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- (71) Applicant: **GLYCOS BIOTECHNOLOGIES, INC.**
[US/US]; 711 Leverkuhn Street, Houston, TX 77007 (US).
- (72) Inventors: **BREDOW, Sebastian**; 9502 Pemberton Trace,
Houston, TX 77025 (US). **DONESKE, Stephanie**; 5119
Azalea Meadow Lane, Katy, TX 77494 (US). **LI, Mai**;
4723 Marbrook Meadow Lane, Katy, TX 77494 (US).
ZHOU, Huaijin; 4217 Gibson Street, Unit B, Houston,
TX 77007 (US). **MONTICELLO, Daniel, J.**; 152 N. Mill
Trace, The Woodlands, TX 77381 (US). **CAMPBELL,
Paul**; 3806 Wickersham Lane, Houston, TX 77027 (US).
- (74) Agents: **VANSTONE, Darlene, A.** et al.; Elmore Patent
Law Group, 484 Groton Road, Westford, MA 01886 (US).

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(54) Title: MICROORGANISMS AND PROCESSES FOR THE CONVERSION OF GLYCEROL TO ISOPRENE

(57) Abstract: The present invention provides non-naturally occurring microorganisms that have been modified to produce isoprene from glycerol. The microorganisms include one of several glycerol dissimilation pathways and one of several isoprene production pathways.



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MICROORGANISMS AND PROCESSES FOR THE CONVERSION OF GLYCEROL TO ISOPRENE

RELATED APPLICATION

[001] This application claims the benefit of U.S. Provisional Application No. 61/741,460, filed on July 20, 2012. The entire teachings of the above application are incorporated herein by reference.

FIELD OF THE INVENTION

[002] The present disclosure generally relates to the use of a non-naturally occurring microorganism for the production of isoprene from glycerol. More specifically, the present disclosure relates to non-naturally occurring microorganisms that have been modified to overexpress genes of the glycerol dissimilation pathway, overexpress genes of the methylerythritol pathway or mevalonate pathway, and reduce or eliminate expression of genes that lead to the formation of unwanted co-products.

BACKGROUND OF THE INVENTION

[003] Currently, many high-value chemicals or fuels are typically manufactured by chemical synthesis from hydrocarbons, including petroleum oil and natural gas. Also, high value chemicals may be produced as “by-products” during the processing of crude oil into usable fractions. For example, isoprene has typically been produced during the catalytic cracking of crude oil. However, as catalytic crackers have shifted their focus from crude oil to natural gas, there is now a reduced source of the four and five carbon chain molecules that are found in crude oil, but not natural gas.

[004] Being a short-chain carbon source, isoprene is a useful early or initial component for synthesizing a variety of chemicals. Isoprene may be used as a monomer or co-monomer. Examples of chemicals that can be produced using isoprene include polyisoprene, polybutylene, styrene-isoprene-styrene block co-polymers, and others. An example of an industry that uses isoprene is the synthetic rubber industry.

[005] Given the demand for and the many uses of isoprene, a new method of isoprene production is desired. Also, as the concerns of energy security, increasing oil and natural gas prices, and global warming escalate, the chemical production industry is seeking ways to

replace chemicals made from non-renewable feedstocks with chemicals produced from renewable feedstocks using environmentally friendly practices.

[006] Glycerol is gaining prominence as a useful carbon source for large-scale fermentations. While glycerol may be produced from petrochemical feedstocks, it is also
5 produced as a co-product of the oleochemical and biodiesel industries, generally through acid splitting or transesterification of oils and fats from animal or vegetable origin. With the growth of the oleochemical and biodiesel industries, there is now an abundance of glycerol available for use as a carbon source in fermentations.

[007] While examples of the conversion of glycerol to isoprene via fermentation are
10 known, these approaches suffer from low carbon flux from glycerol to the important metabolic intermediate, dimethylallyl diphosphate. Furthermore, co-products produced from the glycerol reduce the yield of final product, isoprene, while complicating separations and recovery steps.

[008] Thus, there is a need and a desire for non-naturally occurring microorganisms for
15 the efficient conversion of glycerol to isoprene.

SUMMARY OF THE INVENTION

[009] Embodiments of the present invention generally provide non-naturally occurring microorganisms that produce isoprene from glycerol via the methylerythritol pathway or mevalonate pathway and methods of producing isoprene from glycerol using said non-
20 naturally occurring microorganisms.

[010] In some aspects, the present invention improves the carbon flux from glycerol to dimethylallyl diphosphate, and further conversion from dimethylallyl diphosphate to isoprene, by (i) improving the rate at which glycerol is converted into the glycolytic intermediate dihydroxyacetone phosphate, (ii) improving the rate of conversion of
25 glyceraldehyde-3-phosphate (G3P) and pyruvate (PYR) into dimethylallyl diphosphate or improving the rate of conversion of acetyl-CoA to dimethylallyl diphosphate, and (iii) reducing or eliminating the production of undesirable co-products from the glycerol, such as acetate, lactate, ethanol, and succinate.

[011] In one embodiment, the non-naturally occurring microorganism includes a glycerol dissimilation pathway that comprises either a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and the dihydroxyacetone kinase are encoded by an exogenous nucleic acid. In certain embodiments, each of the glycerol kinase and glycerol-3-phosphate dehydrogenase or the glycerol dehydrogenase and dihydroxyacetone kinase is encoded by exogenous nucleic acids. The microorganism also includes an isoprene pathway comprising an acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, a isopentenyl diphosphate isomerase, a 2-methyl-3-buten-2-ol synthase, and a 2-methyl-3-buten-2-ol dehydratase, wherein one or more of the acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase, 2-methyl-3-buten-2-ol synthase, and 2-methyl-3-buten-2-ol dehydratase are encoded by an exogenous nucleic acid. In certain embodiments, each of the enzymes in the isoprene pathway listed above is encoded by exogenous nucleic acids. The glycerol dissimilation and isoprene pathways are expressed in sufficient quantities to produce isoprene from glycerol.

[012] In another embodiment, the non-naturally occurring microorganism includes a glycerol dissimilation pathway that comprises either a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and the dihydroxyacetone kinase are encoded by an exogenous nucleic acid. In certain embodiments, each of the glycerol kinase and glycerol-3-phosphate dehydrogenase or the glycerol dehydrogenase and dihydroxyacetone kinase is encoded by exogenous nucleic acids. The microorganism also includes an isoprene pathway comprising an acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, a isopentenyl diphosphate isomerase, a 3-methyl-2-buten-1-ol synthase, a 2-methyl-3-buten-2-ol isomerase, and a 2-methyl-3-buten-2-ol dehydratase, wherein one or more of the acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase,

hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase, 3-methyl-2-buten-1-ol synthase, 2-methyl-3-buten-2-ol isomerase, and 2-methyl-3-buten-2-ol dehydratase are encoded by an exogenous nucleic acid. In certain embodiments, each of the enzymes in the isoprene pathway listed above are encoded by exogenous nucleic acids. The glycerol dissimilation and isoprene pathways are expressed in sufficient quantities to produce isoprene from glycerol.

[013] In another embodiment, the non-naturally occurring microorganism includes a glycerol dissimilation pathway that comprises either a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and the dihydroxyacetone kinase are encoded by an exogenous nucleic acid. In certain embodiments, each of the glycerol kinase and glycerol-3-phosphate dehydrogenase or the glycerol dehydrogenase and dihydroxyacetone kinase is encoded by exogenous nucleic acids. The microorganism also includes an isoprene pathway comprising an acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, a isopentenyl diphosphate isomerase, and a monoterpene synthase, sesquiterpene synthase or diterpene synthase capable of converting dimethylallyl diphosphate into isoprene, wherein one or more of the acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase and monoterpene synthase, sesquiterpene synthase or diterpene synthase are encoded by an exogenous nucleic acid. In certain embodiments, each of the enzymes in the isoprene pathway listed above are encoded by exogenous nucleic acids. The glycerol dissimilation and isoprene pathways are expressed in sufficient quantities to produce isoprene from glycerol.

[014] In another embodiment, the non-naturally occurring microorganism includes a glycerol dissimilation pathway that comprises either a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and the dihydroxyacetone kinase are encoded by an exogenous nucleic acid.

In certain embodiments, each of the glycerol kinase and glycerol-3-phosphate dehydrogenase or the glycerol dehydrogenase and dihydroxyacetone kinase is encoded by exogenous nucleic acids. The microorganism also includes an isoprene pathway comprising an acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, an isopentenyl diphosphate isomerase, and an isoprene synthase, wherein one or more of the acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase and isoprene synthase are encoded by an exogenous nucleic acid. In certain embodiments, each of the enzymes in the isoprene pathway listed above are encoded by exogenous nucleic acids. The glycerol dissimilation and isoprene pathways are expressed in sufficient quantities to produce isoprene from glycerol.

[015] In another embodiment, the non-naturally occurring microorganism includes a glycerol dissimilation pathway that comprises either a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and the dihydroxyacetone kinase are encoded by an exogenous nucleic acid. In certain embodiments, each of the glycerol kinase and glycerol-3-phosphate dehydrogenase or the glycerol dehydrogenase and dihydroxyacetone kinase is encoded by exogenous nucleic acids. The microorganism also includes an isoprene pathway comprising an isoprene pathway comprising a 1-deoxyxylulose-5-phosphate synthase, a 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, a 4-diphosphocytidyl-2-C-methylerythritol kinase, a 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, a dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, an isopentenyl diphosphate isomerase, a 2-methyl-3-buten-2-ol synthase, and a 2-methyl-3-buten-2-ol dehydratase, wherein one or more of the 1-deoxyxylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methylerythritol kinase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, isopentenyl diphosphate isomerase, 2-methyl-3-buten-2-ol synthase, and 2-methyl-3-buten-2-ol dehydratase are

encoded by an exogenous nucleic acid. In certain embodiments, each of the enzymes in the isoprene pathway listed above are encoded by exogenous nucleic acids. The glycerol dissimilation and isoprene pathways are expressed in sufficient quantities to produce isoprene from glycerol.

- 5 [016] In another embodiment, the non-naturally occurring microorganism includes a glycerol dissimilation pathway that comprises either a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and the dihydroxyacetone kinase are encoded by an exogenous nucleic acid.
- 10 In certain embodiments, each of the glycerol kinase and glycerol-3-phosphate dehydrogenase or the glycerol dehydrogenase and dihydroxyacetone kinase is encoded by exogenous nucleic acids. The microorganism also includes an isoprene pathway comprising a 1-deoxyxylulose-5-phosphate synthase, a 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a 4-diphosphocytidyl-2-C-methyl-D- erythritol synthase, a 4-diphosphocytidyl-2-C-
- 15 methylerythritol kinase, a 1- hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, a dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, an isopentenyl diphosphate isomerase, a 3-methyl-2-buten-1-ol synthase, a 2-methyl-3-buten-2-ol isomerase, and a 2-methyl-3-buten-2-ol dehydratase, wherein one or more of the 1-deoxyxylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-
- 20 diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methylerythritol kinase, 1-hydroxy-2-methyl-2-(E)-butenyl-4- diphosphate synthase, dimethylallyl-diphosphate/isopentenyl- diphosphate:NAD(P)⁺ oxidoreductase, isopentenyl diphosphate isomerase, 3-methyl-2-buten-1-ol synthase, 2-methyl-3-buten-2-ol isomerase and 2-methyl-3-buten-2-ol dehydratase are encoded by an exogenous nucleic acid. In certain
- 25 embodiments, each of the enzymes in the isoprene pathway listed above are encoded by exogenous nucleic acids. The glycerol dissimilation and isoprene pathways are expressed in sufficient quantities to produce isoprene from glycerol.

- [017] In a further embodiment, the non-naturally occurring microorganism includes a glycerol dissimilation pathway that comprises either a glycerol kinase and a glycerol-3-
- 30 phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and the dihydroxyacetone kinase are encoded by an exogenous nucleic acid.

In certain embodiments, each of the glycerol kinase and glycerol-3-phosphate dehydrogenase or the glycerol dehydrogenase and dihydroxyacetone kinase is encoded by exogenous nucleic acids. The microorganism also includes an isoprene pathway comprising a 1-deoxyxylulose-5-phosphate synthase, a 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, a 4-diphosphocytidyl-2-C-methylerythritol kinase, a 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, a dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, an isopentenyl diphosphate isomerase, and a monoterpene synthase, sesquiterpene synthase, or diterpene synthase capable of converting dimethylallyldiphosphate into isoprene, wherein one or more of the 1-deoxyxylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methylerythritol kinase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, isopentenyl diphosphate isomerase, and the monoterpene synthase, sesquiterpene synthase, or diterpene synthase are encoded by an exogenous nucleic acid. In certain embodiments, each of the enzymes in the isoprene pathway listed above are encoded by exogenous nucleic acids. The glycerol dissimilation and isoprene pathways are expressed in sufficient quantities to produce isoprene from glycerol.

[018] In a further embodiment, the non-naturally occurring microorganism includes a glycerol dissimilation pathway that comprises either a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and the dihydroxyacetone kinase are encoded by an exogenous nucleic acid. In certain embodiments, each of the glycerol kinase and glycerol-3-phosphate dehydrogenase or the glycerol dehydrogenase and dihydroxyacetone kinase is encoded by exogenous nucleic acids. The microorganism also includes an isoprene pathway comprising a 1-deoxyxylulose-5-phosphate synthase, a 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, a 4-diphosphocytidyl-2-C-methylerythritol kinase, a 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, a dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, an isopentenyl diphosphate isomerase, and an isoprene synthase, wherein one or more of the 1-deoxyxylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-

diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methylerythritol kinase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, isopentenyl diphosphate isomerase, and isoprene synthase are encoded by an exogenous nucleic acid. In certain embodiments, each of the enzymes in the isoprene pathway listed above are encoded by exogenous nucleic acids. The glycerol dissimilation and isoprene pathways are expressed in sufficient quantities to produce isoprene from glycerol.

[019] In one embodiment, the non-naturally occurring microorganism converts the dimethylallyl diphosphate produced by the methylerythritol pathway into isoprene using an isoprene synthase polypeptide.

[020] In an additional embodiment, the non-naturally occurring microorganism converts the dimethylallyl diphosphate produced by the methylerythritol pathway into isoprene using a monoterpene synthase, sesquiterpene synthase, or diterpene synthase.

[021] In one embodiment, the non-naturally occurring microorganism converts the dimethylallyl diphosphate produced by the methylerythritol pathway into 3-methyl-2-buten-1-ol by a 3-methyl-2-buten-1-ol synthase, the 3-methyl-2-buten-1-ol is converted to 2-methyl-3-buten-2-ol by a 2-methyl-3-buten-2-ol isomerase, and the 2-methyl-3-buten-2-ol is converted to isoprene by a 2-methyl-3-buten-2-ol dehydratase.

[022] In another embodiment, the non-naturally occurring microorganism converts the dimethylallyl diphosphate produced by the methylerythritol pathway into 2-methyl-3-buten-2-ol by a methylbutenol synthase, and 2-methyl-3-buten-2-ol is converted to isoprene by a 2-methyl-3-buten-2-ol dehydratase.

[023] In some aspects, the present invention improves the carbon flux from glycerol to dimethylallyl diphosphate, and further conversion from dimethylallyl diphosphate to isoprene, by (i) improving the rate at which glycerol is converted into the glycolytic intermediate dihydroxyacetone phosphate, (ii) improving the rate of conversion of acetyl-CoA into dimethylallyl diphosphate, and (iii) reducing or eliminating the production of undesirable co-products from the glycerol.

[024] In an embodiment, the non-naturally occurring microorganism converts the dimethylallyl diphosphate produced by the mevalonate pathway into isoprene using an isoprene synthase polypeptide.

[025] In an additional embodiment, the non-naturally occurring microorganism converts
5 the dimethylallyl diphosphate produced by the mevalonate pathway into isoprene using a monoterpene synthase, sesquiterpene synthase, or diterpene synthase.

[026] In one embodiment, the non-naturally occurring microorganism converts the dimethylallyl diphosphate produced by the mevalonate pathway into 3-methyl-2-buten-1-ol by a 3-methyl-2-buten-1-ol synthase, the 3-methyl-2-buten-1-ol is converted to 2-methyl-3-
10 buten-2-ol by a 2-methyl-3-buten-2-ol isomerase, and the 2-methyl-3-buten-2-ol is converted to isoprene by a 2-methyl-3-buten-2-ol dehydratase.

[027] In another embodiment, the non-naturally occurring microorganism converts the dimethylallyl diphosphate produced by the mevalonate pathway into 2-methyl-3-buten-2-ol by a methylbutenol synthase, and 2-methyl-3-buten-2-ol is converted to isoprene by a 2-
15 methyl-3-buten-2-ol dehydratase.

[028] Embodiments of the invention also provide methods of producing isoprene comprising culturing the microorganisms described herein under suitable conditions in a medium containing glycerol for a sufficient period of time to produce isoprene from the glycerol. The isoprene is then recovered.

20 BRIEF DESCRIPTION OF THE DRAWINGS

[029] So that the manner in which the above recited features of the present disclosure can be understood in detail, a more particular description of the disclosure, briefly summarized above, may be had by reference to embodiments, some of which are illustrated in the appended drawings. It is to be noted, however, that the appended drawings illustrate
25 only typical embodiments of this invention and are therefore not to be considered limiting of its scope, for the invention may admit to other equally effective embodiments.

[030] Figure 1 shows: A. A glycerol dissimilation pathway that uses glycerol kinase and either an aerobic glycerol-3-phosphate dehydrogenase (encoded by *glpD* in *Escherichia coli*) or an anaerobic glycerol-3-phosphate dehydrogenase (encoded by *glpABC* in

Escherichia coli); and B. A glycerol dissimilation pathway that uses glycerol dehydrogenase (*gldA*) and either a phosphoenolpyruvate-dependent dihydroxyacetone kinase (*dhaKLM*) or an adenosine triphosphate- (ATP-) dependent dihydroxyacetone kinase (*dhaKL*).

[031] Figure 2 shows a methylerythritol phosphate metabolic pathway for the conversion of glyceraldehyde-3-phosphate and pyruvate to dimethylallyl diphosphate, comprising the enzymes: 1-deoxy-D-xylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, and isopentenyl diphosphate isomerase.

[032] Figure 3 shows a metabolic pathway for the conversion of acetyl-CoA to dimethylallyl diphosphate, comprising the enzymes: acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentenyl diphosphate isomerase.

[033] Figure 4 shows: A. Conversion of dimethylallyl diphosphate to isoprene in a single enzymatic step; B. Conversion of dimethylallyl diphosphate into isoprene in a three-step enzymatic pathway comprising a 3-methyl-2-buten-1-ol synthase, a 2-methyl-3-buten-2-ol isomerase, and a 2-methyl-3-buten-2-ol dehydratase; and C. Conversion of dimethylallyl diphosphate into isoprene in a two-step enzymatic pathway comprising a 2-methyl-3-buten-2-ol isomerase, and a 2-methyl-3-buten-2-ol dehydratase.

[034] Figure 5 shows a gas chromatogram of a 1-milliliter sample of the headspace of a 20-milliliter vial containing *E. coli* strain BL21 harboring plasmid pJ404-LDI cultured overnight on 1 mM 3-methyl-2-buten-1-ol in Luria Bertani broth supplemented with 100 µg/ml ampicillin. Peak 1 is 3-methyl-2-buten-1-ol, with a retention time of 3.96 minutes. Peak 2 is 2-methyl-3-buten-2-ol, with a retention time of 2.96 minutes. Peak 3 is isoprene, with a retention time of 2.49 minutes.

[035] Figure 6 shows a gas chromatogram of a 1-milliliter sample of the headspace of a 20-milliliter vial containing *E. coli* strain BL21 harboring plasmid pJ404-LDI cultured

overnight on 1 mM 2-methyl-3-buten-2-ol in Luria Bertani broth supplemented with 100 µg/ml ampicillin. Peak 1 is 2-methyl-3-buten-2-ol, with a retention time of 2.96 minutes. Peak 2 is isoprene, with a retention time of 2.49 minutes.

[036] Figure 7 shows a fermentation of glycerol to lactic acid using *E. coli* strain LA02
5 harboring plasmid pZS-adhEp.glpK.glpD.

[037] Figure 8 shows a codon-optimized nucleic acid sequence [SEQ ID NO: 33], including an artificial ribosome binding site and an amino-terminal 6-histidine epitope tag, for the linalool dehydratase-isomerase of *Castellaniella defragrans* strain 65Phen.

[038] Figure 9 shows a codon-optimized nucleic acid sequence [SEQ ID NO: 34],
10 including an artificial ribosome binding site and an amino-terminal 6-histidine epitope tag, for strawberry alcohol acyltransferase.

[039] Figure 10 shows a gas chromatogram of a 1-milliliter sample of the headspace of a 20-milliliter vial containing 1 mM 3-methyl-2-buten-1-ol in Luria Bertani broth. Peak 1 is 3-methyl-2-buten-1-ol, with a retention time of 3.96 minutes.

15 [040] Figure 11 shows the results of GC/MS analysis of authentic isoprene.

[041] Figure 12 shows the results of GC/MS analysis of the peak at 2.49 minutes from Example 1, verifying the identity of the peak as isoprene.

[042] Figure 13 shows a gas chromatogram of a 1-milliliter sample of the headspace of a 20-milliliter vial containing 1 mM 2-methyl-3-buten-2-ol in Luria Bertani broth. Peak 1 is 2-
20 methyl-3-buten-2-ol, with a retention time of 2.96 minutes.

[043] Figure 14 shows the DNA sequence [SEQ ID NO: 35] of plasmid pGA31R-mcs.

[044] Figure 15 shows the DNA sequence [SEQ ID NO: 36] of plasmid pGE21R-mcs.

[045] Figure 16 shows the codon-optimized sequence [SEQ ID NO: 37] of the *mvaE* and *mvaS* genes of *Enterococcus faecalis* ATCC 700802, including incorporated ribosome
25 binding sites and flanking restriction endonuclease sites used in subsequent cloning steps.

[046] Figure 17 shows the codon-optimized sequence [SEQ ID NO: 38] of the synthetic operon encoding the mevalonate kinase gene of *Methanocaldococcus jannaschi*, the

phosphomevalonate kinase gene of *Enterococcus faecalis* ATCC 700802, the mevalonate pyrophosphate decarboxylase gene of *Saccharomyces cerevisiae* S288C, and the isopentenyl diphosphate isomerase gene of *E. coli* MG1655, including incorporated ribosome binding sites and flanking restriction endonuclease sites used in subsequent cloning steps.

5 [047] Figure 18 shows the codon-optimized sequence [SEQ ID NO: 39] of the synthetic isoprene synthase gene of *Populus alba*, without the encoded N-terminal chloroplast transit peptide but containing a ribosome binding site and flanking restriction endonuclease sites used in subsequent cloning steps.

[048] Figure 19 shows a cloning strategy for the production of plasmids pGB1004 and
10 pGB1012.

[049] Figure 20 shows a cloning strategy for the production of plasmid pGB1008 and pGB1012.

[050] Figure 21 shows a cloning strategy for the production of plasmid pGB1030.

[051] Figure 22 shows the codon-optimized sequence [SEQ ID NO: 40] of the 3-methyl-
15 2-buten-1-ol synthase gene of *Pinus sabiniana* and the isopentenyl diphosphate isomerase gene of *Haematococcus pluvialis*, including ribosome binding sites and flanking restriction endonuclease sites used in subsequent cloning steps.

DETAILED DESCRIPTION

[052] It is to be understood that both the foregoing general description and the following
20 detailed description are exemplary and explanatory only, and are not restrictive of the invention, as claimed. In this application, the use of the singular includes the plural, the word “a” or “an” means “at least one”, and the use of “or” means “and/or”, unless specifically stated otherwise. Furthermore, the use of the term “including”, as well as other forms, such as “includes” and “included”, is not limiting. Also, terms such as “element” or “component”
25 encompass both elements or components comprising one unit and elements or components that comprise more than one unit unless specifically stated otherwise.

[053] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of

documents, cited in this application, including, but not limited to, patents, patent applications, articles, books, and treatises, are hereby expressly incorporated herein by reference in their entirety for any purpose. In the event that one or more of the incorporated literature and similar materials defines a term in a manner that contradicts the definition of that term in this
5 application, this application controls.

[054] As used herein, the term "non-naturally occurring" when used in reference to a microbial organism or microorganism of the invention is intended to mean that the microbial organism has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic
10 alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microbial genetic material. Such modifications include, for example, coding regions and functional fragments thereof, for heterologous, homologous or both heterologous and homologous polypeptides for the referenced species. Additional
15 modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. Exemplary metabolic polypeptides include enzymes or proteins within a glycerol dissimilation or isoprene biosynthetic pathway.

[055] A metabolic modification refers to a biochemical reaction that is altered from its naturally occurring state. Therefore, non-naturally occurring microorganisms can have
20 genetic modifications to nucleic acids encoding metabolic polypeptides or, functional fragments thereof. Exemplary metabolic modifications are disclosed herein.

[056] As used herein, the term "isolated" when used in reference to a microbial organism is intended to mean an organism that is substantially free of at least one component as the referenced microbial organism is found in nature. The term includes a microbial
25 organism that is removed from some or all components as it is found in its natural environment. The term also includes a microbial organism that is removed from some or all components as the microbial organism is found in non-naturally occurring environments. Therefore, an isolated microbial organism is partly or completely separated from other substances as it is found in nature or as it is grown, stored or subsisted in non-naturally
30 occurring environments. Specific examples of isolated microbial organisms include partially

pure microbes, substantially pure microbes and microbes cultured in a medium that is non-naturally occurring.

[057] As used herein, the terms "microbe," "microbial," "microbial organism" or "microorganism" is intended to mean any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea and eubacteria of all species as well as eukaryotic microorganisms such as yeast and fungi. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

[058] "Exogenous" as it is used herein is intended to mean that the referenced molecule or the referenced activity is introduced into the host microbial organism. The molecule can be introduced, for example, by introduction of an encoding nucleic acid into the host genetic material such as by integration into a host chromosome or as non-chromosomal genetic material such as a plasmid. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the microbial organism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into the host reference organism. The source can be, for example, a homologous or heterologous encoding nucleic acid that expresses the referenced activity following introduction into the host microbial organism. Therefore, the term "endogenous" refers to a referenced molecule or activity that is present in the host. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the microbial organism. The term "heterologous" refers to a molecule or activity derived from a source other than the referenced species whereas "homologous" refers to a molecule or activity derived from the host microbial organism. Accordingly, exogenous expression of an encoding nucleic acid of the invention can utilize either or both a heterologous or homologous encoding nucleic acid.

[059] The non-naturally occurring microbial organisms of the invention can contain stable genetic alterations, which refer to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will

persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

[060] Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein, are described with reference to a suitable host organism such as *Escherichia coli* and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes for a desired metabolic pathway. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

[061] An ortholog is a gene or genes that are related by vertical descent and are responsible for substantially the same or identical functions in different organisms. For example, mouse epoxide hydrolase and human epoxide hydrolase can be considered orthologs for the biological function of hydrolysis of epoxides. Genes are related by vertical descent when, for example, they share sequence similarity of sufficient amount to indicate they are homologous, or related by evolution from a common ancestor. Genes can also be considered orthologs if they share three-dimensional structure but not necessarily sequence similarity, of a sufficient amount to indicate that they have evolved from a common ancestor to the extent that the primary sequence similarity is not identifiable. Genes that are orthologous can encode proteins with sequence similarity of about 25% to 100% amino acid sequence identity. Genes encoding proteins sharing an amino acid similarity less than 25% can also be considered to have arisen by vertical descent if their three-dimensional structure also shows similarities.

[062] Orthologs include genes or their encoded gene products that through, for example, evolution, have diverged in structure or overall activity. For example, where one species encodes a gene product exhibiting two functions and where such functions have been separated into distinct genes in a second species, the three genes and their corresponding

products are considered to be orthologs. For the production of a biochemical product, those skilled in the art will understand that the orthologous gene harboring the metabolic activity to be introduced or disrupted is to be chosen for construction of the non-naturally occurring microorganism. An example of orthologs exhibiting separable activities is where distinct activities have been separated into distinct gene products between two or more species or within a single species.

[063] In contrast, paralogs are homologs related by, for example, duplication followed by evolutionary divergence and have similar or common, but not identical functions. Paralogs can originate or derive from, for example, the same species or from a different species. For example, microsomal epoxide hydrolase (epoxide hydrolase I) and soluble epoxide hydrolase (epoxide hydrolase II) can be considered paralogs because they represent two distinct enzymes, co-evolved from a common ancestor, that catalyze distinct reactions and have distinct functions in the same species. Paralogs are proteins from the same species with significant sequence similarity to each other suggesting that they are homologous, or related through co-evolution from a common ancestor. Groups of paralogous protein families include HipA homologs, luciferase genes, peptidases, and others.

[064] A nonorthologous gene displacement is a nonorthologous gene from one species that can substitute for a referenced gene function in a different species. Substitution includes, for example, being able to perform substantially the same or a similar function in the species of origin compared to the referenced function in the different species. Although generally, a nonorthologous gene displacement will be identifiable as structurally related to a known gene encoding the referenced function, less structurally related but functionally similar genes and their corresponding gene products nevertheless will still fall within the meaning of the term as it is used herein. Functional similarity requires, for example, at least some structural similarity in the active site or binding region of a nonorthologous gene product compared to a gene encoding the function sought to be substituted. Therefore, a nonorthologous gene includes, for example, a paralog or an unrelated gene.

[065] Therefore, in identifying and constructing the non-naturally occurring microbial organisms of the invention having glycerol dissimilation and/or isoprene biosynthetic capability, those skilled in the art will understand with applying the teaching and guidance provided herein to a particular species that the identification of metabolic modifications can

include identification and inclusion or inactivation of orthologs. To the extent that paralogs and/or nonorthologous gene displacements are present in the referenced microorganism that encode an enzyme catalyzing a similar or substantially similar metabolic reaction, those skilled in the art also can utilize these evolutionally related genes. Orthologs, paralogs and nonorthologous gene displacements can be determined by methods well known to those skilled in the art.

[066] As defined herein, a knocked-out gene is a gene whose encoded product, *e.g.*, a protein or polypeptide, does not or substantially does not perform its usual function or any function. A knocked-out gene can be created through deletion, disruption, insertion, or mutation. As defined herein, microorganisms that lack one or more indicated knocked-out genes are also considered to have knock outs of the indicated gene(s). The microorganisms themselves may also be referred to as knock outs of the indicated gene(s). Such knock outs can also be conditional or inducible, using techniques that are well-known to those of skill in the art. Also contemplated are “knock ins”, in which a gene, or one or more segments of a gene, are introduced into the microorganism in place of, or in addition to, the endogenous copy of the gene. Once again, many techniques for creating knock in microorganisms are known to those of ordinary skill in the art.

[067] As defined herein, nucleic acids or enzymes described herein as being “from” or “derived from” certain organisms include codon-optimized versions of those nucleic acids or enzymes.

[068] The methods and techniques utilized for culturing or generating the microorganisms disclosed herein are known to the skilled worker trained in microbiological and recombinant DNA techniques. Methods and techniques for growing microorganisms (*e.g.*, bacterial cells), transporting isolated DNA molecules into the host cell and isolating, cloning and sequencing isolated nucleic acid molecules, knocking out expression of specific genes, *etc.*, are examples of such techniques and methods. These methods are described in many items of the standard literature, which are incorporated herein in their entirety: “Basic Methods In Molecular Biology” (Davis, *et al.*, eds. McGraw-Hill Professional, Columbus, OH, 1986); Miller, “Experiments in Molecular Genetics” (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1972); Miller, “A Short Course in Bacterial Genetics” (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992); Singer and Berg, “Genes

and Genomes” (University Science Books, Mill Valley, CA, 1991); “Molecular Cloning: A Laboratory Manual,” 2nd Ed. (Sambrook, *et al.*, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989); “Handbook of Molecular and Cellular Methods in Biology and Medicine” (Kaufman, *et al.*, eds., CRC Press, Boca Raton, FL, 1995); “Methods in Plant
5 Molecular Biology and Biotechnology” (Glick and Thompson, eds., CRC Press, Boca Raton, FL, 1993); and Smith-Keary, “Molecular Genetics of *Escherichia coli*” (The Guilford Press, New York, NY, 1989).

[069] Embodiments of the present invention provide non-naturally occurring microorganisms for the production of isoprene having improved rates of glycerol
10 dissimilation, improved rates of conversion of glyceraldehyde-3-phosphate and pyruvate to dimethylallyl diphosphate (also referred to as dimethylallyl pyrophosphate or DMAPP), and reduced amounts of co-products such as acetate, lactate, ethanol, and succinate. As shown in Figure 1, glycerol can be converted to glycerol-3-phosphate by glycerol kinase, and from glycerol phosphate to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate
15 dehydrogenase. Alternatively, in a second glycerol dissimilation pathway, glycerol can be converted to dihydroxyacetone by glycerol dehydrogenase, and from dihydroxyacetone to dihydroxyacetone phosphate by a dihydroxyacetone kinase. In both pathways, the dihydroxyacetone phosphate then enters glycolysis to be converted to glyceraldehyde-3-phosphate and pyruvate. A methylerythritol phosphate pathway then converts the
20 glyceraldehyde-3-phosphate and pyruvate to dimethylallyl diphosphate. A series of one or more steps then converts the dimethylallyl diphosphate into isoprene or another suitable isoprenoid molecule.

[070] Additional embodiments of the present invention provide non-naturally occurring microorganisms for the production of isoprene having improved rates of glycerol
25 dissimilation, improved rates of conversion of acetyl-CoA to dimethylallyl diphosphate, and reduced amounts of co-products such as acetate, lactate, ethanol, and succinate. As shown in Figure 1, glycerol can be converted to glycerol-3-phosphate by glycerol kinase, and from glycerol phosphate to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase. Alternatively, in a second glycerol dissimilation pathway, glycerol can be
30 converted to dihydroxyacetone by glycerol dehydrogenase, and from dihydroxyacetone to dihydroxyacetone phosphate by a dihydroxyacetone kinase. In both pathways, the dihydroxyacetone phosphate then enters glycolysis to be converted to pyruvate, followed by

conversion to acetyl-CoA. A mevalonate pathway then converts the acetyl-CoA to dimethylallyl diphosphate. A series of one or more steps then converts the dimethylallyl diphosphate into isoprene or another suitable isoprenoid molecule.

[071] The rate of glycerol dissimilation may be increased through overexpression of genes encoding a glycerol dissimilation pathway. An example of a glycerol dissimilation pathway includes the enzymes glycerol kinase and glycerol-3-phosphate dehydrogenase (Figure 1. A.).

[072] Glycerol kinase (E.C. No. 2.7.1.30) converts glycerol to glycerol-3-phosphate. This enzyme activity is encoded by the *E. coli* gene *glpK*, Table 1, below. Other comparable enzyme activities and the corresponding genes are known in other organisms. For example, *Mus musculus*, *Saccharomyces cerevisiae*, *Klebsiella pneumoniae* and *Bacillus subtilis* all possess glycerol kinase activities. Additional examples of glycerol kinase enzymes are presented in Table 1.

TABLE 1		
Locus	GenBank Accession No.	Organism
GLPK_ECOLI	P0A6F3	<i>Escherichia coli</i> K-12
GLPK_KLEP7	A6TFR2	<i>Klebsiella pneumoniae</i> (ATCC 700721)
GLPK_BACSU	P18157	<i>Bacillus subtilis</i>
GLPK_MOUSE	Q64516	<i>Mus musculus</i>
GLPK_YEAST	P32190	<i>Saccharomyces cerevisiae</i>

[073] Glycerol-3-phosphate dehydrogenase (E.C. Nos. 1.1.1.8, 1.1.1.94, and 1.1.5.3) converts glycerol-3-phosphate to dihydroxyacetone phosphate with the concomitant reduction of an NAD(P)⁺ molecule to NAD(P)H or a quinone to a quinol. Glycerol-3-phosphate dehydrogenase enzymes belonging to all three E.C. classes are known. The *Saccharomyces cerevisiae* genes *GPD1* and *GPD2* encode glycerol-3-phosphate dehydrogenases of E.C. No. 1.1.1.8. The *Candida versatilis* gene *CvGPD1* encodes a glycerol-3-phosphate dehydrogenase of E.C. No. 1.1.1.94. The *E. coli* gene *glpD* encodes a quinone-dependent glycerol-3-phosphate dehydrogenase of E.C. No. 1.1.5.3. The *E. coli* enzyme GlpD is generally active under aerobic conditions. The *E. coli* operon *glpABC* also encodes a quinone-dependent glycerol-3-phosphate dehydrogenase of E.C. No. 1.1.5.3, but this GlpABC enzyme is generally active under anaerobic conditions. Additional examples of glycerol-3-phosphate dehydrogenase enzymes are presented in Table 2, below.

TABLE 2		
Locus	GenBank Accession No.	Organism
<i>E.C. No. 1.1.1.8</i>		
GPD1L_MOUSE	Q3ULJ0	<i>Mus musculus</i>
GPDA_ARATH	Q9SCX9	<i>Arabidopsis thaliana</i>
GPD1_SCHPO	P21696	<i>Schizosaccharomyces pombe</i>
GPD2_SCHPO	Q09845	<i>Schizosaccharomyces pombe</i>
GPD1_YEAST	Q00055	<i>Saccharomyces cerevisiae</i>
GPD2_YEAST	P41911	<i>Saccharomyces cerevisiae</i>
<i>E.C. No. 1.1.1.94</i>		
GPDA1_LACDA	Q93FD0	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>
GPDA_LACLA	Q9CFX6	<i>Lactococcus lactis</i> subsp. <i>Lactis</i>
GPDA_RHIE6	B3PQA3	<i>Rhizobium etli</i> (strain CIAT 652)
GPDA_AGR5	Q8UC48	<i>Agrobacterium tumefaciens</i> (strain C58)
<i>E. C. No. 1.1.5.3</i>		
GLPA_ECOLI	P0A9C0	<i>Escherichia coli</i>
GLPB_ECOLI	P13033	<i>Escherichia coli</i>
GLPC_ECOLI	P0A996	<i>Escherichia coli</i>
GLPD_ECOLI	P13035	<i>Escherichia coli</i>
GLPD_BACSU	P18158	<i>Bacillus subtilis</i>

[074] Another example of a glycerol dissimilation pathway includes the enzymes glycerol dehydrogenase and dihydroxyacetone kinase (Figure 1. B.).

[075] Glycerol dehydrogenase (E.C. No. 1.1.1.6) converts glycerol to dihydroxyacetone with the concomitant reduction of an NAD(P)⁺ molecule to NAD(P)H or a quinone to a quinol. The *E. coli* gene *gldA* encodes a glycerol dehydrogenase of E.C. No. 1.1.1.6. Similar enzymes are known from *Klebsiella pneumoniae* and *Citrobacter freundii*. Additional examples of glycerol dehydrogenase enzymes are presented in Table 3, below.

TABLE 3		
Locus	GenBank Accession No.	Organism
GLDA_ECOLI	P0A9S5	<i>Escherichia coli</i> K-12
B2ZPN8_KLEPN	B2ZPN8	<i>Klebsiella pneumoniae</i>
C4IIN3_CLOBU	C4IIN3	<i>Clostridium butyricum</i> E4 str. BoNT E BL5262
GLDA_CITFR	P45511	<i>Citrobacter freundii</i>
GLDA_PSEPU	P50173	<i>Pseudomonas putida</i>

[076] Dihydroxyacetone kinase (E.C. No. 2.7.1.29) converts dihydroxyacetone to dihydroxyacetone phosphate. The *E. coli* operon *dhaKLM* encodes a dihydroxyacetone kinase that uses phosphoenolpyruvate as the phosphate donor, resulting in the conversion of dihydroxyacetone and phosphoenolpyruvate (PEP) to dihydroxyacetone phosphate and pyruvate. The *Citrobacter freundii* operon *dhaKL* encodes a dihydroxyacetone kinase that

uses adenosine-5'-triphosphate (ATP) as the phosphate donor, resulting in the conversion of dihydroxyacetone and ATP to dihydroxyacetone phosphate and adenosine-5'-diphosphate (ADP). Additional examples of glycerol dehydrogenase enzymes are presented in Table 4, below.

TABLE 4		
Locus	GenBank Accession No.	Organism
DHAK_ECOLI	P76015	<i>Escherichia coli</i> K-12
DHAL_ECOLI	P76014	<i>Escherichia coli</i> K-12
DHAM_ECOLI	P37349	<i>Escherichia coli</i> K-12
DHAK_CITFR	P45510	<i>Citrobacter freundii</i>
Q1KLC3_CITFR	Q1KLC3	<i>Citrobacter freundii</i>
DAK1_YEAST	P54838	<i>Saccharomyces cerevisiae</i>

5

[077] In some embodiments, a non-naturally occurring microbial organism of the invention is generated from a host that inherently contains the enzymatic capability to convert glycerol to dihydroxyacetone phosphate; however, increased rates of conversion of glycerol to dihydroxyacetone phosphate are desirable. Increased synthesis of dihydroxyacetone phosphate from glycerol can be achieved by, for example, overexpression of nucleic acids encoding one or more of the above-described glycerol dissimilation pathway enzymes or proteins. Overexpression of the glycerol dissimilation pathway enzyme or enzymes can be achieved by, for example, the exogenous expression of the endogenous gene or genes, or the exogenous expression of the heterologous gene or genes of a glycerol dissimilation pathway.

10 An expression vector can be constructed to express one or more endogenous or heterologous genes of the glycerol dissimilation pathway, as exemplified herein, operably linked to expression control sequences functional in the host organism. Expression vectors applicable for use in the microbial host organisms of the invention include, for example, plasmids, phage vectors, viral vectors, episomes and artificial chromosomes, including vectors and

15 selection sequences or markers operable for stable integration into a host chromosome. When two or more exogenous encoding nucleic acids are to be co-expressed, both nucleic acids can be inserted, for example, into a single expression vector or in separate expression vectors. In addition, a non-naturally occurring organism can be generated by mutagenesis of an endogenous gene that results in an increase in activity of an enzyme in the glycerol

20 dissimilation pathway.

25

[078] Glycerol crosses the microbial cell membrane and enters the cytoplasm through a process of facilitated diffusion. For example, the GlpF polypeptide of *E. coli*, encoded by the

gene *glpF* (Table 3, below), is a transmembrane channel protein that facilitates the entry of glycerol into the *E. coli* cell. *glpF* mutants have impaired growth on low concentrations of glycerol (Richey, D.P., and E.C.C. Lin. 1972. Importance of facilitated diffusion for effective utilization of glycerol by *Escherichia coli*. J. Bact. 112: 784-790).

- 5 Overexpression of a glycerol facilitator may lead to improved rates of glycerol dissimilation. Examples of glycerol facilitator proteins are listed in Table 5, below.

TABLE 5		
Locus	GenBank Accession No.	Organism
NP_418362	NP_418362	<i>Escherichia coli</i> K-12
AAO37928	AAO37928	<i>Borrelia hermsii</i> DAH
CAH19327	CAH19327	<i>Yersinia pseudotuberculosis</i> IP 32953
BAB61772	BAB61772	<i>Thermus aquaticus</i>
AAO57623	AAO57623	<i>Pseudomonas syringae</i> pv. tomato str. DC3000
CAY72431	CAY72431	<i>Erwinia pyrifoliae</i> DSM 12163

- [079] The rate of dimethylallyl diphosphate formation from glyceraldehyde-3-phosphate and pyruvate may be increased through overexpression of one or more genes encoding
- 10 enzymes of the methylerythritol pathway (Figure 2): 1-deoxy-D-xylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺
- 15 oxidoreductase, or isopentenyl diphosphate isomerase.

- [080] 1-deoxy-D-xylulose-5-phosphate synthase (E.C. No. 2.2.1.7) converts glyceraldehyde-3-phosphate and pyruvate to 1-deoxy-D-xylulose-5-phosphate and carbon dioxide. This enzyme activity is encoded by the *E. coli* gene *dxs*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of
- 20 1-deoxy-D-xylulose-5-phosphate synthase enzymes are presented in Table 6, below.

TABLE 6		
Locus	GenBank Accession No.	Organism
DXS_ECOLI	P77488	<i>Escherichia coli</i> K-12
DXS_BACSU	P54523	<i>Bacillus subtilis</i>
DXS_MYCTU	P0A554	<i>Mycobacterium tuberculosis</i>
B4YSH1_SALMI	B4YSH1	<i>Salvia miltiorrhiza</i>
DXS_HAEIN	P45205	<i>Haemophilus influenzae</i> str. ATCC 51907

- [081] 1-deoxy-D-xylulose-5-phosphate reductoisomerase (E.C. No. 1.1.1.267) converts 1-deoxy-D-xylulose-5-phosphate to 2-C-methyl-D-erythritol-4-phosphate with the concomitant oxidation of NAD(P)H and H⁺ to NAD(P)⁺. This enzyme activity is encoded by the *E. coli* gene *dxr*. Other comparable enzyme activities and the corresponding genes are
- 5 known in other organisms. Additional examples of 1-deoxy-D-xylulose-5-phosphate reductoisomerase enzymes are presented in Table 7, below.

TABLE 7		
Locus	GenBank Accession No.	Organism
DXR_ECOLI	P45568	<i>Escherichia coli</i> K-12
DXR_BACSU	O31753	<i>Bacillus subtilis</i>
DXR_MYCTU	P64012	<i>Mycobacterium tuberculosis</i>
O96693_PLAFA	O96693	<i>Plasmodium falciparum</i>
Q5J7B3_GINBI	Q5J7B3	<i>Ginkgo biloba</i>

- [082] 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (E.C. No. 2.7.7.60) converts 2-C-methyl-D-erythritol-4-phosphate and cytidine-5'-triphosphate (CTP) to 4-
- 10 diphosphocytidyl-2-C-methyl-D-erythritol with the release of inorganic pyrophosphate. This enzyme activity is encoded by the *E. coli* gene *ispD*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase enzymes are presented in Table 8, below.

TABLE 8		
Locus	GenBank Accession No.	Organism
ISPD_ECOLI	Q46893	<i>Escherichia coli</i> K-12
ISPD_BACSU	Q06755	<i>Bacillus subtilis</i>
ISPD_MYCTU	P96864	<i>Mycobacterium tuberculosis</i>
A9ZN10_HEVBR	A9ZN10	<i>Hevea brasiliensis</i>
ISPD_ARATH	P69834	<i>Arabidopsis thaliana</i>

- [083] 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (E.C. No. 2.7.1.148) converts 4-diphosphocytidyl-2-C-methyl-D-erythritol and ATP to 2-phospho-4-diphosphocytidyl-2-C-methyl-D-erythritol and ADP. This enzyme activity is encoded by the *E. coli* gene *ispE*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
- 20 enzymes are presented in Table 9, below.

TABLE 9		
Locus	GenBank Accession No.	Organism
ISPE_ECOLI	P62615	<i>Escherichia coli</i> K-12
ISPE_BACSU	P37550	<i>Bacillus subtilis</i>

ISPETHET2	Q72GN2	<i>Thermus thermophilus</i> str. ATCC BAA-163
A6XAA8_GINBI	A6XAA8	<i>Ginkgo biloba</i>
A6XAA9_BINBI	A6XAA9	<i>Ginkgo biloba</i>
ISPE_ARATH	O81014	<i>Arabidopsis thaliana</i>

[084] 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (E.C. No. 4.6.1.12) converts 2-phospho-4-diphosphocytidyl-2-C-methyl-D-erythritol to 2-C-methyl-D-erythritol-2,4-cyclodiphosphate and cytidine-5'-monophosphate (CMP). This enzyme activity is encoded by the *E. coli* gene *ispF*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase enzymes are presented in Table 10, below.

TABLE 10		
Locus	GenBank Accession No.	Organism
ISPF_ECOLI	P62617	<i>Escherichia coli</i> K-12
ISPF_BACSU	Q06756	<i>Bacillus subtilis</i>
ISPF_ENTFA	Q839V8	<i>Enterococcus faecalis</i>
A9ZN13_HEVBR	A9ZN13	<i>Hevea brasiliensis</i>
A1KXW3_HEVBR	A1KXW3	<i>Hevea brasiliensis</i>

[085] 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (E.C. No. 1.17.7.1) converts one molecule of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate and two reduced ferredoxins to 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate and two oxidized ferredoxins with the concomitant release of one water molecule. This enzyme activity is encoded by the *E. coli* gene *ispG*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase enzymes are presented in Table 11, below.

TABLE 11		
Locus	GenBank Accession No.	Organism
ISPG_ECOLI	P62620	<i>Escherichia coli</i> K-12
ISPG_BACSU	P54482	<i>Bacillus subtilis</i>
ISPG_THEEB	Q8DK70	<i>Thermosynechococcus elongatus</i> str. BP-1
ISPG_CLOAB	Q97156	<i>Clostridium acetobutylicum</i>
ISPG_AGR5	P58665	<i>Agrobacterium tumefaciens</i> str. C58

[086] Dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase (E.C. No. 1.17.1.2) converts 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate into isopentenyl diphosphate and dimethylallyl diphosphate. This enzyme activity is encoded by the *E. coli* gene *ispH*, where the enzyme catalyzes the conversion of 1-hydroxy-2-methyl-2-

(E)-butenyl-4-diphosphate into isopentenyl diphosphate and dimethylallyl diphosphate in a ratio of approximately 5 or 6 to 1 (for the *E. coli* enzyme; Rohdich, F., Hecht, S., Gärtner, K., Adam, P., Kreiger, C., Amslinger, S., Arigoni, D., Bacher, A. and W. Eisenreich. 2002. Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein. Proc. Natl. Acad. Sci. USA 99: 1158-1163). In other organisms, where other comparable enzyme activities and the corresponding genes are known, the comparable enzyme may produce isopentenyl diphosphate and dimethylallyl diphosphate in different ratios. Additional examples of dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase enzymes are presented in Table 12, below.

TABLE 12		
Locus	GenBank Accession No.	Organism
ISPH_ECOLI	P62623	<i>Escherichia coli</i> K-12
ISPH_BACSU	P54473	<i>Bacillus subtilis</i>
ISPH_ZYMMO	Q5NP61	<i>Zymomonas mobilis</i>
ISPH_AQUAE	O67625	<i>Aquifex aeolicus</i>
A7AME9_BABBO	A7AME9	<i>Babesia bovis</i>

[087] Isopentenyl diphosphate isomerase (E.C. No. 5.3.3.2) isomerizes isopentenyl diphosphate to dimethylallyl diphosphate in a reversible reaction. This enzyme activity is encoded by the *E. coli* gene *idi*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of isopentenyl diphosphate isomerase enzymes are presented in Table 13, below.

TABLE 13		
Locus	GenBank Accession No.	Organism
IDI_ECOLI	Q46822	<i>Escherichia coli</i> K-12
IDI2_BACSU	P50740	<i>Bacillus subtilis</i>
IDI2_STRC1	Q9KWG2	<i>Streptomyces</i> sp. str. CL190
O81660_HAEPL	O81660	<i>Haematococcus pluvialis</i>
IDI1_YEAST	P15496	<i>Saccharomyces cerevisiae</i>
IDI2_STAA9	A5IVC7	<i>Staphylococcus aureus</i> str. JH9
IDI2_STAAM	P65102	<i>Staphylococcus aureus</i> ATCC 700699

[088] The rate of dimethylallyl diphosphate formation from acetyl-CoA may be increased through overexpression of one or more genes encoding enzymes of the mevalonate pathway (Figure 3): acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, or isopentenyl diphosphate isomerase.

[089] Acetyl-CoA C-acetyltransferase (E.C. No. 2.3.1.9) converts two molecules of acetyl-CoA to acetoacetyl-CoA and CoASH. This enzyme activity is encoded by the *E. coli* gene *atoB*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of acetyl-CoA C-acetyltransferase are presented in

5 Table 14, below.

TABLE 14		
Locus	GenBank Accession No.	Organism
<i>E.C. No. 2.3.1.9</i>		
ATOB_ECOLI	P76461	<i>Escherichia coli</i> K-12
THLA_STAAM	Q99WM3	<i>Staphylococcus aureus</i> (ATCC 700699)
AF290094	AF290094	<i>Enterococcus faecalis</i>
Q8NN21_CORGL	Q8NN21	<i>Corynebacterium glutamicum</i>
THL_BACSU	P45855	<i>Bacillus subtilis</i>
THIC_MOUSE	Q8CAY6	<i>Mus musculus</i>
THIC_MOUSE	Q8CAY6	<i>Mus musculus</i>
QCR1_YEAST	P07256	<i>Saccharomyces cerevisiae</i>
THLA_CLOAB	P45359	<i>Clostridium acetobutylicum</i>
THIC_HUMAN	Q9BWD1	<i>Homo sapiens</i>

[090] Hydroxymethylglutaryl-CoA synthase (E.C. No. 2.3.3.10) converts one molecule of acetyl-CoA and one molecule of acetoacetyl-CoA to (S)-3-hydroxy-3-methylglutaryl-CoA and CoASH. This enzyme is encoded by the *Saccharomyces cerevisiae* locus

10 HMCS_YEAST. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of hydroxymethylglutaryl-CoA synthase are presented in Table 15, below.

TABLE 15		
Locus	GenBank Accession No.	Organism
<i>E.C. No. 2.3.3.10</i>		
HMCS1_MOUSE	Q8JZK9	<i>Mus musculus</i>
Q9FD87_STAAU	Q9FD87	<i>Staphylococcus aureus</i>
Q9FD71_ENTFA	Q9FD71	<i>Enterococcus faecalis</i>
B8ZMJ0_STRPJ	B8ZMJ0	<i>Streptococcus pneumoniae</i> (strain ATCC 700669)
A9ZMZ6_HEVBR	A9ZMZ6	<i>Hevea brasiliensis</i>
HMCS_YEAST	P54839	<i>Saccharomyces cerevisiae</i>

[091] Hydroxymethylglutaryl-CoA reductase converts (S)-3-hydroxy-3-methylglutaryl-CoA to mevalonic acid with the concomitant oxidation of two molecules of NAD(P)H. This enzyme is encoded by the *Saccharomyces cerevisiae* locus HMDH1_YEAST. Other comparable enzyme activities and the corresponding genes are known in other organisms.

Additional examples of hydroxymethylglutaryl-CoA reductase are presented in Table 16, below.

TABLE 16		
Locus	GenBank Accession No.	Organism
<i>E.C. No. 1.1.1.34</i>		
HMDH_METJA	Q58116	<i>Methanocaldococcus jannaschii</i>
Y1225_METJA	Q58622	<i>Methanocaldococcus jannaschii</i>
AF290094	AF290094	<i>Enterococcus faecalis</i>
HMDH1_HEVBR	P29057	<i>Hevea brasiliensis</i>
HMDH1_ARATH	P14891	<i>Arabidopsis thaliana</i>
A5IVX2_STAA9	A5IVX2	<i>Staphylococcus aureus</i> (strain JH9)
HMDH1_YEAST	P12683	<i>Saccharomyces cerevisiae</i>
HMDH2_YEAST	P12684	<i>Saccharomyces cerevisiae</i>
D1XUL0_9ACTO	D1XUL0	<i>Streptomyces sp. ACTE</i>
HMDH_ARCFU	O28538	<i>Archaeoglobus fulgidus</i>
D4GU18_HALVD	D4GU18	<i>Haloferax volcanii</i> (strain ATCC 29605)
HMDH_SULSO	O08424	<i>Sulfolobus solfataricus</i>
M24015	M24015	<i>Pseudomonas mevalonii</i>

[092] In some instances, the acetyl-CoA C-acetyltransferase and

5 hydroxymethylglutaryl-CoA reductase activities may be encoded by a single gene. One such example is the *mvaE* gene of *Enterococcus faecalis* (Hedl, M., Sutherlin, A., Wilding, E. I., Mazzulla, M., McDevitt, D., Lane, P., Burgner, J. W. II, Lehnbeuter, K. R., Stauffacher, C. V., Gwynn, M. N., and V. W. Rodwell. 2002. *Enterococcus faecalis* acetoacetyl-Coenzyme A thiolase/3-hydroxy-3-methylglutaryl-coenzyme A reductase, a dual-function protein of
10 ispentenyl diphosphate biosynthesis. J. Bacteriol. 184: 2116 – 2122).

[093] Mevalonate kinase (E.C. No. 2.7.1.36) converts mevalonic acid and ATP to 5-phosphomevalonate and ADP. This enzyme is encoded by the KIME_YEAST locus of *Saccharomyces cerevisiae*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of mevalonate kinase are presented in

15 Table 17, below.

TABLE 17		
Locus	GenBank Accession No.	Organism
<i>E.C. No. 2.7.1.36</i>		
KIME_METJA	Q58487	<i>Methanocaldococcus jannaschii</i>
D4V248_ENTFA	D4V248	<i>Enterococcus faecalis</i> PC1.1
KIME_ARATH	P46086	<i>Arabidopsis thaliana</i>
A5IQE4_STAA9	A5IQE4	<i>Staphylococcus aureus</i> (strain JH9)
KIME_YEAST	P07277	<i>Saccharomyces cerevisiae</i>
B8ZLF2_STRPJ	B8ZLF2	<i>Streptococcus pneumoniae</i> (strain ATCC 700669)
KIME_ARCFU	O27995	<i>Archaeoglobus fulgidus</i>
D4GWT5_HALVD	D4GWT5	<i>Haloferax volcanii</i> (strain ATCC 29605)
Q980D2_SULSO	Q980D2	<i>Sulfolobus solfataricus</i>

- [094] Phosphomevalonate kinase (E.C. No. 2.7.4.2) converts 5-phosphomevalonate and ATP to 5-diphosphomevalonate and ADP. This enzyme is encoded by the ERG8_YEAST locus of *Saccharomyces cerevisiae*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of phosphomevalonate kinase are presented in Table 18, below.

TABLE 18		
Locus	GenBank Accession No.	Organism
<i>E.C. No. 2.7.4.2</i>		
D4V246_ENTFA	D4V246	<i>Enterococcus faecalis</i> PC1.1
A9ZN02_HEVBR	A9ZN02	<i>Hevea brasiliensis</i>
Q2FJ51_STAA3	Q2FJ51	<i>Staphylococcus aureus</i> (strain USA300)
ERG8_YEAST	P24521	<i>Saccharomyces cerevisiae</i>

- [095] Diphosphomevalonate decarboxylase (E.C. No. 4.1.1.33) converts 5-diphosphomevalonate and ATP to ADP, phosphate, isopentenyl diphosphate and carbon dioxide. This enzyme is encoded by the MVD1_YEAST locus of *Saccharomyces cerevisiae*. Other comparable enzyme activities and the corresponding genes are known in other organisms. Additional examples of diphosphomevalonate decarboxylase are presented in Table 19, below.

TABLE 19		
Locus	GenBank Accession No.	Organism
<i>E.C. No. 4.1.1.33</i>		
D4V247_ENTFA	D4V247	<i>Enterococcus faecalis</i> PC1.1
A9ZN03_HEVBR	A9ZN03	<i>Hevea brasiliensis</i>
O23722_ARATH	O23722	<i>Arabidopsis thaliana</i>
A5IQE5_STAA9	A5IQE5	<i>Staphylococcus aureus</i> (strain JH9)
MVD1_YEAST	P32377	<i>Saccharomyces cerevisiae</i>
B8ZLF3_STRPJ	B8ZLF3	<i>Streptococcus pneumoniae</i> (strain ATCC 700669)
D0KQL8_SULS9	D0KQL8	<i>Sulfolobus solfataricus</i> (strain 98/2)

- [096] Isopentenyl diphosphate can be converted to dimethylallyl diphosphate by isopentenyl diphosphate isomerase (Table 13, above).

- [097] Dimethylallyl diphosphate may be converted to isoprene by several routes, including, but not limited to: 1) direct conversion of dimethylallyl diphosphate to isoprene by an isoprene synthase; 2) direct conversion of dimethylallyl diphosphate to isoprene by a mutant terpene synthase, for example a myrcene synthase or farnesene synthase, with substrate specificity altered from its natural substrate (geranyl diphosphate or farnesyl

diphosphate, respectively) to dimethylallyl diphosphate; 3) a three-step isoprene biosynthetic pathway comprising the steps of direct conversion of dimethylallyl diphosphate to 3-methyl-2-buten-1-ol followed by enzymatic isomerization to 2-methyl-3-buten-2-ol and dehydration of 2-methyl-3-buten-2-ol to isoprene; or 4) a two-step isoprene biosynthetic pathway

comprising the steps of direct conversion of dimethylallyl diphosphate to 2-methyl-3-buten-2-ol followed by dehydration of 2-methyl-3-buten-2-ol to isoprene. These routes are depicted in Figure 4.

[098] As used herein, isoprene synthases are nuclearly encoded, naturally occurring polypeptides found in some plant plastids, particularly in the chloroplast, that convert dimethylallyl diphosphate to isoprene, and derivatives (mutants) of polypeptides that naturally convert dimethylallyl diphosphate to isoprene (Figure 4. A.). Isoprene synthases are characterized, in part, by an amino-terminal plastid targeting sequence (“transit peptide”) that routes the polypeptide to the chloroplast. Upon translocation into the chloroplast, the transit peptide may be cleaved from the polypeptide to yield a mature protein that is smaller in molecular weight than the precursor protein. Isoprene synthases have been found in several species of poplar tree (*Populus spp.*) and in kudzu (*Pueraria montana var. lobata*) with activities verified *in vitro*. Additional coding sequences predicted to encode isoprene synthases have been identified in other plant species. Exemplary isoprene synthase genes are presented in Table 20, below.

TABLE 20

Locus	GenBank Accession No.	Organism
ISPS_POPCN	Q9AR86	<i>Populus canescens</i>
ISPS_POPAL	Q50L36	<i>Populus alba</i>
ISPS_PUEML	Q6EJ97	<i>Pueraria montana var. lobata</i>
ISPS_POPTM	Q7XAS7	<i>Populus tremuloides</i>
A0PFK2_POPNI	A0PFK2	<i>Populus nigra</i>
A9Q7C9_POPAL	A9Q7C9	<i>Populus alba</i>
D8UY75_POPAL	D8UY75	<i>Populus alba</i>
D8UY76_POPAL	D8UY76	<i>Populus alba</i>
H2CSU6_ROBPS	H2CSU6	<i>Robinia pseudoacacia</i>

[099] For overexpression of an heterologous isoprene synthase in a microbial organism, it is preferable to express a truncated isoprene synthase that approximates the mature form found in nature, rather than the precursor form. Essentially, the sequence encoding the transit peptide is removed from the isoprene synthase coding sequence. While visual inspection may allow one skilled in the art to select where to truncate the isoprene synthase coding

sequence, computer-based algorithms such as ChloroP 1.1 can be used to help predict which amino acids belong to the transit peptide (Emanuelsson, O., Nielsen, H., G. von Heijne. 1999. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci. 8: 978-984). H2CSU6 from *Robinia pseudoacacia* is
 5 predicted to be a truncated isoprene synthase coding region. It lacks an amino-terminal methionine and a proposed chloroplast transit peptide.

[0100] The direct conversion of dimethylallyl diphosphate to isoprene may be catalyzed by a mutant terpene synthase, for example a myrcene synthase or farnesene synthase, with substrate specificity altered from its natural substrate (geranyl diphosphate or farnesyl
 10 diphosphate, respectively) to dimethylallyl diphosphate (Figure 4. A.). Table 21, below, provides examples of terpene synthases for use in the conversion of dimethylallyl diphosphate to isoprene.

TABLE 21		
Locus	GenBank Accession No.	Organism
<i>Myrcene Synthase</i> (E.C. No. 4.2.3.15)		
MYRS_OCIBA	Q5SBP1	<i>Ocimum basilicum</i>
MYRS1_ARATH	Q9ZUH4	<i>Arabidopsis thaliana</i>
MYRS2_ARATH	Q9LRZ6	<i>Arabidopsis thaliana</i>
MYRS_QUEIL	Q93X23	<i>Quercus ilex</i>
TPSD2_ABIGR	Q24474	<i>Abies grandis</i>
Q58GE8_IPSPI	Q58GE8	<i>Ips pini</i>
<i>(E)-β-Ocimene Synthase</i> (E.C. No. 4.2.3.B40)		
Q5UB07_MEDTR	Q5UB07	<i>Medicago truncatula</i>
Q672F7_LOTJA	Q672F7	<i>Lotus japonicas</i>
Q84NC8_ANTMA	Q84NC8	<i>Antirrhinum majus</i>
<i>β-Farnesene Synthase</i> (E.C. No. 4.2.3.47)		
FARS_ZEAMM	C7E5V7	<i>Zea mays</i> subsp. <i>Mexicana</i>
FARS_MAIZE	Q2NM15	<i>Zea mays</i>
TPSBF_MENPI	O48935	<i>Mentha piperita</i>
ACSS_MAIZE	Q84ZW8	<i>Zea mays</i>
FARS_ARTAN	Q9FXY7	<i>Artemisia annua</i>
FARS_ZEAMH	C7E5V8	<i>Zea mays</i> subsp. <i>Huehuetenangensis</i>
FARS_ZEADI	C7E5V9	<i>Zea diploperennis</i>
FARS_ZEAPE	C7E5W0	<i>Zea perennis</i>
FARS_CITJU	Q94JS8	<i>Citrus junos</i>
D7PCH9_PINSY	D7PCH9	<i>Pinus sylvestris</i>
Q0J7R9_ORYSJ	Q0J7R9	<i>Oryza sativa</i> subsp. <i>japonica</i>

[0101] Dimethylallyl diphosphate may be converted to isoprene by a three-step isoprene
 15 biosynthetic pathway. As used herein, enzyme names are defined as follows (Figure 4. B.). A 3-methyl-2-buten-1-ol synthase or 3-methyl-2-buten-1-ol synthase is an enzyme that

catalyzes the conversion of dimethylallyl diphosphate to 3-methyl-2-buten-1-ol, also referred to herein as prenyl. A 2-methyl-3-buten-2-ol isomerase is an enzyme that catalyzes the isomerization of 3-methyl-2-buten-1-ol to 2-methyl-3-buten-2-ol. A 2-methyl-3-buten-2-ol dehydratase is an enzyme that catalyzes the conversion of 2-methyl-3-buten-2-ol to isoprene.

- 5 [0102] In a first step, dimethylallyl diphosphate is converted to 3-methyl-2-buten-1-ol by a 3-methyl-2-buten-1-ol synthase. In a second step, 3-methyl-2-buten-1-ol is converted to 2-methyl-3-buten-2-ol by a 2-methyl-3-buten-2-ol isomerase. In a third step, 2-methyl-3-buten-2-ol is converted to isoprene by a 2-methyl-3-buten-2-ol dehydratase. The second and third steps may be catalyzed by a single, bi-functional enzyme with both 2-methyl-3-buten-2-ol isomerase and 2-methyl-3-buten-2-ol dehydratase activities.

- [0103] The conversion of dimethylallyl diphosphate to 3-methyl-2-buten-1-ol (prenyl) may be catalyzed by a phosphatase. Examples of such phosphatases include enzymes encoded by the *Bacillus subtilis* genes *yqkG* (*nudF*) and *yhfR* (Withers, S.T., Gottlieb, S.S., Lieu, B., Newman, J.D. and J.D. Keasling. 2007. Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity. Appl. Env. Microbiol. 73: 6277-6283), although other known phosphatases and coding sequences with predicted phosphatase activity, for example, the *ytjC* gene of *E. coli*, may be used. Table 22, below, provides examples of phosphatases for use in the conversion of dimethylallyl diphosphate to prenyl.

TABLE 22		
Locus	GenBank Accession No.	Organism
BAA12639 (<i>YqkG</i>)	BAA12639	<i>Bacillus subtilis</i>
CAA74541 (<i>YhfR</i>)	CAA74541	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> Strain 168
GPMB_ECOLI (<i>YtjC</i>)	P0A7A2	<i>Escherichia coli</i> K-12

20

- [0104] The conversion of dimethylallyl diphosphate to 3-methyl-2-buten-1-ol may be catalyzed by a terpene synthase, e.g., a geraniol synthase or farnesol synthase or mutants thereof, for example. Table 23, below, provides examples of terpene synthases for use in the conversion of dimethylallyl diphosphate to 3-methyl-2-buten-1-ol.

TABLE 23		
Locus	GenBank Accession No.	Organism
<i>Geraniol Synthase</i>		
AAR11765	AAR11765	<i>Ocimum basilicum</i>
ABB30216	ABB30216	<i>Perilla citriodora</i>

ABB30217	ABB30217	<i>Perilla citriodora</i>
ABB30218	ABB30218	<i>Perilla frutescens</i>
CAE52821	CAE52821	<i>Cinnamomum tenuipile</i>
<i>Farnesol Synthase</i>		
ACSS_MAIZE	Q84ZW8	<i>Zea mays</i>
ABJ16554	ABJ16554	<i>Oryza sativa</i>

[0105] 3-methyl-2-buten-1-ol is isomerized to 2-methyl-3-buten-2-ol by a 2-methyl-3-buten-2-ol isomerase. As used herein, a 2-methyl-3-buten-2-ol isomerase is an enzyme that converts 3-methyl-2-buten-1-ol (prenol) to 2-methyl-3-buten-2-ol in a reversible reaction.

- 5 An example of such an enzyme is the linalool dehydratase-isomerase of *Castellaniella defragrans* strain 65Phen, GenBank accession number FR669447. This enzyme catalyzes the isomerization of 3-methyl-2-buten-1-ol to 2-methyl-3-buten-2-ol and the dehydration of 2-methyl-3-buten-2-ol to isoprene (Example 2, below, and Figure 5). Orthologs, paralogs and nonorthologous gene displacements of linalool dehydratase-isomerase can be determined by
- 10 methods well known to those skilled in the art.

- [0106] As used herein, a 2-methyl-3-buten-2-ol dehydratase is an enzyme that converts 2-methyl-3-buten-2-ol to isoprene. An example of such an enzyme is the linalool dehydratase-isomerase of *Castellaniella defragrans* strain 65Phen, GenBank accession number FR669447. This enzyme is capable of catalyzing the dehydration of 2-methyl-3-buten-2-ol to
- 15 isoprene (Example 3, below, and Figure 6). Orthologs, paralogs and nonorthologous gene displacements of linalool dehydratase-isomerase can be determined by methods well known to those skilled in the art. For example, inspection of nucleic acid or amino acid sequences for two polypeptides will reveal sequence identity and similarities between the compared sequences. Based on such similarities, one skilled in the art can determine if the similarity is
- 20 sufficiently high to indicate the proteins are related through evolution from a common ancestor. Algorithms well known to those skilled in the art, such as Align, BLAST, Clustal W and others compare and determine a raw sequence similarity or identity, and also determine the presence or significance of gaps in the sequence which can be assigned a weight or score. Such algorithms also are known in the art and are similarly applicable for determining
- 25 nucleotide sequence similarity or identity. Parameters for sufficient similarity to determine relatedness are computed based on well known methods for calculating statistical similarity, or the chance of finding a similar match in a random polypeptide, and the significance of the match determined. A computer comparison of two or more sequences can, if desired, also be

optimized visually by those skilled in the art. Related gene products or proteins can be expected to have a high similarity, for example, 25% to 100% sequence identity. Proteins that are unrelated can have an identity that is essentially the same as would be expected to occur by chance, if a database of sufficient size is scanned (about 5%). Sequences between 5% and 24% may or may not represent sufficient homology to conclude that the compared sequences are related. Additional statistical analysis to determine the significance of such matches given the size of the data set can be carried out to determine the relevance of these sequences.

[0107] Exemplary parameters for determining relatedness of two or more sequences using the BLAST algorithm, for example, can be as set forth below. Briefly, amino acid sequence alignments can be performed using BLASTP version 2.0.8 (Jan. 5, 1999) and the following parameters: Matrix: 0 BLOSUM62; gap open: 11; gap extension: 1; x_dropoff: 50; expect: 10.0; wordsize: 3; filter: on. Nucleic acid sequence alignments can be performed using BLASTN version 2.0.6 (Sep. 16, 1998) and the following parameters: Match: 1; mismatch: -2; gap open: 5; gap extension: 2; x_dropoff: 50; expect: 10.0; wordsize: 11; filter: off. Those skilled in the art will know what modifications can be made to the above parameters to either increase or decrease the stringency of the comparison, for example, and determine the relatedness of two or more sequences.

[0108] 2-methyl-3-buten-2-ol dehydratase enzyme activity has also been identified in *Aquicola tertiaricarbonis* (Schuster, J., Schäfer, F., Hübler, N., Brandt, A., Rosell, M., Härtig, C., Harms, h., Müller, R. H. and T. Rohwerder. 2012. Bacterial degradation of *tert*-amyl alcohol proceeds via hemiterpene 2-methyl-3-buten-2-ol by employing the tertiary alcohol desaturase function of the Rieske nonheme mononuclear iron oxygenase MdpJ. J. Bact. 194: 972-981). The sequence of this 2-methyl-3-buten-2-ol dehydratase has not been reported.

[0109] Dimethylallyl diphosphate may be converted to isoprene by a two-step isoprene biosynthetic pathway (Figure 4. C.). As used herein, a 2-methyl-3-buten-2-ol synthase is an enzyme that catalyzes the conversion of dimethylallyl diphosphate to 2-methyl-3-buten-2-ol.

[0110] As used herein, a 2-methyl-3-buten-2-ol synthase (also referred to as MBO synthase) is a nuclearily encoded, naturally occurring polypeptide found in some plant plastids, particularly in the chloroplast, that converts dimethylallyl diphosphate to 2-methyl-3-buten-2-ol, and derivatives (mutants) of polypeptides that naturally convert dimethylallyl

diphosphate to 2-methyl-3-buten-2-ol. MBO synthases are characterized, in part, by an amino-terminal plastid targeting sequence that routes the polypeptide to the chloroplast. Upon translocation into the chloroplast, the transit peptide may be cleaved from the polypeptide to yield a mature protein that is smaller in molecular weight than the precursor protein. For overexpression of an exogenous MBO synthase in a microbial organism, it is preferable to express a truncated MBO synthase that approximates the mature form found in nature, rather than the precursor form. Essentially, the sequence encoding the transit peptide is removed from the MBO synthase coding sequence. While visual inspection may allow one skilled in the art to select where to truncate the isoprene synthase coding sequence, computer-based algorithms such as ChloroP 1.1 can be used to help predict which amino acids belong to the transit peptide (Emanuelsson, O., Nielsen, H., G. von Heijne. 1999. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci. 8: 978-984). An example of an MBO synthase is found in *Pinus sabiniana*, with the GenBank accession number AEB53064.1.

- 15 [0111] The conversion of dimethylallyl diphosphate to 2-methyl-3-buten-2-ol may be catalyzed by a terpene synthase, e.g., a linalool synthase (E.C. No. 4.2.3.25 or 4.2.3.26) or nerolidol synthase or mutants thereof, for example. Table 24, below, provides examples of terpene synthases for use in the conversion of dimethylallyl diphosphate to 2-methyl-3-buten-2-ol.

TABLE 24

Locus	GenBank Accession No.	Organism
<i>S-Linalool Synthase</i>		
LIS_CLABR	Q96376	
LINS_ARATH	Q84UV0	<i>Clarkia breweri</i>
C0KWV3_9LAMI	C0KWV3	<i>Arabidopsis thaliana</i>
C0KWV5_PERFR	C0KWV5	<i>Perilla setoyensis</i>
C0KWV7_PERFR	C0KWV7	<i>Perilla frutescens</i> var. <i>hirtella</i>
D4N3A0_9ERIC	D4N3A0	<i>Perilla frutescens</i> var. <i>hirtella</i>
D4N3A1_9ERIC	D4N3A1	<i>Actinidia arguta</i>
		<i>Actinidia polygama</i>
<i>R-Linalool Synthase</i>		
LLOS1_ARTAN	Q9SPN0	<i>Artemesia annua</i>
LLOS_OCIBA	Q5SBP3	<i>Ocimum basilicum</i>
LLOS5_ARTAN	Q9SPN1	
LLOS_MENAQ	Q8H2B4	<i>Artemesia annua</i>
		<i>Mentha aquatica</i>

Q1XBU5_SOLLC	Q1XBU5	<i>Solanum lycopersicum</i>
(3S,6E)-Nerolidol Synthase		
Q5UB06_MEDTR	Q5UB06	<i>Medicago trunculata</i>

[0112] The conversion of 2-methyl-3-buten-2-ol to isoprene may be catalyzed by a 2-methyl-3-buten-2-ol dehydratase as described above. The 2-methyl-3-buten-2-ol dehydratase may be a bi-functional enzyme with both 2-methyl-3-buten-2-ol isomerase and 2-methyl-3-buten-2-ol dehydratase activities, such as the linalool dehydratase-isomerase described above, or the enzyme may encode only the 2-methyl-3-buten-2-ol dehydratase activity without a 2-methyl-3-buten-2-ol isomerase activity.

[0113] The conversion of glycerol to undesirable co-products may be reduced or eliminated by mutations of one or more genes. In general, expression of these genes may result in the routing of carbon from glycerol to undesirable co-products, or create an unfavorable reduction / oxidation state within the host cell, reducing the yield of isoprene from glycerol. Examples of desirable genes / enzyme activities to be reduced or eliminated include: lactate dehydrogenase (for example, the *ldhA* or *lldD* genes of *E. coli*), succinate dehydrogenase (for example, the *frdABCD* genes of *E. coli*), acetate kinase (the *ackA* gene of *E. coli*), phosphate acetyltransferase (the *pta* gene of *E. coli*), pyruvate oxidase (the *poxB* gene of *E. coli*), or alcohol dehydrogenase (the *adhE* gene of *E. coli*).

STRAINS, PLASMIDS AND GENE ABBREVIATIONS

[0114] In the following examples of embodiments of the current invention, the common *E. coli* cloning strains DH10B and DH5 α (GC5) were used during construction of all vectors. For testing the plasmid constructs, either BL21(DE3), MG1655, or the MG1655 derivative strain LA02 was used as the host strain. DNA acquired through complete synthesis was received already transformed in DH10B. Vectors that were constructed in house were transformed into chemically competent DH5 α cells (GC5, Gene Choice, available from Sigma-Aldrich Co. LLC). Wild type MG1655 was obtained from the University of Wisconsin *E. coli* Genome Project (<https://www.genome.wisc.edu>). MG1655 was made electrocompetent and electroporated following the protocol from the MicroPulser Electroporation Apparatus Operating Instructions and Applications Guide (Bio-Rad catalog

number 165-2100), except that LB without salt was used to grow up the culture in making cells electrocompetent. Strain genotypes are found in Table 25.

TABLE 25 – STRAINS		
Strain	Genotype	Reference
MG1655	Wild type <i>E. coli</i> ($F^- \lambda^- ilvR^- rfb-50 rph-I$)	ATCC [†] 47076
LA02	MG1655 $\Delta ackA \Delta pta \Delta frdA \Delta adhE$	This work.
BL21(DE3)	<i>fhuA2 [lon] ompT gal (λ DE3) [dcn] $\Delta hsdS$ λ DE3 = λ sBamHIo $\Delta EcoRI-B$ int::(<i>lacI</i>::<i>PlacUV5</i>::<i>T7 gene1</i>) <i>i21 $\Delta nin5$</i></i>	New England Biolabs, Inc. (www.neb.com)
DH10B	<i>E. coli</i> (F^- endA1 recA1 galE15 galK16 nupG rpsL $\Delta lacX74 \Phi 80 lacZ \Delta M15$ araD139 Δ (ara,leu)7697 mcrA Δ (mrr-hsdRMS-mcrBC) λ^-)	DNA2.0, www.dna20.com
GC5 (DH5 α)	<i>E. coli</i> ($F^- \Phi 80 lacZ \Delta M15 \Delta$ (lacZYA-argF)U169 recA1 endA1 hsdR17(rk ⁻ , mk ⁺) phoA supE44 thi-1 gyrA96 relA1 λ^- tonA)	Sigma-Aldrich Co. LLC, www.sigmaaldrich.com
TRANSFORMAX [™] EC100D [™] <i>pir</i> ⁺	$F^- mcrA \Delta$ (mrr-hsdRMS-mcrBC) $\Phi 80 dlacZ \Delta M15 \Delta lacX74$ recA1 endA1 araD139 Δ (ara, leu)7697 galU galK λ^- rpsL (<i>Str</i> ^R) nupG <i>pir</i> ⁺ (DHFR)	Epicentre Biotechnologies (illumina, Inc.; www.epibio.com)

[†] American Type Culture Collection

TABLE 26 – PLASMIDS		
Plasmid	Features	Source
pZS.glpK.glpD	<i>E. coli</i> <i>glpK</i> and <i>glpD</i> genes under the control of $P_{LtetO-1}$ (<i>tetR</i> , <i>oriR</i> SC101*)	Mazumdar, S., Clomburg, J. M., and R. Gonzalez. 2010. <i>Escherichia coli</i> strains engineered for homofermentative production of D-lactic acid from glycerol. Appl. Env. Microbiol. 76: 4327-4336.
pZS.adhEp.glpK.glpD	<i>E. coli</i> <i>glpK</i> and <i>glpD</i> genes under the control of <i>adhE</i> promoter (<i>tetR</i> , <i>oriR</i> SC101*)	This work
pR6Kan	Kanamycin-resistant derivative of pGP704	Orchard, S. S., and H. Goodrich- Blair. 2005. Pyrimidine nucleoside salvage confers an advantage to <i>Xenorhabdus</i> <i>nematophila</i> in its host interactions. Appl. Environ. Microbiol. 71:6254-6259
pR6KT- Δ ldhA	pR6Kan derivative for deleting <i>ldhA</i> in <i>E. coli</i>	Glycos Biotechnologies, Inc.
pR6KT- Δ frdA	pR6Kan derivative for deleting <i>frdA</i> in <i>E. coli</i>	Glycos Biotechnologies, Inc.
pR6KT- Δ ackA Δ pta	pR6Kan derivative for deleting <i>ackA</i> and <i>pta</i> in <i>E. coli</i>	Glycos Biotechnologies, Inc.
pR6KT- Δ adhE1Kb	pR6Kan derivative for deleting <i>adhE</i> in <i>E. coli</i>	Glycos Biotechnologies, Inc.
pJexpress401	Bacterial expression vector, Rep _{pUC} Km ^R	DNA2.0 (www.dna20.com)
pJexpress404	Bacterial expression vector, Rep _{pUC} Ap ^R	DNA2.0 (www.dna20.com)
pJ404-LDI		Glycos Biotechnologies, Inc.
pJ404-SAAT		Glycos Biotechnologies, Inc.
pJ401-MBO1.IDI		Glycos Biotechnologies, Inc.
pJ401-MBO1.IDI.LDI		Glycos Biotechnologies, Inc.
pGA31R-MCS	Rep _{p15A} Cm ^R tetR MCS	DNA2.0 (www.dna20.com)
pGE21R-MCS	Rep _{ColE1} Km ^R tetR $P_{LtetO-1}$ MCS T1	DNA2.0 (www.dna20.com)
pJ248-mvaES	Rep _{pUC} Ap ^R Gent ^R mvaES _{Ef,optEc}	DNA2.0 (www.dna20.com)
pJ241-MK.PMK.MPD.IDI	Rep _{pUC} Km ^R T1 $P_{LtetO-1}$ MK _{Mj,optEc} PMK _{Ef,optEc} MPD _{Sc,optEc} IDI _{Ec,optEc} T1	DNA2.0 (www.dna20.com)
pUC57-ispS	Rep _{ColE1} Ap ^R P_{lac} ispS _{Pa,optEc}	GenScript, Inc.
pGB1004	Rep _{ColE1} Km ^R tetR $P_{LtetO-1}$ ispS _{Pa,optEc} T1	Glycos Biotechnologies, Inc.
pGB1008	Rep _{p15A} Cm ^R tetR $P_{LtetO-1}$ mvaES _{Ef,optEc} T1	Glycos Biotechnologies, Inc.

pGB1012	Rep _{ColE1} Km ^R tetR P _{LtetO-1} ispS _{Pa,optEc} IDI _{Ec,optEc} T1	Glycos Biotechnologies, Inc.
pGB1030	Rep _{p15A} Cm ^R tetR P _{LtetO-1} mvaES _{Ef,optEc} T1 P _{LtetO-1}	Glycos Biotechnologies, Inc.
pGB1030-GlyOH	MK _{Mj,optEc} PMK _{Ef,optEc} MPD _{Sc,optEc} IDI _{Ec,optEc} T1	Glycos Biotechnologies, Inc.
	pGB1030 P _{adhEp}	

[0115] Table 26 Notes: Ef = *Enterococcus faecalis* ATCC 700802; Mj = *Methanocaldococcus jannaschii*; Sc = *Saccharomyces cerevisiae* S288C; Ec = *Escherichia coli* MG1655; Pa = *Populus alba*; optEC = codon-optimized for expression in *E. coli*; MCS = multiple cloning site; Rep = origin of replication (ColE1, p15A or pUC); P_{LtetO-1} = anhydrotetracycline-inducible promoter; P_{adhEp} = constitutive promoter fragment from *E. coli* *adhE* gene; T1 = terminator T1 of the *rrnB* operon of *E. coli*.

EXAMPLE 1

IMPROVING GLYCEROL DISSIMILATION

[0116] This working example demonstrates the improvement of glycerol dissimilation by overexpression of *glpK* and *glpD* in engineered *E. coli*. In this example, the rate of production of lactic acid is used as a proxy for flux of glycerol through the key metabolic intermediate dihydroxyacetone phosphate.

[0117] The *E. coli* strain used was LA02 (MG1655 Δ *ackA* Δ *pta* Δ *frdA* Δ *adhE*) containing plasmid pZS.adhEp.glpK.glpD. The strain was constructed as follows.

[0118] To create the basic suicide vector used to delete genes from the *E. coli* chromosome, the R6K γ origin of replication, kanamycin marker and multiple cloning site of plasmid pR6Kan (Orchard, S. S., and H. Goodrich-Blair. 2005. Pyrimidine nucleoside salvage confers an advantage to *Xenorhabdus nematophila* in its host interactions. Appl. Environ. Microbiol. 71:6254-6259) were amplified by polymerase chain reaction (PCR) using the following primers:

Primer 1: 5'- ATCGAGCTCAACCATCATCGATGAATTGC -3' [SEQ ID NO: 1]

Primer 2: 5'- TCTGGTACCCTGAAGCTTGCATGCC -3' [SEQ ID NO: 2]

[0119] The first primer introduces a SacI restriction enzyme site while the second primer introduces a KpnI restriction enzyme site.

[0120] An approximately 500 base pair (bp) fragment upstream of the *E. coli ldhA* gene was PCR amplified using the following primers:

5 Primer 1: 5'- ATAGAGCTCCCACCAGATAACGG -3' [SEQ ID NO: 3]

Primer 2: 5'- GCGCTCGAGTTTCATAAGACTTTCTCC -3' [SEQ ID NO: 4]

[0121] The first primer introduces a SacI restriction enzyme site while the second primer introduces a XhoI restriction site.

10 [0122] An approximately 500 base pair fragment downstream of the *E. coli ldhA* gene was PCR amplified using the following primers:

Primer 1: 5'- GAC CTC GAG CTG GTT TAA TCT TGC CG -3' [SEQ ID NO: 5]

Primer 2: 5'- CGT GGT ACC TTC TTT CAG CAT TTC G -3' [SEQ ID NO: 6]

[0123] The first primer introduces a XhoI restriction enzyme site while the second primer introduces a SacI restriction site.

15 [0124] The three PCR products were restriction-digested with SacI, XhoI and/or KpnI restriction enzymes as appropriate, and the resulting fragments were used in a trimolecular ligation reaction with the QUICK LIGASE KIT™ (New England Biolabs, Ipswich, MA). Restriction enzymes and buffer components were removed from the ligation reaction using the DNA CLEAN AND CONCENTRATOR KIT™ (Zymo Research, Irvine, CA) before
20 electroporation into TransforMax EC100D *pir*⁺ electrocompetent cells (Epicentre Biotechnologies, Madison, WI). Transformants were selected on LB plates containing 50 µg/ml kanamycin and the correct plasmid identified by restriction digestion. The resulting intermediate plasmid was then further modified by introduction of the *tetRA* locus as a counterselectable marker as follows: the intermediate plasmid was restriction digested using
25 BamHI, while the *tetRA* locus was PCR amplified using primers P7 and P8; the two DNA fragments were joined using the IN-FUSION CLONING SYSTEM™ (Clontech, Mountain View, CA), diluted five-fold with water, then electroporated into TransforMax EC100D *pir*⁺ electrocompetent cells. Transformants were selected on LB plates containing 50 µg/ml

kanamycin and the correct plasmid identified by restriction digestion. This plasmid is named pR6KT- Δ ldhA.

[0125] Plasmid pR6KT- Δ frdA was constructed as follows. An approximately 448 base pair fragment upstream of the *E. coli frdA* gene was PCR amplified using the following primers:

Primer 1: 5'- ATTGAGCTCGGTTACCGTCGCC -3' [SEQ ID NO: 7]

Primer 2: 5'- GCCATTCGCCTTCTCGAGCACGACATTCCTCC -3' [SEQ ID NO: 8]

[0126] The first primer introduces a SacI restriction enzyme site while the second primer introduces a XhoI restriction site.

[0127] An approximately 471 base pair fragment downstream of the *E. coli frdA* gene was PCR amplified using the following primers:

Primer 1: 5'- GGAGGAATCTCGTGCTCGAGAAGGCGAATGGC -3' [SEQ ID NO: 9]

Primer 2: 5'- AGCGGTACCACAGTTGATGCAACC -3' [SEQ ID NO: 10]

[0128] The first primer introduces a XhoI restriction enzyme site while the second primer introduces a KpnI restriction site. The two resulting PCR products were joined using overlap extension PCR (OE-PCR) with the following primers:

Primer 1: 5'- ATTGAGCTCGGTTACCGTCGCC -3' [SEQ ID NO: 11]

Primer 2: 5'- AGCGGTACCACAGTTGATGCAACC -3' [SEQ ID NO: 12]

[0129] The resulting PCR product was digested with SacI and KpnI. Plasmid pR6KT- Δ ldhA was restriction digested with SacI and KpnI followed by purification by agarose gel electrophoresis. The band corresponding to the vector backbone was recovered from the agarose using the GEL DNA RECOVERY KIT™ (Zymo Research). The two restriction-digested DNA fragments corresponding to the vector backbone, and the fused upstream and downstream regions of *frdA*, were used in a ligation reaction with the QUICK LIGASE KIT™ (New England Biolabs). Restriction enzymes and buffer components were removed

from the ligation reaction using the DNA CLEAN AND CONCENTRATOR KIT™ (Zymo Research) before electroporation into TransforMax EC100D *pir*⁺ electrocompetent cells (Epicentre Biotechnologies). Transformants were selected on LB plates containing 50 µg/ml kanamycin and the correct plasmid identified by restriction digestion.

- 5 [0130] pR6KT-ΔackAΔpta. Plasmid pR6KT-ΔackAΔpta was constructed as follows. An approximately 462 base pair fragment upstream of the *E. coli ackA* gene was PCR amplified using the following primers:

Primer 1: 5'-ACGGAGCTCATCTTGATAACGCGAT-3' [SEQ ID NO: 13]

10 Primer 2: 5'-TACCTCGAGCATGGAAGTACCTATAATTG-3' [SEQ ID NO: 14]

[0131] The first primer introduces a SacI restriction enzyme site while the second primer introduces a XhoI restriction enzyme site. The resulting PCR product was restriction digested with SacI and XhoI.

- 15 [0132] An approximately 473 base pair fragment downstream of the *E. coli pta* gene was PCR amplified using the following primers:

Primer 1: 5'-GGATAAACCGTGTCCCTCGAGCAGCAGTAATCTCG-3' [SEQ ID NO: 15]

Primer 2: 5'-ACTGGTACCTCTAGAAGTTCATCAGGCC-3' [SEQ ID NO: 16]

- 20 [0133] The first primer introduces a XhoI restriction enzyme site while the second primer introduces a KpnI restriction enzyme site. The resulting PCR product was restriction digested with XhoI and KpnI.

- [0134] Plasmid pR6KT-ΔldhA was restriction digested with SacI and KpnI followed by purification by agarose gel electrophoresis. The band corresponding to the vector backbone was recovered from the agarose using the GEL DNA RECOVERY KIT™ (Zymo Research).
- 25 The three restriction-digested DNA fragments corresponding to the vector backbone, the upstream region of *ackA*, and the downstream region of *pta* were used in a trimolecular ligation reaction with the QUICK LIGASE KIT™ (New England Biolabs, Ipswich, MA). Restriction enzymes and buffer components were removed from the ligation reaction using

the DNA CLEAN AND CONCENTRATOR KIT™ (Zymo Research) before electroporation into TransforMax EC100D *pir*⁺ electrocompetent cells (Epicentre Biotechnologies). Transformants were selected on LB plates containing 50 µg/ml kanamycin and the correct plasmid identified by restriction digestion.

- 5 [0135] Plasmid pR6KT-Δ*adhE*1kb was constructed as follows. An approximately 1000 base pair fragment upstream of the *E. coli adhE* gene was PCR amplified using the following primers:

Primer 1: 5'- ATAGAGCTCAGCGAACAATGCAATAGCC -3' [SEQ ID NO: 17]

10 Primer 2: 5'- CAGCGCTACTGATTAAGCTGTACAAGTAACAGCCATA
ATGCTCTCC -3' [SEQ ID NO: 18]

[0136] The first primer introduces a *SacI* restriction enzyme site while the second primer introduces a *BsrGI* restriction site.

[0137] An approximately 1000 base pair fragment downstream of the *E. coli adhE* gene was PCR amplified using the following primers:

15 Primer 1: 5'- GGAGAGCATTATGGCTGTTACTTGTACAGCTTAATCAGT
AGCGCTG -3' [SEQ ID NO: 19]

Primer 2: 5'- ACAGCATGCGAACACCACTTCCAGAGC -3' [SEQ ID NO: 20]

[0138] The first primer introduces a *BsrGI* restriction enzyme site while the second primer introduces a *SphI* restriction site. The two resulting PCR products were joined using
20 overlap extension PCR (OE-PCR) with the following primers:

Primer 1: 5'- ATAGAGCTCAGCGAACAATGCAATAGCC -3' [SEQ ID NO:21]

Primer 2: 5'- ACAGCATGCGAACACCACTTCCAGAGC -3' [SEQ ID NO:22]

[0139] The resulting PCR product and plasmid pR6KT-Δ*ldhA* were restriction digested with *SacI* and *SphI* followed by purification by agarose gel electrophoresis. The bands
25 corresponding to the PCR fragment and vector backbone were recovered from the agarose using the GEL DNA RECOVERY KIT™ (Zymo Research). The two restriction-digested DNA fragments corresponding to the vector backbone, and the fused upstream and

downstream regions of *adhE*, were used in a ligation reaction with the QUICK LIGASE KIT™ (New England Biolabs). Restriction enzymes and buffer components were removed from the ligation reaction using the DNA CLEAN AND CONCENTRATOR KIT™ (Zymo Research) before electroporation into TransforMax EC100D *pir*⁺ electrocompetent cells
5 (Epicentre Biotechnologies). Transformants were selected on LB plates containing 50 µg/ml kanamycin and the correct plasmid identified by restriction digestion

[0140] Gene deletions in *E. coli* were made using the approach of Metcalf and colleagues (Metcalf, W.W., Jiang, W., Daniels, L.L., Kim, S.K., Haldiman, A. and B.L. Wanner. 1996. Conditionally replicative and conjugative plasmids carrying *lacZα* for cloning, mutagenesis,
10 and allele replacement in bacteria. Plasmid 35: 1-13). *E. coli* MG1655 strains with various genes deleted were made using suicide vectors. pR6KT-based gene knock-out plasmids were purified from TransforMax EC100D using standard plasmid DNA purification techniques and concentrated to approximately 1 µg/ml or greater. Concentrated plasmid DNA was electroporated into the *pir*⁻ recipient strain. Transformants were selected on LB agar plates
15 containing 15 µg/ml tetracycline and 2.5 mM Na₄P₂O₇. After confirming integration by testing for resistance to tetracycline, integration into the correct locus was confirmed by PCR. Transformants with the knock-out plasmid integrated into the proper locus were restreaked onto LB agar plates without antibiotic selection to provide the opportunity for chromosomal rearrangement to resolve the gene duplication. Individual colonies from the LB agar plate
20 were then restreaked onto tetracycline-sensitive-selection agar (TSS) and incubated for two or more days at 30 °C. Large colonies from the TSS agar plates were then tested for sensitivity to tetracycline and kanamycin using LB agar plates containing either tetracycline (15 µg/ml) or kanamycin (50 µg/ml). As resolution of the gene duplication can either generate a gene deletion or recreate the wild-type locus, colonies sensitive to both kanamycin
25 and tetracycline were then tested for the desired gene deletion using PCR. Further confirmation was provided by restriction digest of the PCR product with either XhoI or BsrGI (as appropriate).

[0141] TSS agar plates were made as follows. 4.347 g NaH₂PO₄ was mixed with 100 mL distilled water. To this solution, the following chemicals were added: 3 ml of fusaric acid, 2
30 mg/ml; 2.5 mL ZnCl₂, 20 mM; and 0.5 ml anhydrotetracycline, 5 mg/mL. This buffer solution was sterilized by nanofiltration. 2.5 g tryptone, 2.5 g yeast extract, 5 g sodium chloride, and 7.5 g agar were mixed with 400 mL distilled water and autoclaved. Once the

agar solution cooled to approximately 45 °C, it was mixed with 100 mL of the buffer solution. This final buffer/agar mixture was then poured into 100 mm-diameter petri plates.

[0142] Using the above gene deletion method, the *ackA/pta*, *frdA* and *adhE* genes were sequentially deleted from MG1655 to create LA02.

5 [0143] Plasmid pZS.adhEp.glpK.glpD was constructed by inserting an *adhE* promoter at the XhoI and KpnI restriction sites of plasmid pZS.glpK.glpD, replacing the PLteto-1 promoter. The *adhE* promoter was used because it is a constitutive promoter. The *adhE* promoter was amplified by PCR using genomic DNA of *E. coli* MG1655 as the template and the following primers:

10 [0144] Primer 1: 5'- CGCCTCGAGGGTTAGCTCCGAAGCAAAAGC -3' [SEQ ID NO: 23]

[0145] Primer 2: 5'- CGCGGTACCAATGCTCTCCTGATAATGTAAAC -3' [SEQ ID NO: 24]

[0146] The resulting PCR product was digested with XhoI and KpnI. Likewise,
15 pZS.glpK.glpD was digested with XhoI and KpnI. The two restriction digested fragments corresponding to the vector backbone and the *adhE* promoter was purified by agarose gel electrophoresis and fused together in a ligation reaction using the QUICK LIGASE KIT™ (NEB). The ligation reaction was purified using the DNA CLEAN AND
20 CONCENTRATOR KIT™ (Zymo Research) to reduce PEG concentrations before electroporation into GC5 electrocompetent cells. Transformants were selected on LB plates containing 20 µg/ml chloramphenicol and the correct plasmid identified by restriction digestion.

[0147] Strain LA02 was transformed with plasmid pZS.adhEp.glpK.glpD using
electroporation. A single colony was cultured for 16 hours at 37 °C in 5 mL of LB medium
25 supplemented with 20 µg/mL chloramphenicol. 0.5 mL of the culture was added to 0.5-mL of sterile glycerol, mixed well, and stored at -80 °C.

[0148] All experiments were routinely started from strains stored at -80 °C as glycerol stocks. LA02 not bearing plasmids was streaked from the appropriate glycerol stock onto LB-agar plates (LB medium is 10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone,

supplemented with 15 g/L agar). LA02 (pZS.adhEp.glpK.glpD) was streaked from the appropriate glycerol stock onto LB-agar plates supplemented with 20 µg/mL chloramphenicol. Individual colonies of LA02 (pZS.adhEp.glpK.glpD) were used to inoculate 5 mL of Luria Bertani broth supplemented with 20 µg/mL chloramphenicol, 20 g/L glycerol and 5 g/L CaCO₃ in a 25-mL Erlenmeyer flask. Flasks were incubated for 16 hours at 37 °C in a Lab Companion SI-600R incubating shaker set at 175 rpm.

[0149] The 16-hour cultures were used to seed 400-mL cultures of Luria Bertani broth supplemented with 50 g/L CaCO₃, 20 µg/mL chloramphenicol, and 60 g/L glycerol in a 0.5-L working volume fermentor (Ward's Natural Science, Rochester, NY) with independent control of temperature, pH, and stirrer speed. All experimental cultures were seeded with sufficient cells to achieve an initial optical density at 600 nm of 0.1. Temperature was maintained at 37 °C. pH was maintained at 7.0 using 5 N NaOH. The stirrer speed was maintained at 200 rpm. The cultures were aerated at 10 mL/min using air, sufficient to achieve a k_La of 100 h⁻¹. 5-mL samples were withdrawn from the cultures at 0-, 24-, 48- and 72-hour time points. The samples were used to measure cell growth, glycerol consumption, and metabolite production. Cell growth was determined by measuring the optical absorbance of a 1 to 10 dilution of the culture at 600 nm in a Biochrom Libra S22 spectrophotometer.

[0150] Glycerol consumption and metabolite production were quantified by HPLC analysis. All samples were filtered through a 0.22 µm polyvinylidene fluoride syringe filter (Millipore) prior to HPLC analysis. Routinely, 10-µL of filtered fermentation medium was injected onto an HPLC (LC-10AD vp, Shimadzu, Kyoto, Japan] fitted with a Rezex ROA-Organic Acid H⁺ (8%) 150 x 7.8 mm column (Phenomenex, Torrance, CA) at 65 °C with a mobile phase of 2.5 mM H₂SO₄ operated under isocratic conditions at a flow rate of 0.6 mL/minute. Metabolites were detected via a refractive index detector (RID-10A, Shimadzu).

[0151] Typical results are presented in Figure 7. Over the 72-hour period, LA02 (pZS.adhEp.glpK.glpD) consumed glycerol at the average rate of 1.2 mM/L/h/OD and lactic acid was produced at the average rate of 0.94 mM/L/h/OD. The final titer of lactic acid reached 79.32 g/L, while approximately 103 g/L glycerol was consumed.

EXAMPLE 2

MICROORGANISM FOR THE PRODUCTION OF ISOPRENE
FROM 3-METHYL-2-BUTEN-1-OL

[0152] This working example shows the production of isoprene from 3-methyl-2-buten-
5 1-ol by a non-naturally occurring microorganism expressing one or more exogenous genes of
an isoprene biosynthetic pathway.

[0153] The plasmid pJ404-LDI was constructed by DNA2.0 (Menlo Park, CA) using the
codon-optimized sequence of the linalool dehydratase-isomerase (LDI) of *Castellaniella*
defragrans strain 65Phen (Figure 8). The LDI coding sequence was codon-optimized for
10 expression in *E. coli* using the proprietary algorithms of DNA2.0, synthesized and inserted
into the plasmid expression vector pJexpress404. The resulting plasmid, pJ404-LDI, was
electroporated into *E. coli* BL21(DE3) electrocompetent cells.

[0154] Plasmid pJ404-SAAT was constructed by DNA2.0 (Menlo Park, CA) using the
codon-optimized sequence of the strawberry acyl-CoA transferase (SAAT) (Figure 9). The
15 SAAT coding sequence was codon-optimized for expression in *E. coli* using the proprietary
algorithms of DNA2.0, synthesized and inserted into the plasmid expression vector
pJexpress404. The resulting plasmid, pJ404-SAAT, was electroporated into *E. coli*
BL21(DE3) electrocompetent cells. pJ404-SAAT was used as a negative control.

[0155] Transformants of BL21(DE3) harboring either pJ404-LDI or pJ404-SAAT were
20 selected on Luria-Bertani (LB)-agar plates (10 g/L yeast extract, 5 g/L Bacto Tryptone, 10
g/L sodium chloride, 15 g/L Bacto Agar) containing 100 µg/ml ampicillin. The flask was
incubated for 16 hours at 37 °C in a rotary shaking incubator. After 16 hours, the culture was
diluted using LB broth containing 100 µg/ml ampicillin to an optical density of 0.16 at 600
nm.

25 [0156] A single colony of BL21(DE3) harboring pJ404-LDI or pJ404-SAAT from the
LB-agar plates was used to inoculate 10 milliliter aliquots of LB broth (10 g/L yeast extract,
5 g/L Bacto Tryptone, 10 g/L sodium chloride) containing 100 µg/ml ampicillin contained in
125-mL Erlenmeyer flasks. Flasks were incubated for 16 hours at 37 °C in a rotary shaking
incubator. After 16 hours, the resulting cultures were diluted using fresh LB broth containing

100 µg/ml ampicillin to an optical density of 0.16 at 600 nm. 50 milliliters of the diluted cultures were placed in 300-mL Erlenmeyer flasks and incubated at 37 °C in a rotary shaking incubator until the optical density at 600 nm reached approximately 0.6, typically 90 minutes. Four milliliters of the resulting cultures were then placed into 20-mL gas chromatography headspace vials. 3-methyl-2-buten-1-ol was added to a final concentration of 1 mM, IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to 1 mM, and the cultures were grown for an additional 16 hours at 37 °C with shaking.

[0157] Isoprene was measured using headspace analysis on an Agilent 7890A GC equipped with a CTC-PAL autosampler and a FID. Headspace vials (20 mL) were incubated at 50 °C with agitation at 500 rpm for 2 minutes. Then 1 mL of the headspace was removed using a heated headspace syringe at 50 °C and injected into the GC inlet (250 °C, split of 20:1). Samples were analyzed using a FID detector set at 300 °C, with a helium carrier gas flow rate of 2 mL/min through a DB-624 30 m x 530 µm x 3 µm column (J&W Scientific), and an oven program of 85 °C for 5.25 minutes. The isoprene concentration in samples was calculated from calibration curves generated from isoprene calibration gas standards analyzed under the same GC/FID method. The isoprene product was also confirmed by headspace GC/MS using an Agilent 7890A GC equipped with a 5975C MSD and a CTC-PAL autosampler. Headspace vials were incubated at 85 °C with agitation at 600 rpm for 5 minutes. Then 1 mL of the headspace was removed using a heated headspace syringe at 85 °C and injected into the GC inlet (250 °C, split of 25:1). The GC/MS method used helium as the carrier gas at 1 mL/min through a HP-5MS 30 m x 250 µm x 0.25 µm column (J&W Scientific), an oven program of 35 °C for 4 minutes, then ramped 25 °C/min to 150 °C, a MS source temperature of 230 °C, and a quadrupole temperature of 150 °C. The mass spectrometer was operated in scan mode from 25 to 160 mass units. The isoprene peak was identified by the NIST 11 MS Library, as well as comparison against an authentic sample (135 ppm isoprene, 135 ppm carbon dioxide in dry nitrogen gas, Matheson TRIGAS, Houston, TX).

[0158] 3-methyl-2-buten-1-ol and 2-methyl-3-buten-1-ol were measured using headspace analysis on an Agilent 7890A GC equipped with a CTC-PAL autosampler and a FID. Headspace vials (20 mL) were incubated at 85 °C with agitation at 600 rpm for 5 minutes. Then 1 mL of the headspace was removed using a heated headspace syringe at 85 °C and injected into the GC inlet (250 °C, split of 25:1). Samples were analyzed using a FID

detector set at 350 °C, with a helium carrier gas flow rate of 3 ml/min through at DB-624 30 m x 530 µm x 3 µm column (J&W Scientific), and an oven program of 90 °C, then ramping 20 °C/min to 230 °C for 3 minutes. The 3-methyl-2-buten-1-ol and 2-methyl-3-buten-2-ol concentrations in samples were calculated from calibration curves generated from diluted standards of each compound analyzed under the same GC/FID method.

[0159] The results of this example are presented in Figure 5 and Figure 10. LB broth containing 1 mM 3-methyl-2-buten-1-ol with *E. coli* cells omitted showed a peak at 3.96 minutes corresponding to 3-methyl-2-buten-1-ol (Figure 10). Similarly, cultures containing 1 mM 3-methyl-2-buten-1-ol and BL21(DE3) cells harboring pJ404-SAAT showed a peak at 3.96 minutes corresponding to 3-methyl-2-buten-1-ol, and an additional peak corresponding to the aldehyde 3-methyl-2-butenal (prenal, data not shown). In contrast, cultures containing 1 mM 3-methyl-2-buten-1-ol and BL21(DE3) cells harboring pJ404-LDI converted 3-methyl-2-buten-1-ol to 2-methyl-3-buten-2-ol and isoprene, corresponding to peaks at 2.96 minutes and 2.49 minutes, respectively (Figure 5). This demonstrates that *E. coli* cells harboring pJ404-LDI isomerize 3-methyl-2-buten-1-ol to 2-methyl-3-buten-2-ol and dehydrate 2-methyl-3-buten-2-ol to isoprene. Figure 11 presents the GC/MS analysis of an authentic isoprene sample; Figure 12 presents the GC/MS analysis of the peak with a 2.49 minute retention time, with the same fragmentation pattern as authentic isoprene shown in Figure 5.

EXAMPLE 3

MICROORGANISM FOR THE PRODUCTION OF ISOPRENE FROM 2-METHYL-3-BUTEN-2-OL

[0160] This working example shows the production of isoprene from 2-methyl-3-buten-2-ol by a non-naturally occurring microorganism expressing one or more exogenous genes of an isoprene biosynthetic pathway.

[0161] A single colony of BL21(DE3) harboring pJ404-LDI or pJ404-SAAT from LB-agar plates was used to inoculate 10 milliliter aliquots of LB broth (10 g/L yeast extract, 5 g/L Bacto Tryptone, 10 g/L sodium chloride) containing 100 µg/ml ampicillin contained in 125-mL Erlenmeyer flasks. Flasks were incubated for 16 hours at 37 °C in a rotary shaking incubator. After 16 hours, the cultures were diluted using fresh LB broth containing 100 µg/ml ampicillin to an optical density of 0.16 at 600 nm. 50 milliliters of the diluted cultures

were placed in 300-mL Erlenmeyer flasks and incubated at 37 °C in a rotary shaking incubator until the optical density at 600 nm reached approximately 0.6, typically 90 minutes. Four milliliters of the cultures were then placed into 20-mL gas chromatography headspace vials. 2-methyl-3-buten-2-ol was added to a final concentration of 1 mM. IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to 1 mM. Cultures containing 2-methyl-3-buten-2-ol were grown for 16 hours at 37 °C with shaking.

[0162] Isoprene, 3-methyl-2-buten-1-ol and 2-methyl-3-buten-2-ol were measured as above. The identity of the isoprene peak was also verified using GC/MS, as described above.

[0163] The results of this example are presented in Figure 6 and Figure 13. LB broth containing 1 mM 2-methyl-3-buten-2-ol with *E. coli* cells omitted showed a peak at 2.96 minutes corresponding to 2-methyl-3-buten-2-ol (Figure 13). Similarly, cultures containing 1 mM 2-methyl-3-buten-2-ol and BL21(DE3) cells harboring pJ404-SAAT showed a peak at 2.96 minutes corresponding to 2-methyl-3-buten-2-ol (data not shown). In contrast, cultures containing 1 mM 2-methyl-3-buten-2-ol and BL21(DE3) cells harboring pJ404-LDI converted 2-methyl-3-buten-2-ol to isoprene, corresponding to the peak at 2.49 minutes (Figure 6). This demonstrates that *E. coli* cells harboring pJ404-LDI dehydrate 3-methyl-2-buten-1-ol to isoprene.

EXAMPLE 4

MICROORGANISM FOR THE PRODUCTION OF ISOPRENE FROM GLYCEROL

[0164] The following prophetic example demonstrates a non-naturally occurring microorganism engineered for the efficient conversion of glycerol to isoprene.

[0165] Plasmid pGA31R-MCS was constructed entirely by DNA synthesis by DNA2.0 (Menlo Park, CA), with the nucleotide sequence presented in Figure 14. Plasmid pGE21R-MCS was constructed by replacing the chloramphenicol resistance marker and the p15A origin of replication of pGA31R-MCS with a kanamycin resistance marker and a ColE1 origin. The sequence of pGE21R-MCS is presented in Figure 15.

[0166] Plasmid pJ248-*mvaES* was constructed by DNA2.0 using the codon-optimized sequence of the *mvaE* and *mvaS* genes of *Enterococcus faecalis* ATCC 700802 (the codon-optimized sequences of *mvaE* and *mvaS* are as presented in Figure 16). The *mvaE* and *mvaS*

genes of *Enterococcus faecalis* ATCC 700802 were codon-optimized for expression in *E. coli* using the proprietary algorithms of DNA2.0, synthesized and inserted in the plasmid pJ248. Unique ribosomal binding sites were included in front of each gene, along with flanking endonuclease restriction sites for use in plasmid construction.

- 5 [0167] Plasmid pJ241-MK.PMK.MPD.IDI containing a codon-optimized synthetic operon was constructed entirely by DNA synthesis by DNA2.0, with the nucleotide sequence presented in Figure 17. The sequence of the synthetic operon, codon-optimized for expression in *E. coli*, encodes the mevalonate kinase gene of *Methanocaldococcus jannaschi*, the phosphomevalonate kinase of *Enterococcus faecalis* ATCC 700802, the mevalonate
10 pyrophosphate decarboxylase of *Saccharomyces cerevisiae* S288C, and the isopentenyl diphosphate isomerase gene of *E. coli* MG1655, including incorporated ribosomal binding sites and flanking restriction endonuclease sites used in subsequent cloning steps.

[0168] Plasmid pUC57-ispS was synthesized by Genscript (Piscataway, NJ). The ispS coding region, with an associated ribosome binding site, is presented in Figure 18.

- 15 [0169] Plasmid pGB1004 was constructed by inserting a PCR product encoding the codon-optimized *ispS* gene of *P. alba* into the KpnI and NcoI restriction endonuclease sites of plasmid pGE21R-MCS (Figure 19). The PCR product encoding the *ispS* gene was amplified from the plasmid pUC57-ispS using AccuPrimer Pfx polymerase with the following oligonucleotide primers:

- 20 Primer 1: 5'- ATAGGTACCATTAAAGAGGAGAAAATATAATGGAAGCTCG
TCGCTCCG -3' [SEQ ID NO: 25]
Primer 2: 5'- ATACCATGGACGTTTAGCGTTCAAACGGCAGG -3' [SEQ ID
NO: 26]

- Primer 1 incorporates a ribosomal binding site in front of the start codon of *ispS* and a KpnI
25 restriction site. Primer 2 incorporates an NcoI restriction site after the *ispS* stop codon. The PCR and plasmid pGE21R-MCS were digested with KpnI and NcoI, followed by gel purification. The fragments were cloned together using standard cloning techniques. Figure 18 shows the codon-optimized sequence of the synthetic isoprene synthase gene of *Populus alba*, containing a ribosomal binding site and flanking restriction endonuclease sites used in
30 subsequent cloning steps, but without the N-terminal transit peptide.

[0170] Plasmid pGB1012 was constructed by inserting a PCR product encoding the *idi* gene of *E. coli* into the NcoI site of pGB1004 (Figure 19). The PCR product encoding the *idi* gene was amplified from the plasmid pJ241-MK.PMK.MPD.IDI using AccuPrime Pfx polymerase with the following primers:

5 Primer 1: 5'- CGCTAAACGTCCATGTAAGGAGATATAAAAATGCAAACCG -3'
 SEQ ID NO: 27]

 Primer 2: 5'- TGATGCCTCTAGCCCATG -3' [SEQ ID NO: 28]

Primer 1 maintains the ribosomal binding site in front of the start codon of *idi*. Primer 1 and Primer 2 also include appropriate vector-overlapping 5' sequences for use with the In-Fusion Advantage PCR Cloning Kit (Clontech). The PCR product was gel-purified, as was pGB1004 linearized with the restriction endonuclease NcoI. Fragments were directionally joined together using the In-Fusion cloning kit and GC5 competent cells, following the manufacturer's directions. Transformants were screened, and the proper plasmid was identified through agarose gel electrophoresis of restriction endonuclease-digested plasmid DNAs.

[0171] Plasmid pGB1008 was constructed by cloning the optimized *mvaES* genes from pJ248-mvaES into pGA31R-MCS as a KpnI/MluI DNA fragment using standard cloning techniques (Figure 20).

[0172] Plasmid pGB1030 was created through the following process (Figure 21). pGB1008 was digested with the restriction endonucleases NcoI and MluI; the resulting 6.8 kb DNA fragment was gel-purified. Plasmid pJ241-MK.PMK.MPD.IDI was digested with the restriction endonucleases NcoI and MluI; the resulting 4.1 kb containing the synthetic operon was gel-purified. The fragments were ligated together using the NEB Quick Ligation Kit (New England Biolabs) and transformed into GC5 competent cells. Transformants were screened, and the proper plasmid was identified through agarose gel electrophoresis of restriction endonuclease-digested plasmid DNAs.

[0173] Plasmid pGB1030-GlyOH is created from plasmid pGB1030 and plasmid pZS-adhEp.glpK.glpD. A PCR fragment encoding a fragment of the *E. coli adhE* promoter, *glpK*, *glpD*, and the T1 terminator can be amplified from pZS-adhEp.glpK.glpD using Phusion Polymerase (NEB, Ipswich, MA) and the following primers:

Primer 1: 5'- TTTCGCCAGATATCGACGTCGGTTAGCTCCGAAGCAAAAGC -
3' [SEQ ID NO: 29]

Primer 2: 5'- ACAGGTTTGATGACGAACCCGTACCCTAGGTCTAGG -3' [SEQ
ID NO: 30]

5 [0174] The primers are designed to provide overlapping ends for use in an In-Fusion
reaction, with the first primer remaking the AatII site. The second primer destroys the AatII
site by replacing it with an AvrII site. The resulting 3.6 kB fragment is gel extracted and
joined to pGB1030 fragment linearized with AatII using the IN-FUSION HD KIT™. The
fragments are joined together using the IN-FUSION ADVANTAGE PCR CLONING KIT™
10 (Clontech Laboratories, Inc., Mountain View, CA), then transformed into chemically
competent *E. coli* GC5 cells (Gene Choice, available from Sigma-Aldrich Co. LLC)
following the manufacturer's directions. Transformants are screened, and the proper plasmid
is identified through agarose gel electrophoresis of restriction endonuclease-digested plasmid
DNAs.

15 [0175] Plasmids pGB1012 and pGB1030-GlyOH are co-transformed into MG1655 using
electroporation, generating the strain herein referred to as MG1655(1012/1030-GlyOH). This
combination of plasmids provides a mevalonate-based pathway for production of isoprene.

[0176] Seed cultures of MG1655(1012/1030-GlyOH) are prepared as follows: the
cultures (stored as glycerol stocks at -80 °C) are used to inoculate 5 ml (LB medium as
20 described above, containing appropriate amounts of chloramphenicol and kanamycin) seed
cultures in 15 ml culture tubes and grown aerobically at 37 °C and 175 rpm for 16 hours.
After 16 hours, the seed cultures are diluted into LB supplemented with appropriate
antibiotics, 20 g/l glycerol, and 100 µg/l anhydrotetracycline to achieve an initial optical
density of 0.3 at 600 nm. 10-ml aliquots of each diluted culture are placed in three 20-ml
25 headspace vials; the diluted cultures are incubated at 37 °C and 175 rpm. At the end of 1, 2
and 3 hours of incubation, one of the headspace vials is removed from the shaking incubator,
and the isoprene concentration is estimated by manually exposing a solid-phase
microextraction fiber (85 µm Carboxen/PDMS) to sample the headspace. The fiber is
desorbed at 300°C for 30 seconds prior to insertion into the headspace vial, exposed in the
30 vial at ~37°C for 60 seconds to extract the volatiles, and immediately desorbed in the injector
of an Agilent 5890 Series II GC at 200°C for 30 seconds (splitless injection, purge valve

closed). The initial hold is at 30°C for 5 minutes, followed by a ramp at 20°C/min to 230°C, with a final hold of 2 minutes. The carrier gas is helium, the FID detector temperature is kept at 250°C, and the column is a Rtx-5 (30 m x 530 µm x 3 µm). The samples are compared to a commercial isoprene standard. The concentration of isoprene in the samples may be
 5 calculated by comparison to calibration curves generated from diluted standards analyzed under the same GC/FID method.

EXAMPLE 5

MICROORGANISM FOR THE PRODUCTION OF ISOPRENE FROM DIMETHYLALLYL DIPHOSPHATE

10 [0177] This prophetic example demonstrates how one may produce isoprene with a non-naturally occurring microorganism expressing an MBO synthase, a 2-methyl-3-buten-2-ol isomerase, and a 2-methyl-3-buten-2-ol dehydratase.

[0178] The *Tps-MBO1* gene of *Pinus sabiniana* (GenBank Accession No. JF19039) and the *idi* gene of *Haematococcus pluvialis* are codon-optimized for expression in *E. coli* using
 15 the proprietary algorithms of DNA2.0 (including ribosome binding sites, a KpnI restriction site on the 5' end and an NcoI restriction site on the 3' end, Figure 22), synthesized and inserted into the plasmid vector pJexpress401 to produce pJ401-MBO1.IDI.

[0179] The codon-optimized *ldi* gene may be PCR amplified from pJ404-LDI using Phusion Polymerase (NEB) and the following primers:

20 5'- CGAAGCATAACCATGTAGGAGGTAAAACATATGCACCACC -3' [SEQ ID NO: 31]

5'- GCCCTTGGGGCCATGGTTACTTGCCCGCCAGCTTC -3' [SEQ ID NO: 32]

[0180] pJ401-MBO1.IDI is linearized by digestion with NcoI and purified by gel electrophoresis. The fragments are joined together using the IN-FUSION ADVANTAGE
 25 PCR CLONING KIT™ (Clontech Laboratories, Inc., Mountain View, CA), then transformed into chemically competent *E. coli* GC5 cells (GENE CHOICE™, available from Sigma-Aldrich Co. LLC) following the manufacturer's directions. Transformants are screened, and the proper plasmid is identified through agarose gel electrophoresis of restriction

endonuclease-digested plasmid DNAs. The proper plasmid is then transformed into electrocompetent *E. coli* BL21(DE3). The resulting plasmid is designated pJ401-MBO1.IDI.LDI.

[0181] The production of isoprene by BL21(DE3) harboring plasmid pJ401-MBO1.IDI.LDI may be assayed as follows. A single colony of BL21(DE3) harboring pJ401-MBO1.IDI.LDI or pJ404-LDI from LB-agar plates are used to inoculate 10 milliliter aliquots of LB broth (10 g/L yeast extract, 5 g/L Bacto Tryptone, 10 g/L sodium chloride) containing 20 g/L glycerol and 100 µg/ml ampicillin contained in 125-mL Erlenmeyer flasks. Flasks are incubated for 16 hours at 37 °C in a rotary shaking incubator. After 16 hours, the cultures are diluted using fresh LB broth containing 20 g/L glycerol and 100 µg/ml ampicillin to an optical density of 0.16 at 600 nm. 50 milliliters of the diluted cultures are placed in 300-mL Erlenmeyer flasks and incubated at 37 °C in a rotary shaking incubator until the optical density at 600 nm reaches approximately 0.6, typically 90 minutes. Four milliliters of the cultures are then placed into 20-mL gas chromatography headspace vials. IPTG (Isopropyl β-D-1-thiogalactopyranoside) is added to 1 mM. Cultures are grown for 16 hours at 37 °C with shaking.

[0182] Isoprene is measured using headspace analysis on an Agilent 7890A GC equipped with a CTC-PAL autosampler and a FID. Headspace vials (20 mL) are incubated at 50 °C with agitation at 500 rpm for 2 minutes. Then 1 mL of the headspace is removed using a heated headspace syringe at 50 °C and injected into the GC inlet (250 °C, split of 20:1). Samples are analyzed using a FID detector set at 300 °C, with a helium carrier gas flow rate of 2 ml/min through a DB-624 30 m x 530 µm x 3 µm column (J&W Scientific), and an oven program of 85 °C for 5.25 minutes. The isoprene concentration in samples is calculated from calibration curves generated from isoprene calibration gas standards analyzed under the same GC/FID method. The isoprene product is also confirmed by headspace GC/MS using an Agilent 7890A GC equipped with a 5975C MSD and a CTC-PAL autosampler. Headspace vials are incubated at 85 °C with agitation at 600 rpm for 5 minutes. Then 1 mL of the headspace is removed using a heated headspace syringe at 85 °C and injected into the GC inlet (250 °C, split of 25:1). The GC/MS method uses helium as the carrier gas at 1 mL/min through a HP-5MS 30 m x 250 µm x 0.25 µm column (J&W Scientific), an oven program of 35 °C for 4 minutes, then ramped 25 °C/min to 150 °C, a MS source temperature of 230 °C, and a quadrupole temperature of 150 °C. The mass spectrometer is operated in scan mode

from 25 to 160 mass units. The isoprene peak is identified by the NIST 11 MS Library, as well as comparison against an authentic sample (135 ppm isoprene, 135 ppm carbon dioxide in dry nitrogen gas, Matheson TRIGAS, Houston, TX).

[0183] 2-methyl-3-buten-1-ol is measured using headspace analysis on an Agilent 7890A
5 GC equipped with a CTC-PAL autosampler and a FID. Headspace vials (20 mL) are incubated at 85 °C with agitation at 600 rpm for 5 minutes. Then 1 mL of the headspace is removed using a heated headspace syringe at 85 °C and injected into the GC inlet (250 °C, split of 25:1). Samples are analyzed using a FID detector set at 350 °C, with a helium carrier gas flow rate of 3 mL/min through a DB-624 30 m x 530 µm x 3 µm column (J&W
10 Scientific), and an oven program of 90 °C, then ramping 20 °C/min to 230 °C for 3 minutes. The 2-methyl-3-buten-2-ol concentration in samples is calculated from calibration curves generated from diluted standards of the compound analyzed under the same GC/FID method.

[0184] The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or
15 unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

[0185] While the foregoing is directed to embodiments of the present invention, other and
20 further embodiments of the invention may be devised without departing from the basic scope thereof, and the scope thereof is determined by the claims that follow.

CLAIMS

What is claimed is:

1. A non-naturally occurring microorganism comprising:
a glycerol dissimilation pathway comprising a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and dihydroxyacetone kinase are encoded by an exogenous nucleic acid; and
an isoprene pathway comprising an acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, a isopentenyl diphosphate isomerase, a 2-methyl-3-buten-2-ol synthase, and a 2-methyl-3-buten-2-ol dehydratase, wherein one or more of the acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase, 2-methyl-3-buten-2-ol synthase, and 2-methyl-3-buten-2-ol dehydratase are encoded by an exogenous nucleic acid,
wherein the glycerol dissimilation pathway and the isoprene pathway are expressed in sufficient amounts to produce isoprene from glycerol.
2. The non-naturally occurring microorganism according to claim 1 wherein the microorganism further comprises an exogenous nucleic acid encoding a glycerol facilitator protein in sufficient amounts to improve the rate of glycerol dissimilation as compared to a parental strain without the exogenous nucleic acid.
3. The non-naturally occurring microorganism according to claim 1 wherein the microorganism has been modified to have reduced or eliminated activity of one or more of the following enzymes: lactate dehydrogenase, succinate dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, and alcohol dehydrogenase.
4. A non-naturally occurring microorganism comprising:
a glycerol dissimilation pathway comprising a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase,

wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and dihydroxyacetone kinase are encoded by an exogenous nucleic acid; and

an isoprene pathway comprising an acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, a isopentenyl diphosphate isomerase, a 3-methyl-2-buten-1-ol synthase, a 2-methyl-3-buten-2-ol isomerase, and a 2-methyl-3-buten-2-ol dehydratase, wherein one or more of the acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase, 3-methyl-2-buten-1-ol synthase, 2-methyl-3-buten-2-ol isomerase, and 2-methyl-3-buten-2-ol dehydratase are encoded by an exogenous nucleic acid,

wherein the glycerol dissimilation pathway and the isoprene pathway are expressed in sufficient amounts to produce isoprene from glycerol.

5. The non-naturally occurring microorganism according to claim 4 wherein the microorganism further comprises an exogenous nucleic acid encoding a glycerol facilitator protein in sufficient amounts to improve the rate of glycerol dissimilation as compared to a parental strain without the exogenous nucleic acid.

6. The non-naturally occurring microorganism according to claim 4 wherein the microorganism has been modified to have reduced or eliminated activity of one or more of the following enzymes: lactate dehydrogenase, succinate dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, and alcohol dehydrogenase.

7. A non-naturally occurring microorganism comprising:
a glycerol dissimilation pathway comprising a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and dihydroxyacetone kinase are encoded by an exogenous nucleic acid; and
an isoprene pathway comprising an acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, a isopentenyl

diphosphate isomerase, and a monoterpene synthase, sesquiterpene synthase or diterpene synthase capable of converting dimethylallyl diphosphate into isoprene, wherein one or more of the acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase and monoterpene synthase, sesquiterpene synthase or diterpene synthase are encoded by an exogenous nucleic acid,

wherein the glycerol dissimilation pathway and the isoprene pathway are expressed in sufficient amounts to produce isoprene from glycerol.

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8. The non-naturally occurring microorganism according to claim 7 wherein the monoterpene synthase, sesquiterpene synthase, or diterpene synthase is a myrcene, ocimene, or farnesene synthase with altered substrate specificity.

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9. The non-naturally occurring microorganism according to claim 7 wherein the microorganism further comprises an exogenous nucleic acid encoding a glycerol facilitator protein in sufficient amounts to improve the rate of glycerol dissimilation as compared to a parental strain without the exogenous nucleic acid.

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10. The non-naturally occurring microorganism according to claim 7 wherein the microorganism has been modified to have reduced or eliminated activity of one or more of the following enzymes: lactate dehydrogenase, succinate dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, and alcohol dehydrogenase.

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11. A non-naturally occurring microorganism comprising:
a glycerol dissimilation pathway comprising a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and dihydroxyacetone kinase are encoded by an exogenous nucleic acid; and
an isoprene pathway comprising an acetyl-CoA C-acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, a isopentenyl diphosphate isomerase, and an isoprene synthase, wherein one or more of the acetyl-CoA C-

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acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl diphosphate isomerase and isoprene synthase are encoded by an exogenous nucleic acid,

5 wherein the glycerol dissimilation pathway and the isoprene pathway are expressed in sufficient amounts to produce isoprene from glycerol.

12. The non-naturally occurring microorganism according to claim 11 wherein the microorganism further comprises an exogenous nucleic acid encoding a glycerol facilitator
10 protein in sufficient amounts to improve the rate of glycerol dissimilation as compared to a parental strain without the exogenous nucleic acid.

13. The non-naturally occurring microorganism according to claim 11 wherein the microorganism has been modified to have reduced or eliminated activity of one or more of
15 the following enzymes: lactate dehydrogenase, succinate dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, and alcohol dehydrogenase.

14. The non-naturally occurring microorganism according to claim 11 wherein the isoprene synthase is an isoprene synthase from *Populus alba*, *Robinia pseudoacacia* or the
20 genus *Wisteria*.

15. A non-naturally occurring microorganism comprising:
 a glycerol dissimilation pathway comprising a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol
25 dehydrogenase, and dihydroxyacetone kinase are encoded by an exogenous nucleic acid; and
 an isoprene pathway comprising a 1-deoxyxylulose-5-phosphate synthase, a 1- deoxy-D-xylulose-5-phosphate reductoisomerase, a 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, a 4-diphosphocytidyl-2-C-methylerythritol kinase, a 1- hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, a dimethylallyl-diphosphate/isopentenyl-
30 diphosphate:NAD(P)⁺ oxidoreductase, an isopentenyl diphosphate isomerase, a 2-methyl-3-buten-2-ol synthase, and a 2-methyl-3-buten-2-ol dehydratase, wherein one or more of the 1-deoxyxylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-

diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methylerythritol kinase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, dimethylallyl-diphosphate/isopentenyl- diphosphate:NAD(P)⁺ oxidoreductase, isopentenyl diphosphate isomerase, 2-methyl-3-buten-2-ol synthase, and 2-methyl-3-buten-2-ol

5 dehydratase are encoded by an exogenous nucleic acid,

wherein the glycerol dissimilation pathway and the isoprene pathway are expressed in sufficient amounts to produce isoprene from glycerol.

16. The non-naturally occurring microorganism according to claim 15 wherein the
10 microorganism further comprises an exogenous nucleic acid encoding a glycerol facilitator protein in sufficient amounts to improve the rate of glycerol dissimilation as compared to a parental strain without the exogenous nucleic acid.

17. The non-naturally occurring microorganism according to claim 15 wherein the
15 microorganism has been modified to have reduced or eliminated activity of one or more of the following enzymes: lactate dehydrogenase, succinate dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, and alcohol dehydrogenase.

18. A non-naturally occurring microorganism comprising:
20 a glycerol dissimilation pathway comprising a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and dihydroxyacetone kinase are encoded by an exogenous nucleic acid; and
an isoprene pathway comprising a 1-deoxyxylulose-5-phosphate synthase, a 1-
25 deoxy-D-xylulose-5-phosphate reductoisomerase, a 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, a 4-diphosphocytidyl-2-C-methylerythritol kinase, a 1- hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, a dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, an isopentenyl diphosphate isomerase, a 3-methyl-2-buten-1-ol synthase, a 2-methyl-3-buten-2-ol isomerase, and a 2-methyl-3-buten-2-ol
30 dehydratase, wherein one or more of the 1-deoxyxylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4- diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methylerythritol kinase, 1-hydroxy-2-methyl-2-(E)-butenyl-4- diphosphate synthase, dimethylallyl-diphosphate/isopentenyl-

diphosphate:NAD(P)⁺ oxidoreductase, isopentenyl diphosphate isomerase, 3-methyl-2-buten-1-ol synthase, 2-methyl-3-buten-2-ol isomerase and 2-methyl-3-buten-2-ol dehydratase are encoded by an exogenous nucleic acid,

wherein the glycerol dissimilation pathway and the isoprene pathway are expressed in sufficient amounts to produce isoprene from glycerol.

19. The non-naturally occurring microorganism according to claim 18 wherein the microorganism further comprises an exogenous nucleic acid encoding a glycerol facilitator protein in sufficient amounts to improve the rate of glycerol dissimilation as compared to a parental strain without the exogenous nucleic acid.

20. The non-naturally occurring microorganism according to claim 19 wherein the microorganism has been modified to have reduced or eliminated activity of one or more of the following enzymes: lactate dehydrogenase, succinate dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, and alcohol dehydrogenase.

21. A non-naturally occurring microorganism comprising:
a glycerol dissimilation pathway comprising a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and dihydroxyacetone kinase are encoded by an exogenous nucleic acid; and
an isoprene pathway comprising a 1-deoxyxylulose-5-phosphate synthase, a 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, a 4-diphosphocytidyl-2-C-methylerythritol kinase, a 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, a dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, an isopentenyl diphosphate isomerase, and a monoterpene synthase, sesquiterpene synthase, or diterpene synthase capable of converting dimethylallyldiphosphate into isoprene, wherein one or more of the 1-deoxyxylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methylerythritol kinase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, isopentenyl diphosphate

isomerase, and the monoterpene synthase, sesquiterpene synthase, or diterpene synthase are encoded by an exogenous nucleic acid,

wherein the glycerol dissimilation pathway and the isoprene pathway are expressed in sufficient amounts to produce isoprene from glycerol.

5

22. The non-naturally occurring microorganism according to claim 21 wherein the monoterpene synthase, sesquiterpene synthase, or diterpene synthase is a myrcene, ocimene, or farnesene synthase with altered substrate specificity.

10 23. The non-naturally occurring microorganism according to claim 21 wherein the microorganism further comprises an exogenous nucleic acid encoding a glycerol facilitator protein in sufficient amounts to improve the rate of glycerol dissimilation as compared to a parental strain without the exogenous nucleic acid.

15 24. The non-naturally occurring microorganism according to claim 21 wherein the microorganism has been modified to have reduced or eliminated activity of one or more of the following enzymes: lactate dehydrogenase, succinate dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, and alcohol dehydrogenase.

20 25. A non-naturally occurring microorganism comprising:
 a glycerol dissimilation pathway comprising a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase, wherein one or more of the glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, and dihydroxyacetone kinase are encoded by an exogenous nucleic acid; and
 25 an isoprene pathway comprising a 1-deoxyxylulose-5-phosphate synthase, a 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, a 4-diphosphocytidyl-2-C-methylerythritol kinase, a 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, a dimethylallyl-diphosphate/isopentenyl-diphosphate:NAD(P)⁺ oxidoreductase, an isopentenyl diphosphate isomerase, and an isoprene
 30 synthase, wherein one or more of the 1-deoxyxylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methylerythritol kinase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, dimethylallyl-diphosphate/isopentenyl-

diphosphate:NAD(P)⁺ oxidoreductase, isopentenyl diphosphate isomerase, and isoprene synthase are encoded by an exogenous nucleic acid,

wherein the glycerol dissimilation pathway and the isoprene pathway are expressed in sufficient amounts to produce isoprene from glycerol.

5

26. The non-naturally occurring microorganism according to claim 25 wherein the microorganism further comprises an exogenous nucleic acid encoding a glycerol facilitator protein in sufficient amounts to improve the rate of glycerol dissimilation as compared to a parental strain without the exogenous nucleic acid.

10

27. The non-naturally occurring microorganism according to claim 25 wherein the isoprene synthase is an isoprene synthase from *Populus alba*, *Robinia pseudoacacia* or the genus *Wisteria*.

15

28. The non-naturally occurring microorganism according to claim 25 wherein the microorganism has been modified to have reduced or eliminated activity of one or more of the following enzymes: lactate dehydrogenase, succinate dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, and alcohol dehydrogenase.

20

29. A method for producing isoprene comprising culturing the non-naturally occurring microorganism of any of claims 1-28 under suitable conditions in a medium comprising glycerol for a sufficient period of time to produce isoprene from the glycerol and recovering the isoprene.

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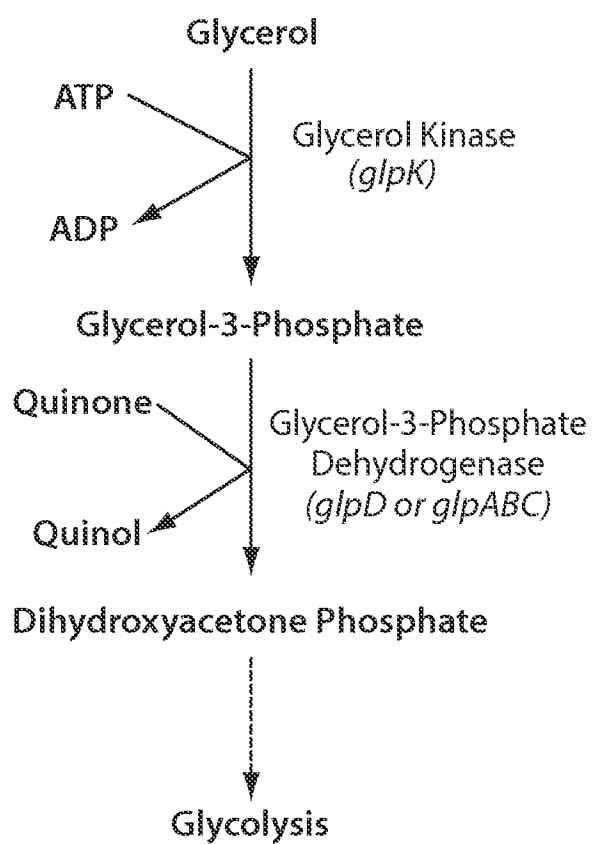


Fig. 1A

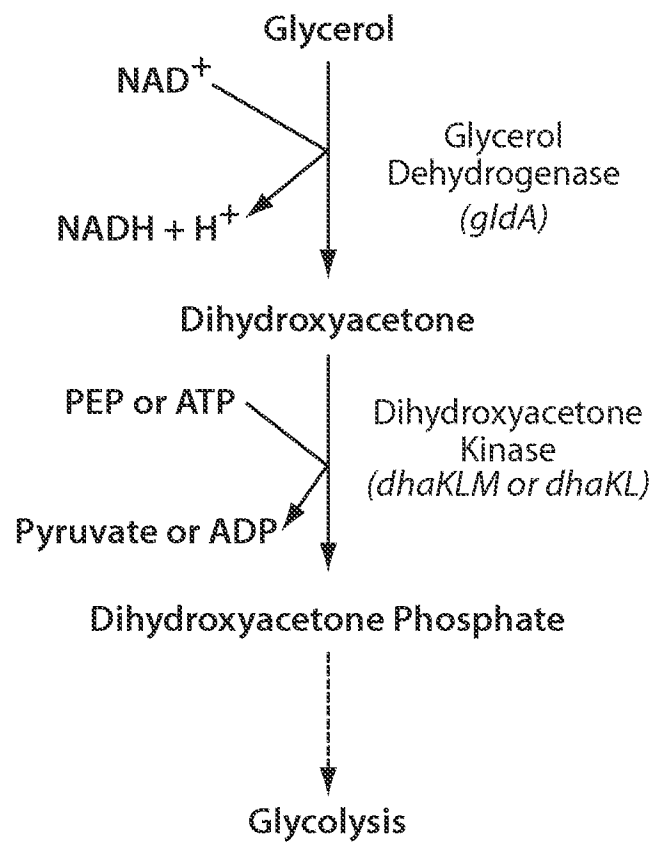


Fig. 1B

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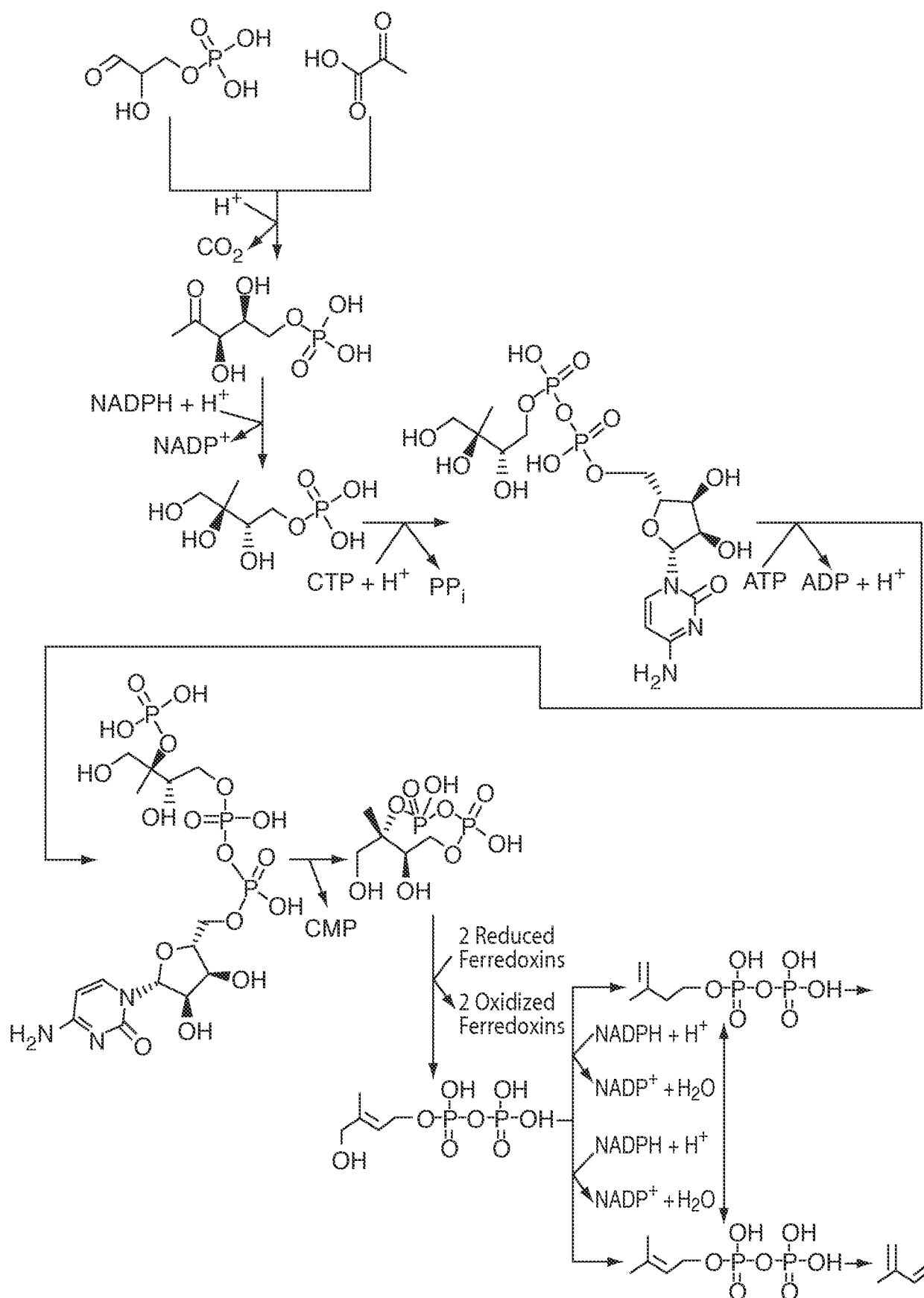
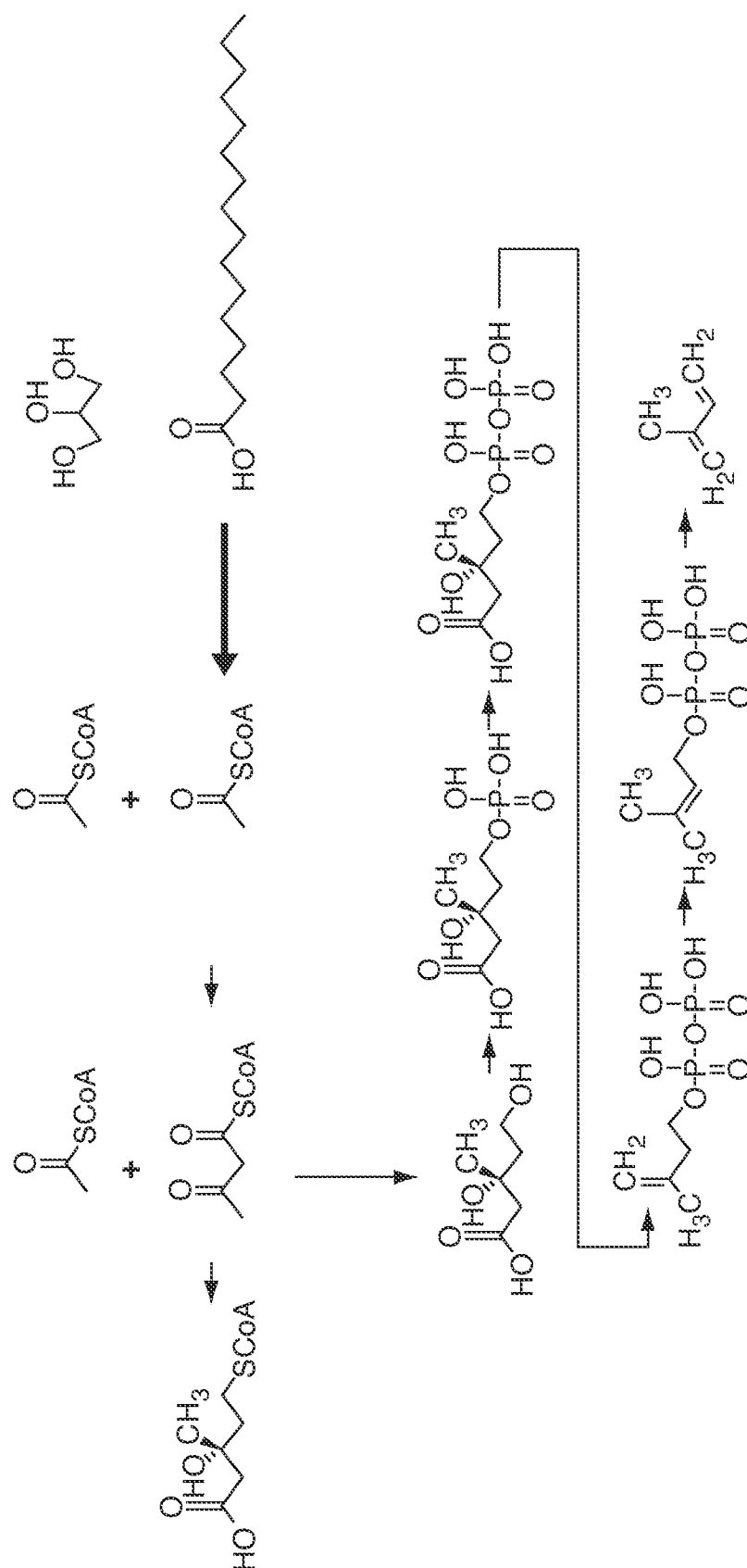


Fig. 2



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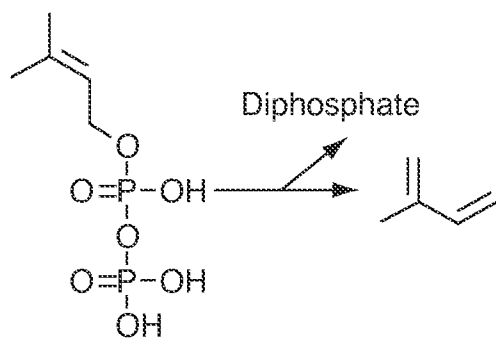


Fig. 4A

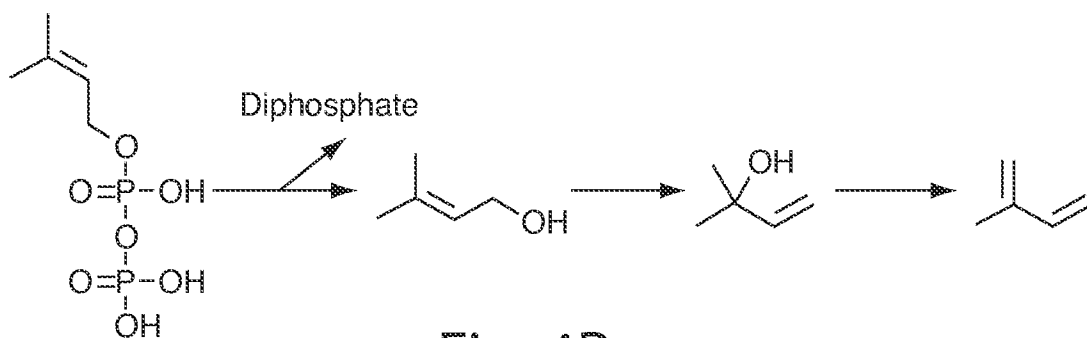


Fig. 4B

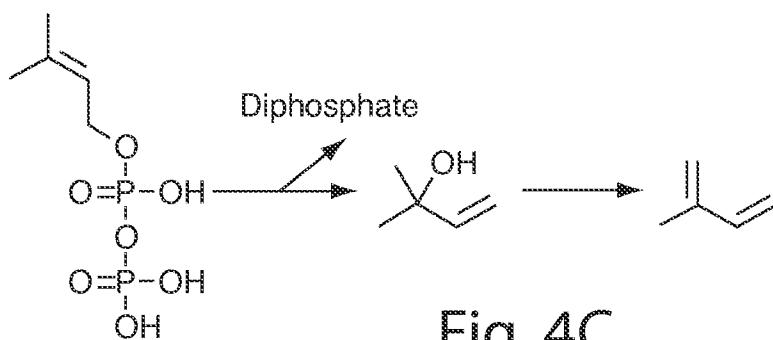


Fig. 4C

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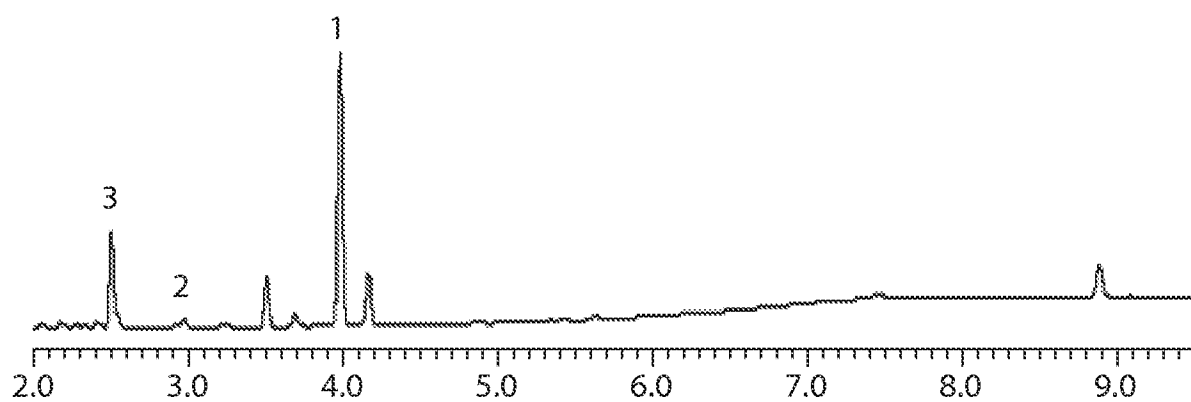


Fig. 5

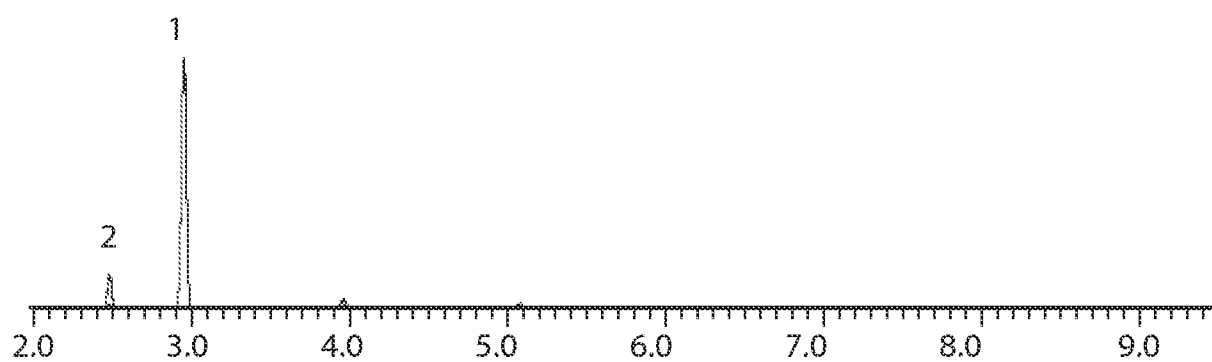


Fig. 6

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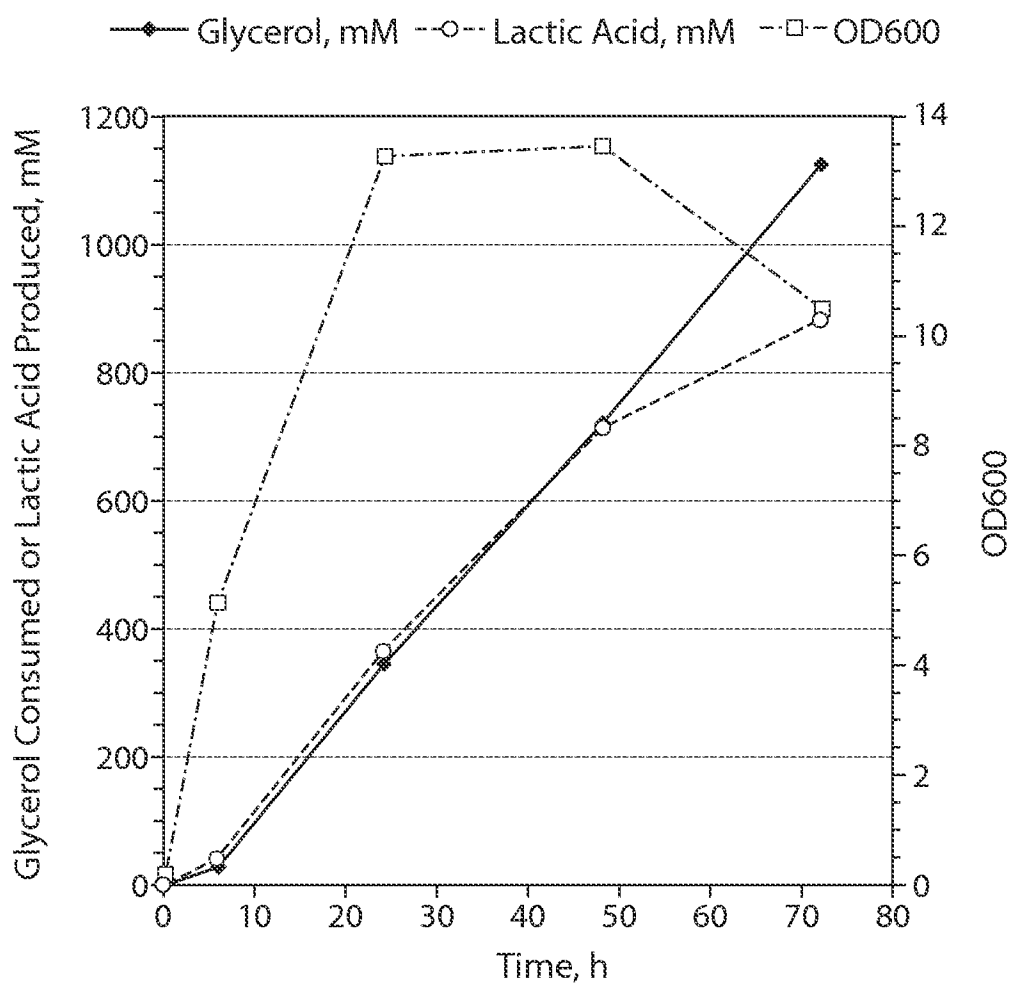


Fig. 7

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[SEQ ID NO: 33]

Fig. 8

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[SEQ ID NO:34]

Fig. 9

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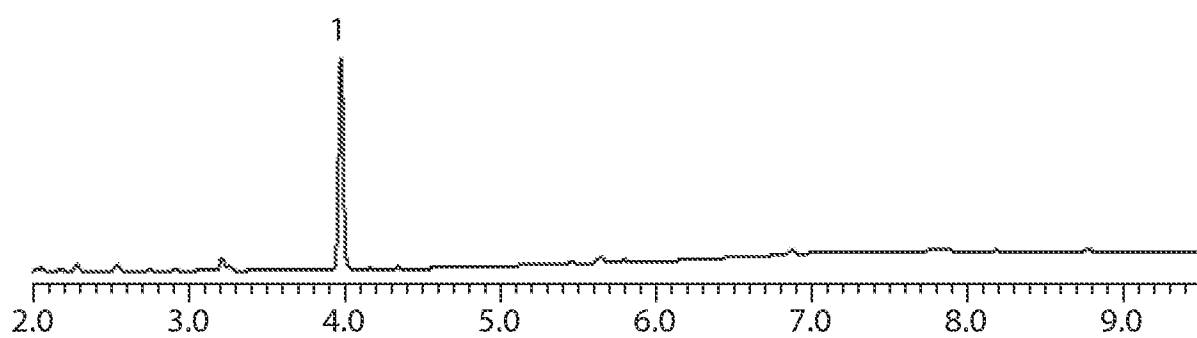


Fig. 10

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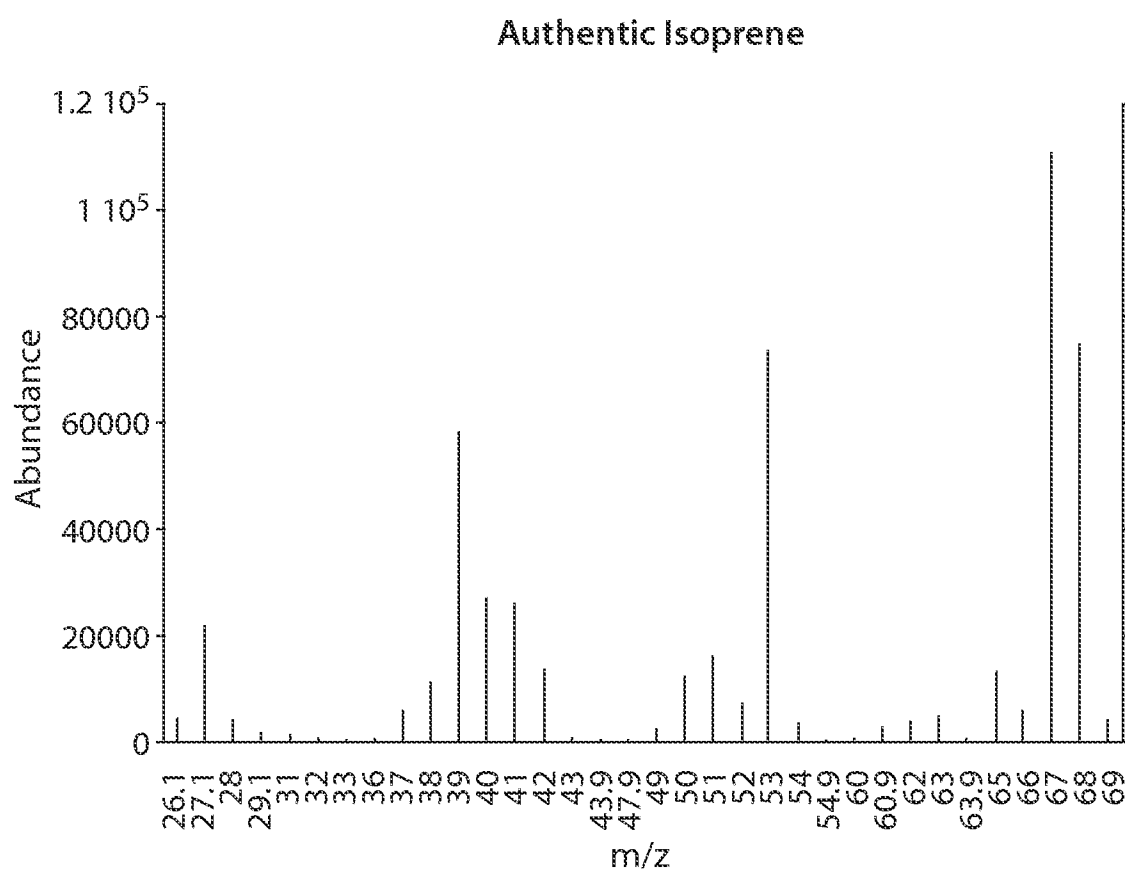


Fig. 11

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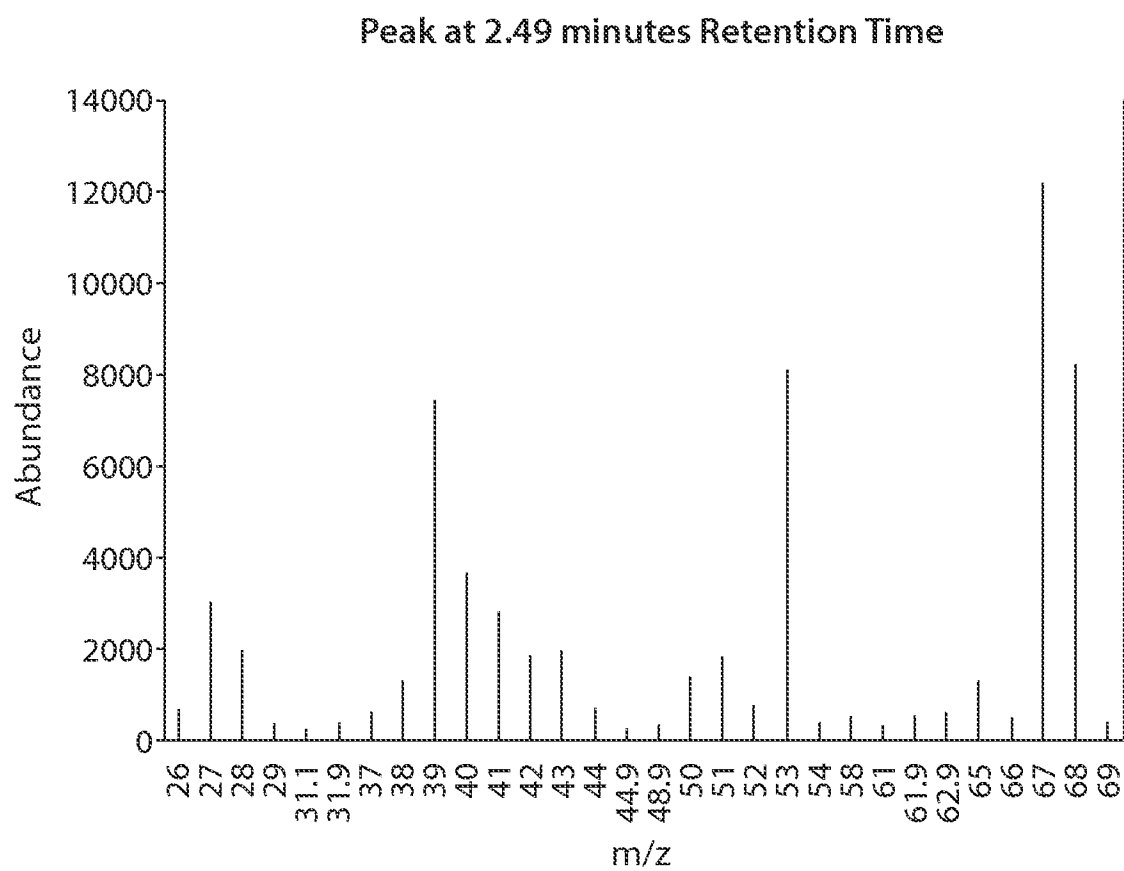


Fig. 12

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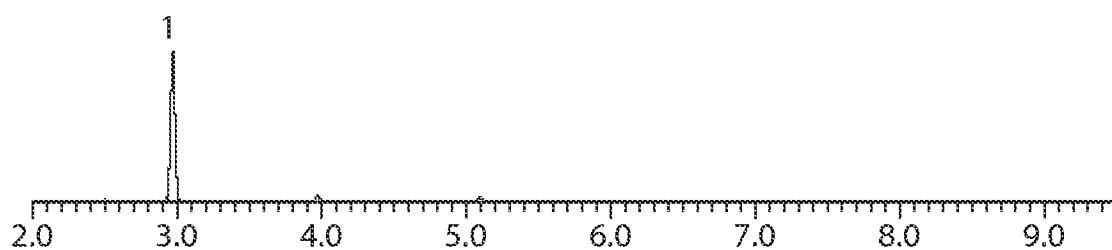


Fig. 13

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Fig. 14

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Fig. 15

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TGCCCCGTATACAGATTACCCACGCGGCGAGAATAGACAATGCTCTCTTCATAACGGGCCAAGATACGCTCTTGCTCAGCC
TCGGTTTGGTCGCTAATCTTGCCAGCAGTGCCTTTTTGCCCATCTTCGTATACGGGATGTGAAACGCCAAGGCGTCATA
GTCCGCGAAGTCCAAACCCGTGGCGTTTTTGTGTTTCATCCAGACTTGTGCAAAGCTCTGAATGTAAGTCTCGTTTCGACA
GTGGACCGTCCACCATCGGGTACGGATGGCCCCGTGGAGCCGAGAAGTCGTAGATATCCTGAGTCAACATCACATTATCT
TCTTTTCAGCGCCAGGATGCGCGGTTTCGCTCGCGACCAGCATCGCCACTGCACCAGCGCCCTGCGTTGGTTCGCCACCGCT
GTTTCAGGCCGTACTTCGCGATGTCTGCCGCCACAACCAACACCTTTTTGTCTGGGTGCAGTGCAACGTGGTTTTTAGCCA
GCTGCAAACCCGCCGTTCGCCCGTAACACGCTTCTTTGATTTTCGAAAGAACGCGCGAAAGGTTGGATACCCATCAGACGG
TGCAGAACAACGGCTGCTGCTTTGGATTTCATCAATGCTAGATTCCGTACCCACGATGACCATGTCAATGGCCTCTTTATC
CTCTTTGGTCAGGATCGCCTCAGCCGCGTTGGCCGCAAAGGTAACGATATCCTGCGAAATCGGATTAAGTCCATCTGAT
CCTGGCCAATGCCAATGTGGAATTTACCCGGGTCAACGTTACGAGCTTCTGCCAGCGCAGTCATATCGATGTAGTACGGT
GGGACGAAGAAGCTGATTTTGTGATGCCGATGGTCATATATTTATCTCTCTCATTGCTTGGCGAGATCATTCAGAAT
TGCCATTGCGCGGCTCTGGTTTCATGGTCTTTTGGCGCTTCAGCTGTTGGGCGACCGCCTCCACTTCTTTGCCGGTTGCAC
CGACCGTCATTGCCAGGCTACGCGCTTGCAACGCCATGTGACCTTTTTGAATACCCTCGCTCACCAGGGGCACGCAGTGCC
GCCAGGTTTTTGGCGCAAACCGACAGCAGCAACCACGCGGACAGCTCCTTCGCATCGGTAACAGCCAGCAGGTCCGCTGC
CGCCTGGGACTTCGGCAAGACTTTGGTCGCACCACCCACGGTGGCCAGTGCCAACGGAACGCTGATCTCGCCGATCAGTT
GCTCACCATCCAGGGTCCACGAGGTCAGGCCCTGGTAACGGCCCTCTTTAACAGCAAATGCGTGGCAGGAAGCGCTCACC
GCACGGGTGTCAATGCCCCGTGGCCAGAACGACCGCTTCAATGCCATTTCATGATACCCCTATTTGTGGGTAACCGCGCGATA
CGGGTCCAGGCTCGCGTAGCGGCTGGCCAGCACAATTTCTCGCGGATTTACGACCATTTGCTACCCCTTGCTCAGACGAG
ACACTGGAATCGCGGTTTTTCATCGTAACCTACCGATTTCGGTGGCGTAGTTGCTCAAAATGCTGAACAGAATCTTTTGTCT
GCGAACCATTTCGCGAAACAGTTCGCCACACCCCTCCAGCATTCGATTAACGATATTGGCACCCATCGCGTCTTCACGTC
CACCAGAAAGTCCACGCTAACAAAGCTCTCATCAAACGTGCGGTATTGCAGGTCACGCAGACCACCACCGCGCTTGACGA
TGCTCGGATAGCTCAGTCCGCGCTGTTGGAAAACCTCCGCCCTCAGTACTTGCAGCTTGTGATCAAGGACTCCGGGTCT
GCCACATCATAAAAGACAATTTGGCCACGCATCAGACGCTGCTGATTGACCGTTTTGAAGCCCTGAGCGATCTTTGCACC
GTTGCTCAGTGCTGCAATAACGCTAGGTTCTTCGGTTGCCATCGGAACCAGGTAATCGGTTTTTCATCAACCGTCAGGTGCA
GGCCAACACCCATCGGCACTTCGGTTTTGCTGATCTGGTTCTCAATCATGTGATTAGCAATCTGGCTGCTCAGCGCGGTG
TTTTCAAATTTCTTTCTGGTATCCGCGCTAATCTGACCCCTATTTCAGCAGGCTGGCCAAACGCTCCTCCGGGCTCATTTG
GTAAAAACGTGAATTTCTTCTGTTGCGGACGTTCCAACAGCATGGCCAGACCCAGGCCACCGCCAATGCACAGGCTGG
CAACGCGTATTTCTTCTCCTTCTGATTTCAGTTGGTAGCTCAGGAGGTCAGCAGACGAGCGCCGTGGCGCCGATTGCA
TGACCCAAGCTGATACCGCCACCGTAAATGTTAACTTTCTCCTCCGGCAGGGCCAACTCAGCTGCACAACGATGCTAGT
TGCTGCAAACGCCCTCGTTAATCTCGTACAGATCAATCTCCTCCGTGGTCAGTGGTTACGGGCCAGCAATTTCTGAATTG
CCTTAATCGGGCTGATACCCATATATGCCGGATCGATACCCACTTCAACGCTGTCACGGATAATCGCCAGGTACGGCAGA
CCGTGAGCCTCTGCGTACTCCTGGCTTGGGATAATCAGGGCGCTCGCACCATCATTGATGGTGGTCCGCTTACCGGCCGT
AACCCTACCGTCTCTTTGAAAACGGTTTTTCAGAGTACCCAGCTTTTCCACGCTAGAAATTAGGGCGGATACCCCTCATCTT
TCTCAACCAGCGTGCCGCTCACCTCCAACGGCGCGATTTCTGCTGCGAAGATACCCCTCTGCTTGTGCTTGGCGCGCTTTC
AGCTGGGAATGCACGCTAAACTGGTCTTGTCTTTCACGAGTGACGTGGTACTTCTCCGCCACATTCTCCGAGTCAGGCC
CATCGCTGACCGCTGAACGCATCGGTTCAGACCGTCTGATCATGCTGGAAAACGGCGCGTCATAGCTTTTCAGTTTCAT
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ATCAGCTGTTTTTGGCAAGATAACCGCCTTCATACCGCTGCCACAAACCTCATTACCGTCATAGCCGGGATCTCGTGGCT
CAAGCCAGAGTTAATCGCAATCTGACGCGCAGGGTTCGACCATTGCCGGCTTGCAGGACGTTACCGAAGATCACCTGAT
CAATTTCTCGCTGATGGTGAATGACGCTTCAGCAACTGGGTCTGACATGGGTGCCAGATCGACTGCGCTAACTTGG
CTCAGAGAACCCTTATACTTGGCGATTGGCGTGGCGAGGGCGTCGATGATTACTACAGTTTTTCATTATATTTCTCCTCT
TTAATGGTACC [SEQ ID NO: 37]

Fig. 16

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ACGCGTGCTAGAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTTCGTTTTATCTGTTGTTTGTTCGGTG
AACGCTCTCCTGAGTAGGACAAATCCGCCGCCCTAGATCTCTAGATTCCCTATCAGTGATAGAGATTGACATCCCTATCA
GTGATAGAGATACTGAGCACATCAGCAGGACGCACTGACCTGTACAGAAGGAGATATACATATGATCATCGAAACCCCAT
CCAAAGTAATCCTGTTCCGCGAGCAGCGGTTGTTTATGGTTACCGTGCCATCAGCATGGCAATTGATCTGACCAGCACC
ATTGAGATCAAGGAAACGCAAGAGGACGAAATCATCTGAATCTGAACGATCTGAACAAATCGCTGGGTCTGAATCTGAA
CGAAATCAAGAATATCAATCCGAACAATTTTGGCGACTTCAAGTATTGTCTGTGCGCTATCAAGAACACCCTGGACTACC
TGAATATCGAACCGAAAACCTGGTTTTAAGATCAACATCAGCTCCAAAATCCCGATTAGCTGTGGCCTGGGTCTAGCGCG
AGCATTACGATCGGCACGATTAAAGCGGTTTTCTGGCTTCTACAATAAAGAGTTGAAAGACGACGAAATCCGGAAGCTGGG
CTACATGGTTGAGAAGGAAATCCAGGGTAAAGCATCCATCAGGATACCAGCACTATTACGTACAAAGGTATTCTGGAAA
TCAAGAATAACAAATTCGCAAAATCAAAGGTGAGTTCGAGGAATTTCTGAAGAACTGCAAAATTTCTGATCGTTTTATGCG
GAGAAACGCAAGAAGAAAACCGCCGAATTTGGTGAACGAAGTCGCAAAAATCGAGAACAAGATGAGATTTTCAAGGAAAT
CGATAAAGTGATTGACGAGCGCTGAAGATTAAGAATAAAGAGGACTTTGGTAAGTTGATGACCAAAAATCAGGAGCTGC
TGAAGAAGCTGAACATTAGCACCCCGAAGCTGGACCGCATCGTTGATATTGGCAACCGTTTTGGCTTCGGTGCTAAACTG
ACCGGCGCTGGCGGTGGTGCTGCGTCATTATCTGGTTAACGAAGAGAAAAGAAAGGAAGTGTGAAAGAGCTGAACAA
AGAGGACGTACGCATTTTCAACTGTCGTATGATGAATTGAAGAAGGAGATATACCATGATCGAAGTAACTACCCAGGCA
AACTGTTTCATCGCCGGCGAGTATGCAGTGGTTGAGCCGGGTCAACCGCGGATTATTGTGCGGCTGGACCAGTTTCGTACG
GTCACGGTAGAAGAAACACCGATGAGGGCAGCATCCAGAGCGCGCAATACAGCAGCCTGCCGATTTCGTTGGACCCGTCG
CAATGGTGAACCTGGTACTGATATCCGTGAAAATCCGTTCCATTACGTTCTGGCCGCTATTACCTGACCGAGAAGTATG
CACAAGAACAGAATAAGGAACTGAGCTTCTACCACCTGAAAGTGACCAGCGAGCTGGATTTCGAGCAACGGCCGCAATAC
GGTTTGGGCAGCAGCGGTGCAGTCACCGTCGGTACGGTGAAGGCCCTGAATATCTTTTACGATCTGGGTCTGGAAAACGA
GGAAATCTTTAAGCTGTCCGCGCTGGCGCACCTGGCAGTGCAGGGTAATGGTAGCTGCGGTGACATCGCAGCGCTCTTGT
ACGGTGGCTGGATCGCATTTAGCACTTTTGACCACGATTGGGTCAACCAGAAGGTGCGGACCGAGACTCTGACCGATCTG
CTGGCTATGGACTGGCCGGAGCTGATGATTTTCCCTCTGAAAGTCCCGAAAACAAATTGCGTCTGCTGATTGGCTGGACGGG
TTCGCTGCGAGCACCTCTGACCTGGTTGATCGCGTACACCAGTCTAAAGAAGAGAAAACAGCGCGGTATGAGCAATTCC
TGATGAAATCCCGTCTGTCTGCGAAACCATGATTAACGGTTTCAATACCGCAAGATTAGCGTGATTCAAAAACAGATC
ACCAAGAACCGTCAACTGTTGGCAGAAGTACGAGCCTGACGGCGGTTGTCATTGAAACCGAGGCGCTGAAAAACTTGTG
TGACCTGGCGGAGAGCTATACGGGTGCCGCTAAAAGCAGCGGTGCTGGTGGCGGCGACTGCGGTATTGTTATCTTCCGCC
AGAAGTCTGGTATCTTCCGCTGATGACCGCGTGGGAGAAGGACGGCATCACCGCGCTGCCGTTGCACGTTTACACCTAT
GGTCAGAAAGAATGCAAGAGAAAACACGAGAGCAAGCGTTAAAGGAGGTATAAAAAATGACCGTCTACACCGCCAGCGTC
ACCGCACAGTAACATCGCGACGTTGAAGTACTGGGGTAAACGTGATACCAAGCTGAACCTGCCGACGAACAGCAGCAT
CAGCGTTACCCTGTCCCAGGACGATCTGCGTACGCTGACGAGCGCAGCTACTGCGCCGGAGTTGCAACGTGACACCCTGT
GGCTGAACGGCGAACCAGCATAGCATTGACAACGAACGTACGCAAAACTGCCTGCGCGACCTGCCCAATTGCGCAAGAA
ATGGAATCTAAAGATGCAAGCCTGCCTACCCTGAGCCAGTGAAGCTGCACATTGTGAGCGAGAACAATTTTCCGACGGC
GGCAGGCCTGGCAAGCTCTGCCGACGGCTTTGCAGCACTGGTCAGCGCCATCGCGAAATTGTACCAATTGCCGCAATCCA
CCTCGGAGATTTCTCGCATCGCTCGTAAAGGCAGCGGACGCGCTGCCGCTCTCTGTTTGGCGGTTATGTTGCCCTGGGAA
ATGGGCAAGGCCGAGGACGGTCACGATTCGATGGCTGTCCAGATTGCCGACAGCAGCGATTGGCCGCAATGAAGGCGTG
CGTTCTGGTCTGTCCGACATCAAGAAGGACGTGAGCAGCACCAGGGTATGCAACTGACGGTCGCTACCAGCGAGCTGT
TCAAAGAGCGCATTGAGCAGCTCGTTCCGAAGCGTTTCGAAGTCATGCGCAAGCGATCGTAGAGAAAAGACTTTGCTACG
TTTGGCAAGAAACCATGATGGAATCCAATAGCTTCCACGCGACCTGTCTGGATTCTTTTCCGCCGATCTTCTATATGAA
CGATACGAGCAACGCATCATCTCCTGGTGTACACGATCAATCAGTTTTATGGCGAAACCATTTGTCGCATACACCTTCG
ATGCGGGTCCGAACGCAGTCTGTACTACCTGGCAGAAAACGAAAGCAAGCTGTTGCGCTTCATTACAAAACCTGTTTGGC
AGCGTGCCGGGTTGGGACAAAAGTTACAGACGGAACAGCTGAGGCATTTAACCACAGTTTCGAGAGCAGCAATTTTCAC
CGCTCGTGAATTGGATCTGGAGCTGCAAAAGGACGTGGCGCGTGTATTCTGACCCAAGTTGGTTCTGGCCCCGAAGAAA
CGAACGAGTCTCTGATCGATGCGAAAACCGGCTGCCGAAGGAGTAGGAAGGAGATATAAAAATGCAAAACCGAACACGTA
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GTGTCTGGACTAATAGCGTGTGCGGTCAACCGCAACTGGGTGAAAGCAATGAGGACGCGAGTATTTCGTCTGTGCTTAT
GAATTGGGTGTGAAATCACCCCGCTGAAAGCATTTATCCGGACTTCCGTTACCGTGCCACCGATCCGAGCGGTATCGT
TGAACCAAGTGTGTCGGTCTTTGCGGCACGTACGACCAGCGCTGCAATCAACGACGACGAGGTGATGGACTACC
AGTGGTGTGATCTGGCCGACGTTCTGCATGGCATCGATGCCACCCGCTGGGCTTTTCTCCGTGGATGGTGTGACGGCG
ACCAACCGTGAGGCGCGTAAACGTTTGAGCGCGTTACCCAACTGAAGTAACCATGGGCTAGAGGCATCAAATAAAACGA
AAGGCTCAGTCGAAAGACTGGGCCTTTTCGTTTTATCTGTTGTTTGTGCGGTGAACGCTCTCTGAGTAGGACAAATCCGCC
GCCCTAGACCTAGG [SEQ ID NO: 38]

Fig. 17

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ggatccatggaagctcgtcgtccgcgaactatgaaccgaacagctgggattatgactacctgctgttctgataccga
cgaaagcatcgaagtgtataaagataaagccaaaaaactggaagcagaagttcgtcgcgaaatcaacaacgaaaaagcag
aatttctgacgctgctggaactgatcgataacgtgcagcgtctgggcctgggttatcgcttcgaaagcgatatccgtggc
gogctggaccgctttgttagttccggcggtttcgatgctgtcaccaaaacgtcgtcgtgcatggcaccgctgagctttcg
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cggcaaattccgtgtcatgttatatgcgcaccaaaaggtattctgaagaactggcgacggaaagtgttatgaacctgatc
gatgaaacctggaagaaaatgaacaaagaaaaactgggcggtctctgtttgcgaaaccgttcgttgaaaccgctatcaa
cctggcgctcagagtcattgtacgtaccacaatggtgatgcccatacctcgccggacgaactgacctgtaaaccgtgtgc
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Fig. 18

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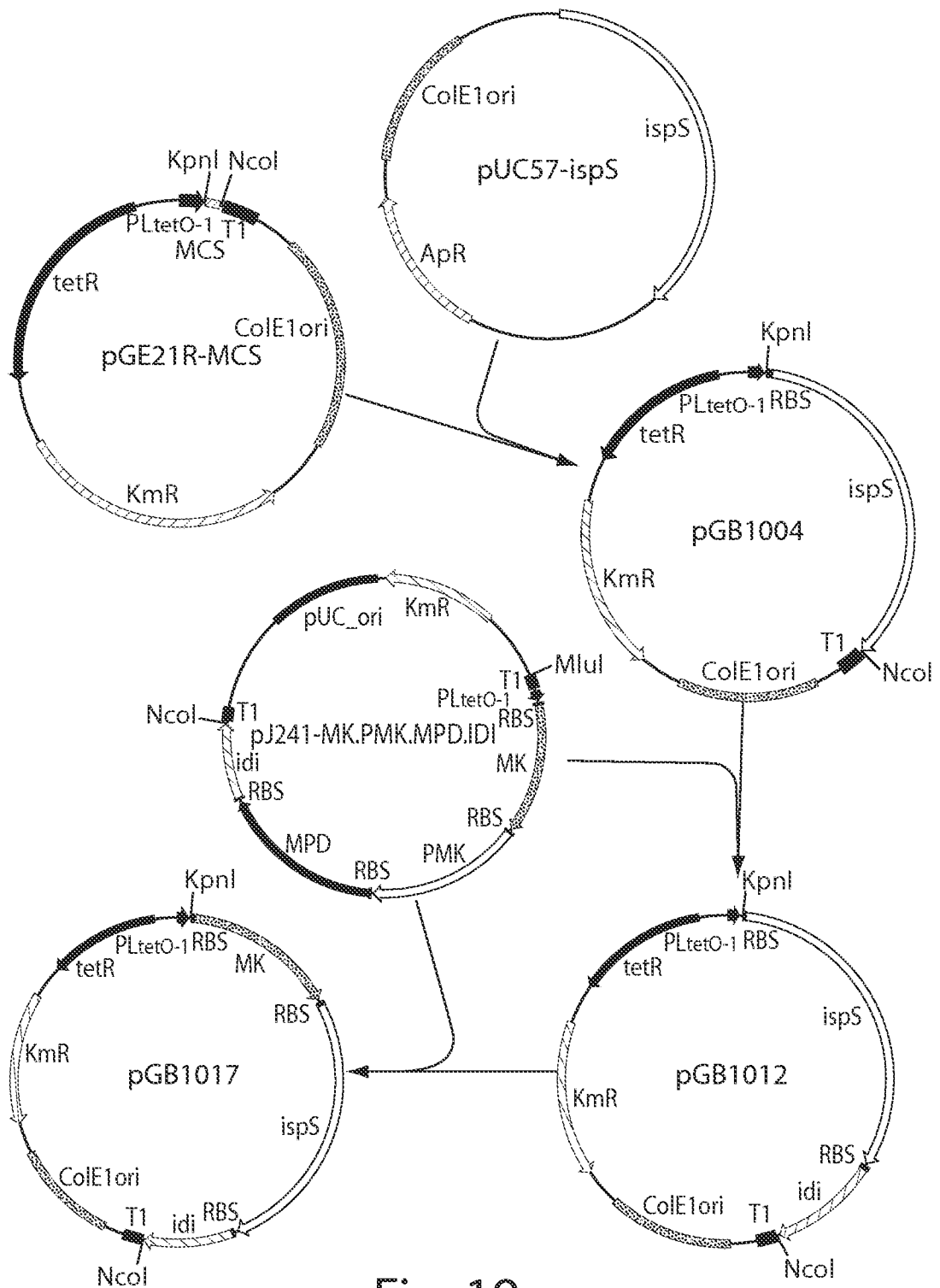


Fig. 19

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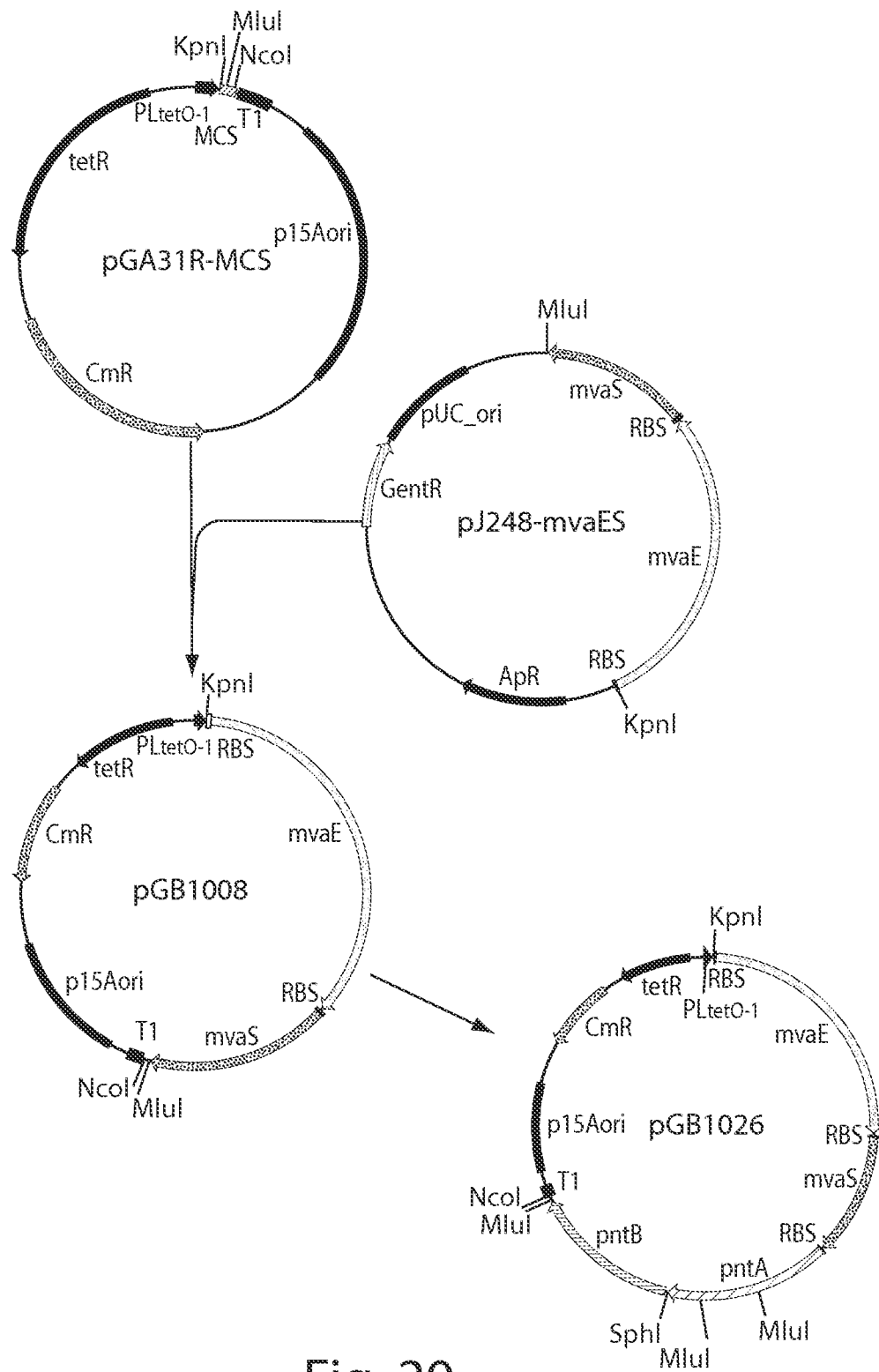


Fig. 20

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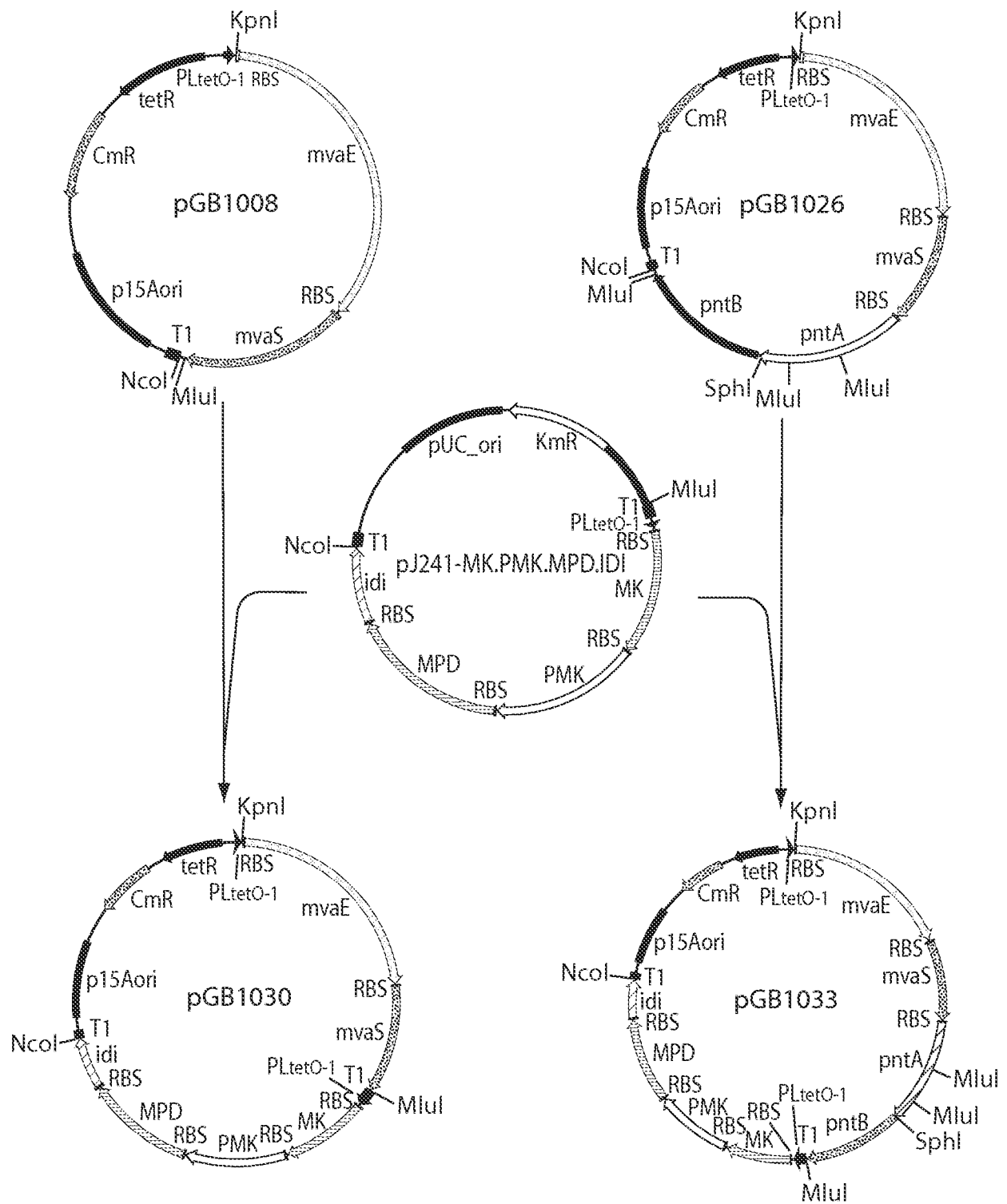


Fig. 21

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GGTACCAGGAGGTAAAACATATGTTCGTCTAAAGTTAAAGTCGTCCGCCGCACCATTCTACT
AGCATCCGTATGTGTTCGTATTACCACCGAGAGCGGCCGAAGGCGTTCAGCGTCGTATCGCGAA
CCACCACAGCAATCTGTGGGACGATAACTTCATCCAGAGCCTGAGCACGCCGTACGGTGCAA
TCTCTTATCACGAGAGCGCGCAGAACTGATTGGTGAGGTTAAAGAAATGATCAACAGCATC
AGCCTGAAGGACGGTGAGCTGATTACCCCAAGCAATGATCTGCTGATGCGCTTGTCCATTGT
GGACTCCATCGAACGCTTGGGTATCGATCGCCACTTCAAAGCGAGATTAAGTCTGCTTTGG
ACTATGTCTATTCTTATTGGAATGAGAAGGGCATCGGTTGGGGTCGTGACAGCGTGGTGGA
GACCTGAATAGCACCGCACTGGGTCTGCGCACGTTGCGTCTGCATGGTTACCCAGTCAGCAG
CGACGTTTTGCAACACTTCAAAGAGCAGAAGGGTCAGTTCGCGTGCAGCGCGATTACAGACCG
AAGGTGAAATCCGTTCCGTCTTGAACCTGTTCCGCGCTAGCCAAATTGCGTTTCCGGGCGAA
AAGGTTATGGAAGAGGCGGAAGTGTTCAGCACGATCTATCTGAAAGAGGCGATCCTGAAACT
GCCGGTGTGCGGCCTGAGCCGTGAGATTTTCGTACGTTCTGGAGTACGGTTGGCACATCAATC
TGCCGCGCTTGAAGCACGTAATTACATTGACGTGTTTGGCGAGGACCCGATTTATCTGACG
CCAAACATGAAAACCCAAAACTGCTGGAGCTGGCCAAGCTGGAGTTCAACATGTTCCACTC
TCTGCAACAACAGGAGCTGAAGCTGCTGAGCCGTTGGTGGAAGGATAGCGGTTTCAGCCAGA
TGACGTTTCCGCGCCATCGTCACGTTGAGTACTACACGCTGGCTAGCTGTATTGATAGCGAG
CCGCAACATAGCAGCTTCCGTCTGGGCTTTGCGAAGATCTTTCACCTGGCAACCGTCCTGGA
TGACATTTACGATACCTTCGGTACTATGGACGAGTTGGAGCTGTTACGCGCGCAGTCAAGC
GCTGGCATCCTAGCGCCACCGAATGGCTGCCGGAGTATATGAAAGGCGTTTACATGGTCCTG
TACGAAACGGTTAACGAGATGGCGGGTGAGGCGGAGAAGAGCCAAGGTCGCGATACCTTGAA
CTACGGTCGTAATGCCCTGGAAGCGTATATTGATGCAAGCATGGAAGAAGCTAAGTGGATCT
TTAGCGGTTTTCTGCCGACGTTCAAGAATATCTGGACAATGGCAAAGTGAGCTTTGGTTAC
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GGAGATTGACTTTCCGAGCCGTCTGAATGATGTCGCGTCTAGCATTTTGCGCCTGAAGGGTG
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CAAACCTGCTGAAAGAGCTGAACTGGGAATATCTGCGTCTGACAGCAATGTGCCGATCACCA
GCAAGAAGCATGCGTTTGATATCCTGCGTGCCCTTCTATCACCTGTACAAATACCGTGATGGT
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TGTGAACTCCGCTCAACAACCGTCGTGCGCCCATGCCCGCCTGCAATTTTCGTCCGCGCAGCA
TGCAGCTGCTGGCCGAAGATCGTACCGACCACATGCGTGGTGCTAGTACGTGGGCGGGCGGT
CAATCCCAAGATGAACTGATGCTGAAAGACGAATGCATTCTGGTCGATGCGGATGACAACAT
CACCGGTCATGTGAGCAAACCTGGAATGTCATAAGTTTCTGCCGCACCAGCCGGCGGGTCTGC
TGCACCGTGCAATTCTCTGTTTTCTGTTCGATGACCAGGGTCGCCCTGCTGCTGCAGCAACGT
GCCCCGAGCAAGATTACCTTTCCGTCTGTTTGGACCAATACGTGCTGTTACATCCGCTGCA
CGGCCAGACCCCGGATGAAGTGGATCAGCTGTGCGCAAGTGGCTGATGGCACGGTTCCGGGTG
CAAAGCGGCGGCAATTCTGTAAGCTGGAACATGAACTGGGTATCCCGGCACACCAGCTGCCG
GCAAGTGCATTTTCGTTTCTGACCCGCCTGCATTATTGTGCTGCCGATGTTACAGCCGGCGGC
AACCCAATCAGCCCTGTGGGGCGAACACGAAATGGATTACATTCTGTTTCATCCGTGCTAACG
TGACCCTGGCGCCGAATCCGGATGAAGTGGACGAAGTTCGTTATGTACGCAGGAAGAAGT
CGCCAGATGATGCAACCGGATAACGGTCTGCAATGGTCCCCGTGGTTTTCGATTATCGCTGC
GCGTTTTCTGGAACGTTGGTGGGCCGATCTGGACGCAGCACTGAACACCGATAAACACGAAG
ACTGGGGCACCGTCCATCACATCAACGAAGCATAACCATGG [SEQ ID NO: 40]

Fig. 22