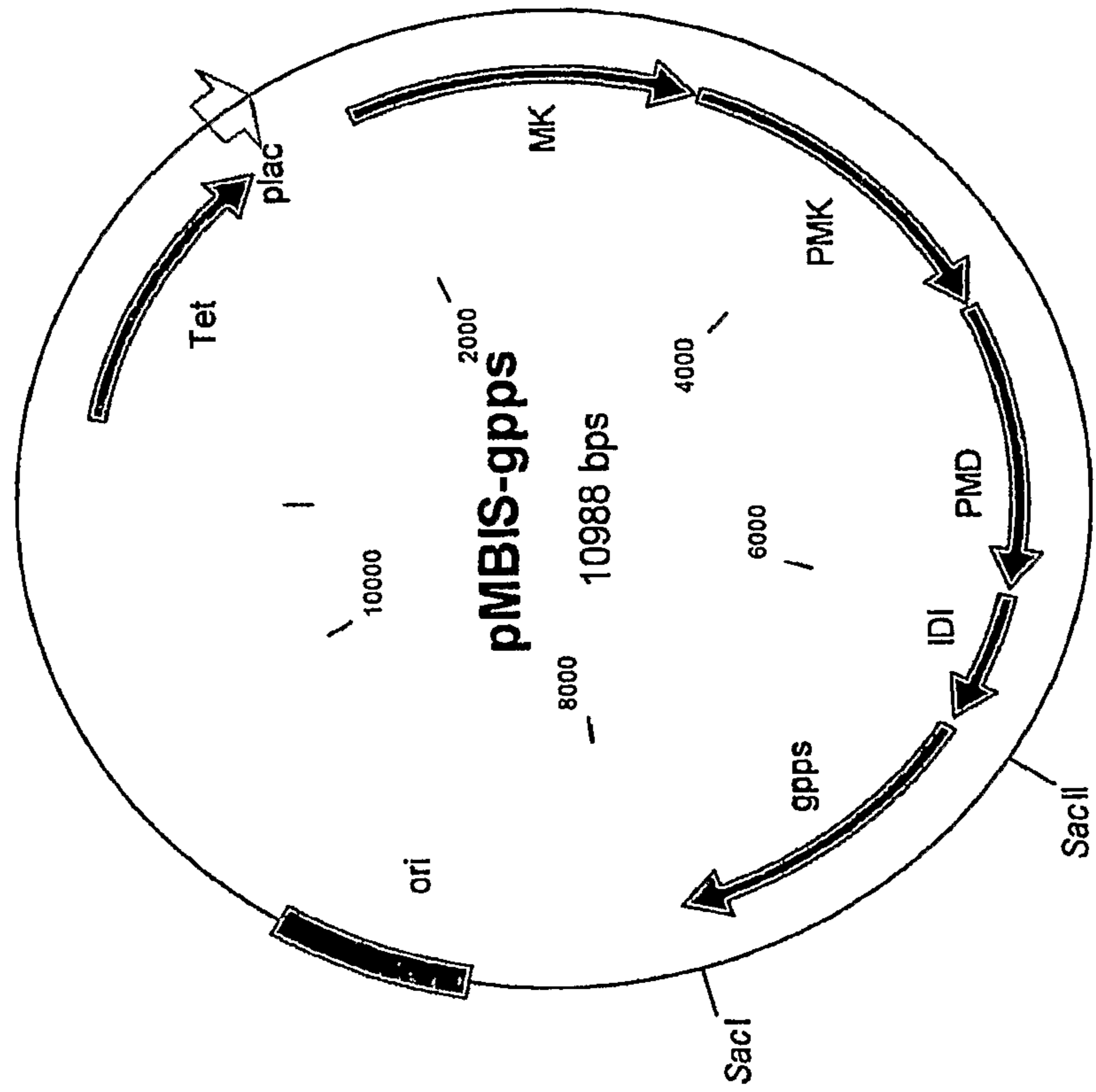


Figure 6



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Figure 7

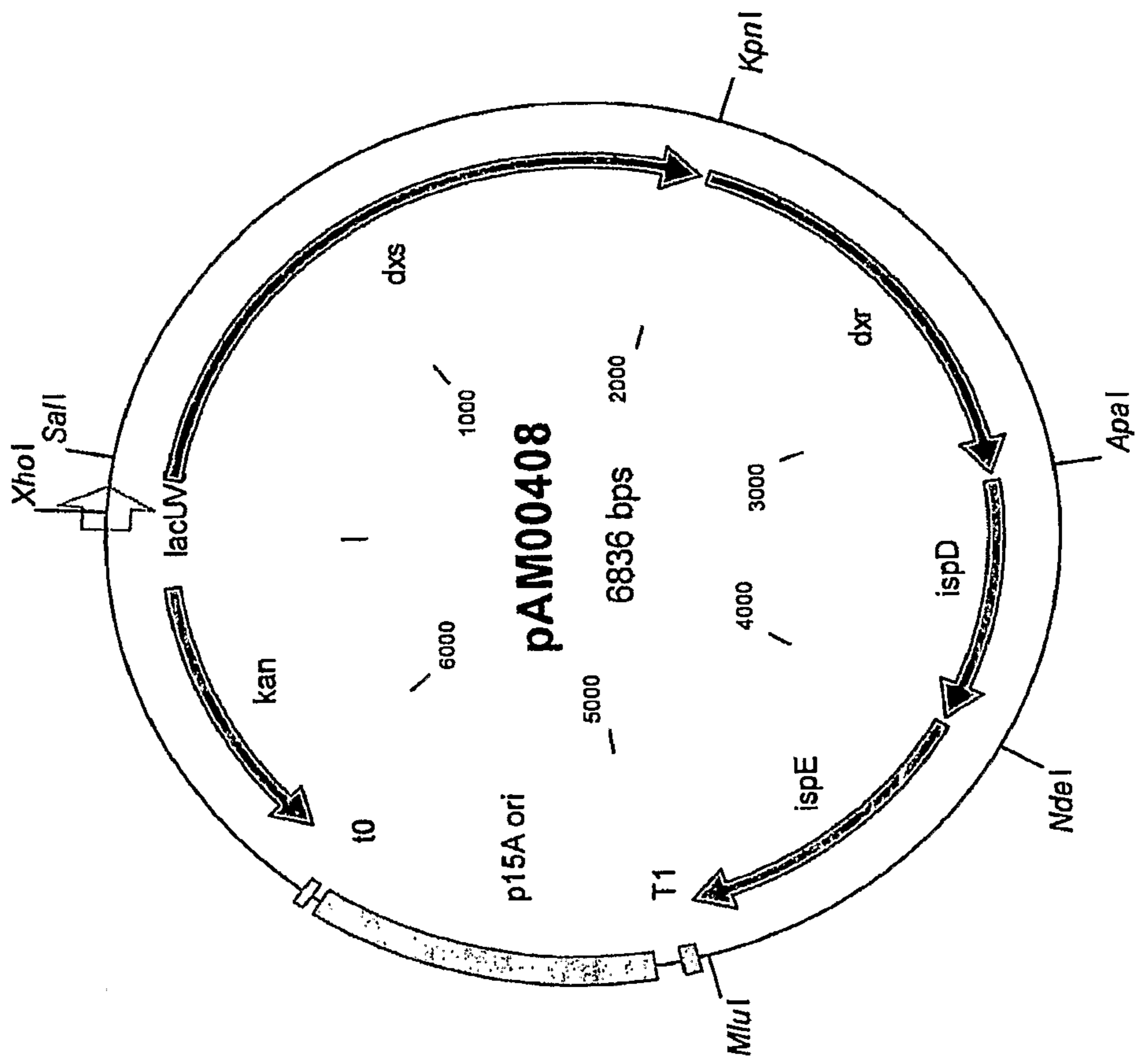


Figure 8

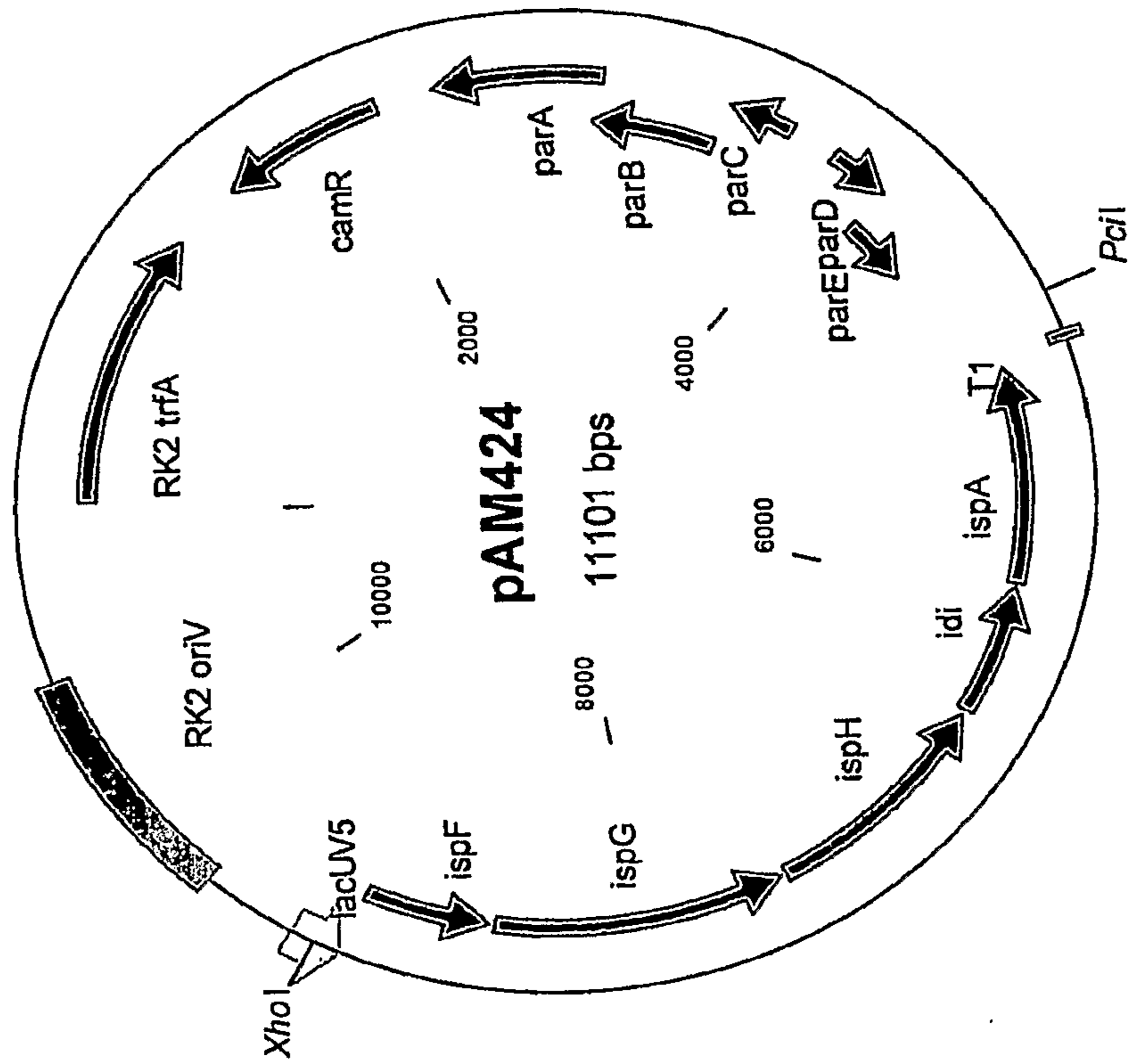


Figure 9

pTrc99A-ADS: ~1.6 kb ADS insert at *NcoI XmaI* site
pTrc99A-FSA: ~1.7 kb FSA insert at *NcoI SacI* site
pTrc99A-GTS: ~1.8 kb GTS insert at *XmaI XbaI* site
pTrc99A-APS: ~1.9 kb APS insert at *XmaI XbaI* site
pTrc99A-TS: ~1.9 kb TS insert at *XmaI XbaI* site
pTrc99A-BPS: ~1.7 kb BPS insert at *XmaI XbaI* site **pTrc99A-**
PHS: ~1.9 kb PHS insert at *XmaI XbaI* site
pTrc99A-SS: ~1.8 kb SS insert at *XmaI XbaI* site
pTrc99A-LLS: ~1.7 kb LLS insert at *NcoI XmaI* site
pTrc99A-CS: ~1.8 kb CS insert at *NcoI XmaI* site
pTrc99A-LMS: ~1.9 kb LMS insert at *XbaI PstI* site
pAM373: ~1.7 kb FSA insert at *NcoI SacI* site

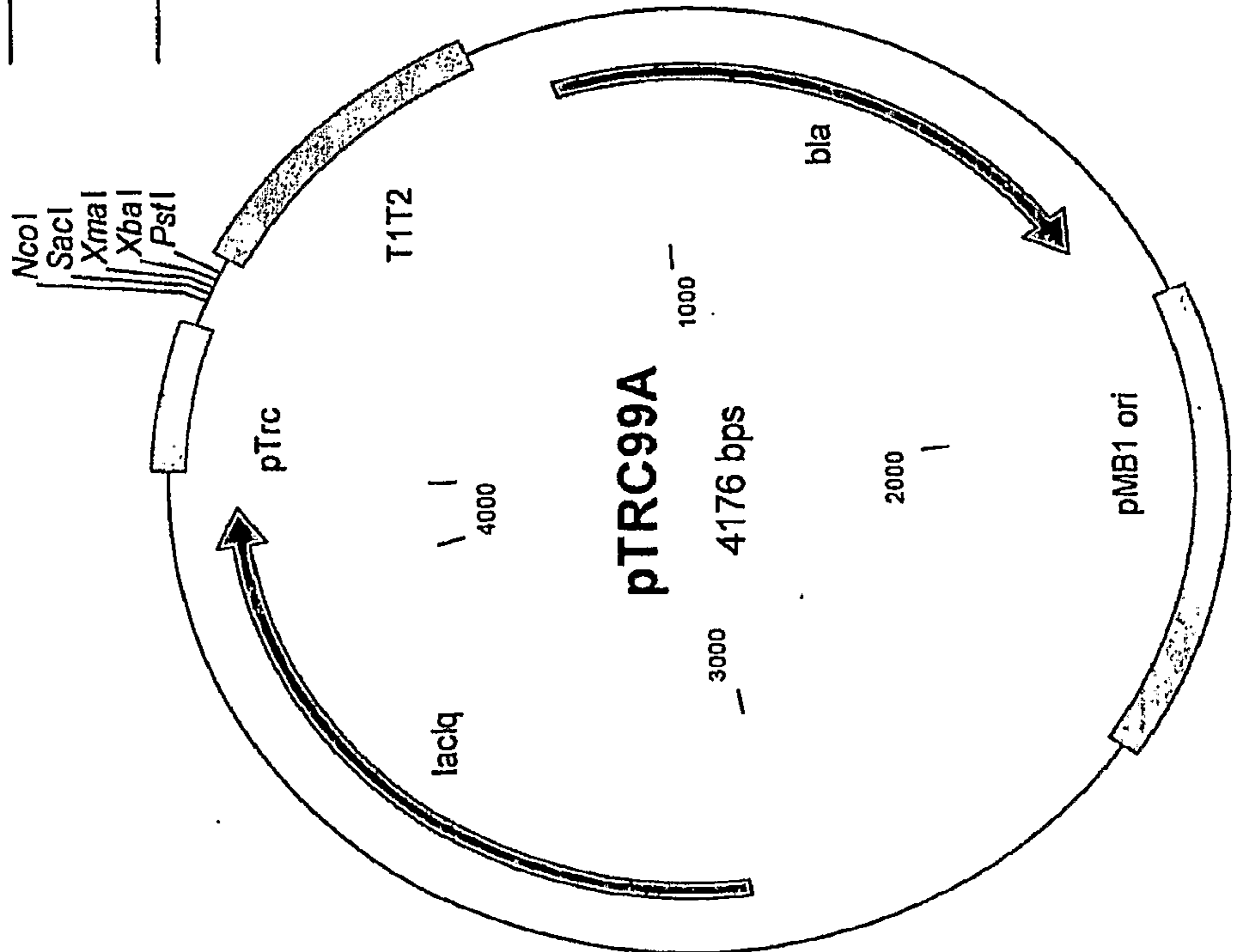


Figure 10

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