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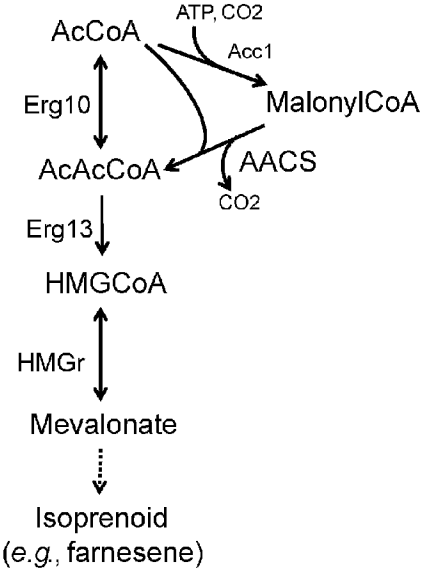

(57) Abrégé/Abstract:
Provided herein are compositions and methods for the heterologous production of acetyl-CoA-derived isoprenoids in a host cell. In some embodiments, the host cell is genetically modified to comprise a heterologous nucleotide sequence encoding an acetaldehyde dehydrogenase, acetylating (ADA, E.C. 1.2.1.10) and an MEV pathway comprising an NADH-using HMG-CoA reductase. In some embodiments, the host cell is genetically modified to comprise a heterologous nucleotide sequence encoding an ADA and an MEV pathway comprising an acetoacetyl-CoA synthase. In some embodiments, the genetically modified host cell further comprises one or more heterologous nucleotide sequences encoding a phosphoketolase and a phosphotransacetylase. In some embodiments, the genetically modified host cell further comprises a functional disruption of the native PDH-bypass. The compositions and methods described herein provide an energy-efficient yet redox balanced route for the heterologous production of acetyl-CoA-derived isoprenoids.

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(54) Title: PRODUCTION OF ACETYL-COENZYME A DERIVED ISOPRENOIDS

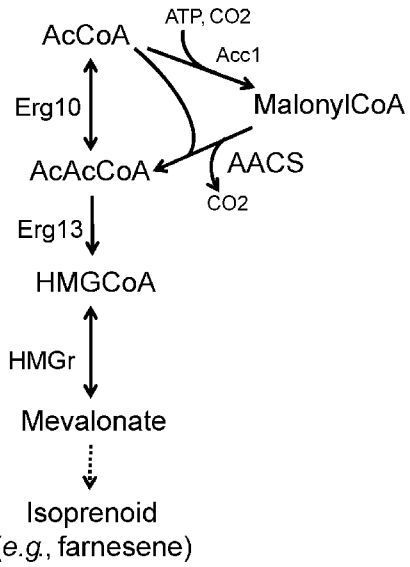

FIG. 5

(57) Abstract: Provided herein are compositions and methods for the heterologous production of acetyl-CoA-derived isoprenoids in a host cell. In some embodiments, the host cell is genetically modified to comprise a heterologous nucleotide sequence encoding an acetaldehyde dehydrogenase, acetylating (ADA, E.C. 1.2.1.10) and an MEV pathway comprising an NADH-using HMG-CoA reductase. In some embodiments, the host cell is genetically modified to comprise a heterologous nucleotide sequence encoding an ADA and an MEV pathway comprising an acetoacetyl-CoA synthase. In some embodiments, the genetically modified host cell further comprises one or more heterologous nucleotide sequences encoding a phosphoketolase and a phosphotransacetylase. In some embodiments, the genetically modified host cell further comprises a functional disruption of the native PDH-bypass. The compositions and methods described herein provide an energy-efficient yet redox balanced route for the heterologous production of acetyl-CoA-derived isoprenoids.

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PRODUCTION OF ACETYL-COENZYME A DERIVED ISOPRENOIDS

1. FIELD OF THE INVENTION

[0002] The present disclosure relates to compositions and methods for producing acetyl-CoA derived isoprenoids in engineered host cells.

2. BACKGROUND

[0003] Acetyl coenzyme A (acetyl-CoA) is a key intermediate in the synthesis of essential biological compounds, including polyketides, fatty acids, isoprenoids, phenolics, alkaloids, vitamins, and amino acids. Among the metabolites derived from acetyl-CoA are primary and secondary metabolites, including compounds of industrial utility. Isoprenoids, for example, are used in pharmaceutical products and as biofuels, food additives, and other specialty chemicals. An isoprenoid product is typically composed of repeating five carbon isopentenyl diphosphate (IPP) units, although irregular isoprenoids and polyterpenes have been reported. In nature, isoprenoids are synthesized by consecutive condensations of their precursor IPP and its isomer dimethylallyl pyrophosphate (DMAPP). Two pathways for these precursors are known. Prokaryotes, with some exceptions, typically employ the deoxyxylulose-5-phosphate (DXP) pathway to convert pyruvate and glyceraldehyde 3-phosphate (G3P) to IPP and DMAPP. Eukaryotes, with the exception of plants, generally use the mevalonate-dependent (MEV) pathway to convert acetyl-CoA to IPP, which is subsequently isomerized to DMAPP.

[0004] The unicellular fungus *Saccharomyces cerevisiae* and its close relatives use two endogenous pathways to generate acetyl-CoA. One pathway takes place in the mitochondrial matrix, where the PDH complex catalyzes the oxidative decarboxylation of pyruvate, generated from glucose via glycolysis, to acetyl CoA. The PDH complex consists of 60 polypeptide chains – 24 chains of the lipoamide reductase-transacetylase, 12 chains of dihydrolipyl dehydrogenase, and 24 chains of pyruvate decarboxylase. This massive complex converts pyruvate to acetyl-CoA, generating NADH as a byproduct. The resulting acetyl-CoA can then be completely oxidized to CO_2 and H_2O via the citric acid cycle for energy generation, or be used for biosynthetic reactions that are performed in the mitochondria.

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[0005] The acetyl-CoA generated in the mitochondria is unable to cross the mitochondrial membrane into the cytosol. Thus, to generate cytosolic acetyl-CoA, which is needed for the biosynthesis of important primary and secondary metabolites, *S. cerevisiae* uses an independent mechanism located in the cytosol known as the "PDH-bypass." This multi-step pathway catalyzes: (1) the decarboxylation of pyruvate into acetaldehyde by pyruvate decarboxylase (PDC, EC 4.1.1.1); (2) the conversion of acetaldehyde into acetate by acetaldehyde dehydrogenase (ACDH, EC 1.2.1.5 and EC 1.2.1.4), which reduced one $NADP^+$ to one NADPH; and (3) the synthesis of acetyl-CoA from acetate and CoA by acetyl-CoA synthetase (ACS, EC 6.2.1.1), which hydrolyzes 1 ATP to 1 AMP, the energetic equivalent of hydrolyzing 2 ATP to 2 ADP.

[0006] Since nature provides only low yield sources for the extraction of many acetyl-CoA derived biomolecules, fermentative production using genetically modified microorganisms has become a promising alternative for their production. However, utilization of the native acetyl-CoA pathway for production of the acetyl-CoA intermediate has certain limitations. For example, isoprenoid production via the native MEV pathway requires three acetyl-CoA molecules and the oxidation of two NADPH for each molecule of mevalonate generated, as shown in **FIG. 1**. While the PDH-bypass generates one NADPH per acetyl-CoA produced, two ATP equivalents are expended in the process. Thus, while the generation of NADPH is beneficial with regard to the cofactor requirements of the native MEV pathway, the expenditure of six ATP equivalents per mevalonate generated results in an energetically inefficient reaction, as more carbon source must be diverted to ATP synthesis, *e.g.,* via the TCA cycle and oxidative phosphorylation, at the expense of product yield.

[0007] Thus, one of the challenges in designing a production host that efficiently produces acetyl-CoA derived compounds is to optimize acetyl-CoA production such that the ATP requirements are minimized, while also meeting the co-factor and requirements of the biosynthetic pathway. The compositions and methods provided herein address this need and provide related advantages as well.

3. SUMMARY OF THE INVENTION

[0008] The compositions and methods described herein provide for the energetically efficient and co-factor balanced production of acetyl-CoA derived isoprenoids. By utilizing a heterologous acylating acetaldehyde dehydrogenase (alternately referred to as "acetylaldehyde dehydrogenase, acetylating," "acetylaldehyde dehydrogenase, acylating," or "ADA" (EC 1.2.1.10)) as an alternative to the PDH-bypass for cytosolic production of acetyl-CoA, two equivalents of ATP are saved per molecule of acetyl-CoA produced. ADA

converts acetaldehyde directly to acetyl-CoA without expenditure of ATP, and reduces one NAD^+ to one NADH in the process.

[0009] While the ATP savings gained from replacement of the PDH-bypass with ADA can be utilized towards higher product yields, there are potential shortcomings associated with the use of ADA in combination with the native mevalonate pathway. First, inactivation of the native PDH-bypass removes one source of NADPH, while the reaction catalyzed by ADA produces NADH. Thus, the replacement of the PDH-bypass with ADA, without further pathway modification, introduces a redox imbalance in isoprenoid synthesis, which consumes NADPH.

[0010] Secondly, ADA catalyzes the following reversible reaction:

$$\text{Acetaldehyde} + NAD^+ + \text{Coenzyme A} \Leftrightarrow \text{Acetyl-CoA} + NADH + H^+$$

The native PDH-bypass reaction for forming acetyl-CoA is thermodynamically favorable because the reaction is coupled to the hydrolysis of ATP to AMP. In contrast, the ADA reaction is not coupled to ATP, and is much closer to equilibrium than the native PDH-bypass reactions for forming Acetyl-CoA. Thus, the reaction catalyzed by ADA has a lower a thermodynamic driving force behind the conversion of acetaldehyde to acetyl-CoA, and without further pathway modification, the theoretical energy gains of ADA may not be realized.

[0011] The compositions and methods described herein address these shortcomings. In some embodiments, to address the redox imbalance introduced by replacement of the PDH-bypass with ADA, the genetically modified host cells further utilize an NADH-using enzyme in the isoprenoid pathway to consume ADA-generated NADH. Thus, the pool of NADH generated by the ADA-mediated conversion of acetaldehyde to acetyl-CoA can be utilized directly towards isoprenoid synthesis. In some embodiments, the NADH-using enzyme is an enzyme that is non-native to the isoprenoid pathway. For example, the NADH-using enzyme can replace an NADPH-using enzyme that is native to the isoprenoid pathway. In particular embodiments, the NADH-using enzyme is an NADH-using 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) that converts HMG-CoA to mevalonate.

[0012] In some embodiments, to address the lower thermodynamic driving force behind the ADA reaction, the genetically modified host cells further utilize, as a first step in the mevalonate pathway, a thermodynamically favorable reaction immediately downstream of acetyl-CoA to provide a pull on the ADA reaction. In some embodiments, the formation of acetoacetyl-CoA from acetyl-CoA is catalyzed by an acetoacetyl-CoA synthase (AACS;

alternately referred to as an acetyl-CoA:malonyl-CoA acyltransferase). The reaction catalyzed by AACS is thermodynamically more favorable than the reaction catalyzed by the acetyl-CoA thiolase of the native mevalonate pathway, due to the hydrolysis of 1 ATP resulting from the generation of malonyl-CoA by acetyl-CoA carboxylase (**FIG. 5**). Thus, AACS provides a stronger pull on acetyl-CoA to drive the ADA reaction forward.

[0013] The advantages of utilizing a heterologous ADA in combination with these modifications are exemplified by the improved theoretical yield of the sesquiterpene farnesene in host cells comprising a MEV pathway. Isoprenoid production via the native mevalonate pathway is illustrated in **FIG. 1** and **FIG. 2.** As indicated in **FIG. 3**, when cytosolic acetyl-CoA is synthesized from glucose using only the chemical reactions which occur in the native yeast metabolic network, the maximum possible stoichiometric yield for conversion of glucose to farnesene via the mevalonate pathway is 23.6 wt%, with 4.77 molecules of glucose being required for the synthesis of each molecule of farnesene. 27 ATP are required per molecule of farnesene, 18 of which are consumed in the synthesis of cytosolic acetyl-CoA from acetaldehyde via the PDH-bypass. However, by including the reactions catalyzed by ADA and NADH-using HMG-CoA reductase into the metabolic network for mevalonate production, as illustrated in **FIG. 4**, the maximum theoretical stoichiometric yield is improved to 25.2 wt%. In particular, ADA converts acetaldehyde to acetyl-CoA without any ATP input; this reduces the ATP equivalents required for farnesene synthesis to 9, resulting in a savings of 18 ATP equivalents per molecule of farnesene produced (2 ATP equivalents per acetyl-CoA x 9 acetyl-CoAs per 1 farnesene). This savings in ATP usage during acetyl-CoA production eliminates the cell's need for oxygen to run the TCA cycle for farnesene production. The oxygen requirement for conversion of glucose to farnesene decreases from 7.8 molecules of O_2 per glucose consumed to 6, thereby reducing a major production cost of providing oxygen to fermenters at scale. In addition, redox imbalance is alleviated by co-introduction of an NADH-using HMG-CoA reductase, which consumes NADH generated by ADA.

[0014] As indicated in **FIG. 4**, there remains a stoichiometric excess of ATP in a strain that comprises both an ADA and an NADH-using HMG-CoA reductase, which can be used by the cell for maintenance and growth. Alternatively, some of this excess ATP can be utilized towards improving the kinetics of acetoacetyl-CoA production, by introducing an acetoacetyl-CoA synthase (AACS). As illustrated in **FIG. 5**, AACS is an enzyme which synthesizes acetoacteyl-CoA from malonyl-CoA and acetyl-CoA. Malonyl-CoA synthesis requires an energetic input of 1 ATP per molecule of acetyl-CoA converted (catalyzed by

acetyl-CoA carboxylase, thereby improving the thermodynamic driving force of acetoacetyl-CoA synthesis from acetyl-CoA. Importantly, this does not affect the maximum stoichiometric yield of farnesene from sugar or the oxygen demand of the pathway, as there is still excess ATP available in this strain design, as illustrated in **FIG. 6**.

[0015] As shown in **FIG. 7**, additional efficiencies can be gained via the introduction of phosphoketolase (PK) and phosphotransacetylase (PTA) enzymes. PK and PTA catalyze the reactions to convert fructose-6-phosphate (F6P) or xyulose-5-phosphate (X5P) to acetyl-CoA. With these metabolic pathways available, at optimality, the reaction network is able to reach 29.8 wt% mass yield or greater, a significant increase in maximum theoretical yield. This solution involves diverting carbon away from lower glycolysis (G3P → pyruvate), which results in less ATP and NADH generation, both of which are already in excess in a network comprising the ADA and NADH-using HMG-CoA reductase modifications. One benefit of reducing flux through lower glycolysis is that less CO_2 is produced in converting pyruvate into acetaldehyde, and thus more carbon can be captured in the end product, thereby increasing the maximum theoretical yield of the network. A second benefit is that less NADH is produced, and therefore significantly less oxygen is needed to reoxidize it. In particular, the oxygen demand at optimality is only 1.84 molecules of O_2 per glucose consumed. The redox impact of the addition of PK and PTA to an ADA background is visible even at low yields in the microscale, as illustrated in **FIG. 13,** where glycerol production returns to wild-type levels.

[0016] Thus, provided herein are genetically modified host cells and methods of their use for the production of acetyl-CoA-derived isoprenoids. In one aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: (a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate; and (b) a heterologous nucleic acid encoding an acylating acetylaldehyde dehydrogenase.

[0017] In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an acetyl-CoA:malonyl-CoA acyltransferase (*i.e.,* an acetoacetyl-CoA synthase (AACS)).

[0018] In some embodiments, the one or more enzymes of the MEV pathway comprise an NADH-using enzyme that converts HMG-CoA to mevalonate. In some embodiments, the one or more enzymes of the MEV pathway comprise an NADH-using HMG-CoA reductase.

[0019] In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding a phosphoketolase. In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding a phosphotransacetylase.

[0020] In some embodiments, the amino acid sequence of the ADA is at least 80% identical to SEQ ID NO:2. In some embodiments, the amino acid sequence of the acetyl-CoA:malonyl-CoA acyltransferase is at least 80% identical to SEQ ID NO:16. In some embodiments, the amino acid sequence of the NADH-using HMG-CoA reductase is at least 80% identical to SEQ ID NO:20. In some embodiments, the amino acid sequence of the phosphoketolase is at least 80% identical to SEQ ID NO:12. In some embodiments, the amino acid sequence of the phosphotransacetylase is at least 80% identical to SEQ ID NO:14.

[0021] In some embodiments, the genetically modified host cell further comprises a functional disruption of one or more enzymes of the native pyruvate dehydrogenase (PDH)-bypass. In some embodiments, the one or more enzymes of the PDH-bypass are selected from acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6). In some embodiments, ACS1 is functionally disrupted. In some embodiments, ACS2 is functionally disrupted. In some embodiments, ALD6 is functionally disrupted. In some embodiments, ACS1 and ACS2 are functionally disrupted. In some embodiments, ACS1, ACS2 and ALD6 are functionally disrupted.

[0022] In some embodiments, the genetically modified host cell further comprises a functional disruption of one or more enzymes having alcohol dehydrogenase (ADH) activity. In some embodiments, the one or more enzymes having ADH activity are selected from alcohol dehydrogenase 1 (ADH1), alcohol dehydrogenase 3 (ADH3), alcohol dehydrogenase 4 (ADH4), and alcohol dehydrogenase 5 (ADH5).

[0023] In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetoacetyl-CoA with acetyl-CoA to form HMG-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts HMG-CoA to mevalonate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-

pyrophosphate to isopentenyl pyrophosphate. In some embodiments, the one or more enzymes of the MEV pathway are selected from HMG-CoA synthase, mevalonate kinase, phosphomevalonate kinase and mevalonate pyrophosphate decarboxylase.

[0024] In some embodiments, the host cell comprises a plurality of heterologous nucleic acids encoding all of the enzymes of the MEV pathway. In some embodiments, the one or more heterologous nucleic acids encoding one or more enzymes of the MEV pathway are under control of a single transcriptional regulator. In some embodiments, the one or more heterologous nucleic acids encoding one or more enzymes of the MEV pathway are under control of multiple heterologous transcriptional regulators.

[0025] In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding an enzyme that can convert isopentenyl pyrophosphate (IPP) into dimethylallyl pyrophosphate (DMAPP). In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding an enzyme that can condense IPP and/or DMAPP molecules to form a polyprenyl compound. In some embodiments, the genetically modified host cell further comprise a heterologous nucleic acid encoding an enzyme that can modify IPP or a polyprenyl to form an isoprenoid compound. In some embodiments, the enzyme that can modify IPP or a polyprenyl to form an isoprenoid compound is selected from the group consisting of carene synthase, geraniol synthase, linalool synthase, limonene synthase, myrcene synthase, ocimene synthase, α-pinene synthase, β-pinene synthase, γ-terpinene synthase, terpinolene synthase, amorphadiene synthase, α-farnesene synthase, β-farnesene synthase, farnesol synthase, nerolidol synthase, patchouliol synthase, nootkatone synthase, and abietadiene synthase. In some embodiments, the isoprenoid is selected from the group consisting of a hemiterpene, monoterpene, diterpene, triterpene, tetraterpene, sesquiterpene, and polyterpene. In some embodiments, the isoprenoid is a C_5-C_{20} isoprenoid. In some embodiments, the isoprenoid is selected from the group consisting of abietadiene, amorphadiene, carene, α-farnesene, β-farnesene, farnesol, geraniol, geranylgeraniol, isoprene, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, β-pinene, sabinene, γ-terpinene, terpinolene, and valencene.

[0026] In some embodiments, the genetically modified host cell is a yeast cell. In some embodiments, the yeast is *Saccharomyces cerevisiae*.

[0027] In another aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: (a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate; (b) a heterologous nucleic acid encoding an acetylaldehyde dehydrogenase,

acetylating (ADA); (c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6); (d) a heterologous nucleic acid encoding a phosphoketolase (PK); and (e) a heterologous nucleic acid encoding a phosphoketolase (PTA).

[0028] In another aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: (a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate, wherein the one or more enzymes comprise a NADH-using HMG-CoA reductase; (b) a heterologous nucleic acid encoding an acetylaldehyde dehydrogenase, acetylating (ADA); and (c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6).

[0029] In another aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: (a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate, wherein the one or more enzymes comprise a NADH-using HMG-CoA reductase; (b) a heterologous nucleic acid encoding an acetylaldehyde dehydrogenase, acetylating (ADA); (c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6); (d) a heterologous nucleic acid encoding a phosphoketolase (PK); and (e) a heterologous nucleic acid encoding a phosphoketolase (PTA).

[0030] In another aspect, provided herein is genetically modified host cell capable of producing an isoprenoid, the cell comprising: (a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate, wherein the one or more enzymes comprise an acetyl-CoA:malonyl-CoA acyltransferase; (b) a heterologous nucleic acid encoding acetylaldehyde dehydrogenase, acetylating (ADA); and (c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6).

[0031] In another aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: (a) one or more heterologous nucleic acids encoding a plurality of enzymes of a mevalonate (MEV) pathway for making isopentenyl

pyrophosphate, wherein the plurality of enzymes comprise an acetyl-CoA:malonyl-CoA acyltransferase and an NADH-using HMG-CoA reductase; (b) a heterologous nucleic acid encoding an acetylaldehyde dehydrogenase, acetylating (ADA); (c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6); (d) a heterologous nucleic acid encoding a phosphoketolase (PK); and (e) a heterologous nucleic acid encoding a phosphoketolase (PTA).

[0032] In another aspect, provided herein is a method for producing an isoprenoid, the method comprising: (a) culturing a population of genetically modified yeast cells described herein in a medium with a carbon source under conditions suitable for making said isoprenoid compound; and (b) recovering said isoprenoid compound from the medium.

4. BRIEF DESCRIPTION OF THE FIGURES

[0033] **FIG. 1** provides a schematic representation of the mevalonate ("MEV") pathway for the production of isopentenyl diphosphate ("IPP").

[0034] **FIG. 2** provides a schematic representation of the conversion of IPP and dimethylallyl pyrophosphate ("DMAPP") to geranyl pyrophosphate ("GPP"), farnesyl pyrophosphate ("FPP"), and geranylgeranyl pyrophosphate ("GGPP").

[0035] **FIG. 3** provides a schematic representation of the optimal flow of carbon and the metabolic requirements and yields in the conversion of glucose to farnesene via the mevalonate pathway, wherein cytosolic acetyl-CoA is generated via the "wild-type" PDH-bypass.

[0036] **FIG. 4** provides a schematic representation of the optimal flow of carbon and the metabolic requirements and yields in the conversion of glucose to farnesene via the mevalonate pathway, wherein cytosolic acetyl-CoA is generated via ADA, and the mevalonate pathway comprises an NADH-using HMGr instead of an NADPH-using HMGr.

[0037] **FIG. 5** provides a schematic representation of farnesene production from acetyl-CoA, wherein acetoacteyl-CoA (AcAcCoA) is synthesized from malonyl-CoA and acetyl-CoA (AcCoA) by acetoacetyl-CoA synthase (AACS). Malonyl-CoA synthesis requires an energetic input of 1 ATP per molecule of acetyl-CoA converted (catalyzed by acetyl-CoA carboxylase (ACC1)).

[0038] **FIG. 6** provides a schematic representation of the optimal flow of carbon and the metabolic requirements and yields in the conversion of glucose to farnesene via the mevalonate pathway, wherein cytosolic acetyl-CoA is generated via ADA, the mevalonate pathway comprises an NADH-using HMGr instead of an NADPH-using HMGr, and

acetoacteyl-CoA is synthesized from malonyl-CoA and acetyl-CoA by acetoacetyl-CoA synthase.

[0039] **FIG. 7** provides a schematic representation of the optimal flow of carbon and the metabolic requirements and yields in the conversion of glucose to farnesene via the mevalonate pathway, wherein cytosolic acetyl-CoA is generated via ADA, the mevalonate pathway comprises an NADH-using HMGr instead of an NADPH-using HMGr, and phosphoketolase (PK) and phosphotransacetylase (PTA) catalyze the reactions to convert fructose-6-phosphate (F6P) to acetyl-CoA.

[0040] **FIG. 8** provides the NADPH-specific or NADH-specific activities (measured as nmol/mg/min) of hydroxymethylglutaryl-CoA reductases from *Sacchormyces cerevisiae* (Sc. tHMG-CoA reductase), *Pseudomonas mevalonii* (Pm.), *Delftia acidovorans* (Da.) and *Silicibacter pomeroyi* (Sp.).

[0041] **FIG. 9** provides cell densities (measured as OD_{600}) after 24 and 48 hours for *S. cerevisiae* (Sc.) strains comprising a heterologous MevT pathway comprising an NADPH-using HMG-CoA reductase (Sc. tHMG-CoA reductase) or an NADH-using HMG-CoA reductase (Pm. – *Pseudomonas mevalonii; Da. -- Delftia acidovorans; Sp. -- Silicibacter pomeroyi*) in a wild-type ADH1, and an ADH1 knockout (*adh1*Δ) background, respectively.

[0042] **FIG. 10** provides glycerol production (measured as g/L) after 24 and 48 hours for *S. cerevisiae* (Sc.) strains a heterologous MevT pathway comprising comprising an NADPH-using HMG-CoA reductase (Sc. tHMG-CoA reductase) or an NADH-using HMG-CoA reductase (Pm. – *Pseudomonas mevalonii; Da. -- Delftia acidovorans; Sp. -- Silicibacter pomeroyi*) in both a wild-type ADH1 and ADH1 knockout background.

[0043] **FIG. 11** provides mevlonate production (measured as g/L) after 24 and 48 hours for *S. cerevisiae* (Sc.) strains comprising an NADPH-using HMG-CoA reductase (Sc. tHMG-CoA reductase) or an NADH-using HMG-CoA reductase (Pm. – *Pseudomonas mevalonii; Da. -- Delftia acidovorans; Sp. -- Silicibacter pomeroyi*) in both a wild-type ADH1 and ADH1 knockout (*adh1*Δ) background.

[0044] **FIG. 12** provides farnesene production and cell densities of *S. cerevisiae* strains comprising: (**A**) heterologously expressed ADA (Dz.eutE) coupled with *acs1*Δ *acs2* Δ *ald6*Δ and an MEV pathway comprising either an NADPH-using HMG-CoA reductase or an NADH-using HMG-CoA reductase; (**B**) an intact (wild-type) PDH-bypass and an MEV pathway comprising either an NADPH-using HMG-CoA reductase or an NADH-using HMG-CoA reductase. Columns indicated as "Empty" represent wells with media only (no cells).

[0045] **FIG. 13** provides glycerol production (top panels) and glucose consumption (lower panels) by: (**A**) a wild-type strain (Y968); a strain heterologously expressing ADA (Dz.eutE) (Y12869); and (**B**) a strain heterologously expressing ADA (Dz.eutE), phosphoketolase (PK) and phosphotransacetylase (PTA) (Y12745).

[0046] **FIG. 14** provides mevalonate production by *S. cerevisiae* strains comprising either an intact (wild-type) PDH-bypass or heterologously expressed ADA (Dz.eutE) coupled with *acs1*Δ *acs2* Δ *ald6*Δ; and an MEV pathway comprising either ERG10 (acetyl-CoA thiolase) or nphT7 (acetoacetyl-CoA synthase).

5. DETAILED DESCRIPTION OF THE EMBODIMENTS

5.1 Terminology

[0047] As used herein, the term "heterologous" refers to what is not normally found in nature. The term "heterologous nucleotide sequence" refers to a nucleotide sequence not normally found in a given cell in nature. As such, a heterologous nucleotide sequence may be: (a) foreign to its host cell (*i.e.,* is "exogenous" to the cell); (b) naturally found in the host cell (*i.e.,* "endogenous") but present at an unnatural quantity in the cell (*i.e.,* greater or lesser quantity than naturally found in the host cell); or (c) be naturally found in the host cell but positioned outside of its natural locus. The term "heterologous enzyme" refers to an enzyme that is not normally found in a given cell in nature. The term encompasses an enzyme that is: (a) exogenous to a given cell (*i.e.*, encoded by a nucleotide sequence that is not naturally present in the host cell or not naturally present in a given context in the host cell); and (b) naturally found in the host cell (*e.g.*, the enzyme is encoded by a nucleotide sequence that is endogenous to the cell) but that is produced in an unnatural amount (*e.g.*, greater or lesser than that naturally found) in the host cell.

[0048] On the other hand, the term "native" or "endogenous" as used herein with reference to molecules, and in particular enzymes and nucleic acids, indicates molecules that are expressed in the organism in which they originated or are found in nature, independently of the level of expression that can be lower, equal, or higher than the level of expression of the molecule in the native microorganism. It is understood that expression of native enzymes or polynucleotides may be modified in recombinant microorganisms.

[0049] As used herein, to "functionally disrupt" or a "functional disruption" *e.g.*, of a target gene, for example, one or more genes of the PDH-bypass, means that the target gene is altered in such a way as to decrease in the host cell the activity of the protein encoded by the target gene. Similarly, to "functionally disrupt" or a "functional disruption" *e.g.*, of a target protein, for example, one or more enzymes of the PDH-bypass, means that the target protein

is altered in such a way as to decrease in the host cell the activity of the protein. In some embodiments, the activity of the target protein encoded by the target gene is eliminated in the host cell. In other embodiments, the activity of the target protein encoded by the target gene is decreased in the host cell. Functional disruption of the target gene may be achieved by deleting all or a part of the gene so that gene expression is eliminated or reduced, or so that the activity of the gene product is eliminated or reduced. Functional disruption of the target gene may also be achieved by mutating a regulatory element of the gene, *e.g.,* the promoter of the gene so that expression is eliminated or reduced, or by mutating the coding sequence of the gene so that the activity of the gene product is eliminated or reduced. In some embodiments, functional disruption of the target gene results in the removal of the complete open reading frame of the target gene.

[0050] As used herein, the term "parent cell" refers to a cell that has an identical genetic background as a genetically modified host cell disclosed herein except that it does not comprise one or more particular genetic modifications engineered into the modified host cell, for example, one or more modifications selected from the group consisting of: heterologous expression of an ADA, heterologous expression of an NADH-using HMG-CoA reductase, heterologous expression of an AACS, heterologous expression of a phosphoketolase, heterologous expression of a phosphotrancacetylase, and heterologous expression of one or more enzymes of the mevalonate pathway.

[0051] As used herein, the term "production" generally refers to an amount of an isoprenoid produced by a genetically modified host cell provided herein. In some embodiments, production is expressed as a yield of isoprenoid by the host cell. In other embodiments, production is expressed as a productivity of the host cell in producing the isoprenoid.

[0052] As used herein, the term "productivity" refers to production of an isoprenoid by a host cell, expressed as the amount of isoprenoid produced (by weight) per amount of fermentation broth in which the host cell is cultured (by volume) over time (per hour).

[0053] As used herein, the term "yield" refers to production of an isoprenoid by a host cell, expressed as the amount of isoprenoid produced per amount of carbon source consumed by the host cell, by weight.

5.2 Genetically Modified Microbes Producing Acetyl-CoA Derived Isoprenoids

5.2.1 Host Cells

[0054] Host cells useful compositions and methods provided herein include archae, prokaryotic, or eukaryotic cells.

[0055] Suitable prokaryotic hosts include, but are not limited, to any of a variety of gram-positive, gram-negative, or gram-variable bacteria. Examples include, but are not limited to, cells belonging to the genera: *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azobacter*, *Bacillus*, *Brevibacterium*, *Chromatium*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Mesorhizobium*, *Methylobacterium*, *Microbacterium*, *Phormidium*, *Pseudomonas*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Rhodococcus*, *Salmonella*, *Scenedesmun*, *Serratia*, *Shigella*, *Staphlococcus*, *Strepromyces*, *Synnecoccus*, and *Zymomonas*. Examples of prokaryotic strains include, but are not limited to: *Bacillus subtilis*, *Bacillus amyloliquefacines*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum*, *Clostridium beigerinckii*, *Enterobacter sakazakii*, *Escherichia coli*, *Lactococcus lactis*, *Mesorhizobium loti*, *Pseudomonas aeruginosa*, *Pseudomonas mevalonii*, *Pseudomonas pudica*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Salmonella enterica*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, and *Staphylococcus aureus*. In a particular embodiment, the host cell is an *Escherichia coli* cell.

[0056] Suitable archae hosts include, but are not limited to, cells belonging to the genera: *Aeropyrum*, *Archaeglobus*, *Halobacterium*, *Methanococcus*, *Methanobacterium*, *Pyrococcus*, *Sulfolobus*, and *Thermoplasma*. Examples of archae strains include, but are not limited to: *Archaeoglobus fulgidus*, *Halobacterium sp.*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*, *Pyrococcus horikoshii*, *Pyrococcus abyssi*, and *Aeropyrum pernix*.

[0057] Suitable eukaryotic hosts include, but are not limited to, fungal cells, algal cells, insect cells, and plant cells. In some embodiments, yeasts useful in the present methods include yeasts that have been deposited with microorganism depositories (*e.g.* IFO, ATCC, *etc.*) and belong to the genera *Aciculoconidium, Ambrosiozyma, Arthroascus, Arxiozyma, Ashbya, Babjevia, Bensingtonia, Botryoascus, Botryozyma, Brettanomyces, Bullera, Bulleromyces, Candida, Citeromyces, Clavispora, Cryptococcus, Cystofilobasidium,*

Debaryomyces, Dekkara, Dipodascopsis, Dipodascus, Eeniella, Endomycopsella, Eremascus, Eremothecium, Erythrobasidium, Fellomyces, Filobasidium, Galactomyces, Geotrichum, Guilliermondella, Hanseniaspora, Hansenula, Hasegawaea, Holtermannia, Hormoascus, Hyphopichia, Issatchenkia, Kloeckera, Kloeckeraspora, Kluyveromyces, Kondoa, Kuraishia, Kurtzmanomyces, Leucosporidium, Lipomyces, Lodderomyces, Malassezia, Metschnikowia, Mrakia, Myxozyma, Nadsonia, Nakazawaea, Nematospora, Ogataea, Oosporidium, Pachysolen, Phachytichospora, Phaffia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Saccharomycodes, Saccharomycopsis, Saitoella, Sakaguchia, Saturnospora, Schizoblastosporion, Schizosaccharomyces, Schwanniomyces, Sporidiobolus, Sporobolomyces, Sporopachydermia, Stephanoascus, Sterigmatomyces, Sterigmatosporidium, Symbiotaphrina, Sympodiomyces, Sympodiomycopsis, Torulaspora, Trichosporiella, Trichosporon, Trigonopsis, Tsuchiyaea, Udeniomyces, Waltomyces, Wickerhamia, Wickerhamiella, Williopsis, Yamadazyma, Yarrowia, Zygoascus, Zygosaccharomyces, Zygowilliopsis, and *Zygozyma*, among others.

[0058] In some embodiments, the host microbe is *Saccharomyces cerevisiae, Pichia pastoris, Schizosaccharomyces pombe, Dekkera bruxellensis, Kluyveromyces lactis* (previously called *Saccharomyces lactis*), *Kluveromyces marxianus, Arxula adeninivorans*, or *Hansenula polymorpha (*now known as *Pichia angusta)*. In some embodiments, the host microbe is a strain of the genus *Candida*, such as *Candida lipolytica, Candida guilliermondii, Candida krusei, Candida pseudotropicalis*, or *Candida utilis*.

[0059] In a particular embodiment, the host microbe is *Saccharomyces cerevisiae*. In some embodiments, the host is a strain of *Saccharomyces cerevisiae* selected from the group consisting of Baker's yeast, CBS 7959, CBS 7960, CBS 7961, CBS 7962, CBS 7963, CBS 7964, IZ-1904, TA, BG-1, CR-1, SA-1, M-26, Y-904, PE-2, PE-5, VR-1, BR-1, BR-2, ME-2, VR-2, MA-3, MA-4, CAT-1, CB-1, NR-1, BT-1, and AL-1. In some embodiments, the host microbe is a strain of *Saccharomyces cerevisiae* selected from the group consisting of PE-2, CAT-1, VR-1, BG-1, CR-1, and SA-1. In a particular embodiment, the strain of *Saccharomyces cerevisiae* is PE-2. In another particular embodiment, the strain of *Saccharomyces cerevisiae* is CAT-1. In another particular embodiment, the strain of *Saccharomyces cerevisiae* is BG-1.

[0060] In some embodiments, the host microbe is a microbe that is suitable for industrial fermentation. In particular embodiments, the microbe is conditioned to subsist under high solvent concentration, high temperature, expanded substrate utilization, nutrient limitation, osmotic stress due to sugar and salts, acidity, sulfite and bacterial contamination,

or combinations thereof, which are recognized stress conditions of the industrial fermentation environment.

5.2.2 Heterologous ADA for Acetyl-CoA Production

[0061] In one aspect, provided herein is a genetically modified host cell capable of producing an acetyl-CoA derived isoprenoid, the cell comprising one or more heterologous nucleotide sequences encoding acylating acetaldehyde dehydrogenase (alternately referred to as "acetylaldehyde dehydrogenase, acetylating," "acetylaldehyde dehydrogenase, acylating," or ADA (EC 1.2.1.10)).

[0062] Proteins capable of catalyzing this reaction that are useful for the compositions and methods provided herein include the following four types of proteins:

[0063] (1) Bifunctional proteins that catalyze the reversible conversion of acetyl-CoA to acetaldehyde, and the subsequent reversible conversion of acetaldehyde to ethanol. An example of this type of protein is the AdhE protein in *E. coli* (Gen Bank No: NP_415757). AdhE appears to be the evolutionary product of a gene fusion. The NH_2-terminal region of the AdhE protein is highly homologous to aldehyde:NAD^+ oxidoreductases, whereas the COOH-terminal region is homologous to a family of Fe^{2+}-dependent ethanol:NAD^+ oxidoreductases (Membrillo-Hernandez *et al.*, (2000) *J. Biol. Chem.* 275: 33869-33875). The *E. coli* AdhE is subject to metal-catalyzed oxidation and therefore oxygen-sensitive (Tamarit *et al.* (1998) *J. Biol. Chem.* 273:3027-32).

[0064] (2) Proteins that catalyze the reversible conversion of acetyl-CoA to acetaldehyde in strictly or facultative anaerobic microbes but do not possess alcohol dehydrogenase activity. An example of this type of protein has been reported in *Clostridium kluyveri* (Smith *et al.* (1980) *Arch. Biochem. Biophys.* 203: 663-675). An ADA has been annotated in the genome of *Clostridium kluyveri* DSM 555 (accession no: EDK33116). A homologous protein AcdH is identified in the genome of *Lactobacillus plantarum* (accession no: NP_784141). Another example of this type of protein is the *ald* gene product in *Clostridium beijerinckii* NRRL B593 (Toth *et al.* (1999) *Appl. Environ. Microbiol.* 65: 4973-4980, accession no: AAD31841).

[0065] (3) Proteins that are involved in ethanolamine catabolism. Ethanolamine can be utilized both as carbon and nitrogen source by many enterobacteria (Stojiljkovic *et al.* (1995) *J. Bacteriol.* 177: 1357-1366). Ethanolamine is first converted by ethanolamine ammonia lyase to ammonia and acetaldehyde, subsequently, acetaldehyde is converted by ADA to acetyl-CoA. An example of this type of ADA is the EutE protein in *Salmonella typhimurium* (Stojiljkovic *et al.* (1995) *J. Bacteriol.* 177: 1357-1366, accession no:

AAL21357; *see* also U18560.1). *E. coli* is also able to utilize ethanolamine (Scarlett *et al.* (1976) *J. Gen. Microbiol.* 95:173-176) and has an EutE protein (accession no: AAG57564; *see* also EU897722.1) which is homologous to the EutE protein in *S. typhimurium*.

[0066] (4) Proteins that are part of a bifunctional aldolase-dehydrogenase complex involved in 4-hydroxy-2-ketovalerate catabolism. Such bifunctional enzymes catalyze the final two steps of the meta-cleavage pathway for catechol, an intermediate in many bacterial species in the degradation of phenols, toluates, naphthalene, biphenyls and other aromatic compounds (Powlowski and Shingler (1994) *Biodegradation* 5, 219-236). 4-Hydroxy-2-ketovalerate is first converted by 4-hydroxy-2-ketovalerate aldolase to pyruvate and acetaldehyde, subsequently acetaldehyde is converted by ADA to acetyl-CoA. An example of this type of ADA is the DmpF protein in *Pseudomonas sp* CF600 (accession no: CAA43226) (Shingler *et al.* (1992) *J. Bacteriol.* 174:71 1-24). *E. coli* has a homologous MphF protein (Ferrandez *et al.* (1997) J. Bacteriol. 179: 2573-2581 , accession no: NP_414885) to the DmpF protein in *Pseudomonas sp.* CF600.

[0067] In some embodiments, an ADA (or nucleic acid sequence encoding such activity) useful for the compositions and methods described herein is selected from the group consisting of *Escherichia coli* adhE, *Entamoeba histolytica* adh2, *Staphylococcus aureus* adhE, *Piromyces sp.E2* adhE, *Clostridium kluyveri* (EDK33116), *Lactobacillus plantarum* acdH, and *Pseudomonas putida* (YP 001268189), as described in International Publication No. WO 2009/013159. In some embodiments, the ADA is selected from the group consisting of *Clostridium botulinum* eutE (FR745875.1), *Desulfotalea psychrophila* eutE (CR522870.1), *Acinetobacter sp. HBS-2* eutE (ABQ44511.2), *Caldithrix abyssi* eutE (ZP_09549576), and *Halorubrum lacusprofundi* ATCC 49239 (YP_002565337.1).

[0068] In particular embodiments, the ADA useful for the compositions and methods provided herein is eutE from *Dickeya zeae*. A representative eutE nucleotide sequence of *Dickeya zeae* includes accession number NC_012912.1:1110476..1111855 and SEQ ID NO: 1 as provided herein. A representative eutE protein sequence of *Dickeya zeae* includes accession number YP_003003316, and SEQ ID NO: 2 as provided herein.

[0069] ADAs also useful in the compositions and methods provided herein include those molecules which are said to be "derivatives" of any of the ADAs described herein. Such a "derivative" has the following characteristics: (1) it shares substantial homology with any of the ADAs described herein; and (2) is capable of catalyzing the conversion of acetaldehyde to acetyl-CoA. A derivative of an ADA is said to share "substantial homology"

with ADA if the amino acid sequences of the derivative is at least 80%, at least 85% and more preferably at least 90%, and most preferably at least 95%, the same as that of any of the ADAs described herein.

5.2.2.1 Methods for Identifying Functional ADAs

[0070] In another aspect, provided herein is a screening method for ADAs with elevated *in vivo* performance. In this screening method, ADAs with elevated *in vivo* performance are identified by their ability to rescue engineered host cells from cell death. The engineered host cells comprise a heterologous pathway for the production of a cytosolic acetyl-CoA derived secondary metabolite, *e.g.,* an isoprenoid. In some embodiments, the engineered host cells further comprise a functionally disrupted PDH-bypass pathway, and a weakly active ADA, wherein the combined activities of the functionally disrupted PDH-bypass pathway and the weakly active ADA do not produce enough cytosolic acetyl-CoA to meet the requirements for production of both: (1) the cytosolic acetyl-CoA derived secondary metabolite; and (2) the cytosolic acetyl-CoA derived primary metabolites required for cell survival, health, and/or growth. For survival, health, and/or growth, the host cell thus requires an active ADA that enables production of an elevated pool of cytosolic acetyl-CoA.

[0071] In some embodiments, the method of screening for ADAs with elevated *in vivo* performance comprises: (a) expressing a control ADA in a host cell having a functionally disrupted PDH-bypass pathway to produce an elevated level of a cytosolic acetyl-CoA derived secondary metabolite, wherein production of the elevated level of the cytosolic acetyl-CoA derived secondary metabolite reduces the viability of the host cell compared to a parent cell not producing the elevated level of the cytosolic acetyl-CoA derived secondary metabolite; and

(b) expressing in the host cell a test ADA instead of the control ADA; whereby an increase in viability of the host cell expressing the test ADA compared to the host cell expressing the control ADA identifies the test ADA as having improved *in vivo* performance compared to the control ADA.

[0072] In some embodiments, production of the elevated level of a cytosolic acetyl-CoA derived secondary metabolite in the host cell is inducible. Induction may occur in response to an inducing agent (*e.g.*, galcatose) or specific growth condition (*e.g.,* growth temperature). When grown in the absence of the inducing agent, the ADA activity of the host cell is sufficient to enable production of the cytosolic acetyl-CoA required by the host cell for survival. However, when grown in the presence of the inducing agent, the ADA activity of the host cell is not sufficient to enable production of both the cytosolic acetyl-CoA required

by the host cell for survival and the elevated level of the cytosolic acetyl-CoA derived secondary metabolite. In the latter case, the host cell thus requires for survival a more active ADA that enables production of an elevated pool of cytosolic acetyl-CoA. The production of the cytosolic acetyl-CoA derived secondary metabolite in the host cell may range from about 10% to at least about 1,000-fold, or more, higher than the production of the cytosolic acetyl-CoA derived secondary metabolite in the parent cell.

[0073] The reduced viability of the host cell expressing the control ADA compared to the parent cell may range from decreased cell growth to lethality. Thus, in some embodiments, the host cell expressing the control ADA produces a reduced number of progeny cells in a liquid culture or on an agar plate compared to the parent cell. In other embodiments, the host cell expressing the control ADA produces no progeny cells in a liquid culture or on an agar plate compared to the parent cell. Accordingly, the increase in viability of the host cell expressing the test ADA instead of the control ADA may be apparent in liquid culture by a higher number of progeny cells, or on an agar plate by a larger colony size, compared to the number of progeny cells or colony size produced by the host cell expressing the control ADA.

[0074] Production of the elevated level of the cytosolic acetyl-CoA derived secondary metabolite in the host cell may be effected by modifying the expression and/or activity of an enzyme involved in the production of the cytosolic acetyl-CoA derived secondary metabolite or its precursors in the host cell. In some such embodiments, the expression and/or activity of an enzyme of the MEV or DXP pathway is modified. In some such embodiments, the expression and/or activity of a HMG-CoA reductase and/or a mevalonate kinase is modified.

[0075] The control ADA and test ADA may be naturally occurring ADAs or non-naturally occurring ADAs. In some embodiments, the test ADA is a variant of the control ADA that differs from the control ADA by one or more amino acid substitutions, deletions, and/or additions. In some embodiments, the test ADA comprises identical amino acids as the control ADA but the codons encoding these amino acids differ between the test ADA and the control ADA. In some such embodiments, the codons are optimized for usage in the host cell. In some embodiments, the control ADA and/or test ADA is fused to a pyruvate decarboxylase. In some embodiments, expression of the test ADA is under regulatory control of a strong promoter. In some embodiments, expression of the test ADA is under regulatory control of a medium strength promoter. In some embodiments, expression of the test ADA is under regulatory control of a weak promoter.

[0076] The increase in viability of the host cell in the presence of the test ADA may be effected by a test ADA that is more active than the control ADA or by a test ADA that is similarly or less active than the control ADA but that is expressed at a higher level. Identification of test ADAs with increased activity can be accomplished by expressing the control ADA and the test ADA at similar levels in the host cell. This can be accomplished, for example, by placing the nucleotide sequences encoding the control ADA and test ADA in the host cell under the control of the same regulatory elements. In other embodiments in which the method is used, for example, to identify regulatory elements (*e.g.*, promoters) that provide a desired expression level, the test ADA differs from the control ADA not in nucleotide or amino acid sequence but in expression level. In such embodiments, different regulatory elements can be used for the expression of the control ADA and the test ADA, and comparison of host cell viabilities provides information not about the activity of the test ADA but about the strength of the regulatory elements driving the expression of the test ADA.

[0077] To prevent a competitive growth situation in which fast growing false positive host cells comprising a growth promoting mutation rather than an improved ADA variant take over a host cell culture, one embodiment of the screening method involves an agar-plate based selection system. In this embodiment, the host cell is plated on an agar plate, and a host cell comprising a test ADA variant with improved *in vivo* performance is identified by colony growth.

[0078] A substantial advantage of the presently disclosed screening method is its simplicity and capacity for high-throughput implementation. ADA variants are identified simply based on cell viability, making other costly and time consuming screening methods virtually unnecessary. Thus, in one embodiment, the method is used to screen a collection of ADA variants (*e.g.*, a library of mutant ADAs) for ADA variants with improved *in vivo* performance. In such an embodiment, not a single test ADA is expressed in a host cell but a collection of test ADAs are expressed in a collection of host cells. The host cells can then be grown on agar plates, and host cells expressing ADA variants with improved *in vivo* performance can be identified based on colony growth. In some embodiments, the collection of ADA variants comprises from 2 to 5, from 5 to 10, from 10 to 50, from 50 to 100, from 100 to 500, from 500 to 1,000, from 1,000 to 10,000, from 10,000 to 100,000, from 100,000 to 1,000,000, and more, ADA variants.

[0079] Another major advantage of the presently disclosed screening method is its continued capacity to select for better and better ADA variants in an iterative fashion, wherein a test ADA identified in an iteration is used as the control ADA in a subsequent

iteration. Such an embodiment requires, however, that at each iteration the production of the cytosolic acetyl-CoA derived secondary metabolite in the host cell is checked and potentially increased (*e.g.,* by increasing or decreasing expression levels of enzymes, adding or subtracting enzymes, increasing or decreasing copy numbers of genes, replacing promoters controlling expression of enzymes, or altering enzymes by genetic mutation) to a level that causes reduced viability when the host cell expresses the new control ADA (*i.e.*, the test ADA of the previous iteration). Alternatively, or in addition, at each iteration, the expression of the control ADA can be reduced (*e.g.*, by decreasing expression of or by using weaker promoters or by reducing the stability of the control ADA transcript or polypeptide) to provide reduced control ADA activity. In the next iteration, a test ADA can then be identified that has yet increased *in vivo* performance compared to the test ADA of the previous iteration.

[0080] Another major advantage of the presently disclosed screening method is that selection for improved ADAs occurs *in vivo* rather than *in vitro*. As a result, improvements of multiple enzyme properties that enhance the *in vivo* performance of the ADA variant can be obtained.

[0081] Enzymes developed using the presently disclosed screening method can be subjected to additional means of optional screening including but not limited to a fluorescent screen and/or a direct quantitation of the cytosolic acetyl-CoA derived secondary metabolite by gas chromatography. More specifically, this includes a Nile Red-based high throughput fluorescent assay for measuring production of a sesquiterpene such as farnesene, and a gas chromatography (GC)-based direct quantitation method for measuring the titer of a sesquiterpene such as farnesene. The improved enzymes can also be further improved by genetic engineering methods such as induced mutations and the like. As a result, improvements of multiple enzyme properties that enhance the final enzyme performance are successively accomplished, and the most effective enzyme variants are identified.

5.2.3 Functional Disruption of the PDH-bypass

[0082] Acetyl-CoA can be formed in the mitochondria by oxidative decarboxylation of pyruvate catalyzed by the PDH complex. However, due to the inability of *S. cerevisiae* to transport acetyl-CoA out of the mitochondria, the PDH bypass has an essential role in providing acetyl-CoA in the cytosolic compartment, and provides an alternative route to the PDH reaction for the conversion of pyruvate to acetyl-CoA. The PDH bypass involves the enzymes pyruvate decarboxylase (PDC; EC 4.1.1.1), acetaldehyde dehydrogenase (ACDH; EC 1.2.1.5 and EC 1.2.1.4), and acetyl-CoA synthetase (ACS; EC 6.2.1.1). Pyruvate

decarboxylase catalyzes the decarboxylation of pyruvate to acetaldehyde and carbon dioxide. Acetaldehyde dehydrogenase oxidizes acetaldehyde to acetic acid. In *S. cerevisiae*, the family of aldehyde dehydrogenases contains five members. *ALD2* (YMR170c), *ALD3* (YMR169c), and *ALD6* (YPL061w) correspond to the cytosolic isoforms, while *ALD4* (YOR374w) and *ALD5* (YER073w) encode the mitochondrial enzyme. The main cytosolic acetaldehyde dehydrogenase isoform is encoded by *ALD6*. The formation of acetyl-CoA from acetate is catalyzed by ACS and involves hydrolysis of ATP. Two structural genes, *ACS1* and *ACS2,* encode ACS.

[0083] In some embodiments, the genetically modified host cell comprises a functional disruption in one or more genes of the PDH-bypass pathway. In some embodiments, disruption of the one or more genes of the PDH-bypass of the host cell results in a genetically modified microbial cell that is impaired in its ability to catalyze one or more of the following reactions: (1) the decarboxylation of pyruvate into acetaldehyde by pyruvate decarboxylase; (2) the conversion of acetaldehyde into acetate by acetaldehyde dehydrogenase; and (3) the synthesis of acetyl-CoA from acetate and CoA by acetyl-CoA synthetase.

[0084] In some embodiments, compared to a parent cell, a host cell comprises a functional disruption in one or more genes of the PDH-bypass pathway, wherein the activity of the reduced-function or non-functional PDH-bypass pathway alone or in combination with a weak ADA is not sufficient to support host cell growth, viability, and/or health.

[0085] In some embodiments, the activity or expression of one or more endogenous proteins of the PDH-bypass is reduced by at least about 50%. In another embodiment, the activity or expression of one or more endogenous proteins of the PDH-bypass is reduced by at least about 60%, by at least about 65%, by at least about 70%, by at least about 75%, by at least about 80%, by at least about 85%, by at least about 90%, by at least about 95%, or by at least about 99% as compared to a recombinant microorganism not comprising a reduction or deletion of the activity or expression of one or more endogenous proteins of the PDH-bypass.

[0086] As is understood by those skilled in the art, there are several mechanisms available for reducing or disrupting the activity of a protein, such as a protein of the PDH-bypass, including, but not limited to, the use of a regulated promoter, use of a weak constitutive promoter, disruption of one of the two copies of the gene encoding the protein in a diploid yeast, disruption of both copies of the gene in a diploid yeast, expression of an anti-sense nucleic acid, expression of an siRNA, over expression of a negative regulator of the

endogenous promoter, alteration of the activity of an endogenous or heterologous gene, use of a heterologous gene with lower specific activity, the like or combinations thereof.

[0087] In some embodiments, the genetically modified host cell comprises a mutation in at least one gene encoding for a protein of the PDH-bypass, resulting in a reduction of activity of a polypeptide encoded by said gene. In another embodiment, the genetically modified host cell comprises a partial deletion of gene encoding for a protein of the PDH-bypass, resulting in a reduction of activity of a polypeptide encoded by the gene. In another embodiment, the genetically modified host cell comprises a complete deletion of a gene encoding for a protein of the PDH-bypass, resulting in a reduction of activity of a polypeptide encoded by the gene. In yet another embodiment, the genetically modified host cell comprises a modification of the regulatory region associated with the gene encoding a protein of the PDH-bypass, resulting in a reduction of expression of a polypeptide encoded by said gene. In yet another embodiment, the genetically modified host cell comprises a modification of the transcriptional regulator resulting in a reduction of transcription of a gene encoding a protein of the PDH-bypass. In yet another embodiment, the genetically modified host cell comprises mutations in all genes encoding for a protein of the PDH-bypass resulting in a reduction of activity of a polypeptide encoded by the gene(s). In one embodiment, the activity or expression of the protein of the PDH-bypass is reduced by at least about 50%. In another embodiment, the activity or expression of the protein of the PDH-bypass is reduced by at least about 60%, by at least about 65%, by at least about 70%, by at least about 75%, by at least about 80%, by at least about 85%, by at least about 90%, by at least about 95%, or by at least about 99% as compared to a recombinant microorganism not comprising a reduction of the activity or expression of the protein of the PDH-bypass.

[0088] In some embodiments, disruption of one or more genes of the PDH-bypass is achieved by using a "disruption construct" that is capable of specifically disrupting a gene of the PDH-bypass upon introduction of the construct into the microbial cell, thereby rendering the disrupted gene non-functional. In some embodiments, disruption of the target gene prevents the expression of a functional protein. In some embodiments, disruption of the target gene results in expression of a non-functional protein from the disrupted gene. In some embodiments, disruption of a gene of the PDH-bypass is achieved by integration of a "disrupting sequence" within the target gene locus by homologous recombination. In such embodiments, the disruption construct comprises a disrupting sequence flanked by a pair of nucleotide sequences that are homologous to a pair of nucleotide sequences of the target gene locus (homologous sequences). Upon replacement of the targeted portion of the target gene

by the disruption construct, the disrupting sequence prevents the expression of a functional protein, or causes expression of a non-functional protein, from the target gene.

[0089] Disruption constructs capable of disrupting a gene of the PDH-bypass may be constructed using standard molecular biology techniques well known in the art. *See, e.g.,* Sambrook *et al.*, 2001, *Molecular Cloning -- A Laboratory Manual*, 3rd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, and Ausubel *et al.*, *eds.*, Current Edition, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY. Parameters of disruption constructs that may be varied in the practice of the present methods include, but are not limited to, the lengths of the homologous sequences; the nucleotide sequence of the homologous sequences; the length of the disrupting sequence; the nucleotide sequence of the disrupting sequence; and the nucleotide sequence of the target gene. In some embodiments, an effective range for the length of each homologous sequence is 50 to 5,000 base pairs. In particular embodiments, the length of each homologous sequence is about 500 base pairs. For a discussion of the length of homology required for gene targeting, *see* Hasty *et al., Mol Cell Biol* 11:5586-91 (1991). In some embodiments, the homologous sequences comprise coding sequences of the target gene. In other embodiments, the homologous sequences comprise upstream or downstream sequences of the target gene. Is some embodiments, one homologous sequence comprises a nucleotide sequence that is homologous to a nucleotide sequence located 5' of the coding sequence of the target gene, and the other homologous sequence comprises a nucleotide sequence that is homologous to a nucleotide sequence located 3' of the coding sequence of the target gene. In some embodiments, the disrupting sequence comprises a nucleotide sequence encoding a selectable marker that enables selection of microbial cells comprising the disrupting sequence. Thus, in such embodiments, the disruption construct has a dual function, *i.e.,* to functionally disrupt the target gene and to provide a selectable marker for the identification of cells in which the target gene is functionally disrupted. In some embodiments, a termination codon is positioned in-frame with and downstream of the nucleotide sequence encoding the selectable marker to prevent translational read-through that might yield a fusion protein having some degree of activity of the wild type protein encoded by the target gene. In some embodiments, the length of the disrupting sequence is one base pair. Insertion of a single base pair can suffice to disrupt a target gene because insertion of the single base pair in a coding sequence could constitute a frame shift mutation that could prevent expression of a functional protein. In some embodiments, the sequence of the disruption sequence differs from the nucleotide sequence of the target gene located between the homologous sequences by a single base pair.

Upon replacement of the nucleotide sequence within the target gene with the disrupting sequence, the single base pair substitution that is introduced could result in a single amino acid substitution at a critical site in the protein and the expression of a non-functional protein. It should be recognized, however, that disruptions effected using very short disrupting sequences are susceptible to reversion to the wild type sequence through spontaneous mutation, thus leading to restoration of PDH-bypass function to the host strain. Accordingly, in particular embodiments, the disrupting sequences are longer than one to a few base pairs. At the other extreme, a disrupting sequence of excessive length is unlikely to confer any advantage over a disrupting sequence of moderate length, and might diminish efficiency of transfection or targeting. Excessive length in this context is many times longer than the distance between the chosen homologous sequences in the target gene. Thus, in certain embodiments, the length for the disrupting sequence can be from 2 to 2,000 base pairs. In other embodiments, the length for the disrupting sequence is a length approximately equivalent to the distance between the regions of the target gene locus that match the homologous sequences in the disruption construct.

[0090] In some embodiments, the disruption construct is a linear DNA molecule. In other embodiments, the disruption construct is a circular DNA molecule. In some embodiments, the circular disruption construct comprises a pair of homologous sequences separated by a disrupting sequence, as described above. In some embodiments, the circular disruption construct comprises a single homologous sequence. Such circular disruption constructs, upon integration at the target gene locus, would become linearized, with a portion of the homologous sequence positioned at each end and the remaining segments of the disruption construct inserting into and disrupting the target gene without replacing any of the target gene nucleotide sequence. In particular embodiments, the single homologous sequence of a circular disruption construct is homologous to a sequence located within the coding sequence of the target gene.

[0091] Disruption constructs can be introduced into a microbial cell by any method known to one of skill in the art without limitation. Such methods include, but are not limited to, direct uptake of the molecule by a cell from solution, or facilitated uptake through lipofection using, *e.g.,* liposomes or immunoliposomes; particle-mediated transfection; *etc.* See, *e.g.,* U.S. Patent No. 5,272,065; Goeddel et al., eds, 1990, Methods in Enzymology, vol. 185, Academic Press, Inc., CA; Krieger, 1990, Gene Transfer and Expression -- A Laboratory Manual, Stockton Press, NY; Sambrook et al., 1989, Molecular Cloning -- A Laboratory Manual, Cold Spring Harbor Laboratory, NY; and Ausubel et al., eds., Current

Edition, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, NY. Particular methods for transforming yeast cells are well known in the art. *See* Hinnen *et al.,* Proc. Natl. Acad. Sci. USA 75:1292-3 (1978); Cregg *et al.,* Mol. Cell. Biol. 5:3376-3385 (1985). Exemplary techniques include, but are not limited to, spheroplasting, electroporation, PEG 1000 mediated transformation, and lithium acetate or lithium chloride mediated transformation.

5.2.3.1 ALD4 and ALD6

[0092] In some embodiments, one or more genes encoding aldehyde dehydrogenase (ACDH) activity are functionally disrupted in the host cell. In some embodiments, the aldehyde dehydrogenase is encoded by a gene selected from the group consisting of ALD2, ALD3, ALD4, ALD5, ALD6, and homologs and variants thereof.

[0093] In some embodiments, the genetically modified host cell comprises a functional disruption of ALD4. Representative *ALD4* nucleotide sequences of *Saccharomyces cerevisiae* include accession number NM_001183794, and SEQ ID NO:7 as provided herein. Representative Ald4 protein sequences of *Saccharomyces cerevisiae* include accession number NP_015019.1and SEQ ID NO:8 as provided herein.

[0094] In some embodiments, the genetically modified host cell comprises a functional disruption of cytosolic aldehyde dehydrogenase (ALD6). Ald6p functions in the native PDH-bypass to convert acetaldehyde to acetate. Representative *ALD6* nucleotide sequences of *Saccharomyces cerevisiae* include accession number SCU56604, and SEQ ID NO:9 as provided herein. Representative Ald6 protein sequences of *Saccharomyces cerevisiae* include accession number AAB01219 and SEQ ID NO:10 as provided herein.

[0095] As would be understood in the art, naturally occurring homologs of aldehyde dehydrogenase in yeast other than *S. cerevisiae* can similarly be inactivated using the methods described herein.

[0096] As would be understood by one skilled in the art, the activity or expression of more than one aldehyde dehydrogenase can be reduced or eliminated. In one specific embodiment, the activity or expression of ALD4 and ALD6 or homologs or variants thereof is reduced or eliminated. In another specific embodiment, the activity or expression of ALD5 and ALD6 or homologs or variants thereof is reduced or eliminated. In yet another specific embodiment, the activity or expression of ALD4, ALD5, and ALD6 or homologs or variants thereof is reduced or eliminated. In yet another specific embodiment, the activity or expression of the cytosolically localized aldehyde dehydrogenases ALD2, ALD3, and ALD6 or homologs or variants thereof is reduced or eliminated. In yet another specific embodiment,

the activity or expression of the mitochondrially localized aldehyde dehydrogenases, ALD4 and ALD5 or homologs or variants thereof, is reduced or eliminated.

5.2.3.2 ACS1 and ACS2

[0097] In some embodiments, one or more genes encoding acetyl-CoA synthase (ACS) activity are functionally disrupted in the host cell. In some embodiments, the acetyl-CoA synthase is encoded by a gene selected from the group consisting of ACS1, ACS2, and homologs and variants thereof.

[0098] In some embodiments, one or more genes encoding acetyl-CoA synthase (ACS) activity is functionally disrupted in the host cell. ACS1 and ACS2 are both acetyl-CoA synthases that con convert acetate to acetyl-CoA. ACS1 is expressed only under respiratory conditions, whereas ACS2 is expressed constitutively. When ACS2 is knocked out, strains are able to grow on respiratory conditions (*e.g.* ethanol, glycerol, or acetate media), but die on fermentable carbon sources (*e.g.* sucrose, glucose).

[0099] In some embodiments, the genetically modified host cell comprises a functional disruption of ACS1. The sequence of the *ACS1* gene of *S. cerevisiae* has been previously described. *See, e.g.,* Nagasu *et al., Gene* 37 (1-3):247-253 (1985). Representative *ACS1* nucleotide sequences of *Saccharomyces cerevisiae* include accession number X66425, and SEQ ID NO:3 as provided herein. Representative Acs1 protein sequences of *Saccharomyces cerevisiae* include accession number AAC04979 and SEQ ID NO:4 as provided herein.

[00100] In some embodiments, the genetically modified host cell comprises a functional disruption of ACS2. The sequence of the *ACS2* gene of *S. cerevisiae* has been previously described. *See, e.g.,* Van den Berg *et al., Eur. J. Biochem.* 231(3):704-713 (1995). Representative *ACS2* nucleotide sequences of *Saccharomyces cerevisiae* include accession number S79456, and SEQ ID NO:5 as provided herein. Representative Acs2 protein sequences of *Saccharomyces cerevisiae* include accession number CAA97725 and SEQ ID NO:6 as provided herein.

[00101] As would be understood in the art, naturally occurring homologs of acetyl-CoA synthase in yeast other than *S. cerevisiae* can similarly be inactivated using the methods described herein.

[00102] In some embodiments, the host cell comprises a cytosolic acetyl-coA synthase activity that can convert acetate to acetyl-CoA under respiratory conditions (*i.e.*, when the host cell is grown in the presence of e.g. ethanol, glycerol, or acetate). In some such embodiments, the host cell is a yeast cell that comprises ACS1 activity. In other

embodiments, the host cell compared to a parent cell comprises no or reduced endogenous acetyl-CoA synthase activity under respiratory conditions. In some such embodiments, the host cell is a yeast cell that compared to a parent cell comprises no or reduced ACS1 activity.

[00103] In some embodiments, the host cell comprises a cytosolic acetyl-coA synthase activity that can convert acetate to acetyl-CoA under non-respiratory conditions (*i.e.*, when the host cell is grown in the presence of fermentable carbon sources (*e.g.* sucrose, glucose)). In some such embodiments, the host cell is a yeast cell that comprises ACS2 activity. In other embodiments, the host cell compared to a parent cell comprises no or reduced endogenous acetyl-CoA synthase activity under non-respiratory conditions. In some such embodiments, the host cell is a yeast cell that compared to a parent cell comprises no or reduced ACS2 activity.

5.2.4 Phophoketolase (PK) and Phosphotransacetylase (PTA)

[00104] In yeast, acetyl-CoA is biosynthesized from glucose via glycolysis, the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and pyruvate metabolism. However, in this biosynthetic pathway, CO_2 is lost during pyruvate metabolism by pyruvate carboxylase, and in the TCA cycle by pyruvate dehydrogenase and isocitrate dehydrogenase. In an industrial fermentation setting, one benefit of reducing flux through lower glycolysis is that less CO_2 is produced in converting pyruvate into acetaldehyde, and thus more carbon can be captured in the end product, thereby increasing the maximum theoretical yield. A second benefit is that less NADH is produced, and therefore significantly less oxygen is needed to reoxidize it. The loss of carbon atoms can theoretically be avoided by bypassing the TCA cycle. This can be accomplished by using phosphoketolase (PK) (enzyme classes EC 4.1.2.9, EC 4.1.2.22)in conjunction with phosphoacetyltransferase (PTA) (EC 2.3.1.8).

[00105] PK and PTA catalyze the reactions to convert fructose-6-phosphate (F6P) or xylulose-5-phosphate (X5P) to acetyl-CoA (**FIG. 7**). PK draws from the pentose phosphate intermediate xyulose 5-phosphate, or from the upper glycolysis intermediate D-fructose 6-phosphate (F6P); PK splits X5P into glyceraldehyde 3-phosphate (G3P) and acetyl phosphate, or F6P into erythrose 4-phosphate (E4P). PTA then converts the acetyl phosphate into acetyl-CoA. G3P can re-enter lower glycolysis, and E4P can re-enter the pentose phosphate pathway or glycolysis by cycling through the non-oxidative pentose phosphate pathway network of transaldolases and transketolases.

[00106] In some embodiments, the genetically modified host cell provided herein comprises a heterologous nucleotide sequence encoding a phosphoketolase. In some embodiments, the phosphoketolase is from *Leuconostoc mesenteroides* (Lee *et al.*, *Biotechnol*

Lett. 27(12);853-858 (2005). Representative phosphoketolase nucleotide sequences of *Leuconostoc mesenteroides* includes accession number AY804190, and SEQ ID NO: 11 as provided herein. Representative phosphoketolase protein sequences of *Leuconostoc mesenteroides* include accession numbers YP_819405, AAV66077.1 and SEQ ID NO: 12 as provided herein. Other useful phosphoketolases include, but are not limited to, those from *Bifidobacterium dentium* ATCC 27678 (ABIX02000002.1:2350400..2352877; EDT46356.1); *Bifidobacterium animalis* (NC_017834.1:1127580..1130057; YP_006280131.1); and *Bifidobacterium pseudolongum* (AY518216.1:988..3465; AAR98788.1).

[00107] Phosphoketolases also useful in the compositions and methods provided herein include those molecules which are said to be "derivatives" of any of the phosphoketolases described herein. Such a "derivative" has the following characteristics: (1) it shares substantial homology with any of the phosphoketolases described herein; and (2) is capable of catalyzing the conversion of X5P into glyceraldehyde 3-phosphate (G3P) and acetyl phosphate; or F6P into erythrose 4-phosphate (E4P). A derivative of a phosphoketolase is said to share "substantial homology" with the phosphoketolase if the amino acid sequences of the derivative is at least 80%, and more preferably at least 90%, and most preferably at least 95%, the same as that of the phosphoketolase.

[00108] In some embodiments, the genetically modified host cell provided herein comprises a heterologous nucleotide sequence encoding a phosphotransacetylase. In some embodiments, the phosphotransacetylase is from *Clostridium kluyveri*. Representative phosphotransacetylase nucleotide sequences of *Clostridium kluyveri* includes accession number NC_009706.1:1428554..1429555, and SEQ ID NO: 13 as provided herein. Representative phosphotransacetylase protein sequences of *Clostridium kluyveri* include accession number YP_001394780 and SEQ ID NO: 14 as provided herein. Other useful phosphotransacetylases include, but are not limited to, those from *Lactobacillus reuteri* (NC_010609.1:460303..461277; YP_001841389.10); *Bacillus subtilis* (NC_014479.1:3671865..3672836; YP_003868063.1); and *Methanosarcina thermophile* (L23147.1:207..1208; AAA72041.1).

[00109] Phosphotransacetylases also useful in the compositions and methods provided herein include those molecules which are said to be "derivatives" of any of the phosphotransacetylases described herein. Such a "derivative" has the following characteristics: (1) it shares substantial homology with any of the phosphotransacetylases described herein; and (2) is capable of catalyzing the conversion of acetyl phosphate into acetyl-CoA. A derivative of a phosphotransacetylase is said to share "substantial homology"

with the phosphotransacetylase if the amino acid sequences of the derivative is at least 80%, and more preferably at least 90%, and most preferably at least 95%, the same as that of the phosphotransacetylase.

5.2.5 MEV Pathway

[00110] In some embodiments, the host cell comprises one or more heterologous enzymes of the MEV pathway. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetoacetyl-CoA with acetyl-CoA to form HMG-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts HMG-CoA to mevalonate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate.

[00111] In some embodiments, the one or more enzymes of the MEV pathway are selected from the group consisting of acetyl-CoA thiolase, acetoacetyl-CoA synthase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase and mevalonate pyrophosphate decarboxylase. In some embodiments, with regard to the enzyme of the MEV pathway capable of catalyzing the formation of acetoacetyl-CoA, the genetically modified host cell comprises either an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA, *e.g.,* acetyl-CoA thiolase; or an enzyme that condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA, *e.g.,* acetoacetyl-CoA synthase. In some embodiments, the genetically modified host cell comprises both an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA, *e.g.,* acetyl-CoA thiolase; and an enzyme that condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA, *e.g.,* acetoacetyl-CoA synthase.

[00112] In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding more than one enzyme of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding two enzymes of the MEV pathway. In some embodiments, the host cell comprises

one or more heterologous nucleotide sequences encoding an enzyme that can convert HMG-CoA into mevalonate and an enzyme that can convert mevalonate into mevalonate 5-phosphate. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding three enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding four enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding five enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding six enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding seven enzymes of the MEV pathway. In some embodiments, the host cell comprises a plurality of heterologous nucleic acids encoding all of the enzymes of the MEV pathway.

[00113] In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding an enzyme that can convert isopentenyl pyrophosphate (IPP) into dimethylallyl pyrophosphate (DMAPP). In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding an enzyme that can condense IPP and/or DMAPP molecules to form a polyprenyl compound. In some embodiments, the genetically modified host cell further comprise a heterologous nucleic acid encoding an enzyme that can modify IPP or a polyprenyl to form an isoprenoid compound.

5.2.5.1 Conversion of Acetyl-CoA to Acetoacetyl-CoA

[00114] In some embodiments, the genetically modified host cell comprises a heterologous nucleotide sequence encoding an enzyme that can condense two molecules of acetyl-coenzyme A to form acetoacetyl-CoA, *e.g.,* an acetyl-CoA thiolase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (NC_000913 REGION: 2324131.2325315; *Escherichia coli*), (D49362; *Paracoccus denitrificans*), and (L20428; *Saccharomyces cerevisiae*).

[00115] Acetyl-CoA thiolase catalyzes the reversible condensation of two molecules of acetyl-CoA to yield acetoacetyl-CoA, but this reaction is thermodynamically unfavorable; acetoacetyl-CoA thiolysis is favored over acetoacetyl-CoA synthesis. Acetoacetyl-CoA synthase (AACS) (alternately referred to as acetyl-CoA:malonyl-CoA acyltransferase; EC 2.3.1.194) condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA. In contrast to acetyl-CoA thiolase, AACS-catalyzed acetoacetyl-CoA synthesis is essentially an energy-favored reaction, due to the associated decarboxylation of malonyl-CoA. In addition, AACS exhibits no thiolysis activity against acetoacetyl-CoA, and thus the reaction is irreversible.

[00116] In host cells comprising a heterologous ADA and acetyl-CoA thiolase, the reversible reaction catazlyzed by acetyl-CoA thiolase, which favors acetoacetyl-CoA thiolysis, may result in a large acetyl-CoA pool. In view of the reversible activity of ADA, this acetyl-CoA pool may in turn drive ADA towards the reverse reaction of converting acetyl-CoA to acetaldehyde, thereby diminishing the benefits provided by ADA towards acetyl-CoA production. Thus, in some embodiments, in order to provide a strong pull on acetyl-CoA to drive the forward reaction of ADA, the MEV pathway of the genetically modified host cell provided herein utilizes an acetoacetyl-CoA synthase to form acetoacetyl-CoA from acetyl-CoA and malonyl-CoA.

[00117] In some embodiments, the AACS is from *Streptomyces* sp. strain CL190 (Okamura *et al.*, *Proc Natl Acad Sci* USA 107(25):11265-70 (2010). Representative AACS nucleotide sequences of *Streptomyces* sp. strain CL190 include accession number AB540131.1 and SEQ ID NO:15 as provided herein. Representative AACS protein sequences of *Streptomyces* sp. strain CL190 include accession numbers D7URV0, BAJ10048 and SEQ ID NO:16 as provided herein. Other acetoacetyl-CoA synthases useful for the compositions and methods provided herein include, but are not limited to, *Streptomyces sp.* (AB183750; KO-3988 BAD86806); *S. anulatus* strain 9663 (FN178498; CAX48662); *Streptomyces sp.* KO-3988 (AB212624; BAE78983); *Actinoplanes sp.* A40644 (AB113568; BAD07381); *Streptomyces sp.* C (NZ_ACEW010000640; ZP_05511702); *Nocardiopsis dassonvillei* DSM 43111 (NZ_ABUI01000023; ZP_04335288); *Mycobacterium ulcerans* Agy99 (NC_008611; YP_907152); *Mycobacterium marinum* M (NC_010612; YP_001851502); *Streptomyces sp.* Mg1 (NZ_DS570501; ZP_05002626); *Streptomyces sp.* AA4 (NZ_ACEV01000037; ZP_05478992); *S. roseosporus* NRRL 15998 (NZ_ABYB01000295; ZP_04696763); *Streptomyces sp.* ACTE (NZ_ADFD01000030; ZP_06275834); *S. viridochromogenes* DSM 40736 (NZ_ACEZ01000031; ZP_05529691); *Frankia sp.* CcI3 (NC_007777; YP_480101); *Nocardia brasiliensis* (NC_018681; YP_006812440.1); and *Austwickia chelonae* (NZ_BAGZ01000005; ZP_10950493.1). Additional suitable acetoacetyl-CoA synthases include those described in U.S. Patent Application Publication Nos. 2010/0285549 and 2011/0281315.

[00118] Acetoacetyl-CoA synthases also useful in the compositions and methods provided herein include those molecules which are said to be "derivatives" of any of the acetoacetyl-CoA synthases described herein. Such a "derivative" has the following characteristics: (1) it shares substantial homology with any of the acetoacetyl-CoA synthases

described herein; and (2) is capable of catalyzing the irreversible condensation of acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA. A derivative of an acetoacetyl-CoA synthaseis said to share "substantial homology" with acetoacetyl-CoA synthase if the amino acid sequences of the derivative is at least 80%, and more preferably at least 90%, and most preferably at least 95%, the same as that of acetoacetyl-CoA synthase.

5.2.5.2 Conversion of Acetoacetyl-CoA to HMG-CoA

[00119] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can condense acetoacetyl-CoA with another molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), *e.g.,* a HMG-CoA synthase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (NC_001145. complement 19061.20536; *Saccharomyces cerevisiae*), (X96617; *Saccharomyces cerevisiae*), (X83882; *Arabidopsis thaliana*), (AB037907; *Kitasatospora griseola*), (BT007302; *Homo sapiens*), and (NC_002758, Locus tag SAV2546, GeneID 1122571; *Staphylococcus aureus*).

5.2.5.3 Conversion of HMG-CoA to Mevalonate

[00120] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert HMG-CoA into mevalonate, *e.g.,* a HMG-CoA reductase. In some embodiments, HMG-CoA reductase is an NADH-using hydroxymethylglutaryl-CoA reductase-CoA reductase. HMG-CoA reductases (EC 1.1.1.34; EC 1.1.1.88) catalyze the reductive deacylation of (S)-HMG-CoA to (R)-mevalonate, and can be categorized into two classes, class I and class II HMGrs. Class I includes the enzymes from eukaryotes and most archaea, and class II includes the HMG-CoA reductases of certain prokaryotes and archaea. In addition to the divergence in the sequences, the enzymes of the two classes also differ with regard to their cofactor specificity. Unlike the class I enzymes, which utilize NADPH exclusively, the class II HMG-CoA reductases vary in the ability to discriminate between NADPH and NADH. *See, e.g.,* Hedl *et al., Journal of Bacteriology* 186 (7): 1927-1932 (2004). Co-factor specificities for select class II HMG-CoA reductases are provided below.

[00121] **Table 1. Co-factor specificities for select class II HMG-CoA reductases**

Source	Coenzyme specificity	K_m^{NADPH} (μM)	K_m^{NADH} (μM)
P. mevalonii	NADH		80
A. fulgidus	NAD(P)H	500	160

S. aureus	NAD(P)H	70	100
E. faecalis	NADPH	30	

[00122] Useful HMG-CoA reductases for the compositions and methods provided herein include HMG-CoA reductases that are capable of utilizing NADH as a cofactor, *e.g.,* HMG-CoA reductase from *P. mevalonii*, *A. fulgidus* or *S. aureus*. In particular embodiments, the HMG-CoA reductase is capable of only utilizing NADH as a cofactor, *e.g.,* HMG-CoA reductase from *P. mevalonii* , *S. pomeroyi* or *D. acidovorans*.

[00123] In some embodiments, the NADH-using HMG-CoA reductase is from *Pseudomonas mevalonii*. The sequence of the wild-type *mvaA* gene of *Pseudomonas mevalonii*, which encodes HMG-CoA reductase (E.C. 1.1.1.88), has been previously described. *See* Beach and Rodwell, *J. Bacteriol.* 171:2994-3001 (1989). Representative *mvaA* nucleotide sequences of *Pseudomonas mevalonii* include accession number M24015, and SEQ ID NO: 17 as provided herein. Representative HMG-CoA reductase protein sequences of *Pseudomonas mevalonii* include accession numbers AAA25837, P13702, MVAA_PSEMV and SEQ ID NO: 18 as provided herein.

[00124] In some embodiments, the NADH-using HMG-CoA reductase is from *Silicibacter pomeroyi*. Representative *HMG-CoA reductase* nucleotide sequences of *Silicibacter pomeroyi* include accession number NC_006569.1, and SEQ ID NO: 19 as provided herein. Representative HMG-CoA reductase protein sequences of *Silicibacter pomeroyi* include accession number YP_164994 and SEQ ID NO: 20 as provided herein.

[00125] In some embodiments, the NADH-using HMG-CoA reductase is from *Delftia acidovorans*. A representative *HMG-CoA reductase* nucleotide sequences of *Delftia acidovorans* includes NC_010002 REGION: complement(319980..321269), and SEQ ID NO: 21 as provided herein. Representative HMG-CoA reductase protein sequences of *Delftia acidovorans* include accession number YP_001561318 and SEQ ID NO: 22 as provided herein.

[00126] In some embodiments, the NADH-using HMG-CoA reductases is from *Solanum tuberosum* (Crane *et al.*, *J. Plant Physiol.* 159:1301-1307 (2002)).

[00127] NADH-using HMG-CoA reductases also useful in the compositions and methods provided herein include those molecules which are said to be "derivatives" of any of the NADH-using HMG-CoA reductases described herein, *e.g.,* from *P. mevalonii*, *S. pomeroyi* and *D. acidovorans*. Such a “derivative” has the following characteristics: (1) it

shares substantial homology with any of the NADH-using HMG-CoA reductases described herein; and (2) is capable of catalyzing the reductive deacylation of (S)-HMG-CoA to (R)-mevalonate while preferentially using NADH as a cofactor. A derivative of an NADH-using HMG-CoA reductase is said to share "substantial homology" with NADH-using HMG-CoA reductase if the amino acid sequences of the derivative is at least 80%, and more preferably at least 90%, and most preferably at least 95%, the same as that of NADH-using HMG-CoA reductase.

[00128] As used herein, the phrase "NADH-using" means that the NADH-using HMG-CoA reductase is selective for NADH over NADPH as a cofactor, for example, by demonstrating a higher specific activity for NADH than for NADPH. In some embodiments, selectivity for NADH as a cofactor is expressed as a $k_{cat}^{(NADH)}/ k_{cat}^{(NADPH)}$ ratio. In some embodiments, the NADH-using HMG-CoA reductase has a $k_{cat}^{(NADH)}/ k_{cat}^{(NADPH)}$ ratio of at least 5, 10, 15, 20, 25 or greater than 25. In some embodiments, the NADH-using HMG-CoA reductase uses NADH exclusively. For example, an NADH-using HMG-CoA reductase that uses NADH exclusively displays some activity with NADH supplied as the sole cofactor *in vitro* (*see, e.g.,* Example 1 and Section 6.1.1.3 below), and displays no detectable activity when NADPH is supplied as the sole cofactor. Any method for determining cofactor specificity known in the art can be utilized to identify HMG-CoA reductases having a preference for NADH as cofactor, including those described by Kim *et al., Protein Science* 9:1226-1234 (2000); and Wilding *et al., J. Bacteriol.* 182(18):5147-52 (2000).

[00129] In some embodiments, the NADH-using HMG-CoA reductase is engineered to be selective for NADH over NAPDH, for example, through site-directed mutagenesis of the cofactor-binding pocket. Methods for engineering NADH-selectivity are described in Watanabe *et al., Microbiology* 153:3044-3054 (2007), and methods for determining the cofactor specificity of HMG-CoA reductases are described in Kim *et al., Protein Sci.* 9:1226-1234 (2000).

[00130] In some embodiments, the NADH-using HMG-CoA reductase is derived from a host species that natively comprises a mevalonate degradative pathway, for example, a host species that catabolizes mevalonate as its sole carbon source. Within these embodiments, the NADH-using HMG-CoA reductase, which normally catalyzes the oxidative acylation of internalized (R)-mevalonate to (S)-HMG-CoA within its native host cell, is utilized to catalyze the reverse reaction, that is, the reductive deacylation of (S)-HMG-CoA to (R)-mevalonate, in a genetically modified host cell comprising a mevalonate biosynthetic

pathway. Prokaryotes capable of growth on mevalonate as their sole carbon source have been described by: Anderson *et al., J. Bacteriol,* 171(12):6468-6472 (1989); Beach *et al., J. Bacteriol.* 171:2994-3001 (1989); Bensch *et al., J. Biol. Chem.* 245:3755-3762; Fimongnari *et al., Biochemistry* 4:2086-2090 (1965); Siddiqi *et al., Biochem. Biophys. Res. Commun.* 8:110-113 (1962); Siddiqi *et al., J. Bacteriol.* 93:207-214 (1967); and Takatsuji *et al., Biochem. Biophys. Res. Commun.*110:187-193 (1983).

[00131] In some embodiments of the compositions and methods provided herein, the host cell comprises both a NADH-using HMGr and an NADPH-using HMG-CoA reductase. Illustrative examples of nucleotide sequences encoding an NADPH-using HMG-CoA reductase include, but are not limited to: (NM_206548; *Drosophila melanogaster*), (NC_002758, Locus tag SAV2545, GeneID 1122570; *Staphylococcus aureus*), (AB015627; *Streptomyces sp.* KO 3988), (AX128213, providing the sequence encoding a truncated HMG-CoA reductase; *Saccharomyces cerevisiae*), and (NC_001145: complement (115734.118898; *Saccharomyces cerevisiae*).

5.2.5.4 Conversion of Mevalonate to Mevalonate-5-Phosphate

[00132] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert mevalonate into mevalonate 5-phosphate, *e.g.,* a mevalonate kinase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (L77688; *Arabidopsis thaliana*), and (X55875; *Saccharomyces cerevisiae*).

5.2.5.5 Conversion of Mevalonate-5-Phosphate to Mevalonate-5-Pyrophosphate

[00133] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert mevalonate 5-phosphate into mevalonate 5-pyrophosphate, *e.g.,* a phosphomevalonate kinase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (AF429385; *Hevea brasiliensis*), (NM_006556; *Homo sapiens*), and (NC_001145. complement 712315.713670; *Saccharomyces cerevisiae*).

5.2.5.6 Conversion of Mevalonate-5-Pyrophosphate to IPP

[00134] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert mevalonate 5-pyrophosphate into isopentenyl diphosphate (IPP), *e.g.,* a mevalonate pyrophosphate decarboxylase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (X97557;

Saccharomyces cerevisiae), (AF290095; *Enterococcus faecium*), and (U49260; *Homo sapiens*).

5.2.5.7 Conversion of IPP to DMAPP

[00135] In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding an enzyme that can convert IPP generated via the MEV pathway into dimethylallyl pyrophopsphate (DMAPP), *e.g.,* an IPP isomerase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (NC_000913, 3031087.3031635; *Escherichia coli*), and (AF082326; *Haematococcus pluvialis*).

5.2.5.8 Polyprenyl Synthases

[00136] In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding a polyprenyl synthase that can condense IPP and/or DMAPP molecules to form polyprenyl compounds containing more than five carbons.

[00137] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can condense one molecule of IPP with one molecule of DMAPP to form one molecule of geranyl pyrophosphate ("GPP"), *e.g.,* a GPP synthase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (AF513111; *Abies grandis*), (AF513112; *Abies grandis*), (AF513113; *Abies grandis*), (AY534686; *Antirrhinum majus*), (AY534687; *Antirrhinum majus*), (Y17376; *Arabidopsis thaliana*), (AE016877, Locus AP11092; *Bacillus cereus*; ATCC 14579), (AJ243739; *Citrus sinensis*), (AY534745; *Clarkia breweri*), (AY953508; *Ips pini*), (DQ286930; *Lycopersicon esculentum*), (AF182828; *Mentha x piperita*), (AF182827; *Mentha x piperita*), (MPI249453; Mentha x piperita), (PZE431697, Locus CAD24425; *Paracoccus zeaxanthinifaciens*), (AY866498; *Picrorhiza kurrooa*), (AY351862; *Vitis vinifera*), and (AF203881, Locus AAF12843; *Zymomonas mobilis*).

[00138] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can condense two molecules of IPP with one molecule of DMAPP, or add a molecule of IPP to a molecule of GPP, to form a molecule of farnesyl pyrophosphate ("FPP"), *e.g.,* a FPP synthase. Illustrative examples of nucleotide sequences that encode such an enzyme include, but are not limited to: (ATU80605; *Arabidopsis thaliana*), (ATHFPS2R; *Arabidopsis thaliana*), (AAU36376; *Artemisia annua*), (AF461050; *Bos taurus*), (D00694; *Escherichia coli* K-12), (AE009951, Locus AAL95523; *Fusobacterium nucleatum subsp. nucleatum* ATCC 25586), (GFFPPSGEN; *Gibberella fujikuroi*), (CP000009, Locus AAW60034; *Gluconobacter oxydans* 621H), (AF019892;

Helianthus annuus), (HUMFAPS; *Homo sapiens*), (KLPFPSQCR; *Kluyveromyces lactis*), (LAU15777; *Lupinus albus*), (LAU20771; *Lupinus albus*), (AF309508; *Mus musculus*), (NCFPPSGEN; *Neurospora crassa*), (PAFPS1; *Parthenium argentatum*), (PAFPS2; *Parthenium argentatum*), (RATFAPS; *Rattus norvegicus*), (YSCFPP; *Saccharomyces cerevisiae*), (D89104; *Schizosaccharomyces pombe*), (CP000003, Locus AAT87386; *Streptococcus pyogenes*), (CP000017, Locus AAZ51849; *Streptococcus pyogenes*), (NC_008022, Locus YP_598856; *Streptococcus pyogenes* MGAS10270), (NC_008023, Locus YP_600845; *Streptococcus pyogenes* MGAS2096), (NC_008024, Locus YP_602832; *Streptococcus pyogenes* MGAS10750), (MZEFPS; *Zea mays*), (AE000657, Locus AAC06913; *Aquifex aeolicus* VF5), (NM_202836; *Arabidopsis thaliana*), (D84432, Locus BAA12575; *Bacillus subtilis*), (U12678, Locus AAC28894; *Bradyrhizobium japonicum* USDA 110), (BACFDPS; *Geobacillus stearothermophilus*), (NC_002940, Locus NP_873754; *Haemophilus ducreyi* 35000HP), (L42023, Locus AAC23087; *Haemophilus influenzae* Rd KW20), (J05262; *Homo sapiens*), (YP_395294; *Lactobacillus sakei subsp. sakei* 23K), (NC_005823, Locus YP_000273; *Leptospira interrogans serovar Copenhageni str. Fiocruz* L1-130), (AB003187; *Micrococcus luteus*), (NC_002946, Locus YP_208768; *Neisseria gonorrhoeae* FA 1090), (U00090, Locus AAB91752; *Rhizobium sp.* NGR234), (J05091; *Saccharomyces cerevisae*), (CP000031, Locus AAV93568; *Silicibacter pomeroyi* DSS-3), (AE008481, Locus AAK99890; *Streptococcus pneumoniae* R6), and (NC_004556, Locus NP 779706; *Xylella fastidiosa* Temecula1).

[00139] In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding an enzyme that can combine IPP and DMAPP or IPP and FPP to form geranylgeranyl pyrophosphate ("GGPP"). Illustrative examples of nucleotide sequences that encode such an enzyme include, but are not limited to: (ATHGERPYRS; *Arabidopsis thaliana*), (BT005328; *Arabidopsis thaliana)*, (NM_119845; *Arabidopsis thaliana*), (NZ_AAJM01000380, Locus ZP_00743052; *Bacillus thuringiensis serovar israelensis,* ATCC 35646 sq1563), (CRGGPPS; *Catharanthus roseus*), (NZ_AABF02000074, Locus ZP_00144509; *Fusobacterium nucleatum subsp. vincentii,* ATCC 49256), (GFGGPPSGN; *Gibberella fujikuroi*), (AY371321; *Ginkgo biloba*), (AB055496; *Hevea brasiliensis*), (AB017971; *Homo sapiens*), (MCI276129; *Mucor circinelloides f. lusitanicus*), (AB016044; *Mus musculus*), (AABX01000298, Locus NCU01427; *Neurospora crassa*), (NCU20940; *Neurospora crassa*), (NZ_AAKL01000008, Locus ZP_00943566; *Ralstonia solanacearum* UW551), (AB118238; *Rattus norvegicus*), (SCU31632; *Saccharomyces cerevisiae*), (AB016095; Synechococcus elongates), (SAGGPS;

Sinapis alba), (SSOGDS; *Sulfolobus acidocaldarius*), (NC_007759, Locus YP_461832; *Syntrophus aciditrophicus* SB), (NC_006840, Locus YP_204095; *Vibrio fischeri* ES114), (NM_112315; *Arabidopsis thaliana*), (ERWCRTE; *Pantoea agglomerans*), (D90087, Locus BAA14124; *Pantoea ananatis*), (X52291, Locus CAA36538; *Rhodobacter capsulatus*), (AF195122, Locus AAF24294; *Rhodobacter sphaeroides*), and (NC_004350, Locus NP_721015; *Streptococcus mutans* UA159).

5.2.5.9 Terpene Synthases

[00140] In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding an enzyme that can modify a polyprenyl to form a hemiterpene, a monoterpene, a sesquiterpene, a diterpene, a triterpene, a tetraterpene, a polyterpene, a steroid compound, a carotenoid, or a modified isoprenoid compound.

[00141] In some embodiments, the heterologous nucleotide encodes a carene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (AF461460, REGION 43.1926; *Picea abies*) and (AF527416, REGION: 78.1871; *Salvia stenophylla*).

[00142] In some embodiments, the heterologous nucleotide encodes a geraniol synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (AJ457070; *Cinnamomum tenuipilum*), (AY362553; *Ocimum basilicum*), (DQ234300; *Perilla frutescens* strain 1864), (DQ234299; *Perilla citriodora* strain 1861), (DQ234298; *Perilla citriodora* strain 4935), and (DQ088667; *Perilla citriodora*).

[00143] In some embodiments, the heterologous nucleotide encodes a linalool synthase. Illustrative examples of a suitable nucleotide sequence include, but are not limited to: (AF497485; *Arabidopsis thaliana*), (AC002294, Locus AAB71482; *Arabidopsis thaliana*), (AY059757; *Arabidopsis thaliana*), (NM_104793; *Arabidopsis thaliana*), (AF154124; *Artemisia annua*), (AF067603; *Clarkia breweri*), (AF067602; *Clarkia concinna*), (AF067601; *Clarkia breweri*), (U58314; *Clarkia breweri*), (AY840091; *Lycopersicon esculentum*), (DQ263741; *Lavandula angustifolia*), (AY083653; *Mentha citrate*), (AY693647; *Ocimum basilicum*), (XM_463918; *Oryza sativa*), (AP004078, Locus BAD07605; *Oryza sativa*), (XM_463918, Locus XP_463918; *Oryza sativa*), (AY917193; *Perilla citriodora*), (AF271259; *Perilla frutescens*), (AY473623; *Picea abies*), (DQ195274; *Picea sitchensis*), and (AF444798; *Perilla frutescens* var. crispa cultivar No. 79).

[00144] In some embodiments, the heterologous nucleotide encodes a limonene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (+)-limonene synthases (AF514287, REGION: 47.1867; *Citrus limon*) and (AY055214,

REGION: 48.1889; *Agastache rugosa*) and (-)-limonene synthases (DQ195275, REGION: 1.1905; *Picea sitchensis*), (AF006193, REGION: 73.1986; *Abies grandis*), and (MHC4SLSP, REGION: 29.1828; *Mentha spicata*).

[00145] In some embodiments, the heterologous nucleotide encodes a myrcene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (U87908; *Abies grandis*), (AY195609; *Antirrhinum majus*), (AY195608; *Antirrhinum majus*), (NM_127982; *Arabidopsis thaliana* TPS10), (NM_113485; *Arabidopsis thaliana* ATTPS-CIN), (NM_113483; *Arabidopsis thaliana* ATTPS-CIN), (AF271259; *Perilla frutescens*), (AY473626; *Picea abies*), (AF369919; *Picea abies*), and (AJ304839; *Quercus ilex*).

[00146] In some embodiments, the heterologous nucleotide encodes a ocimene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (AY195607; *Antirrhinum majus*), (AY195609; *Antirrhinum majus*), (AY195608; *Antirrhinum majus*), (AK221024; *Arabidopsis thaliana*), (NM_113485; *Arabidopsis thaliana* ATTPS-CIN), (NM_113483; *Arabidopsis thaliana* ATTPS-CIN), (NM_117775; *Arabidopsis thaliana* ATTPS03), (NM_001036574; *Arabidopsis thaliana* ATTPS03), (NM_127982; *Arabidopsis thaliana* TPS10), (AB110642; *Citrus unshiu* CitMTSL4), and (AY575970; *Lotus corniculatus var. japonicus*).

[00147] In some embodiments, the heterologous nucleotide encodes an α-pinene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (+) α-pinene synthase (AF543530, REGION: 1.1887; *Pinus taeda*), (-)α-pinene synthase (AF543527, REGION: 32.1921; *Pinus taeda*), and (+)/(-)α-pinene synthase (AGU87909, REGION: 6111892; *Abies grandis*).

[00148] In some embodiments, the heterologous nucleotide encodes a β-pinene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (-) β-pinene synthases (AF276072, REGION: 1.1749; *Artemisia annua*) and (AF514288, REGION: 26.1834; *Citrus limon*).

[00149] In some embodiments, the heterologous nucleotide encodes a sabinene synthase. An illustrative example of a suitable nucleotide sequence includes but is not limited to AF051901, REGION: 26.1798 from *Salvia officinalis*.

[00150] In some embodiments, the heterologous nucleotide encodes a γ-terpinene synthase. Illustrative examples of suitable nucleotide sequences include: (AF514286, REGION: 30.1832 from *Citrus limon*) and (AB110640, REGION 1.1803 from *Citrus unshiu*).

[00151] In some embodiments, the heterologous nucleotide encodes a terpinolene synthase. Illustrative examples of a suitable nucleotide sequence include, but are not limited to: (AY693650 from *Oscimum basilicum*) and (AY906866, REGION: 10.1887 from *Pseudotsuga menziesii*).

[00152] In some embodiments, the heterologous nucleotide encodes an amorphadiene synthase. An illustrative example of a suitable nucleotide sequence is SEQ ID NO. 37 of U.S. Patent Publication No. 2004/0005678.

[00153] In some embodiments, the heterologous nucleotide encodes a α-farnesene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to DQ309034 from *Pyrus communis cultivar d'Anjou* (pear; gene name AFS1) and AY182241 from *Malus domestica* (apple; gene AFS1). Pechouus *et al.*, *Planta* 219(1):84-94 (2004).

[00154] In some embodiments, the heterologous nucleotide encodes a β-farnesene synthase. Illustrative examples of suitable nucleotide sequences include but is not limited to accession number AF024615 from *Mentha x piperita* (peppermint; gene Tspa11), and AY835398 from *Artemisia annua*. Picaud *et al.*, *Phytochemistry* 66(9): 961-967 (2005).

[00155] In some embodiments, the heterologous nucleotide encodes a farnesol synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to accession number AF529266 from *Zea mays* and YDR481C from *Saccharomyces cerevisiae* (gene Pho8). Song, L., *Applied Biochemistry and Biotechnology* 128:149-158 (2006).

[00156] In some embodiments, the heterologous nucleotide encodes a nerolidol synthase. An illustrative example of a suitable nucleotide sequence includes, but is not limited to AF529266 from *Zea mays* (maize; gene tps1).

[00157] In some embodiments, the heterologous nucleotide encodes a patchouliol synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to AY508730 REGION: 1.1659 from *Pogostemon cablin*.

[00158] In some embodiments, the heterologous nucleotide encodes a nootkatone synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to AF441124 REGION: 1.1647 from *Citrus sinensis* and AY917195 REGION: 1.1653 from *Perilla frutescens*.

[00159] In some embodiments, the heterologous nucleotide encodes an abietadiene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (U50768; *Abies grandis*) and (AY473621; *Picea abies*).

[00160] In some embodiments, the host cell produces a C_5 isoprenoid. These compounds are derived from one isoprene unit and are also called hemiterpenes. An illustrative example of a hemiterpene is isoprene. In other embodiments, the isoprenoid is a C_{10} isoprenoid. These compounds are derived from two isoprene units and are also called monoterpenes. Illustrative examples of monoterpenes are limonene, citranellol, geraniol, menthol, perillyl alcohol, linalool, thujone, and myrcene. In other embodiments, the isoprenoid is a C_{15} isoprenoid. These compounds are derived from three isoprene units and are also called sesquiterpenes. Illustrative examples of sesquiterpenes are periplanone B, gingkolide B, amorphadiene, artemisinin, artemisinic acid, valencene, nootkatone, epi-cedrol, epi-aristolochene, farnesol, gossypol, sanonin, periplanone, forskolin, and patchoulol (which is also known as patchouli alcohol). In other embodiments, the isoprenoid is a C_{20} isoprenoid. These compounds are derived from four isoprene units and also called diterpenes. Illustrative examples of diterpenes are casbene, eleutherobin, paclitaxel, prostratin, pseudopterosin, and taxadiene. In yet other examples, the isoprenoid is a C_{20+} isoprenoid. These compounds are derived from more than four isoprene units and include: triterpenes (C_{30} isoprenoid compounds derived from 6 isoprene units) such as arbrusideE, bruceantin, testosterone, progesterone, cortisone, digitoxin, and squalene; tetraterpenes (C_{40} isoprenoid compounds derived from 8 isoprenoids) such as β-carotene; and polyterpenes (C_{40+} isoprenoid compounds derived from more than 8 isoprene units) such as polyisoprene. In some embodiments, the isoprenoid is selected from the group consisting of abietadiene, amorphadiene, carene, α-farnesene, β-farnesene, farnesol, geraniol, geranylgeraniol, isoprene, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, β-pinene, sabinene, γ-terpinene, terpinolene and valencene. Isoprenoid compounds also include, but are not limited to, carotenoids (such as lycopene, α- and β-carotene, α- and β-cryptoxanthin, bixin, zeaxanthin, astaxanthin, and lutein), steroid compounds, and compounds that are composed of isoprenoids modified by other chemical groups, such as mixed terpene-alkaloids, and coenzyme Q-10.

5.3 Methods of Making Genetically Modified Cells

[00161] Also provided herein are methods for producing a host cell that is genetically engineered to comprise one or more of the modifications described above, *e.g.,* one or more nucleic heterologous nucleic acids encoding one or more enzymes selected from ADA, NADH-using HMG-CoA reductase, AACS, PK, PTA, and other mevalonate pathway enzymes. Expression of a heterologous enzyme in a host cell can be accomplished by introducing into the host cells a nucleic acid comprising a nucleotide sequence encoding the

enzyme under the control of regulatory elements that permit expression in the host cell. In some embodiments, the nucleic acid is an extrachromosomal plasmid. In other embodiments, the nucleic acid is a chromosomal integration vector that can integrate the nucleotide sequence into the chromosome of the host cell.

[00162] Nucleic acids encoding these proteins can be introduced into the host cell by any method known to one of skill in the art without limitation (*see*, for example, Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci.* USA 75:1292-3; Cregg *et al.* (1985) *Mol. Cell. Biol.* 5:3376-3385; Goeddel *et al.* eds, 1990, Methods in Enzymology, vol. 185, Academic Press, Inc. , CA; Krieger, 1990, Gene Transfer and Expression -- A Laboratory Manual, Stockton Press, NY; Sambrook *et al.* , 1989, Molecular Cloning -- A Laboratory Manual, Cold Spring Harbor Laboratory, NY; and Ausubel *et al.* , eds. , Current Edition, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, NY). Exemplary techniques include, but are not limited to, spheroplasting, electroporation, PEG 1000 mediated transformation, and lithium acetate or lithium chloride mediated transformation.

[00163] The copy number of an enzyme in a host cell may be altered by modifying the transcription of the gene that encodes the enzyme. This can be achieved for example by modifying the copy number of the nucleotide sequence encoding the enzyme (*e.g.*, by using a higher or lower copy number expression vector comprising the nucleotide sequence, or by introducing additional copies of the nucleotide sequence into the genome of the host cell or by deleting or disrupting the nucleotide sequence in the genome of the host cell), by changing the order of coding sequences on a polycistronic mRNA of an operon or breaking up an operon into individual genes each with its own control elements, or by increasing the strength of the promoter or operator to which the nucleotide sequence is operably linked. Alternatively or in addition, the copy number of an enzyme in a host cell may be altered by modifying the level of translation of an mRNA that encodes the enzyme. This can be achieved for example by modifying the stability of the mRNA, modifying the sequence of the ribosome binding site, modifying the distance or sequence between the ribosome binding site and the start codon of the enzyme coding sequence, modifying the entire intercistronic region located "upstream of" or adjacent to the 5' side of the start codon of the enzyme coding region, stabilizing the 3'-end of the mRNA transcript using hairpins and specialized sequences, modifying the codon usage of enzyme, altering expression of rare codon tRNAs used in the biosynthesis of the enzyme, and/or increasing the stability of the enzyme, as, for example, via mutation of its coding sequence.

[00164] The activity of an enzyme in a host cell can be altered in a number of ways, including, but not limited to, expressing a modified form of the enzyme that exhibits increased or decreased solubility in the host cell, expressing an altered form of the enzyme that lacks a domain through which the activity of the enzyme is inhibited, expressing a modified form of the enzyme that has a higher or lower Kcat or a lower or higher Km for the substrate, or expressing an altered form of the enzyme that is more or less affected by feed-back or feed-forward regulation by another molecule in the pathway.

[00165] In some embodiments, a nucleic acid used to genetically modify a host cell comprises one or more selectable markers useful for the selection of transformed host cells and for placing selective pressure on the host cell to maintain the foreign DNA.

[00166] In some embodiments, the selectable marker is an antibiotic resistance marker. Illustrative examples of antibiotic resistance markers include, but are not limited to, the *BLA, NAT1, PAT, AUR1-C, PDR4, SMR1, CAT*, mouse dhfr, *HPH, DSDA,* KAN^R, and *SH BLE* gene products. The *BLA* gene product from *E. coli* confers resistance to beta-lactam antibiotics (*e.g.* , narrow-spectrum cephalosporins, cephamycins, and carbapenems (ertapenem), cefamandole, and cefoperazone) and to all the anti-gram-negative-bacterium penicillins except temocillin; the *NAT1* gene product from *S. noursei* confers resistance to nourseothricin; the *PAT* gene product from *S. viridochromogenes* Tu94 confers resistance to bialophos; the *AUR1-C* gene product from *Saccharomyces cerevisiae* confers resistance to Auerobasidin A (AbA); the PDR4 gene product confers resistance to cerulenin; the SMR1 gene product confers resistance to sulfometuron methyl; the CAT gene product from Tn9 transposon confers resistance to chloramphenicol; the mouse dhfr gene product confers resistance to methotrexate; the *HPH* gene product of *Klebsiella pneumonia* confers resistance to Hygromycin B; the *DSDA* gene product of *E. coli* allows cells to grow on plates with D-serine as the sole nitrogen source; the KAN^R gene of the Tn903 transposon confers resistance to G418; and the *SH BLE* gene product from *Streptoalloteichus hindustanus* confers resistance to Zeocin (bleomycin). In some embodiments, the antibiotic resistance marker is deleted after the genetically modified host cell disclosed herein is isolated.

[00167] In some embodiments, the selectable marker rescues an auxotrophy (*e.g.,* a nutritional auxotrophy) in the genetically modified microorganism. In such embodiments, a parent microorganism comprises a functional disruption in one or more gene products that function in an amino acid or nucleotide biosynthetic pathway and that when non-functional renders a parent cell incapable of growing in media without supplementation with one or more nutrients. Such gene products include, but are not limited to, the *HIS3, LEU2, LYS1,*

LYS2, MET15, TRP1, ADE2, and *URA3* gene products in yeast. The auxotrophic phenotype can then be rescued by transforming the parent cell with an expression vector or chromosomal integration construct encoding a functional copy of the disrupted gene product, and the genetically modified host cell generated can be selected for based on the loss of the auxotrophic phenotype of the parent cell. Utilization of the *URA3, TRP1*, and *LYS2* genes as selectable markers has a marked advantage because both positive and negative selections are possible. Positive selection is carried out by auxotrophic complementation of the *URA3, TRP1*, and *LYS2* mutations, whereas negative selection is based on specific inhibitors, *i.e.,* 5-fluoro-orotic acid (FOA), 5-fluoroanthranilic acid, and aminoadipic acid (aAA), respectively, that prevent growth of the prototrophic strains but allows growth of the *URA3, TRP1*, and *LYS2* mutants, respectively. In other embodiments, the selectable marker rescues other non-lethal deficiencies or phenotypes that can be identified by a known selection method.

[00168] Described herein are specific genes and proteins useful in the methods, compositions and organisms of the disclosure; however it will be recognized that absolute identity to such genes is not necessary. For example, changes in a particular gene or polynucleotide comprising a sequence encoding a polypeptide or enzyme can be performed and screened for activity. Typically such changes comprise conservative mutations and silent mutations. Such modified or mutated polynucleotides and polypeptides can be screened for expression of a functional enzyme using methods known in the art.

[00169] Due to the inherent degeneracy of the genetic code, other polynucleotides which encode substantially the same or functionally equivalent polypeptides can also be used to clone and express the polynucleotides encoding such enzymes.

[00170] As will be understood by those of skill in the art, it can be advantageous to modify a coding sequence to enhance its expression in a particular host. The genetic code is redundant with 64 possible codons, but most organisms typically use a subset of these codons. The codons that are utilized most often in a species are called optimal codons, and those not utilized very often are classified as rare or low-usage codons. Codons can be substituted to reflect the preferred codon usage of the host, in a process sometimes called "codon optimization" or "controlling for species codon bias."

[00171] Optimized coding sequences containing codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al.*, 1989, *Nucl Acids Res.* 17: 477-508) can be prepared, for example, to increase the rate of translation or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, as compared with transcripts produced from a non-optimized sequence. Translation stop codons can also be modified to

reflect host preference. For example, typical stop codons for *S. cerevisiae* and mammals are UAA and UGA, respectively. The typical stop codon for monocotyledonous plants is UGA, whereas insects and E. coli commonly use UAA as the stop codon (Dalphin *et al.*, 1996, *Nucl Acids Res.* 24: 216-8).

[00172] Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA molecules differing in their nucleotide sequences can be used to encode a given enzyme of the disclosure. The native DNA sequence encoding the biosynthetic enzymes described above are referenced herein merely to illustrate an embodiment of the disclosure, and the disclosure includes DNA molecules of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the disclosure. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The disclosure includes such polypeptides with different amino acid sequences than the specific proteins described herein so long as the modified or variant polypeptides have the enzymatic anabolic or catabolic activity of the reference polypeptide. Furthermore, the amino acid sequences encoded by the DNA sequences shown herein merely illustrate embodiments of the disclosure.

[00173] In addition, homologs of enzymes useful for the compositions and methods provided herein are encompassed by the disclosure. In some embodiments, two proteins (or a region of the proteins) are substantially homologous when the amino acid sequences have at least about 30%, 40%, 50% 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity. To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In one embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, typically at least 40%, more typically at least 50%, even more typically at least 60%, and even more typically at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a

function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[00174] When "homologous" is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of homology may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art (See, e.g., Pearson W. R., 1994, *Methods in Mol Biol* 25: 365-89).

[00175] The following six groups each contain amino acids that are conservative substitutions for one another: 1) Serine (S), Threonine (T); 2) Aspartic Acid (D), Glutamic Acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Alanine (A), Valine (V), and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[00176] Sequence homology for polypeptides, which is also referred to as percent sequence identity, is typically measured using sequence analysis software. A typical algorithm used comparing a molecule sequence to a database containing a large number of sequences from different organisms is the computer program BLAST. When searching a database containing sequences from a large number of different organisms, it is typical to compare amino acid sequences.

[00177] Furthermore, any of the genes encoding the foregoing enzymes (or any others mentioned herein (or any of the regulatory elements that control or modulate expression thereof)) may be optimized by genetic/protein engineering techniques, such as directed evolution or rational mutagenesis, which are known to those of ordinary skill in the art. Such action allows those of ordinary skill in the art to optimize the enzymes for expression and activity in yeast.

[00178] In addition, genes encoding these enzymes can be identified from other fungal and bacterial species and can be expressed for the modulation of this pathway. A variety of organisms could serve as sources for these enzymes, including, but not limited to,

Saccharomyces spp., including *S. cerevisiae* and *S. uvarum*, *Kluyveromyces spp.*, including *K. thermotolerans*, *K. lactis*, and *K. marxianus*, *Pichia spp.*, *Hansenula spp.*, including *H. polymorpha*, *Candida spp.*, *Trichosporon spp.*, *Yamadazyma spp.*, including *Y. spp. stipitis*, *Torulaspora pretoriensis*, *Issatchenkia orientalis*, *Schizosaccharomyces spp.*, including *S. pombe, Cryptococcus spp., Aspergillus spp., Neurospora spp.*, or *Ustilago spp.* Sources of genes from anaerobic fungi include, but are not limited to, *Piromyces spp.*, *Orpinomyces spp.*, or *Neocallimastix spp.* Sources of prokaryotic enzymes that are useful include, but are not limited to, *Escherichia. coli*, *Zymomonas mobilis*, *Staphylococcus aureus*, *Bacillus spp.*, *Clostridium spp.*, *Corynebacterium spp.*, *Pseudomonas spp.*, *Lactococcus spp.*, *Enterobacter spp.*, and *Salmonella spp.*

[00179] Techniques known to those skilled in the art may be suitable to identify additional homologous genes and homologous enzymes. Generally, analogous genes and/or analogous enzymes can be identified by functional analysis and will have functional similarities. Techniques known to those skilled in the art may be suitable to identify analogous genes and analogous enzymes. For example, to identify homologous or analogous ADA genes, proteins, or enzymes, techniques may include, but are not limited to, cloning a gene by PCR using primers based on a published sequence of an ADA gene/enzyme or by degenerate PCR using degenerate primers designed to amplify a conserved region among ADA genes. Further, one skilled in the art can use techniques to identify homologous or analogous genes, proteins, or enzymes with functional homology or similarity. Techniques include examining a cell or cell culture for the catalytic activity of an enzyme through *in vitro* enzyme assays for said activity (*e.g.* as described herein or in Kiritani, K., *Branched-Chain Amino Acids Methods Enzymology*, 1970), then isolating the enzyme with said activity through purification, determining the protein sequence of the enzyme through techniques such as Edman degradation, design of PCR primers to the likely nucleic acid sequence, amplification of said DNA sequence through PCR, and cloning of said nucleic acid sequence. To identify homologous or similar genes and/or homologous or similar enzymes, analogous genes and/or analogous enzymes or proteins, techniques also include comparison of data concerning a candidate gene or enzyme with databases such as BRENDA, KEGG, or MetaCYC. The candidate gene or enzyme may be identified within the above mentioned databases in accordance with the teachings herein.

5.4 Methods of Producing Isoprenoids

[00180] In another aspect, provided herein is a method for the production of an isoprenoid, the method comprising the steps of: (a) culturing a population of any of the

genetically modified host cells described herein in a medium with a carbon source under conditions suitable for making an isoprenoid compound; and (b) recovering said isoprenoid compound from the medium.

[00181] In some embodiments, the genetically modified host cell comprises one or more modifications selected from the group consisting of: heterologous expression of an ADA, heterologous expression of an NADH-using HMG-CoA reductase, heterologous expression of an AACS, heterologous expression of a phosphoketolase, heterologous expression of a phosphotrancacetylase, and heterologous expression of one or more enzymes of the mevalonate pathway; and the genetically modified host cell produces an increased amount of the isoprenoid compound compared to a parent cell not comprising the one or more modifications, or a parent cell comprising only a subset of the one or more modifications of the genetically modified host cell, but is otherwise genetically identical. In some embodiments, the increased amount is at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or greater than 100%, as measured, for example, in yield, production, productivity, in grams per liter of cell culture, milligrams per gram of dry cell weight, on a per unit volume of cell culture basis, on a per unit dry cell weight basis, on a per unit volume of cell culture per unit time basis, or on a per unit dry cell weight per unit time basis.

[00182] In some embodiments, the host cell produces an elevated level of isoprenoid that is greater than about 10 grams per liter of fermentation medium. In some such embodiments, the isoprenoid is produced in an amount from about 10 to about 50 grams, more than about 15 grams, more than about 20 grams, more than about 25 grams, or more than about 30 grams per liter of cell culture.

[00183] In some embodiments, the host cell produces an elevated level of isoprenoid that is greater than about 50 milligrams per gram of dry cell weight. In some such embodiments, the isoprenoid is produced in an amount from about 50 to about 1500 milligrams, more than about 100 milligrams, more than about 150 milligrams, more than about 200 milligrams, more than about 250 milligrams, more than about 500 milligrams, more than about 750 milligrams, or more than about 1000 milligrams per gram of dry cell weight.

[00184] In some embodiments, the host cell produces an elevated level of isoprenoid that is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-

fold, at least about 2. 5-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, or at least about 1,000-fold, or more, higher than the level of isoprenoid produced by a parent cell, on a per unit volume of cell culture basis.

[00185] In some embodiments, the host cell produces an elevated level of isoprenoid that is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 2. 5-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, or at least about 1,000-fold, or more, higher than the level of isoprenoid produced by the parent cell, on a per unit dry cell weight basis.

[00186] In some embodiments, the host cell produces an elevated level of an isoprenoid that is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 2. 5-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, or at least about 1,000-fold, or more, higher than the level of isoprenoid produced by the parent cell, on a per unit volume of cell culture per unit time basis.

[00187] In some embodiments, the host cell produces an elevated isoprenoid that is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 2. 5-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, or at least about 1,000-fold, or more, higher than the level of isoprenoid produced by the parent cell, on a per unit dry cell weight per unit time basis.

[00188] In most embodiments, the production of the elevated level of isoprenoid by the host cell is inducible by an inducing compound. Such a host cell can be manipulated with ease in the absence of the inducing compound. The inducing compound is then added to induce the production of the elevated level of isoprenoid by the host cell. In other embodiments, production of the elevated level of isoprenoid by the host cell is inducible by changing culture conditions, such as, for example, the growth temperature, media constituents, and the like.

5.4.1 Culture Media and Conditions

[00189] Materials and methods for the maintenance and growth of microbial cultures are well known to those skilled in the art of microbiology or fermentation science (*see*, for example, Bailey *et al*., Biochemical Engineering Fundamentals, second edition, McGraw Hill, New York, 1986). Consideration must be given to appropriate culture medium, pH, temperature, and requirements for aerobic, microaerobic, or anaerobic conditions, depending on the specific requirements of the host cell, the fermentation, and the process.

[00190] The methods of producing isoprenoids provided herein may be performed in a suitable culture medium (*e.g.,* with or without pantothenate supplementation) in a suitable container, including but not limited to a cell culture plate, a flask, or a fermentor. Further, the methods can be performed at any scale of fermentation known in the art to support industrial production of microbial products. Any suitable fermentor may be used including a stirred tank fermentor, an airlift fermentor, a bubble fermentor, or any combination thereof. In particular embodiments utilizing *Saccharomyces cerevisiae* as the host cell, strains can be grown in a fermentor as described in detail by Kosaric, *et al*, in Ullmann's Encyclopedia of Industrial Chemistry, Sixth Edition, Volume 12, pages 398-473, Wiley-VCH Verlag GmbH & Co. KDaA, Weinheim, Germany.

[00191] In some embodiments, the culture medium is any culture medium in which a genetically modified microorganism capable of producing an isoprenoid can subsist, *i.e.*, maintain growth and viability. In some embodiments, the culture medium is an aqueous medium comprising assimilable carbon, nitrogen and phosphate sources. Such a medium can also include appropriate salts, minerals, metals and other nutrients. In some embodiments, the carbon source and each of the essential cell nutrients, are added incrementally or continuously to the fermentation media, and each required nutrient is maintained at essentially the minimum level needed for efficient assimilation by growing cells, for example, in accordance with a predetermined cell growth curve based on the metabolic or respiratory function of the cells which convert the carbon source to a biomass.

[00192] Suitable conditions and suitable media for culturing microorganisms are well known in the art. In some embodiments, the suitable medium is supplemented with one or more additional agents, such as, for example, an inducer (*e.g.*, when one or more nucleotide sequences encoding a gene product are under the control of an inducible promoter), a repressor (*e.g.*, when one or more nucleotide sequences encoding a gene product are under the control of a repressible promoter), or a selection agent (*e.g.*, an antibiotic to select for microorganisms comprising the genetic modifications).

[00193] In some embodiments, the carbon source is a monosaccharide (simple sugar), a disaccharide, a polysaccharide, a non-fermentable carbon source, or one or more combinations thereof. Non-limiting examples of suitable monosaccharides include glucose, galactose, mannose, fructose, ribose, and combinations thereof. Non-limiting examples of suitable disaccharides include sucrose, lactose, maltose, trehalose, cellobiose, and combinations thereof. Non-limiting examples of suitable polysaccharides include starch, glycogen, cellulose, chitin, and combinations thereof. Non-limiting examples of suitable non-fermentable carbon sources include acetate and glycerol.

[00194] The concentration of a carbon source, such as glucose, in the culture medium should promote cell growth, but not be so high as to repress growth of the microorganism used. Typically, cultures are run with a carbon source, such as glucose, being added at levels to achieve the desired level of growth and biomass, but at undetectable levels (with detection limits being about <0.1g/l). In other embodiments, the concentration of a carbon source, such as glucose, in the culture medium is greater than about 1 g/L, preferably greater than about 2 g/L, and more preferably greater than about 5 g/L. In addition, the concentration of a carbon source, such as glucose, in the culture medium is typically less than about 100 g/L, preferably less than about 50 g/L, and more preferably less than about 20 g/L. It should be noted that references to culture component concentrations can refer to both initial and/or ongoing component concentrations. In some cases, it may be desirable to allow the culture medium to become depleted of a carbon source during culture.

[00195] Sources of assimilable nitrogen that can be used in a suitable culture medium include, but are not limited to, simple nitrogen sources, organic nitrogen sources and complex nitrogen sources. Such nitrogen sources include anhydrous ammonia, ammonium salts and substances of animal, vegetable and/or microbial origin. Suitable nitrogen sources include, but are not limited to, protein hydrolysates, microbial biomass hydrolysates, peptone, yeast extract, ammonium sulfate, urea, and amino acids. Typically, the concentration of the nitrogen sources, in the culture medium is greater than about 0.1 g/L, preferably greater than

about 0.25 g/L, and more preferably greater than about 1.0 g/L. Beyond certain concentrations, however, the addition of a nitrogen source to the culture medium is not advantageous for the growth of the microorganisms. As a result, the concentration of the nitrogen sources, in the culture medium is less than about 20 g/L, preferably less than about 10 g/L and more preferably less than about 5 g/L. Further, in some instances it may be desirable to allow the culture medium to become depleted of the nitrogen sources during culture.

[00196] The effective culture medium can contain other compounds such as inorganic salts, vitamins, trace metals or growth promoters. Such other compounds can also be present in carbon, nitrogen or mineral sources in the effective medium or can be added specifically to the medium.

[00197] The culture medium can also contain a suitable phosphate source. Such phosphate sources include both inorganic and organic phosphate sources. Preferred phosphate sources include, but are not limited to, phosphate salts such as mono or dibasic sodium and potassium phosphates, ammonium phosphate and mixtures thereof. Typically, the concentration of phosphate in the culture medium is greater than about 1.0 g/L, preferably greater than about 2.0 g/L and more preferably greater than about 5.0 g/L. Beyond certain concentrations, however, the addition of phosphate to the culture medium is not advantageous for the growth of the microorganisms. Accordingly, the concentration of phosphate in the culture medium is typically less than about 20 g/L, preferably less than about 15 g/L and more preferably less than about 10 g/L.

[00198] A suitable culture medium can also include a source of magnesium, preferably in the form of a physiologically acceptable salt, such as magnesium sulfate heptahydrate, although other magnesium sources in concentrations that contribute similar amounts of magnesium can be used. Typically, the concentration of magnesium in the culture medium is greater than about 0.5 g/L, preferably greater than about 1.0 g/L, and more preferably greater than about 2.0 g/L. Beyond certain concentrations, however, the addition of magnesium to the culture medium is not advantageous for the growth of the microorganisms. Accordingly, the concentration of magnesium in the culture medium is typically less than about 10 g/L, preferably less than about 5 g/L, and more preferably less than about 3 g/L. Further, in some instances it may be desirable to allow the culture medium to become depleted of a magnesium source during culture.

[00199] In some embodiments, the culture medium can also include a biologically acceptable chelating agent, such as the dihydrate of trisodium citrate. In such instance, the

concentration of a chelating agent in the culture medium is greater than about 0.2 g/L, preferably greater than about 0.5 g/L, and more preferably greater than about 1 g/L. Beyond certain concentrations, however, the addition of a chelating agent to the culture medium is not advantageous for the growth of the microorganisms. Accordingly, the concentration of a chelating agent in the culture medium is typically less than about 10 g/L, preferably less than about 5 g/L, and more preferably less than about 2 g/L.

[00200] The culture medium can also initially include a biologically acceptable acid or base to maintain the desired pH of the culture medium. Biologically acceptable acids include, but are not limited to, hydrochloric acid, sulfuric acid, nitric acid, phosphoric acid and mixtures thereof. Biologically acceptable bases include, but are not limited to, ammonium hydroxide, sodium hydroxide, potassium hydroxide and mixtures thereof. In some embodiments, the base used is ammonium hydroxide.

[00201] The culture medium can also include a biologically acceptable calcium source, including, but not limited to, calcium chloride. Typically, the concentration of the calcium source, such as calcium chloride, dihydrate, in the culture medium is within the range of from about 5 mg/L to about 2000 mg/L, preferably within the range of from about 20 mg/L to about 1000 mg/L, and more preferably in the range of from about 50 mg/L to about 500 mg/L.

[00202] The culture medium can also include sodium chloride. Typically, the concentration of sodium chloride in the culture medium is within the range of from about 0.1 g/L to about 5 g/L, preferably within the range of from about 1 g/L to about 4 g/L, and more preferably in the range of from about 2 g/L to about 4 g/L.

[00203] In some embodiments, the culture medium can also include trace metals. Such trace metals can be added to the culture medium as a stock solution that, for convenience, can be prepared separately from the rest of the culture medium. Typically, the amount of such a trace metals solution added to the culture medium is greater than about 1 ml/L, preferably greater than about 5 mL/L, and more preferably greater than about 10 mL/L. Beyond certain concentrations, however, the addition of a trace metals to the culture medium is not advantageous for the growth of the microorganisms. Accordingly, the amount of such a trace metals solution added to the culture medium is typically less than about 100 mL/L, preferably less than about 50 mL/L, and more preferably less than about 30 mL/L. It should be noted that, in addition to adding trace metals in a stock solution, the individual components can be added separately, each within ranges corresponding independently to the amounts of the components dictated by the above ranges of the trace metals solution.

[00204] The culture media can include other vitamins, such as pantothenate, biotin, calcium, pantothenate, inositol, pyridoxine-HCl, and thiamine-HCl. Such vitamins can be added to the culture medium as a stock solution that, for convenience, can be prepared separately from the rest of the culture medium. Beyond certain concentrations, however, the addition of vitamins to the culture medium is not advantageous for the growth of the microorganisms.

[00205] The fermentation methods described herein can be performed in conventional culture modes, which include, but are not limited to, batch, fed-batch, cell recycle, continuous and semi-continuous. In some embodiments, the fermentation is carried out in fed-batch mode. In such a case, some of the components of the medium are depleted during culture, including pantothenate during the production stage of the fermentation. In some embodiments, the culture may be supplemented with relatively high concentrations of such components at the outset, for example, of the production stage, so that growth and/or isoprenoid production is supported for a period of time before additions are required. The preferred ranges of these components are maintained throughout the culture by making additions as levels are depleted by culture. Levels of components in the culture medium can be monitored by, for example, sampling the culture medium periodically and assaying for concentrations. Alternatively, once a standard culture procedure is developed, additions can be made at timed intervals corresponding to known levels at particular times throughout the culture. As will be recognized by those in the art, the rate of consumption of nutrient increases during culture as the cell density of the medium increases. Moreover, to avoid introduction of foreign microorganisms into the culture medium, addition is performed using aseptic addition methods, as are known in the art. In addition, a small amount of anti-foaming agent may be added during the culture.

[00206] The temperature of the culture medium can be any temperature suitable for growth of the genetically modified cells and/or production of isoprenoids. For example, prior to inoculation of the culture medium with an inoculum, the culture medium can be brought to and maintained at a temperature in the range of from about 20°C to about 45°C, preferably to a temperature in the range of from about 25°C to about 40°C, and more preferably in the range of from about 28°C to about 32°C.

[00207] The pH of the culture medium can be controlled by the addition of acid or base to the culture medium. In such cases when ammonia is used to control pH, it also conveniently serves as a nitrogen source in the culture medium. Preferably, the pH is

maintained from about 3.0 to about 8.0, more preferably from about 3.5 to about 7.0, and most preferably from about 4.0 to about 6.5.

[00208] In some embodiments, the carbon source concentration, such as the glucose concentration, of the culture medium is monitored during culture. Glucose concentration of the culture medium can be monitored using known techniques, such as, for example, use of the glucose oxidase enzyme test or high pressure liquid chromatography, which can be used to monitor glucose concentration in the supernatant, *e.g.*, a cell-free component of the culture medium. As stated previously, the carbon source concentration should be kept below the level at which cell growth inhibition occurs. Although such concentration may vary from organism to organism, for glucose as a carbon source, cell growth inhibition occurs at glucose concentrations greater than at about 60 g/L, and can be determined readily by trial. Accordingly, when glucose is used as a carbon source the glucose is preferably fed to the fermentor and maintained below detection limits. Alternatively, the glucose concentration in the culture medium is maintained in the range of from about 1 g/L to about 100 g/L, more preferably in the range of from about 2 g/L to about 50 g/L, and yet more preferably in the range of from about 5 g/L to about 20 g/L. Although the carbon source concentration can be maintained within desired levels by addition of, for example, a substantially pure glucose solution, it is acceptable, and may be preferred, to maintain the carbon source concentration of the culture medium by addition of aliquots of the original culture medium. The use of aliquots of the original culture medium may be desirable because the concentrations of other nutrients in the medium (*e.g.* the nitrogen and phosphate sources) can be maintained simultaneously. Likewise, the trace metals concentrations can be maintained in the culture medium by addition of aliquots of the trace metals solution.

5.4.2 Recovery of isoprenoids

[00209] Once the isoprenoid is produced by the host cell, it may be recovered or isolated for subsequent use using any suitable separation and purification methods known in the art. In some embodiments, an organic phase comprising the isoprenoid is separated from the fermentation by centrifugation. In other embodiments, an organic phase comprising the isoprenoid separates from the fermentation spontaneously. In other embodiments, an organic phase comprising the isoprenoid is separated from the fermentation by adding a deemulsifier and/or a nucleating agent into the fermentation reaction. Illustrative examples of deemulsifiers include flocculants and coagulants. Illustrative examples of nucleating agents include droplets of the isoprenoid itself and organic solvents such as dodecane, isopropyl myristrate, and methyl oleate.

[00210] The isoprenoid produced in these cells may be present in the culture supernatant and/or associated with the host cells. In embodiments where the isoprenoid is associated with the host cell, the recovery of the isoprenoid may comprise a method of permeabilizing or lysing the cells. Alternatively or simultaneously, the isoprenoid in the culture medium can be recovered using a recovery process including, but not limited to, chromatography, extraction, solvent extraction, membrane separation, electrodialysis, reverse osmosis, distillation, chemical derivatization and crystallization.

[00211] In some embodiments, the isoprenoid is separated from other products that may be present in the organic phase. In some embodiments, separation is achieved using adsorption, distillation, gas-liquid extraction (stripping), liquid-liquid extraction (solvent extraction), ultrafiltration, and standard chromatographic techniques.

6. EXAMPLES

6.1 Example 1: Identification and characterization of NADH-specific HMG-CoA Reductases

[00212] This example describes the identification and characterization of HMG-CoA reductases not previously known to have NADH cofactor specificity.

6.1.1 Materials and Methods

6.1.1.1 Strain Engineering

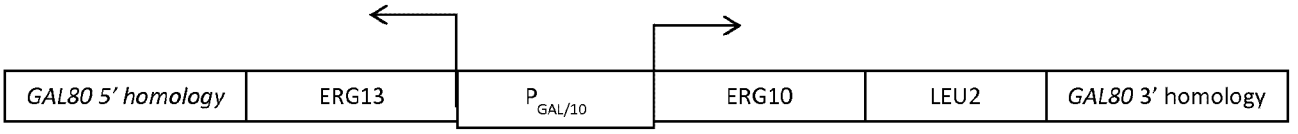
[00213] A wild-type *Saccharomyces cerevisiae* strain, (CEN.PK2, Mat a, *ura3*$^-$, TRP1$^+$, *leu2*$^-$, MAL2-8C, SUC2,) was used as a host for the expression of the mevalonate (MevT) pathway (whereby acetyl-CoA thiolase (ERG10) converts acetyl-CoA to acetoacetyl-CoA; HMG-CoA synthase (ERG13) converts acetoacetyl-CoA into HMG-CoA; and HMG-CoA reductase converts HMG-CoA into mevalonate (**FIG. 1**)).

[00214] This strain was transformed with a plasmid encoding either a heterologous class II HMG-CoA reductase derived from *Staphylococcus aureus* (ZP_06815052), *Herpetosiphon aurantiacus* (YP_001546303), *Pseudomonas mevalonii* (P13702), *Delftia acidovorans* (YP_001561318), *Menthanosaeta thermofila* (YP_843364) *or Silicibacter pomeoyri* (YP_164994); or an N-terminally truncated version of the *Saccharomyces cerevisiae* HMG-CoA reductase (tHMG-CoA reductase) (EEU05004). The class II HMG-CoA reductases were codon optimized for yeast expression and chemically synthesized with c-terminal FLAG-HIS tags, with the exception that the *P. mevalonii* HMG-CoA reductase was synthesized with the following additional modifications:

[00215] *Not*I site—GAL1 promotor—*Nde*I site—[P. *mevalonii* HMG-CoA reductase]---*Eco*RI site—FLAG tag—HIS tag—STOP codon ---PGK1 terminator---*Not*I site

[00216] This DNA was cloned into the *Not*I site of the pBluescript SK+ vector (Stratagene). The yeast Gal7 promoter was PCR amplified using the genomic DNA extract of a wild-type CENPK2 strain as template and using the oligonucleotides YT_164_30_Gal7F (which contains a *Sac*I and a *Not*I restriction site at 5'-end) and YT_164_30_Gal7R (which contains *Nde*I restriction site at 3'-end) (*see* Table 2). The PCR product was cloned onto pCR II-TOPO vector (Invitrogen). Both plasmids were cut using *Sac*I and *Not*I, and the excised Sc.GAL7 promoter was used to swap the Gal1 promoter upstream of the P. *mevalonii* HMG-CoA reductase gene. The resulting plasmid and pAM70 (SEQ ID NO:23), a yeast episomal vector pRS426 with a URA3 marker, were both digested with *Not*I. The plasmid pAM01147 (SEQ ID NO:24) was then constructed by ligating the *Not*I fragment into the *Not*I digested site of pAM70. This plasmid was used as a base plasmid to swap the *P. mevalonii* HMG-CoA reductase coding sequence for any HMG-CoA reductase coding sequence of interest (including the yeast tHMG-CoA reductase) by digesting the plasmid with *Nde*I and *Eco*RI and ligating a digested HMG-CoA reductase coding sequence of interest having *Nde*I and *Eco*RI sites at the 5'- and 3'- ends, respectively. Propagation of plasmid DNA was performed in *Escherichia coli* strain DH5α. Strain Y1389 was then transformed with the plasmids harboring coding sequences for different HMG-CoA reductases, and transformants were selected on CSM media plate without uracil containing 2% glucose. All DNA-mediated transformation into *S. cerevisiae* was conducted using the standard Lithium Acetate procedure as described by Gietz RW and Woods RA, *Guide to Yeast Genetics and Molecular and Cell Biology, Part B*. San Diego, CA: Academic Press Inc. pp. 87–96 (2002).

[00217] Genomic integration of Sc. acetoacetyl-CoA thiolase (ERG10) and Sc.HMG-CoA Synthase (ERG13) was targeted to the Gal80 locus of the host strain using the integration construct shown below (SEQ ID NO:25).



[00218] Each component of the integration construct was PCR amplified using 100ng of Y002 genomic DNA as template. PCR amplification of the upstream GAL80 locus from positions -1000 to -1 was performed with oligonucleotides YT_164_36_001 and YT_164_36_003 (*see* Table 2). PCR amplification of the yeast ERG10 and ERG13 genes

was done using the pair of oligonucleotides YT_164_36_002 and YT_164_36_005 for ERG13 and YT_164_36_006 and YT_164_36_009 for ERG10. The oligonucleotides YT_164_36_004 and YT_164_36_007 were used to amplify the GAL1/10 promoter, while primers YT_164_36_008 and YT_164_36_011 were used to amplify the LEU2 gene. PCR amplification of the downstream GAL80 locus positions 23 to 1000 (after the stop codon) was performed with oligonucleotides YT_164_36_010 and YT_164_36_012. One hundred fmol of each piece of DNA was added in a single tube and assembled by stitching PCR reaction (as described in U.S. Patent No. 8,221,982, the contents of which are hereby incorporated by reference) using the primers YT_164_36_001 and YT_164_36_012. PCR products having the expected molecular weights were gel purified.

[00219] **Table 2. Primers used for strain engineering**

Primer name	SEQ ID NO:	Primer Sequence
YT_164_36_001	SEQ ID NO:26	GCCTGTCTACAGGATAAAGACGGG
YT_164_36_002	SEQ ID NO:27	TCCCGTTCTTTCCACTCCCGTCTATATATATA TCATTGTTATTA
YT_164_36_003	SEQ ID NO:28	TAATAACAATGATATATATATAGACGGGAGT GGAAAGAACGGGA
YT_164_36_004	SEQ ID NO:29	CCAACAAAGTTTAGTTGAGAGTTTCATTTAT ATTGAATTTTCAAAAATTCTTAC
YT_164_36_005	SEQ ID NO:30	GTAAGAATTTTTGAAAATTCAATATAAATGA AACTCTCAACTAAACTTTGTTGG
YT_164_36_006	SEQ ID NO:31	GTCAAGGAGAAAAAACTATAATGTCTCAGA ACGTTTACATTGTATCGACTGCCAGAACCC
YT_164_36_007	SEQ ID NO:32	GGGTTCTGGCAGTCGATACAATGTAAACGTT CTGAGACATTATAGTTTTTTCTCCTTGAC
YT_164_36_008	SEQ ID NO:33	GTGTGCCTTTTGACTTACTTTTACGTTGAGCC ATTAGTATCA
YT_164_36_009	SEQ ID NO:34	TGATACTAATGGCTCAACGTAAAAGTAAGTC AAAAGGCACAC
YT_164_36_010	SEQ ID NO:35	GATATTTCTTGAATCAGGCGCCTTAGACCCC CCAGTGCAGCGAACGTTATAAAAAC
YT_164_36_011	SEQ ID NO:36	GTTTTTATAACGTTCGCTGCACTGGGGGGTC TAAGGCGCCTGATTCAAGAAATATC
YT_164_36_012	SEQ ID NO:37	AAATATGACCCCCAATATGAGAAATTAAGG C
YT_164_30_Gal3F	SEQ ID NO:38	GAGCTCGCGGCCGC GTACATACCTCTCTCCGTATCCTCGTAATCAT TTTCTTGT
YT_164_30_Gal3R	SEQ ID NO:39	CATATGACTATGTGT TGCCCTACCTTTTTACTTTTATTTTCTCTTT
YT_164_30_Gal7F	SEQ ID NO:40	GAGCTCGCGGCCGC GTGTCACAGCGAATTTCCTCACATGTAGGGA CCGAATTGT
YT_164_30_Gal7R	SEQ ID NO:41	CATATGTTTTGAGGGAATATTCAACTGTTTTT

		TTTTATCATGTTGA
RYSE 0	SEQ ID NO:42	GACGGCACGGCCACGCGTTTAAACCGCC
RYSE 19	SEQ ID NO:43	CCCGCCAGGCGCTGGGGTTTAAACACC

[00220] Derivatives of Y1389 transformed with different HMG-CoA reductases (as indicated above) were transformed with the ERG 10/ERG13 integration construct to create the strains listed below in Table 3. Transformants were selected on CSM containing 2% glucose media plate without uracil and leucine. All gene disruptions and replacements were confirmed by phenotypic analysis and colony PCR.

[00221] **Table 3: Strain Description**

Strain #	Descrption	strain # after adh1 Knockout
Y1431	MevT with *S. cerevisae* tHMG-CoA reductase	Y1804
Y1432	MevT with *S. aureus* HMG-CoA reductase	
Y1433	MevT with *P. mevalonii* HMG-CoA reductase	Y1805
Y1435	MevT with *D. acidovorans* HMG-CoA reductase	Y1806
Y1436	MevT with *M. thermofila* HMG-CoA reductase	
Y1486	MevT with *H. aurantiacus* HMG-CoA reductase	
Y1487	MevT with *S. pomeroyi* HMG-CoA reductase	Y1807

[00222] For strains Y1431, Y1433, Y1435 and Y1487, the ADH1 gene was knocked out using the disruption construct shown below (SEQ ID NO:44):

[00223]

ADH1 5' homology	Kan A	ADH1 3' homology

[00224] The disruption construct was generated by the methods of polynucleotide assembly described in U.S. Patent No. 8,221,982. The ADH1 5' homology region of the integration construct was homologous to positions -563 to -77 of the ADH1 coding sequence, and the ADH1 3' homology region was homologous to positions 87 to 538 (after the stop codon of the ADH1 gene). Primers RYSE 0 and RYSE 19 were used to amplify the product. Strain Y1431, Y1433, Y1435 and Y1487 (Table 2) were transformed with the product, and transformants were selected on YPD media plate containing 2% glucose and G418 (*Geneticin)*. The ADH1 gene disruption was confirmed by phenotypic analysis and colony PCR.

6.1.1.2 Cell Culture

[00225] A single colony of a given yeast strain was cultured in 3ml of Yeast Nitrogen Base (YNB) media with 2% sucrose as an overnight starter culture. The next day, production flasks were prepared with an initial OD_{600} of 0.05 diluted from the starter culture in 40 ml

YNB-4% sucrose production culture media in 250ml disposable PETG sterile flasks (Nalgene). The flasks were incubated at 30°C by shaking at 250 RPM for the durations indicated below.

6.1.1.3 HMG-CoA reductase Activity Assay Using Cell-Free Extract

[00226] Yeast cells were grown for 48 hours HMG-CoA reductase activity assays (**FIG. 8**) or 72 hours for mevalonate assays (Table 4) and harvested by centrifugation in a 15mL Falcon tube for 10 minutes at 4000 x g in a swinging bucket rotor JS-5.3 with proper carriage for the Falcon tubes. The cell pellet was resuspended in 1ml and washed once using cold lysis buffer (100 mM Tris pH 7.0 with Mini, EDTA free protease inhibitor tablet (Roche) added, 1 mM DTT and 1 mM EDTA). The cells were then transferred to a 2 mL plastic screw cap microfuge tube with O ring cap (Fisher Brand 520-GRD) and cells were lysed using disruption beads (Disruption beads, 0.5Mm, Fisher) and a bead beater for 1 minute at 6 M/S. The tubes were immediately placed in an ice water bath for at least 5 minutes. Tubes were spun at a minimum of 8000 x g for 20 minutes. The supernatant was then transferred to a new cold tube. Protein concentration was measured using the classic Bradford assay for proteins (Bradford MM A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* **72**, 248-254 (1976)).

[00227] For HMG-CoA reducatase assays, the reaction buffer (100 mM phosphate buffer pH 7.0, 100 mM KCl, 1 mM DTT and 1 mM EDTA) was initially pre-incubated in a 96 well plate at 30 °C. Either NADH or NADPH at a final concentration of 150 μM, a final concentration of 400 μM HMG-CoA and 5mM final concentration of DTT was added to a total volume of 190μl in each well. The assay was initiated by adding ten microliter of cell-free extract diluted to the range of linear activity. The reaction was monitored by measuring the decrease in absorbance of NADPH or NADH at 340nm using Molecular Devices Spectramax M5 plate reader. The slope of the line of absorbance at 340nm along with the protein concentration was used to calculate the specific activity of HMGr for each cell free extract.

6.1.1.4 Organic Acids and Alcohol Measurement

[00228] Samples for organic acids and alcohols assay were prepared by taking 1ml of fermentation broth and transferring the samples to a 1.5 ml eppendorf tubes. Samples were spun for 1min at 13,000 RPM using a table eppendorf centrifuges. The supernatant was then diluted (1:1 v/v) in 15mM sulfuric acid. The mixture was vortexed and centrifuged for 1min at 13,000 RPM. The clarified supernatant was transferred to a vial for HPLC analysis.

[00229] HPLC analysis was performed for glycerol and mevalonate content using HPLC Thermofisher and by ion exclusion chromatography using Column Waters IC-Pak 7.8 mm x 300 mm, 7 μm, 50 Å (Waters) and with refractive index (RI) detection (Thermofisher). Elution was carried out isocratically using a 15mM sulfuric acid aqueous mobile phase with 0.6 mL/min flow rate.

6.1.2 Results

6.1.2.1 Determination of cofactor specificity for Class II HMG-CoA reductases

[00230] As shown in **FIG. 8**, HMG-CoA reductases from *D. acidovorans* and *S. pomeroyi* exhibit high specificity for NADH and high specific activity *in vitro*. These HMG-CoA reductases displayed virtually no specific activity in the presence of NADPH, while specific activity approached 400 nmol/mg/min in the presence of NADH. Similarly, HMG-CoA reductase from *P. mevalonii* demonstrated selectivity for NADH as a cofactor, consistent with previously published reports. *See, e.g.,* Hedl *et al., J. Bacteriol* 186(7):1927-1932 (2004). By contrast, HMG-CoA reductases from *S. cerevisiae, S. aureus* and *H. aurantiacus* showed no measurable activity in the presence of NADH, and HMG-CoA reductase from *M. thermofila* showed barely detectable activity in the presence of both NADPH and NADH. These results indicate that HMG-CoA reductases from *D. acidovorans* and *S. pomeroyi* are NADH-selective HMG-CoA reductases, similar to the HMG-CoA reductase from *P. mevalonii.*

[00231] In addition, Table 4 indicates that strains comprising a MevT pathway comprising an NADH-using HMG-CoA reductase (from *P. mevalonii*, *D. acidovorans* and *S. pomeroyi*, respectively) produced substantially less mevalonate than strains comprising a MevT pathway comprising an NADPH-using HMG-CoA reductase (from *S. cerevisiae, S. aureus* and *H. aurantiacus*, respectively). This suggests that *in vivo*, an additional source of NADH is required to utilize the full catalytic capacity of NADH-using HMG-CoA reductases towards mevalonate and downstream isoprenoid production.

Table 4. Mevalonate production from NADPH-using HMG-CoA reductases vs. NADH-using HMG-CoA reductases

Source of HMG-CoA reductase	Mevalonate production (g/L)	Co-factor specificity
S. cerevisiae	1.11	NADPH
S. aureus	1.74	NADPH
H. aurantiacus	1.84	NADPH
P. mevalonii	0.41	NADH

D. acidovorans	0.42	NADH
S. pomeoyri	0.57	NADH

6.1.2.2 Increased intracellular NADH improves NADH-using HMG-CoA reductase activity

[00232] As indicated in **FIGS. 9-11**, mevalonate production is substantially improved in cells comprising a MevT pathway comprising an NADH-using HMG-CoA reductase when a metabolic perturbation is introduced which increases the intracellular concentration of NADH. ADH1 reduces acetaldehyde to ethanol in an NADH-dependent manner. In an *adh1*Δbackground, host cells suffer reduced growth (**FIG. 9**) and increased glycerol production (**FIG. 10**), which is indicative of redox imbalance likely resulting from the accumulation of intracellular NADH. However, while cells comprising a MevT pathway comprising an NADPH-using HMG-CoA reductase (*S. cerevisiae* (Sc.) tHMG-CoA reductase) display reduced mevalonate production in the *adh1*Δbackground, cells comprising a MevT pathway comprising an NADH-using HMG-CoA reductase ((from *P. mevalonii*, *D. acidovorans* and *S. pomeroyi*, respectively) display substantial improvements in mevalonate production (**FIG. 11**), despite also showing signs of redox stress. These data suggest that NADH-using HMG-CoA reductases are able to utilize increased pools of intracellular NADH to boost mevalonate production. These results also suggest that in the absence of an increased intracellular source of NADH, NADH-using HMG-CoA reductases are cofactor limited.

[00233] Notably, previous published reports have indicated that the HMG-CoA reductase of *P. mevalonii* is utilized in the degradation of mevalonate. *See Anderson et al.,J. Bacteriol.,* (171(12):6468-6472 (1989). *P. mevalonii* is among the few prokaryotes that have been identified as capable of subsisting on mevalonate as its sole carbon source. However, the results presented here demonstrate the unexpected utility of *P. mevalonii* HMG-CoA reductase for use in a biosynthetic pathway for mevalonate.

6.2 Example 2: Improved isoprenoid production and redox balancing with alternate routes to acetyl-CoA and alternate MEV pathway enzymes

[00234] This example demonstrates that mevalonate and downstream isoprenoid production from the MEV pathway can be improved by utilizing alternate routes to cytolsolic acetyl-CoA production, *e.g. via* the heterologous expression of acetaldehyde dehydrogenase, acetylating (ADA, E.C. 1.2.1.10), in lieu of the wild-type PDH-bypass, and in various combinations with alternate MEV pathway enzymes. These results show that the redox imbalance introduced by the replacement of the NADPH-producing PDH-bypass enzymes with NADH-producing ADA can be alleviated in part by combining ADA expression with an

NADH-using HMG-CoA reductase of the MEV pathway, and/or with heterologous expression of phosphoketolase and phosphotransacetylasse, which can also provide an additional alternate route to cytosolic acetyl-CoA production. These results further demonstrate that the catalytic capacity of ADA for providing acetyl-CoA substrate to the MEV pathway is substantially improved by providing a thermodynamically favorable downstream conversion of acetyl-CoA to acetoacetyl-CoA, such as that provided by acetyl-CoA:malonyl-CoA acyltransferase.

6.2.1 Materials and Methods

6.2.1.1 Strain Engineering

[00235] The strains listed in Table 5 were constructed to determine: (1) the effects on cell growth and heterologous isoprenoid production when ADA is paired with an NADH-using HMG-CoA reductase versus an NADPH-using HMG-CoA reductase; (2) the effect of phosphoketolase and phosphotransacetylase expression on the redox imbalance created by the expression of ADA; and (3) the effect of acetoacetyl-CoA synthase expression on mevalonate levels in strains expressing ADA.

Table 5.

Strain Name	Description
Y968	Wildtype CEN.PK2
Y12869	*acs1^acs2^ald6^; 2x Dz.eutE*
Y12746	*acs1^acs2^ald6^; 2x Dz.eutE; 3x Lm.PK; 1x Ck.PTA*
Y12869.ms63908	Y12869 with construct ms63908
Y12869.ms63909	Y12869 with construct ms63909
Y968.ms63908	Y968 with construct ms63908
Y968.ms63909	Y968 with construct ms63909
Y12869.ms63907.ms64472	Y12869.ms63907 with construct ms64472
Y12869.ms63909.ms64472	Y12869.ms63909 with construct ms64472
Y968.ms63907.ms64472	Y968.ms63907 with construct ms64472
Y968.ms63909.ms64472	Y968.ms63909 with construct ms64472

6.2.1.1.1 Y968

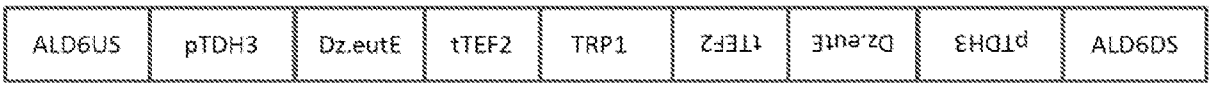
[00236] Y968 is wildtype *Saccharomyces cerevisiae* CEN.PK2, Matalpha. The starting strain for Y12869, Y12746, and all of their derivatives, was *Saccharomyces cerevisiae* strain (CEN.PK2, Mat alpha, ura3-52, trp1-289, leu2-3,122, his3^1), Y003. All DNA-mediated transformation into *S. cerevisiae* was conducted using the standard Lithium Acetate procedure as described by Gietz RW and Woods RA, *Guide to Yeast Genetics and Molecular and Cell Biology. Part B*. San Diego, CA: Academic Press Inc. pp. 87–96 (2002),

and in all cases integration of the constructs were confirmed by PCR amplification of genomic DNA.

6.2.1.1.2 Y12869

[00237] Y12869 was generated through three successive integrations into Y003. First, the gene *ACS2* was deleted by introducing an integration construct (i2235; SEQ ID NO:45) consisting of the native *S. cerevisiae LEU2* gene, flanked by sequences consisting of upstream and downstream nucleotide sequences of the *ACS2* locus. Upon introduction of a *S. cerevisiae* host cell, this construct can integrate by homologous recombination into the *ACS2* locus of the genome, functionally disrupting *ACS2* by replacing the *ACS2* coding sequence with its integrating sequence. Transformants were plated onto CSM –leu plates containing 2% EtOH as the sole carbon source, and were confirmed by PCR amplification. The resulting strain was Y4940.

[00238] Next, ALD6 was deleted and *Dickeya zeae eutE* was introduced in Y4940 with the integration construct (i74804; SEQ ID NO:46) pictured below.



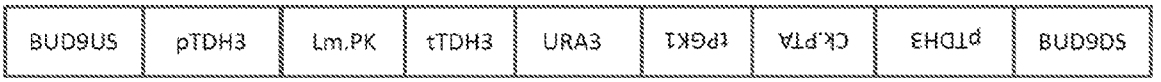
[00239] This integration construct comprises a selectable marker (TRP1), as well as two copies a yeast-codon-optimized sequence encoding the gene *eutE* from *Dickeya zeae* (NCBI Reference Sequence: YP_003003316.1) under control of the *TDH3* promoter (840 basepairs upstream of the native *S. cerevisiae TDH3* coding region), and the *TEF2* terminator (508 basepairs downstream of the native *S. cerevisiae TEF2* coding region). These components are flanked by upstream and downstream nucleotide sequences of the *ALD6* locus. Upon introduction into a host cell, this construct integrates by homologous recombination into the host cell genome, functionally disrupting *ALD6* by replacing the *ALD6* coding sequence with its integrating sequence. The construct was assembled using the methods described in U.S. Patent No. 8,221,982. The construct was transformed into Y4940, and transformants were selected on CSM-TRP plates with 2% glucose and confirmed by PCR amplification. The resulting strain was 12602.

[00240] Next, ACS1 was deleted in Y12602 by introducing an integration construct (i76220; SEQ ID NO:47) consisting of the upstream and downstream nucleotide sequences of *ACS1*, flanking the native *S. cerevisiae HIS3* gene under its own promoter and terminator. Transformants were plated onto CSM –his plates containing 2% glucose as the sole carbon source, and were confirmed by PCR amplification. The resulting strain was Y12747.

[00241] Next, Y12747 was transformed with a PCR product amplified from the native *URA3* sequence. This sequence restores the ura3-52 mutation. *See* Rose and Winston, *Mol Gen Genet* 193:557-560 (1984). Transformants were plated onto CSM-ura plates containing 2% glucose as the sole carbon source, and were confirmed by PCR amplification. The resulting strain was Y12869.

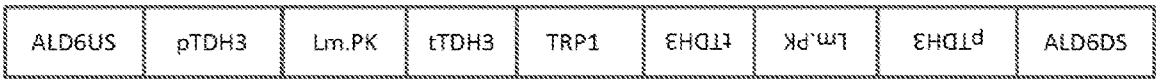
6.2.1.1.3 Y12746

[00242] Y12746 was generated through three successive integrations into Y4940. First, Y4940 was transformed with the integration construct (i73830; SEQ ID NO:48) pictured below.



[00243] This integration construct comprises a selectable marker (URA3); a yeast codon-optimized version of phosphoketolase from *Leuconostoc mesenteroides* (NCBI Reference Sequence YP_819405.1) under the *TDH3* promoter (870 bp upstream of the *TDH3* coding sequence) and *TDH3* terminator (259 bp downstream of the *TDH3* coding sequence); a yeast codon-optimized version of *Clostridium kluyveri* phosphotransacetylase (NCBI Reference Sequence: YP_001394780.1) under control of the *TDH3* promoter (870 bp upstream of the *TDH3* coding sequence) and the *PGK1* terminator (259 bp downstream of the *PGK1* coding sequence); flanked by homologous sequences consisting of the upstream and downstream nucleotide sequences of the *S. cerevisiae BUD9* locus. Upon introduction into a host cell, this construct integrates by homologous recombination into the host cell genome, functionally disrupting *BUD9* by replacing the *BUD9* coding sequence with its integrating sequence. The construct was assembled using the methods described in U.S. Patent No. 8,221,982. Transformants were selected on CSM-URA plates with 2% glucose.

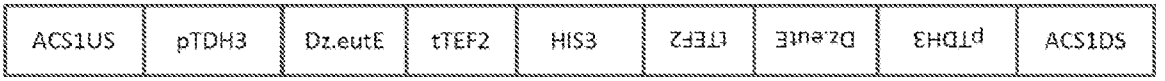
[00244] The resulting strain was transformed with the construct (i74810; SEQ ID NO:49) shown below.



[00245] This construct comprising a selectable marker (*TRP1*); two copies of phosphoketolase from *Leuconostoc mesenteroides* under the *TDH3* promoter (870 bp upstream of the *TDH3* coding sequence) and *TDH3* terminator (259 bp downstream of the *TDH3* coding sequence); flanked by homologous sequences consisting of the upstream and downstream nucleotide sequences of the *ALD6* locus. Upon introduction into a host cell, this construct integrates by homologous recombination into the host cell genome, functionally disrupting *ALD6* by replacing the *ALD6* coding sequence with its integrating sequence. The

construct was assembled using the methods described in U.S. Patent No. 8,221,982. Transformants were selected on CSM-URA plates with 2% glucose and confirmed by PCR amplification.

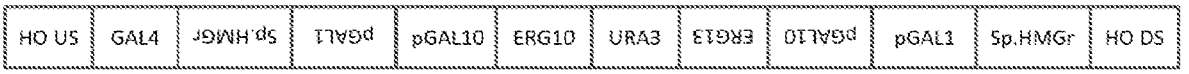
[00246] Finally, the resulting strain was transformed with the construct (i76221; SEQ ID NO:50) shown below.



[00247] This construct comprises a selectable marker (*HIS3*); as well as two copies a yeast-codon-optimized sequence encoding the gene *eutE* from *Dickeya Zeae* (NCBI Reference Sequence: YP_003003316.1) under control of the *TDH3* promoter (840 basepairs upstream of the native *S. cerevisiae TDH3* coding region) and the *TEF2* terminator (508 basepairs downstream of the native *S. cerevisiae TEF2* coding region). These components are flanked by upstream and downstream nucleotide sequences of the *ACS1* locus. Upon introduction into a host cell, this construct integrates by homologous recombination into the host cell genome, functionally disrupting *ACS1* by replacing the *ACS1* coding sequence with its integrating sequence. The construct was assembled using the methods described in U.S. Patent No. 8,221,982. Transformants were selected on CSM-HIS plates with 2% glucose and confirmed by PCR amplification. The resulting strain was Y12746.

6.2.1.1.4 ms63907, ms63908, ms63909, and ms64472 integration constructs

[00248] The ms63907 integration construct (i84022; SEQ ID NO:51) is shown below.

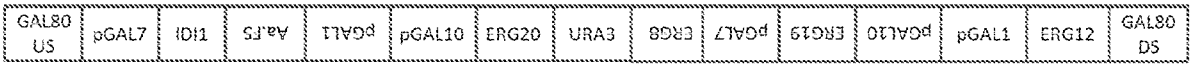


This construct comprises nucleotide sequences that encode a selectable marker (*URA3*); a copy of the native yeast *GAL4* transcription factor under its own promoter; two native yeast enzymes of the mevalonate pathway (*ERG10* which encodes Acetoacetyl-CoA thiolase, and *ERG13*, which encodes HMG-CoA synthase), as well as two copies of a yeast codon-optimized version of *Silicibacter pomeroyi* HMG-CoA reductase, all under galactose-inducible promoters (promoters of the *S. cerevisiae* genes *GAL1* and *GAL10*, flanked by homologous sequences consisting of upstream and downstream nucleotide sequences of the *S. cerevisiae HO* endonuclease locus. Upon introduction into a host cell, the ms63907 construct integrates by homologous integration into the host cell genome, functionally disrupting *HO* by replacing the *HO* coding sequence with its integrating sequence. The construct was assembled using the methods described in U.S. Patent No. 8,221,982. Transformants were selected on CSM-URA plates with 2% glucose and confirmed by PCR amplification.

[00249] The ms63908 integration construct (i84024; SEQ ID NO:52) is identical to ms63907, with two exceptions: first, *ERG10* is replaced by a yeast codon-optimized version of the *nphT7* gene of *Streptomyces sp. CL190* encoding acetyl-CoA:malonyl-CoA acyltransferase (accession no. AB540131.1) fused to the *AHP1* terminator (125 bp downstream of the *AHP1* coding sequence in *S. cerevisiae*); second, the sequences encoding *S. pomeroyi* HMG-CoA reductase are replaced by *tHMGr*, the truncated *HMG1* coding sequence which encodes the native *S. cerevisiae* HMG-CoA reductase.

[00250] The ms63909 integration construct (i84026; SEQ ID NO:53) is identical to ms63907, with one exception: the sequences encoding *S. pomeroyi* HMG-CoA reductase are replaced by *tHMGr*, the truncated *HMG1* coding sequence which encodes the native *S. cerevisiae* HMG-CoA reductase.

[00251] The ms64472 integration construct (i85207; SEQ ID NO:54) is shown below.



This construct comprises nucleotide sequences that encode a selectable marker (*URA3)*; five native yeast enzymes of the ergosterol pathway (*ERG12* which encodes mevalonate kinase, *ERG8* which encodes phosphomevalonate kinase, *ERG19* which encodes mevalonate pyrophosphate decarboxylase, *IDI1* which encodes dimethylallyl diphosphate isomerase, and *ERG20* which encodes farnesyl pyrophosphate synthetase), as well as an evolved, yeast codon-optimized version of *Artemisia annua* farnesene synthase, all under galactose-inducible promoters (Promoters of the *S. cerevisiae* genes *GAL1, GAL10, and GAL7*). These sequences are flanked by homologous sequences consisting of the upstream and downstream nucleotide sequences of *GAL80*. Upon introduction into a host cell, the ms64472 construct integrates by homologous integration into the host cell genome, functionally disrupting *GAL80* by replacing the *GAL80* coding sequence with its integrating sequence. The construct was assembled using the methods described in U.S. Patent No. 8,221,982. Transformants were selected on CSM-URA plates with 2% glucose and confirmed by PCR amplification.

6.2.1.2 Quantitation of mevalonate

[00252] Single colonies were inoculated in wells of a 96-well plate in seed media (15 g/L ammonium sulfate, 8 g/L potassium phosphate, 6.1 g/L magnesium sulfate, 150 mg/L EDTA, 57.5 mg/L zinc sulfate, 4.8 mg/L cobalt chloride, 3.24 mg/L manganese chloride, 5 mg/L copper sulfate, 29.4 mg/L calcium chloride, 27.8 mg/L iron sulfate, 4.8 mg/L sodium molybdate, 0.6 mg/L biotin, 12 mg/L calcium pantothenate, 12 mg/L nicotinic acid, 30 mg/L inositol, 12 mg/L thiamin hydrochloride, 12 mg/L pyridoxine hydrochloride, 0.24 mg/L para-aminobenzoic acid) with 50 mM succinate pH 5.0, and 20 g/L sucrose, and grown at 30C for

three days. Then, 14.4 ul of culture was subcultured into seed media with 50 mM succinate pH 5.0 and 40 g/L galactose, and grown at 30C for 2 days.

[00253] To quantitate secreted mevalonate, whole cell broth was first spun down at 14,000 RPM for 5 min. 10 ul of clarified broth was then incubated with 190 ul of assay buffer (1 mM CoA, 2 mM NAD, purified and lyophilized *Pseudomonas mevalonii* HMG-CoA reductase at 0.2 mg/ml, purified and lyophilized *Pseudomonas mevalonii* HMG-CoA lyase at 0.1 mg/ml, 95 mM TrisCl pH8.5, 20 mM MgCl2, and 5 mM DTT). The sample was incubated for 30 minutes at 30C, then assayed for 340nM absorbance on a Beckman M5 plate reader. Mevalonate concentration was quantitated by plotting onto a standard curve generated with purified mevalonate.

6.2.1.3 Quantitation of farnesene

[00254] Cultures were first grown as described above. To quantitate farnesene, 600 ul of 2-butoxyethanol was added to 150 ul of whole cell broth in three additions of 200 ul each, with 90 seconds of shaking at 1000 rpm on a 96-well plate shaker between each addition. The samples were then incubated for 40 minutes. 8 ul of the 2-butoxyethanol extract was mixed with 200 ul of isopropyl alcohol in a 96-well UV plate (Costar 3635), then read on a plate reader for absorbance 222.

6.2.1.4 Quantitation of optical density

[00255] In a 96-well assay plate, 8 ul of culture was mixed with diluent (20% PEG 200, 20% Ethanol, 2% Triton™ X-114) and incubated for 30 minutes at room temperature. The assay plate was vortexted before measuring OD_{600} on a Beckman M5 plate reader.

6.2.1.5 Batch fermentation

[00256] Inoculum cultures of Y967, Y12869, and Y12746 were grown from single colonies in 5 ml of seed media with 50 mM succinate pH 5.0, and 20 g/L sucrose. After 3 days of growth, the precultures were subcultured into 25 ml of seed media with 50 mM succinate pH 5.0 and 40 g/L sucrose to an initial optical density (OD) of 0.1. After 10 hours, the cultures were subcultured again into 50 ml of seed media with 50 mM succinate pH 5.0 and 40 g/L sucrose to an OD of 0.05. Cultures were grown at 30 °C. When the OD was approximately 3, the 3 flasks were split in half and spun down and the media was discarded. The cultures were resuspended in 1.5 L seed media with 40 g/L glucose (without succinate) and transferred to the fermentor. Fermentation experiments were performed in a 2 L Biostat B plus vessel (Sartorius, Germany). Stirring was controlled at 1200 rpm and the fermentor was continuously sparged with 0.5 L/min air. The pH was maintained at 5.0 with 14.4 M

NH_4OH and the temperature was maintained at 30 °C. Roughly every 1.5 hours, a sample was drawn to measure the OD, dry cell weight, and organic acids and sugars.

6.2.2 Results

6.2.2.1 ADA strains produce more isoprenoid when paired with an NADH-using HMGr versus an NADPH-using HMGr

[00257] **FIG. 12A** shows that strain Y12869, comprising a deletion of the PDH-bypass (*acs1*Δ *acs2* Δ *ald6*Δ) and heterologously expressing ADA (Dz.cutE), produces more farnesene when expressing a MEV pathway comprising an NADH-using HMGr (construct ms63907) than a MEV pathway comprising an NADPH-using HMGr (construct ms63909). In contrast, **FIG. 12B** shows that strain Y968, comprising an intact PDH-bypass, produces more farnesene when paired with an NADPH-using HMGr. These results demonstrate that utilization of ADA for isoprenoid production from the MEV pathway is improved when the MEV pathway comprises an NADH-using HMGr.

6.2.2.2 Expression of ADA causes a redox imbalance which is alleviated when PK and PTA share flux with glycolysis

[00258] Native yeast produce two NADH per glucose consumed through glycolysis. When fermented to ethanol, the two NADH are reoxidized to NAD+. However, a fraction of the glucose is converted to biomass rather than fermented to ethanol, resulting in an excess of NADH. This excess NADH is reoxidized to NAD+ through the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate, which is hydrolyzed to glycerol. Strains which use the acylating acetaldehyde dehydrogenase in place of the native PDH-bypass produce NADH instead of NADPH, resulting in a further excess of NADH. For each glucose converted to biomass, a strain which uses ADA in place of the native PDH-bypass produces exactly twice as much NADH, meaning that twice as much glycerol must be produced in order to reoxidize the excess NADH. As shown in **FIG. 13A**, Y12869 (a strain which uses ADA in the place of the wildtype PDH-bypass) produces twice as much glycerol as Y968 (comprising an intact PDH-bypass) while consuming comparable levels of glucose in a batch glucose fermentation. These results demonstrate that Y12869 is redox imbalanced as predicted by the stoichiometry of the ADA reaction.

[00259] The addition of phosphoketolase and phosphotransacetylase to an ADA strain provides an alternative, non-glycolytic route to generating AcCoA from glucose, reducing the NADH produced through glycolysis and improving redox balance. As shown in **FIG. 13B**, Y12745 (a strain which carries phosphoketolase and phosphotransacetylase in addition to the ADA) produces half as much glycerol as Y12869, while consuming comparable levels of glucose in a batch glucose fermentation.

6.2.2.3 The ATP savings in an ADA strain come at the cost of thermodynamic driving force, which is alleviated by a strong downstream pull on acetyl-CoA

[00260] The native PDH-bypass reaction for forming Acetyl-CoA is thermodynamically favorable because the reaction is coupled to the hydrolysis of ATP to AMP. In contrast, the acylating acetaldehyde dehydrogenase reaction is not coupled to ATP, and is much closer to equilibrium than the native PDH-bypass reactions for forming Acetyl-CoA. When using then native *S. cerevisiae* pathway genes for producing mevalonate, strains using the ADA produce much less mevalonate than strains using the wildtype PDH-bypass despite comparable kinetic properties of ADA and Ald6 *in vitro*. As shown in **FIG. 14** (1st and 2nd column), mevalonate production in an ADA strain (Y12869.ms63909) is only ~30% that of a wildtype equivalent strain (Y968.ms63909), despite sufficient kinetic capacity measured *in vitro*. This result reflects the lack of a thermodynamic driving force behind the conversion of acetaldehyde to acetyl-CoA by ADA.

[00261] The Erg10 acetyl-CoA thiolase catalyzes the formation of acetoacetyl-CoA from two acetyl-CoA, a reaction that is thermodynamically unfavorable. Acetoacetyl-CoA synthase (*i.e.*, acetyl-CoA:malonyl-CoA acyltransferase), encoded by *nphT7*, catalyzes the formation of acetoacetyl-CoA from acetyl-CoA and malonyl-CoA, a reaction that is thermodynamically favorable due to the decarboxylation of malonyl-CoA. Putting this thermodynamically favorable reaction directly downstream of AcCoA production provides a thermodynamic driving force that increases the forward activity of ADA. As shown in **FIG. 14** (3rd and 4th column), when *nphT7* is overexpressed in place of *ERG10*, Y968.ms63908 and Y12869.ms63908 make comparable levels of mevalonate. Moreover, they produce more substantially more mevalonate than equivalent strains which use *ERG10* for the first step of the MEV pathway (Y968.ms63909 and Y12869.63909.).

[00262] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. A genetically modified host cell capable of producing an isoprenoid, the cell comprising:

(a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate; and

(b) a heterologous nucleic acid encoding an acylating acetylaldehyde dehydrogenase (ADA);

wherein the host cell is a *Saccharomyces cerevisiae*.

2. The genetically modified host cell of claim 1, wherein the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA.

3. The genetically modified host cell of claim 1 or claim 2, wherein the one or more enzymes of the MEV pathway comprise an acetyl-CoA:malonyl-CoA acyltransferase.

4. The genetically modified host cell of any one of claims 1 to 3, wherein the one or more enzymes of the MEV pathway comprise an NADH-using enzyme that converts 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate.

5. The genetically modified host cell of any one of claims 1 to 4, wherein the one or more enzymes of the MEV pathway comprise an NADH-using HMG-CoA reductase.

6. The genetically modified host cell of any one of claims 1 to 5, further comprising a heterologous nucleic acid encoding a phosphoketolase (PK).

7. The genetically modified host cell of any one of claims 1 to 6, further comprising a heterologous nucleic acid encoding a phosphotransacetylase (PTA).

8. The genetically modified host cell of any one of claims 1 to 7, further comprising a functional disruption of one or more enzymes of the native pyruvate dehydrogenase (PDH) - bypass.

9. The genetically modified host cell of claim 8, wherein the one or more enzymes of the PDH-bypass are selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6).

10. The genetically modified host cell of claim 9, wherein ACS1 is functionally disrupted.

11. The genetically modified host cell of claim 9, wherein ACS2 is functionally disrupted.

12. The genetically modified host cell of claim 9, wherein ALD6 is functionally disrupted.

13. The genetically modified host cell of claim 9, wherein ACS1 and ACS2 are functionally disrupted.

14. The genetically modified host cell of claim 9, wherein ACS1, ACS2 and ALD6 are functionally disrupted.

15. The genetically modified host cell of any one of claims 1 to 14, further comprising a functional disruption of one or more enzymes having alcohol dehydrogenase (ADH) activity.

16. The genetically modified host cell of claim 15, wherein the one or more enzymes having ADH activity are selected from the group consisting of alcohol dehydrogenase 1 (ADH1), alcohol dehydrogenase 3 (ADH3), alcohol dehydrogenase 4 (ADH4), and alcohol dehydrogenase 5 (ADH5).

17. The genetically modified host cell of any one of claims 1 to 16, wherein the amino acid sequence of the ADA is at least 80% identical to SEQ ID NO:2.

18. The genetically modified host cell of claim 3, wherein the amino acid sequence of the acetyl-CoA:malonyl-CoA acyltransferase is at least 80% identical to SEQ ID NO:16.

19. The genetically modified host cell of claim 5, wherein the amino acid sequence of the NADH-using HMG-CoA reductase is at least 80% identical to SEQ ID NO:20.

20. The genetically modified host cell of claim 6, wherein the amino acid sequence of the phosphoketolase (PK) is at least 80% identical to SEQ ID NO:12.

21. The genetically modified host cell of claim 7, wherein the amino acid sequence of the phosphotransacetylase (PTA) is at least 80% identical to SEQ ID NO:14.

22. The genetically modified host cell of any one of claims 1, 4 to 17, and 19 to 21, wherein the one or more enzymes of the MEV pathway comprise an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA.

23. The genetically modified host cell of any one of claims 1 to 22, wherein the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetoacetyl-CoA with acetyl-CoA to form HMG-CoA.

24. The genetically modified host cell of any one of claims 1 to 23, wherein the one or more enzymes of the MEV pathway comprise an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate.

25. The genetically modified host cell of any one of claims 1 to 24, wherein the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate.

26. The genetically modified host cell of any one of claims 1 to 25, wherein the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate.

27. The genetically modified host cell of any one of claims 1 to 26, wherein the host cell comprises a plurality of heterologous nucleic acids encoding all of the enzymes of the MEV pathway.

28. The genetically modified host cell of any one of claims 1 to 27, wherein the one or more heterologous nucleic acids encoding one or more enzymes of the MEV pathway are under control of a single transcriptional regulator.

29. The genetically modified host cell of any one of claims 1 to 27, wherein the one or more heterologous nucleic acids encoding one or more enzymes of the MEV pathway are under control of multiple heterologous transcriptional regulators.

30. The genetically modified host cell of any one of claims 1 to 29, further comprising a heterologous nucleic acid encoding an enzyme that can convert isopentenyl pyrophosphate (IPP) into dimethylallyl pyrophosphate (DMAPP).

31. The genetically modified host cell of any one of claims 1 to 30, further comprising a heterologous nucleic acid encoding an enzyme that can condense IPP and/or DMAPP molecules to form a polyprenyl compound.

32. The genetically modified host cell of any one of claims 1 to 31, further comprising a heterologous nucleic acid encoding an enzyme that can modify IPP or a polyprenyl to form an isoprenoid compound.

33. The genetically modified host cell of claim 32, wherein the enzyme that can modify IPP or a polyprenyl to form an isoprenoid compound is selected from the group consisting of carene synthase, geraniol synthase, linalool synthase, limonene synthase, myrcene synthase, ocimene synthase, α-pinene synthase, β-pinene synthase, γ-terpinene synthase, terpinolene synthase, amorphadiene synthase, α-farnesene synthase, β-farnesene synthase, farnesol synthase, nerolidol synthase, patchouliol synthase, nootkatone synthase, and abietadiene synthase.

34. The genetically modified host cell of claim 32, wherein the isoprenoid is selected from the group consisting of a hemiterpene, monoterpene, diterpene, triterpene, tetraterpene, sesquiterpene, and polyterpene.

35. The genetically modified host cell of claim 32, wherein the isoprenoid is a sesquiterpene.

36. The genetically modified host cell of claim 32, wherein the isoprenoid is a C_5-C_{20} isoprenoid.

37. The genetically modified host cell of claim 32, wherein the isoprenoid is selected from the group consisting of abietadiene, amorphadiene, carene, α-farnesene, β-farnesene, farnesol, geraniol, geranylgeraniol, isoprene, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, β-pinene, sabinene, γ-terpinene, terpinolene, and valencene.

38. The genetically modified host cell of any one of claims 1 to 37, wherein the yeast is a strain of *Saccharomyces cerevisiae* selected from the group consisting of Baker's yeast, CBS 7959, CBS 7960, CBS 7961, CBS 7962, CBS 7963, CBS 7964, IZ-1904, TA, BG-1, CR-1, SA-1, M-26, Y-904, PE-2, PE-5, VR-1, BR-1, BR-2, ME-2, VR-2, MA-3, MA-4, CAT-1, CB-1, NR-1, BT-1, and AL-1.

39. A genetically modified host cell capable of producing an isoprenoid, the cell comprising:

(a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate;

(b) a heterologous nucleic acid encoding an acylating acetylaldehyde dehydrogenase (ADA);

(c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6)

(d) a heterologous nucleic acid encoding a phosphoketolase (PK); and

(e) a heterologous nucleic acid encoding a phosphoketolase (PTA);

wherein the host cell is a *Saccharomyces cerevisiae*.

40. A genetically modified host cell capable of producing an isoprenoid, the cell comprising:

(a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate, wherein the one or more enzymes comprise a NADH-using HMG-CoA reductase;

(b) a heterologous nucleic acid encoding an acylating acetylaldehyde dehydrogenase (ADA); and

(c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6);

wherein the host cell is a *Saccharomyces cerevisiae*.

41. A genetically modified host cell capable of producing an isoprenoid, the cell comprising:

(a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate, wherein the one or more enzymes comprise a NADH-using HMG-CoA reductase;

(b) a heterologous nucleic acid encoding an acylating acetylaldehyde dehydrogenase (ADA);

(c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6);

(d) a heterologous nucleic acid encoding a phosphoketolase (PK); and

(e) a heterologous nucleic acid encoding a phosphoketolase (PTA);

wherein the host cell is a *Saccharomyces cerevisiae*.

42. A genetically modified host cell capable of producing an isoprenoid, the cell comprising:

(a) one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate, wherein the one or more enzymes comprise an acetyl-CoA:malonyl-CoA acyltransferase;

(b) a heterologous nucleic acid encoding acylating acetylaldehyde dehydrogenase (ADA); and

(c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6);

wherein the host cell is a *Saccharomyces cerevisiae*.

43. A genetically modified host cell capable of producing an isoprenoid, the cell comprising:

(a) one or more heterologous nucleic acids encoding a plurality of enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate, wherein the plurality of enzymes comprise an acetyl-CoA:malonyl-CoA acyltransferase and an NADH-using HMG-CoA reductase;

(b) a heterologous nucleic acid encoding an acylating acetylaldehyde dehydrogenase (ADA);

(c) a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthase 1 (ACS1), acetyl-CoA synthase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6);

(d) a heterologous nucleic acid encoding a phosphoketolase (PK); and

(e) a heterologous nucleic acid encoding a phosphoketolase (PTA);

wherein the host cell is a *Saccharomyces cerevisiae*.

44. A method for producing an isoprenoid comprising:

(a) culturing a population of the genetically modified host cells of any one of claims 1 to 43 in a medium with a carbon source under conditions suitable for making said isoprenoid compound; and

(b) recovering said isoprenoid compound from the medium.

45. The method of claim 44, wherein the isoprenoid compound is produced in an amount greater than about 10 grams per liter of medium.

46. The method of claim 44, wherein the isoprenoid compound is produced in an amount greater than about 50 mg per gram of dry cell weight.

47. The method of any one of claims 44 to 46, wherein the host cell produces an increased amount of an isoprenoid compound compared to a same host cell not comprising the heterologous nucleotide sequence encoding said ADA.

48. The method of claim 47, wherein said increased amount is at least 10%.

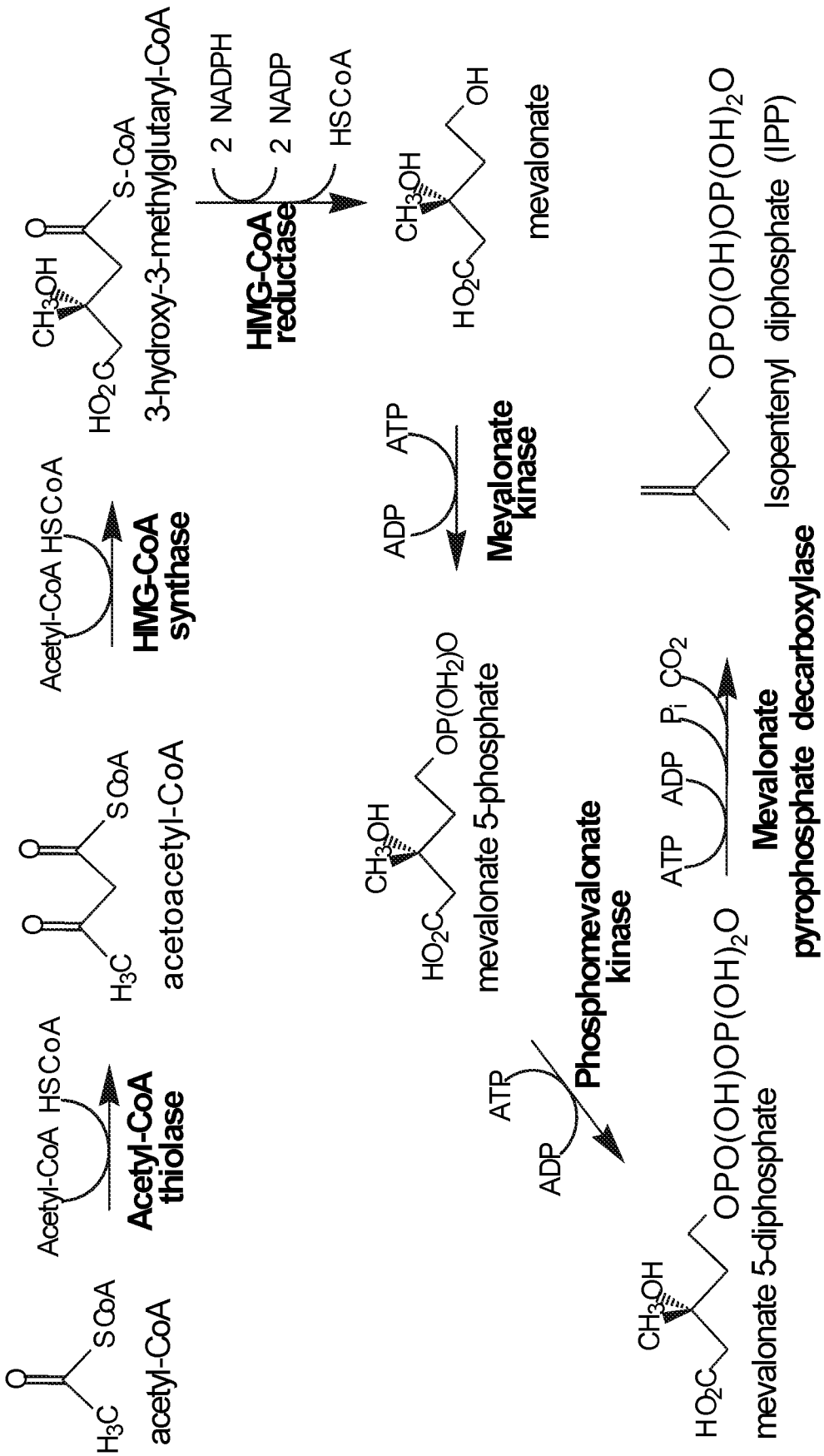

acetyl-CoA
Acetyl-CoA HSCoA
Acetyl-CoA thiolase
acetoacetyl-CoA
Acetyl-CoA HSCoA
HMG-CoA synthase
3-hydroxy-3-methylglutaryl-CoA
2 NADPH
2 NADP
HSCoA
HMG-CoA reductase
mevalonate
ADP ATP
Mevalonate kinase
mevalonate 5-phosphate
ATP
ADP
Phosphomevalonate kinase
mevalonate 5-diphosphate
ATP ADP Pi CO2
Mevalonate pyrophosphate decarboxylase
Isopentenyl diphosphate (IPP)

FIG. 1

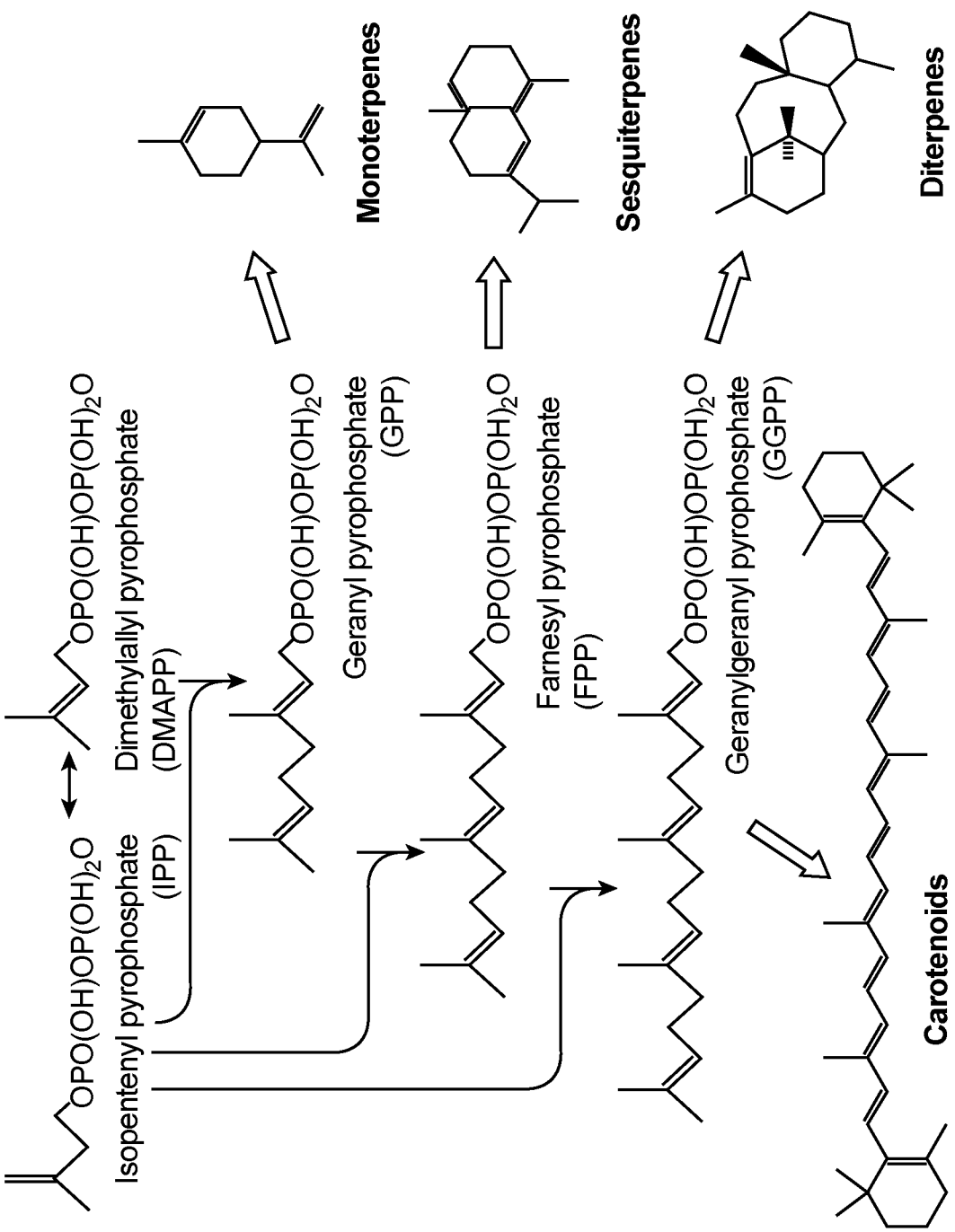

OPO(OH)OP(OH)$_2$O
Isopentenyl pyrophosphate
(IPP)
OPO(OH)OP(OH)$_2$O
Dimethylallyl pyrophosphate
(DMAPP)
OPO(OH)OP(OH)$_2$O
Geranyl pyrophosphate
(GPP)
OPO(OH)OP(OH)$_2$O
Farnesyl pyrophosphate
(FPP)
OPO(OH)OP(OH)$_2$O
Geranylgeranyl pyrophosphate
(GGPP)
Monoterpenes
Sesquiterpenes
Diterpenes
Carotenoids

FIG. 2

Pathway	Carbon in	Carbon out	ATP	NADH	NADPH
Glycolysis	4.8 Glucose	9.6 Pyruvate	+9.6	+9.6	0
PDH bypass	9 Pyruvate	9 Acetyl-CoA	-18	0	+9
TCA	0.6 Pyruvate	-	+0.6	+3	0
Isoprenoid	9 Acetyl-CoA	1 Farnesene	-9	0	-6
Respiration	-	-	+16.8	-12.6	-3
Overall	4.8 Glucose	1 Farnesene	0	0	0

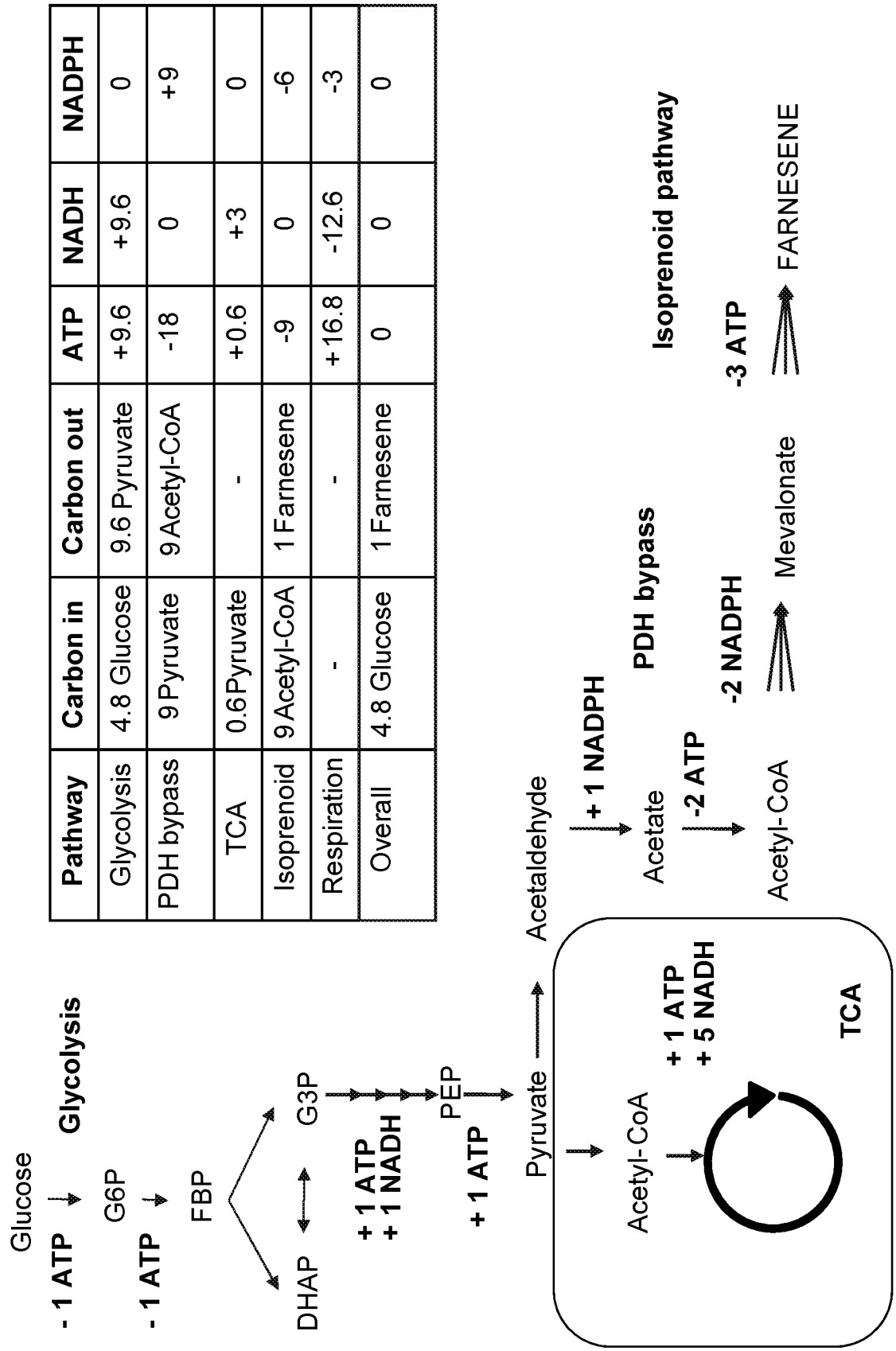


FIG. 3

Pathway	Carbon in	Carbon out	ATP	NADH	NADPH
Glycolysis	4.5 Glucose	9 Pyruvate	+9	+9	0
PDH bypass	9 Pyruvate	9 Acetyl-CoA	0	+9	0
TCA	-	-	0	0	0
Isoprenoid	9 Acetyl-CoA	1 Farnesene	-9	-6	0
Respiration	-	-	+13.5	-12.6	0
Overall	4.5 Glucose	1 Farnesene	+ 14.1	0	0

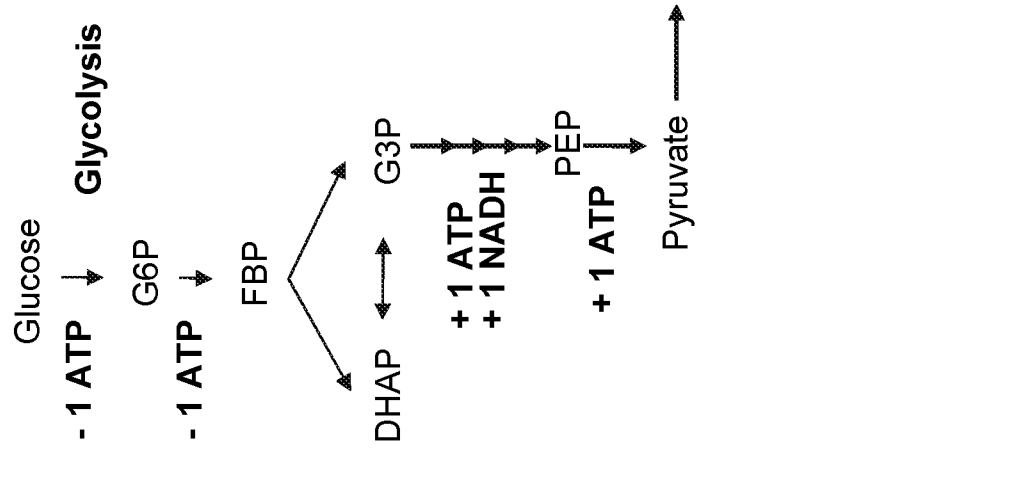


FIG. 4

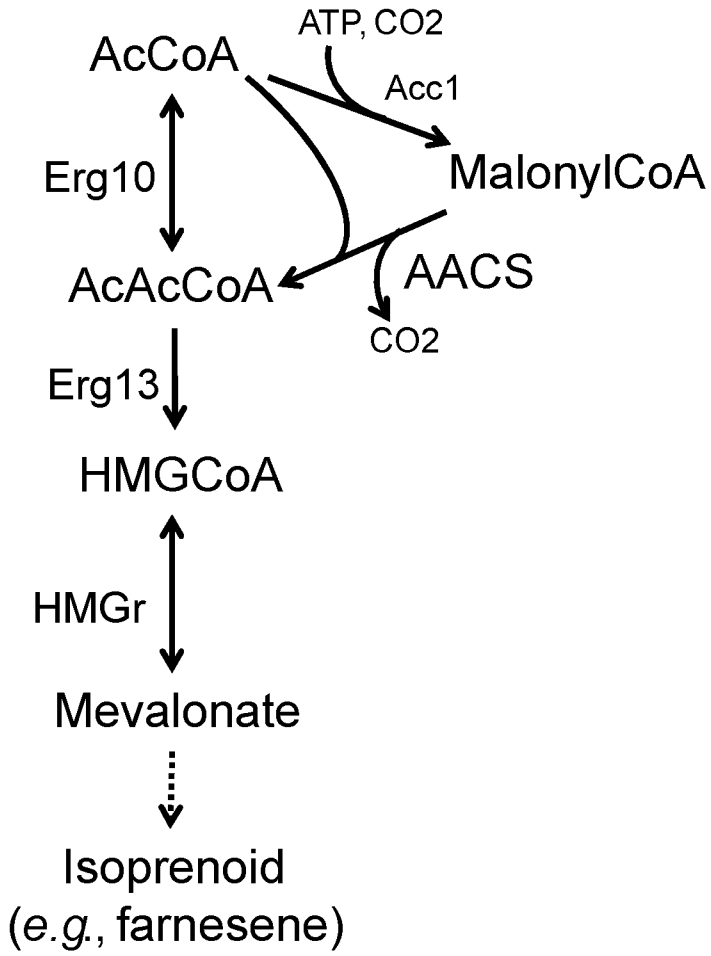

AcCoA
ATP, CO2
Acc1
MalonylCoA
Erg10
AcAcCoA
AACS
CO2
Erg13
HMGCoA
HMGr
Mevalonate
Isoprenoid
(e.g., farnesene)

FIG. 5

Pathway	Carbon in	Carbon out	ATP	NADH	NADPH
Glycolysis	4.5 Glucose	9 Pyruvate	+9	+9	0
PDH bypass	9 Pyruvate	9 Acetyl-CoA	0	+9	0
TCA	-	-	0	0	0
Isoprenoid	9 Acetyl-CoA	1 Farnesene	-12	-6	0
Respiration	-	-	+13.5	-12.6	0
Overall	4.5 Glucose	1 Farnesene	+ 11.1	0	0

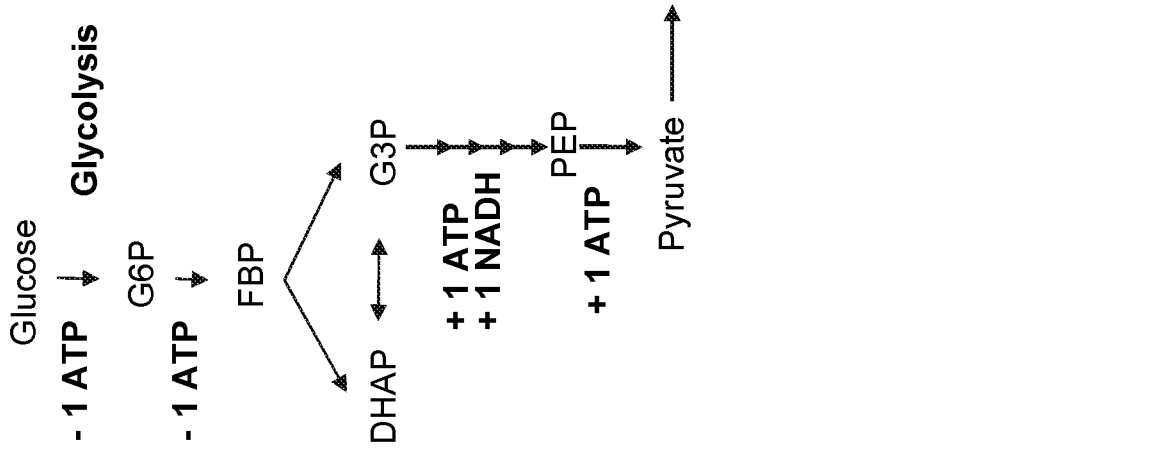


FIG. 6

Pathway	Carbon in	Carbon out	ATP	NADH	NADPH
Glycolysis	3.8 Glucose	4.9 Pyruvate	+4.9	+4.9	0
PDH bypass	4.9 Pyruvate	4.9 Acetyl-CoA	0	+4.9	0
PK/PTA	1.6 F6P	4.9 Acetyl-CoA	0	0	0
Isoprenoid	9 Acetyl-CoA	1 Farnesene	-9	-6	0
Respiration	-	-	+4.2	-3.8	0
Overall	3.8 Glucose	1 Farnesene	0	0	0

Max Stoichiometric Yield = 29.8 wt%

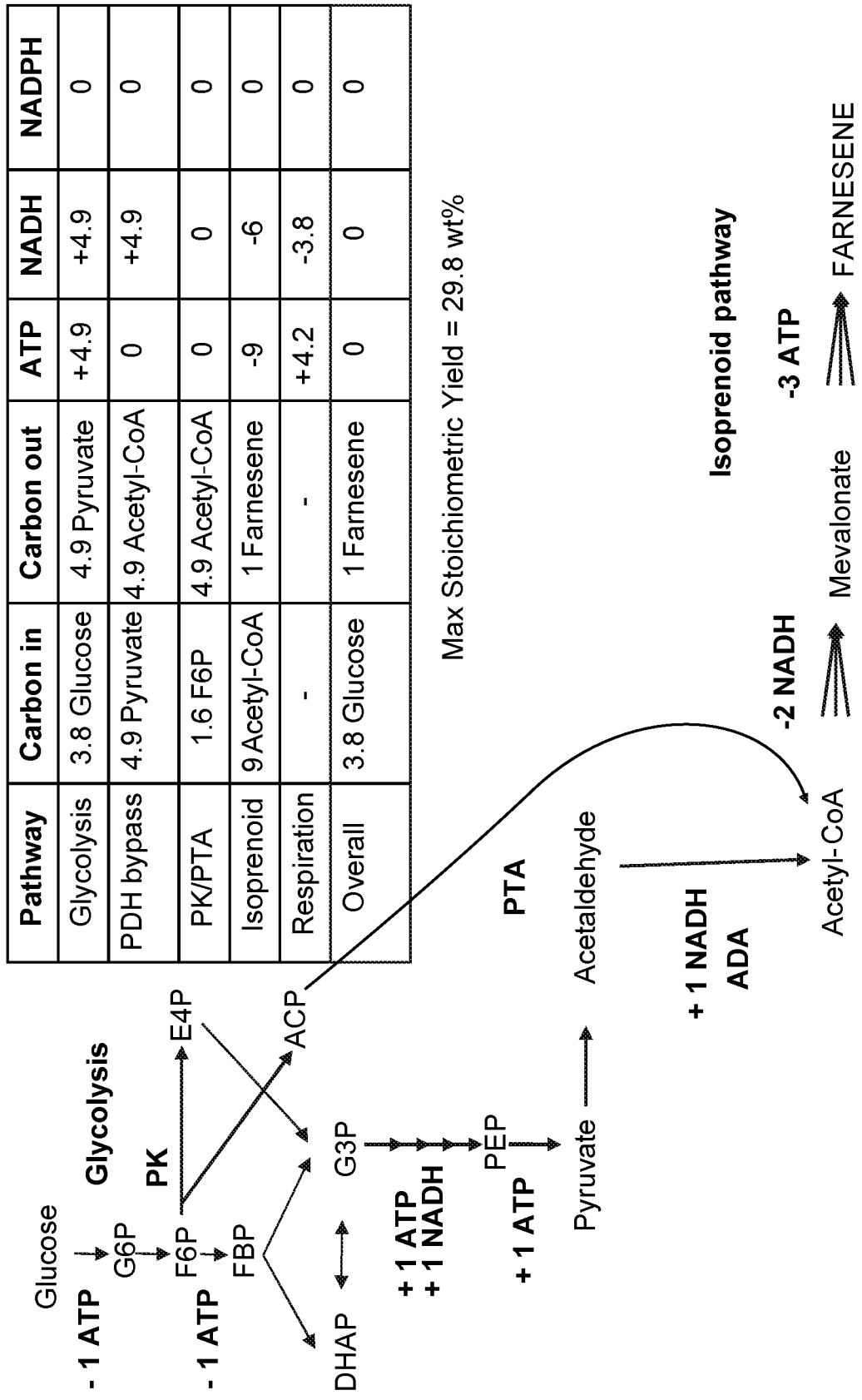


FIG. 7

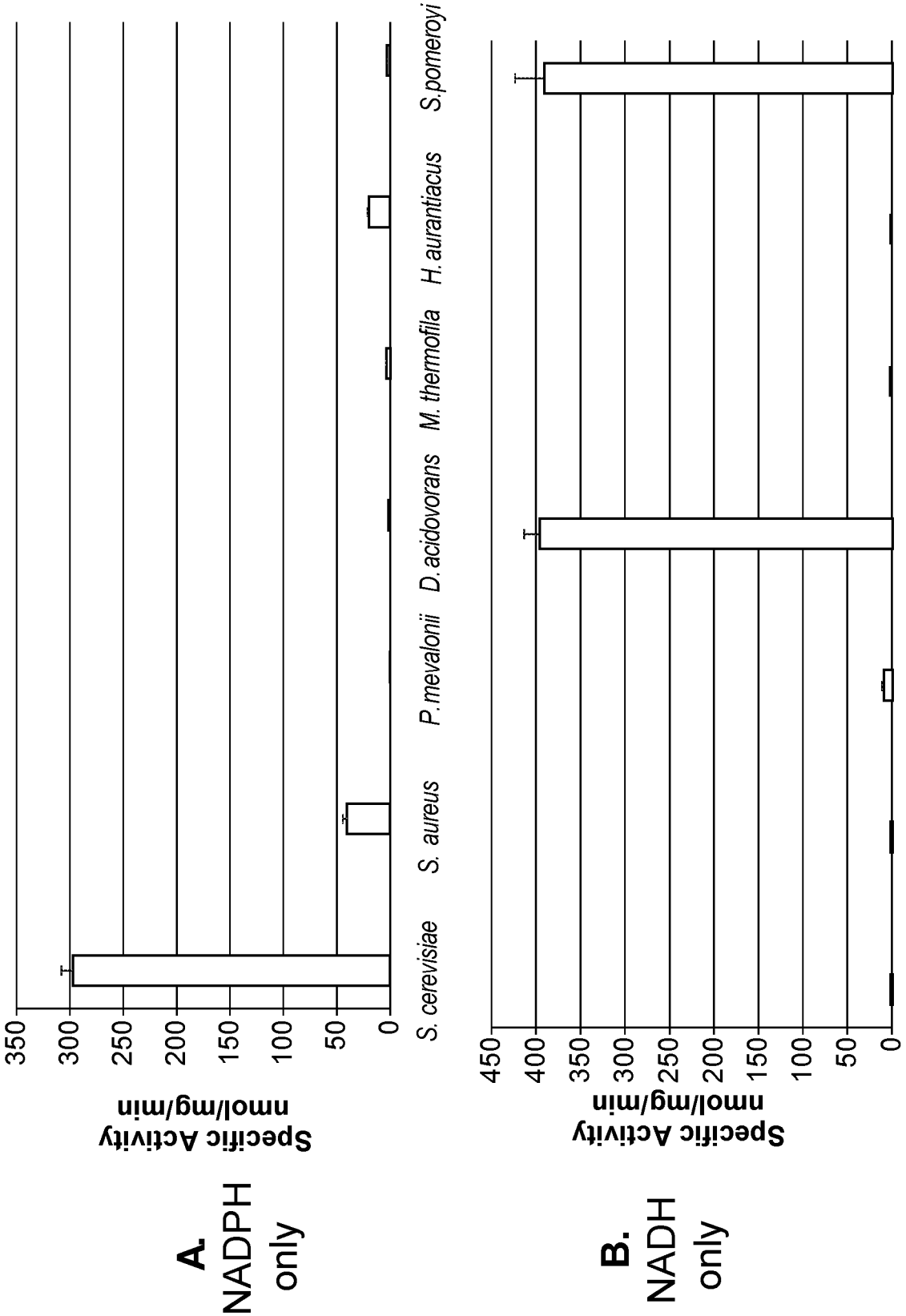

A.
NADPH
only
Specific Activity
nmol/mg/min
350
300
250
200
150
100
50
0
S. cerevisiae
S. aureus
P.mevalonii
D.acidovorans
M. thermofila
H.aurantiacus
S.pomeroyi
B.
NADH
only
Specific Activity
nmol/mg/min
450
400
350
300
250
200
150
100
50
0

FIG. 8

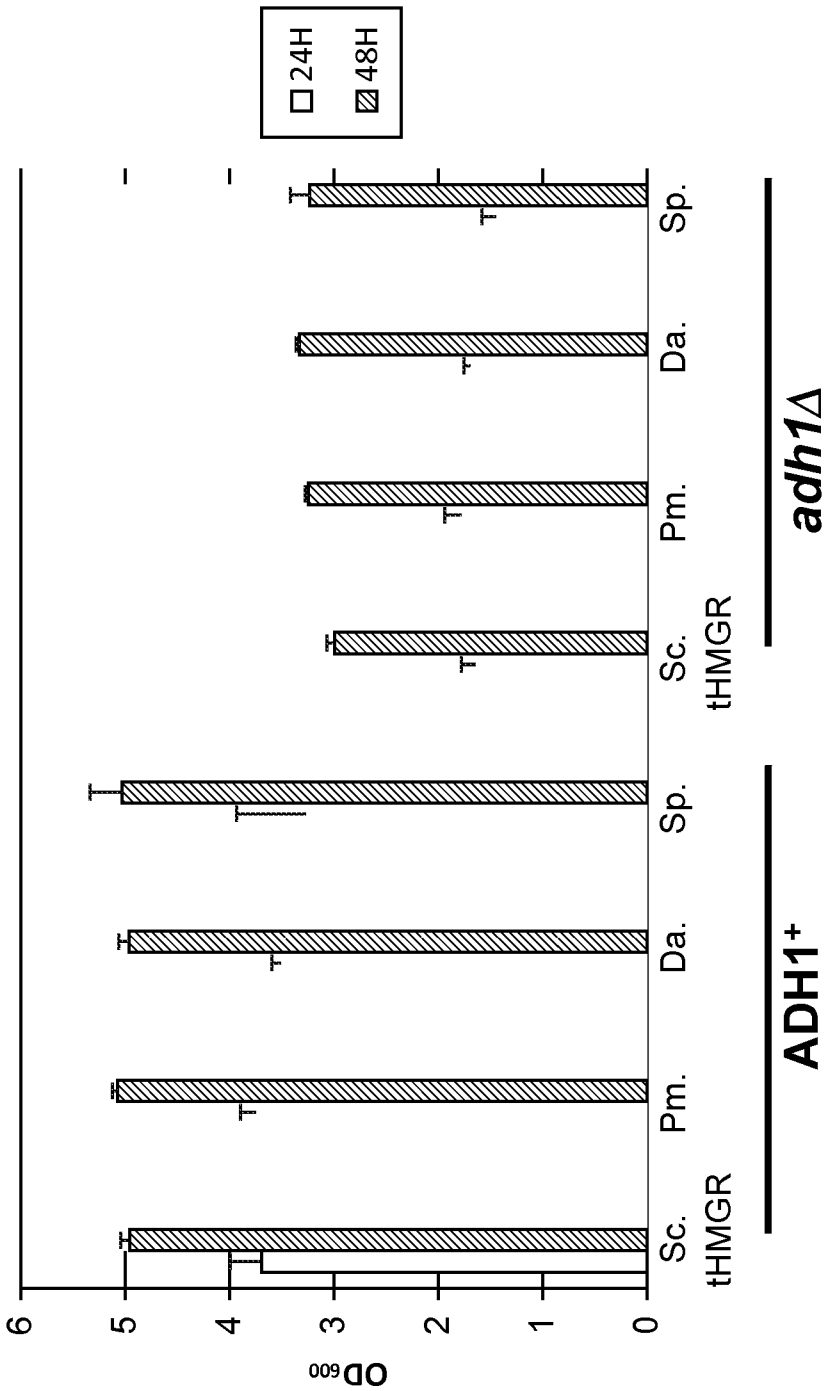


FIG. 9

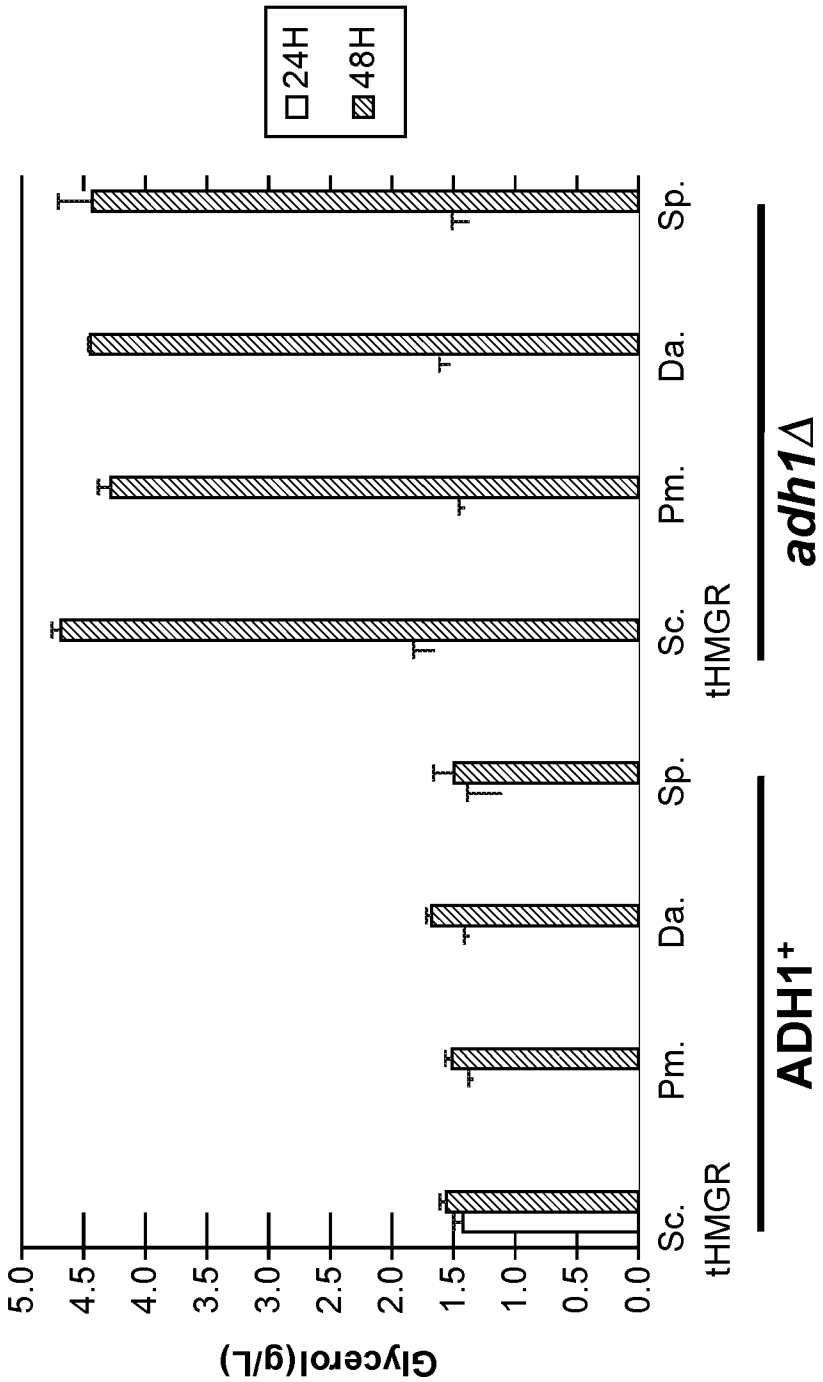

24H
48H
Glycerol(g/L)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Sc. tHMGR
Pm.
Da.
Sp.
Sc. tHMGR
Pm.
Da.
Sp.
ADH1+
adh1Δ

FIG. 10

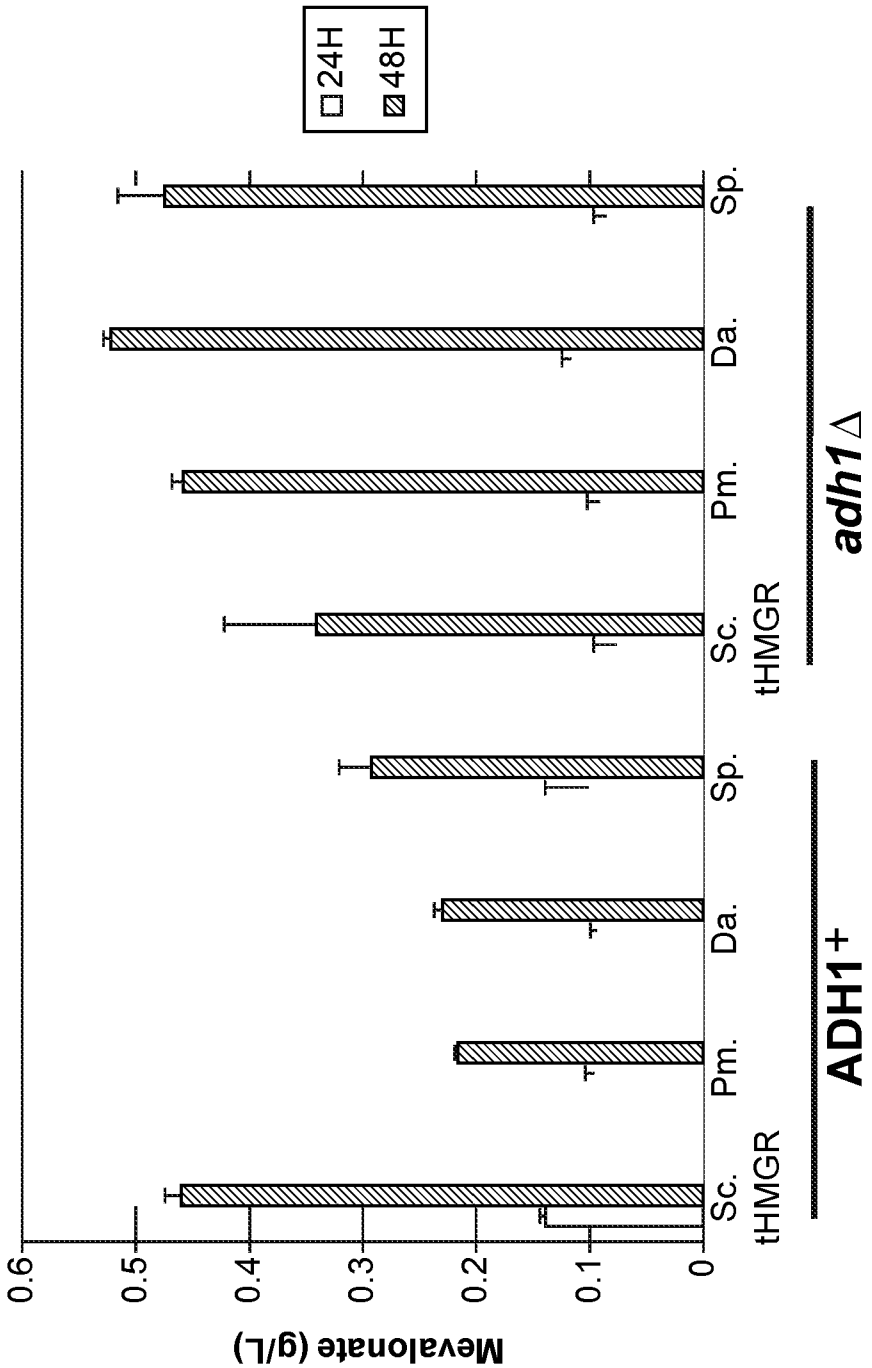
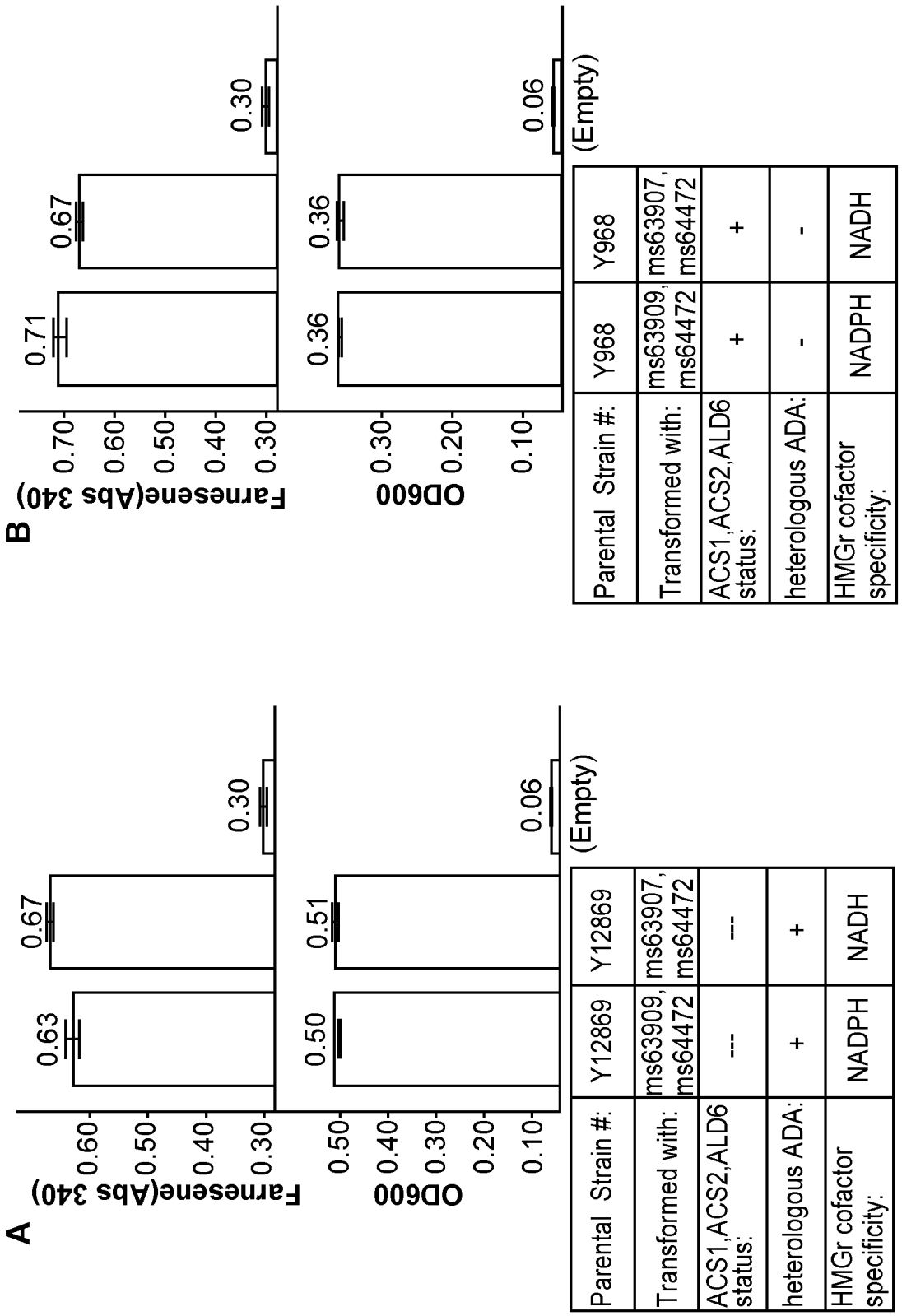

24H
48H
Mevalonate (g/L)
0.6
0.5
0.4
0.3
0.2
0.1
0
Sc. tHMGR
Pm.
Da.
Sp.
ADH1+
Sc. tHMGR
Pm.
Da.
Sp.
adh1Δ

FIG. 11



Parental Strain #:	Y12869	Y12869	Y968	Y968
Transformed with:	ms63909, ms64472	ms63907, ms64472	ms63909, ms64472	ms63907, ms64472
ACS1,ACS2,ALD6 status:	---	---	+	+
heterologous ADA:	+	+	-	-
HMGr cofactor specificity:	NADPH	NADH	NADPH	NADH

FIG. 12

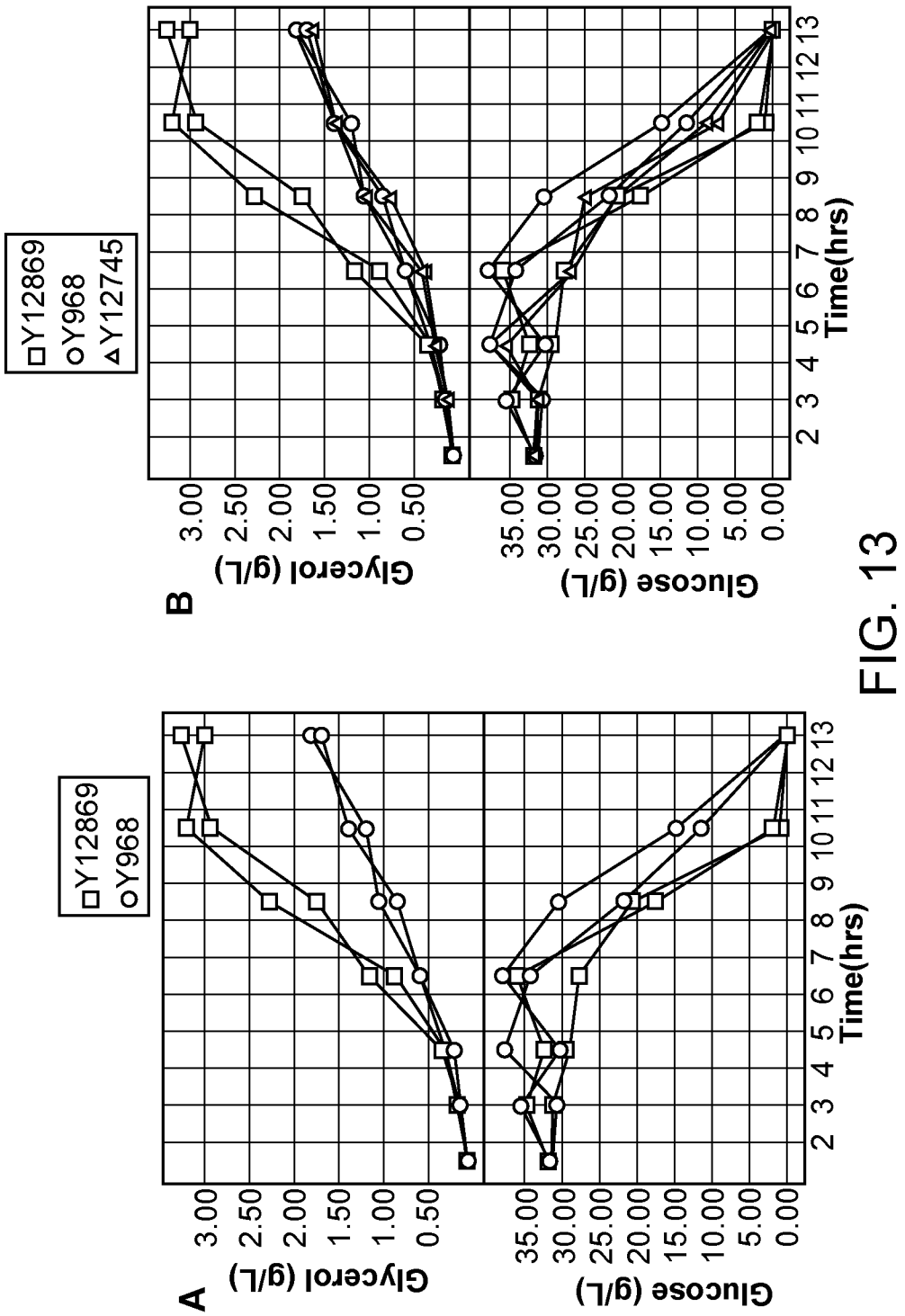
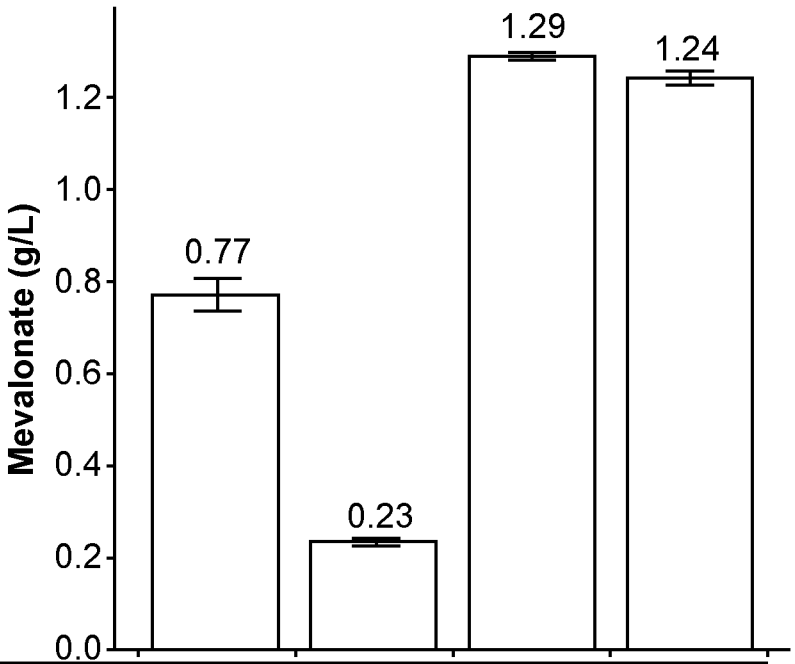
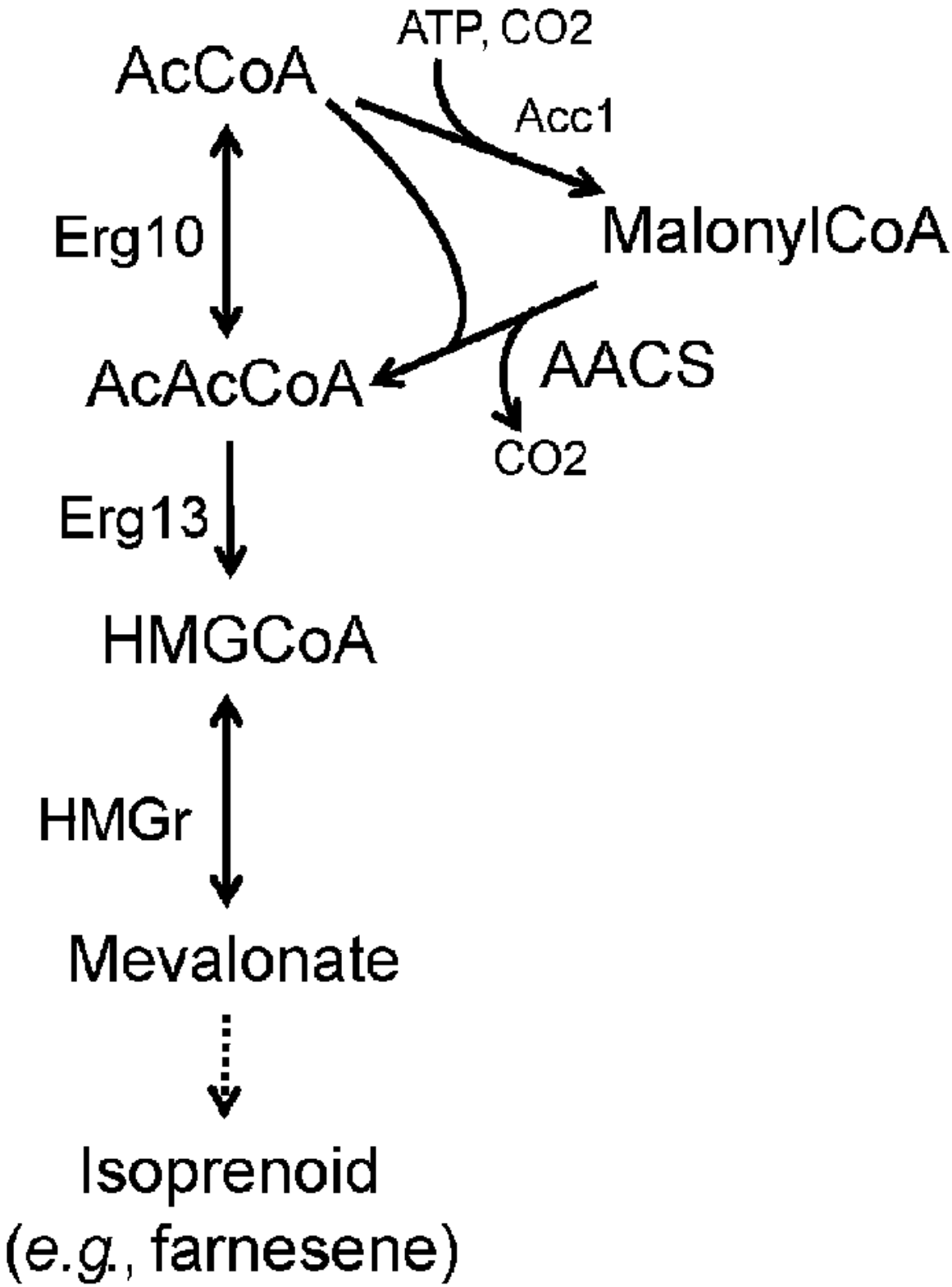

□Y12869
○Y968
△Y12745
B
Glycerol (g/L)
3.00
2.50
2.00
1.50
1.00
0.50
Glucose (g/L)
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
2 3 4 5 6 7 8 9 10 11 12 13
Time(hrs)
□Y12869
○Y968
A
Glycerol (g/L)
Glucose (g/L)
Time(hrs)

FIG. 13



Parental Strain #:	Y968	Y12869	Y968	Y12869
Transformed with:	ms63909	ms63909	ms63908	ms63908
ACS1,ACS2,ALD6 status:	+	---	+	---
heterologous ADA:	-	+	-	+
Acetyl-CoA to acetoacetyl-CoA via:	ERG10	ERG10	nphT7	nphT7

FIG. 14


ATP, CO2
AcCoA
Acc1
Erg10
MalonylCoA
AcAcCoA
AACS
CO2
Erg13
HMGCoA
HMGr
Mevalonate
Isoprenoid
(e.g., farnesene)