Abstract:

Provided herein are compositions and methods for improved production of acetyl-CoA and acetyl-CoA derived compounds in a host cell. In some embodiments, the host cell is genetically modified to comprise a heterologous nucleotide sequence encoding a phosphoketolase (PK), and a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate. In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding a phosphotransacetylase (PTA). In some embodiments, the enzyme that converts acetyl phosphate to acetate is a glycerol-3-phosphatase. In some embodiments, the glycerol-3-phosphatase is GPP/WRH2. In some embodiments, the glycerol-3-phosphatase is GPP2/HOR2. The compositions and methods described herein provide an efficient route for the heterologous production of acetyl-CoA-derived compounds, including but not limited to, isoprenoids, polyketides, and fatty acids.

Title: USE OF PHOSPHOKETOLASE AND PHOSPHOTRANSACETYLASE FOR PRODUCTION OF ACetyl-COENZYME A DERIVED COMPOUNDS

Figure 1

[Continued on nextpage]
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USE OF PHOSPHOKETOLASE AND PHOSPHOTRANSACETYLASE FOR PRODUCTION OF ACETYL-COENZYME A DERIVED COMPOUNDS

[0001] This application claims benefit of priority of U.S. Provisional Application No. 61/800,356, filed on March 15, 2013, the contents of which are hereby incorporated by reference in their entirety.

1. FIELD OF THE INVENTION

[0002] The present disclosure relates to compositions and methods for producing acetyl-CoA derived compounds 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. In yeast, acetyl-CoA is biosynthesized from pyruvate metabolism (FIG. 1). However, in this biosynthetic pathway, CO₂ is lost via the reactions catalyzed by pyruvate carboxylase and/or pyruvate dehydrogenase. In an industrial fermentation setting, one benefit of providing an alternative to pyruvate metabolism and lower glycolysis is that less CO₂ is produced in the decarboxylation of pyruvate, 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. This can be accomplished by expressing phosphoketolase (PK; EC 4.1.2.9) in conjunction with phosphoacetyltransferase (PTA; EC 2.3.1.8).

[0004] PK and PTA catalyze the reactions to convert fructose-6-phosphate (F6P) or xylulose-5-phosphate (X5P) to acetyl-CoA. As shown in FIG. 1, 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) and acetyl phosphate. 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.

[0005] The applicants have previously described the improved efficiency of heterologous isoprenoid production that can be gained with the introduction of PK and PTA enzymes. See U.S. Application No. 13/673,819 (now U.S. Patent No. 8,415,136), filed on...
November 9, 2012, the contents of which are hereby incorporated by reference in their entirety. In particular, 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 the isoprenoid farnesene via the mevalonate pathway is 23.6 wt%. By including the reactions catalyzed by acetaldehyde dehydrogenase, acetylated (ADA; EC 1.2.1.10) and NADH-using HMG-CoA reductase into the metabolic network for mevalonate production, the maximum theoretical stoichiometric yield is improved to 25.2 wt%. With the further introduction of PK and PTA, the reaction network, at optimality, is able to reach 29.8 wt% mass yield or greater, a significant increase in maximum theoretical yield.

Sondregger et al. have also described the benefits of PK and PTA with respect to ethanol production in a xylose-utilizing yeast strain. See Sondregger et al., Applied and Environmental Microbiology 70(5):2892-2897 (2004), the contents of which are hereby incorporated by reference in their entirety. The heterologous phosphoketolase pathway (PK, PTA, and ADA) was introduced in *S. cerevisiae* to address low ethanol yields that result from overexpression of NAD(P)H-dependent xylose reductase and NAD+-dependent xylitol dehydrogenase from *Pichia stipitis*. The different cofactor preferences in the two oxidoreductase reactions caused an anaerobic redox balancing problem that manifested in the extensive accumulation of the reduced reaction intermediate xylitol, and thus, low ethanol yields. Redox metabolism was balanced by introducing the phosphoketolase pathway, which lead to the net reoxidation of one NADH per xylose converted to ethanol, and an improvement in ethanol yield by 25%. However, overexpression of PK also leads to an increase in acetate accumulation and a reduction in fermentation rate. Although some acetate accumulation could be reduced by combining the phosphoketolase pathway with a mutation of ALD6, which converts acetaldehyde to acetate, the flux through the recombinant phosphoketolase pathway was about 30% of the optimum flux that would be required to completely eliminate xylitol and glycerol accumulation. The authors suggested that higher activities of phosphotransacetylase and/or acetaldehyde dehydrogenase may be necessary to prevent phosphoketolase pathway-based acetate formation.

Thus, while the introduction of a heterologous PK pathway can lead to substantial improvements in the yields of acetyl-CoA derived compounds, further improvements in the implementation of this pathway appear to be required to achieve optimal
carbon flux through PK and PTA. The compositions and methods provided herein address this need and provide related advantages as well.

3. SUMMARY OF THE INVENTION

[0008] Provided herein are compositions and methods for the improved utilization of phosphoketolase (PK) and phosphotransacetylase (PTA) for the production of industrially useful compounds. These compositions and methods are based on the surprising discovery that phosphoketolase pathway-based acetate accumulation results from the enzyme-catalyzed hydrolysis of acetyl phosphate, the product of PK catalysis. Hydrolysis of acetyl phosphate is an undesirable side-reaction that can negatively impact production, via depletion of carbon, of any type of product derived from acetyl-CoA, including isoprenoids, polyketides, and fatty acids. By functionally disrupting native enzymes in the host cell that catalyze acetyl phosphate hydrolysis, acetate accumulation is reduced and carbon flux through the PK/PTA pathway towards acetyl-CoA production is increased.

[0009] The compositions and methods provided herein are further based on the unexpected discovery of native enzymes in yeast that catalyze the hydrolysis of acetyl phosphate to acetate, namely GPP1/RHR2, and its closely related homolog GPP2/HOR2. Both of these enzymes have only been previously characterized as having glycerol-1-phosphatase (EC 3.1.3.21; alternately referred to as "glycerol-3-phosphatase") activity, and thus, the promiscuous acetyl-phosphatase activity of these enzymes is unexpected. In cells heterologously expressing PK and PTA, deletion of one or both of the genes encoding RHR2 and HOR2 leads to a reduction in acetate accumulation, with deletion of the gene encoding RHR2 alone leading to a substantial reduction in acetate levels. Moreover, deletion of the RHR2 gene in cells engineered to comprise PK, PTA and a mevalonate pathway resulted in a substantial increase in the production of farnesene, an acetyl-CoA derived isoprenoid.

[0010] Thus, provided herein are genetically modified host cells and methods of their use for the production of industrially useful compounds. In one aspect, provided herein is a genetically modified host cell comprising: a heterologous nucleic acid encoding a phosphoketolase (PK; EC 4.1.2.9); and a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate. In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding a phosphotransacetylase (PTA; EC 2.3.1.8).

[0011] In another aspect, provided herein is a genetically modified host cell comprising: a heterologous nucleic acid encoding a phosphotransacetylase (PTA; EC
2.3.1.8); and a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate. In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding a phosphoketolase (PK; EC 4.1.2.9).

In some embodiments, the enzyme that converts acetyl phosphate to acetate is a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is selected from the group consisting of GPP1/RHR2, GPP2HOR2, and homologues and variants thereof. In some embodiments, the genetically modified host cell comprises a functional disruption of GPP1/RHR2. In some embodiments, the genetically modified host cell comprises a functional disruption of GPP2/HOR2. In some embodiments, the genetically modified host cell comprises a functional disruption of both GPP1/RHR2 and GPP2/HOR2.

In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding an acetylating acetylaldehyde dehydrogenase (ADA; EC 1.2.1.10). 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 synthetase 1 (ACS1), acetyl-CoA synthetase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6).

In some embodiments, the genetically modified host cell is capable of producing a heterologous acetyl-CoA derived compound. In some embodiments, the heterologous acetyl-CoA derived compound is selected from the group consisting of an isoprenoid, a polyketide, and a fatty acid. In particular embodiments, the genetically modified host cell is capable of producing an isoprenoid.

In some embodiments, the genetically modified host cell comprises one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate. In some embodiments, the one or more enzymes of the MEV pathway comprise an NADH-using HMG-CoA reductase. 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. 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. 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 comprises a heterologous nucleic acid encoding an enzyme that can modify IPP or a polyprenyl to form an isoprenoid compound.

[0016] 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, a-pinene synthase, β-pinene synthase, γ-terpinene synthase, terpinolene synthase, amorphadiene synthase, a-farnesene synthase, β-farnesene synthase, farnesol synthase, nerolidol synthase, patchouliol synthase, nootkatone synthase, and abietadiene synthase.

[0017] In some embodiments, the isoprenoid is selected from the group consisting of a hemiterpene, monoterpenes, diterpene, triterpene, tetraterpene, sesquiterpene, and polyterpene. In some embodiments, the isoprenoid is a sesquiterpene. 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, a-farnesene, β-farnesene, farnesol, geraniol, geranylgeraniol, isoprene, linalool, limonene, myrcene, nerolidol, ocimene, patchouliol, β-pinene, sabinene, γ-terpinene, terpinolene, and valencene.
In another aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate; a heterologous nucleic acid encoding a phosphoketolase (PK); a heterologous nucleic acid encoding a phosphotransacetylase (PTA); and a functional disruption of a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is GPP1/RHR2, or a homologue or variant thereof. In some embodiments, the glycerol-1-phosphatase is GPP2/HOR2, or a homologue or variant thereof.

In another aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate; a heterologous nucleic acid encoding an acetylaldehyde dehydrogenase, acetylating (ADA); a heterologous nucleic acid encoding a phosphoketolase (PK); a heterologous nucleic acid encoding a phosphotransacetylase (PTA); and a functional disruption of a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is GPP1/RHR2, or a homologue or variant thereof. In some embodiments, the glycerol-1-phosphatase is GPP2/HOR2, or a homologue or variant thereof.

In another aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate; a heterologous nucleic acid encoding an acetylaldehyde dehydrogenase, acetylating (ADA); a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthetase 1 (ACSI), acetyl-CoA synthetase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6); a heterologous nucleic acid encoding a phosphoketolase (PK); a heterologous nucleic acid encoding a phosphotransacetylase (PTA); and a functional disruption of a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is GPP1/RHR2, or a homologue or variant thereof. In some embodiments, the glycerol-1-phosphatase is GPP2/HOR2, or a homologue or variant thereof.

In another aspect, provided herein is a genetically modified host cell capable of producing an isoprenoid, the cell comprising: 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; a heterologous nucleic acid encoding an acetylaldehyde dehydrogenase, acetylating (ADA); a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthetase 1 (ACSI), acetyl-CoA synthetase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6); a heterologous nucleic acid encoding a phosphoketolase (PK); a heterologous nucleic acid encoding a phosphotransacetylase (PTA); and a functional disruption of a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is GPP1/RHR2, or a homologue or variant thereof. In some embodiments, the glycerol-1-phosphatase is GPP2/HOR2, or a homologue or variant thereof.

[0022] In another aspect, provided herein is genetically modified host cell capable of producing an isoprenoid, the cell comprising: 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; a heterologous nucleic acid encoding an acetylaldehyde dehydrogenase, acetylating (ADA); a functional disruption of at least one enzyme of the native PDH-bypass selected from the group consisting of acetyl-CoA synthetase 1 (ACSI), acetyl-CoA synthetase 2 (ACS2), and aldehyde dehydrogenase 6 (ALD6); a heterologous nucleic acid encoding a phosphoketolase (PK); a heterologous nucleic acid encoding a phosphotransacetylase (PTA); and a functional disruption of a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is GPP1/RHR2, or a homologue or variant thereof. In some embodiments, the glycerol-1-phosphatase is GPP2/HOR2, or a homologue or variant thereof.

[0023] In another aspect, provided herein is a genetically modified host cell capable of producing a polyketide, the cell comprising: one or more heterologous nucleic acids encoding one or more enzymes of polyketide biosynthetic pathway; a heterologous nucleic acid encoding a phosphoketolase (PK); a heterologous nucleic acid encoding a phosphotransacetylase (PTA); and a functional disruption of a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is GPP1/RHR2, or a homologue or variant thereof. In some embodiments, the glycerol-1-phosphatase is GPP2/HOR2, or a homologue or variant thereof.

[0024] In another aspect, provided herein is a genetically modified host cell capable of producing a fatty acid, the cell comprising: one or more heterologous nucleic acids encoding one or more enzymes of fatty acid biosynthetic pathway; a heterologous nucleic
acid encoding a phosphoketolase (PK); a heterologous nucleic acid encoding a phosphotransacetylase (PTA); and a functional disruption of a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is GPP1/RHR2, or a homologue or variant thereof. In some embodiments, the glycerol-1-phosphatase is GPP2/HOR2, or a homologue or variant thereof.

[0025] In some embodiments, the genetically modified host cell provided herein is selected from the group consisting of a bacterial cell, a fungal cell, an algal cell, an insect cell, and a plant cell. In some embodiments, the cell is a yeast cell. In some embodiments, the yeast is *Saccharomyces cerevisiae*.

[0026] In some embodiments, the genetically modified host cell produces an increased amount of an acetyl-CoA derived compound (e.g., an isoprenoid, polyketide, or fatty acid) compared to a yeast cell not comprising a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate.

[0027] In another aspect, provided herein are methods for producing a heterologous acetyl-CoA derived compound, the method comprising: culturing a population of genetically modified host cells, capable of producing a heterologous acetyl-CoA derived compound as described herein, in a medium with a carbon source under conditions suitable for making said heterologous acetyl-CoA derived compound; and recovering said heterologous acetyl-CoA derived compound from the medium. In some embodiments, heterologous acetyl-CoA derived compound is selected from the group consisting of an isoprenoid, a polyketide, and a fatty acid.

[0028] In another aspect, provided herein is a method for increasing the production of acetyl-CoA or an acetyl-CoA derived compound in a host cell, the method comprising: expressing in the host cell a heterologous nucleic acid encoding a phosphoketolase (PK; EC 4.1.2.9); and functionally disrupting an endogenous enzyme that converts acetyl phosphate to acetate. In some embodiments, the method further comprises expressing in the host cell a heterologous nucleic acid encoding a phosphotransacetylase (PTA; EC 2.3.1.8).

[0029] In another aspect, provided herein is a method for increasing the production of acetyl-CoA in a host cell, the method comprising: expressing in the host cell a heterologous nucleic acid encoding a phosphotransacetylase (PTA; EC 2.3.1.8); and functionally disrupting an endogenous enzyme that converts acetyl phosphate to acetate. In some embodiments, the method further comprises expressing in the host cell a heterologous nucleic acid encoding a phosphoketolase (PK; EC 4.1.2.9).
In some embodiments, the enzyme that converts acetyl phosphate to acetate is a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the glycerol-1-phosphatase is selected from GPP1/RHR2, GPP2/HOR2, and homologues and variants thereof. In some embodiments, GPP1/RHR2, or a homologue or variant thereof, is functionally disrupted. In some embodiments, GPP2/HOR2, or a homologue or variant thereof, is functionally disrupted. In some embodiments, both GPP1/RHR2 and GPP2/HOR2, or both a homologue or variant of GPP1/RHR2 and a homologue or variant of GPP2/HOR2, are functionally disrupted. In some embodiments, the host cell is selected from the group consisting of a bacterial cell, a fungal cell, an algal cell, an insect cell, and a plant cell. In some embodiments, the host cell is a yeast cell. In some embodiments, the yeast is *Saccharomyces cerevisiae*. In some embodiments, the host cell produces an increased amount of acetyl-CoA or an acetyl-CoA derived compound compared to a yeast cell not comprising a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate.

4. **BRIEF DESCRIPTION OF THE FIGURES**

**FIG. 1** provides a schematic representation of the pathways involved in the conversion of sugar (glucose and xylose) to acetyl-CoA, and acetyl-CoA derived compounds, in a yeast host cell. The bold arrows indicate the recombinant phosphoketolase pathway. Acetyl phosphate is an intermediate of the phosphoketolase (PK) / phosphotransacetyklase (PTA) pathway to acetyl-CoA, and is hydrolyzed to acetate by RHR2 and HOR2.

Abbreviations: G6P, glucose-6-phosphate; R5P, ribulose-5-phosphate; X5P, xylose-5-phosphate; F6P, fructose-6-phosphate; E4P, erythrose-4-phosphate; FBP, fructose-1,6-biphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; ADA, acetaldehyde dehydrogenase, acetylating; ACP, acetyl phosphate.

**FIG. 2** provides representative enzymes of the mevalonate pathway for isoprenoid production. Abbreviations: AcCoA, acetyl-CoA; AcAcCoA, acetoacetyl-CoA; HMGCoA, 3-hydroxy-3-methylglutaryl-CoA; Mev5P, mevalonate-5-phosphate; Mev5DP, mevalonate-5-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl pyrophosphate; ErglO, acetyl-CoA thiolase; ACC1, acetyl-CoA carboxylase; AACS, acetoacetyl-CoA synthase; Ergl3, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGr, 3-hydroxy-3-methylglutaryl-CoA reductase; Ergl2, mevalonate kinase; Erg8, phosphomevalonate kinase; Ergl9, mevalonate pyrophosphate decarboxylase.
FIGS. 3A-3B provides the sugar consumption (A) and acetate production (B) of wild-type (strain Y967, left) and recombinant yeast cells (middle, right) comprising: a heterologous acetaldehyde dehydrogenase acylating (Dz.eutE) and deletion of the native PDH-bypass (acslA acs2 A ald6A) (strain Y12869; middle); and further comprising a heterologous phosphoketolase (Lm.PK) and phosphotransacetylase (Ck.PTA) (strain Y12746; right).

FIGS. 3C-3D provides the sugar consumption (C) and acetate production (D) of recombinant yeast cells comprising: a heterologous acetaldehyde dehydrogenase acylating (Dz.eutE) and deletion of the native PDH-bypass (acslA acs2 A ald6A) (strain Y12869; left); and further comprising a heterologous phosphoketolase (Lm.PK) (strain Y19390; middle) or phosphotransacetylase (Ck.PTA) (strain Y19391; right).

FIG. 4 provides a demonstration of acetyl phosphate hydrolysis in cell free extracts (CFE) of wild-type S. cerevisiae strain Y967 over a 120 minute timecourse. Shown are CFE only (left); CFE plus 30 mM sodium fluoride, a broad spectrum phosphatase inhibitor (middle); and CFE that has been heat inactivated (right).

FIG. 5 provides results of anion exchange chromatography on Y967 cell free extracts. Protein was eluted with a 0-100% gradient of buffer B (20 mM Tris-Cl pH 7, 1M NaCl, 10% glycerol) over 30 column volumes at a flow rate of 0.5 mL/minute, and 1 mL fractions were collected, analyzed by protein gel electrophoresis (FIG. 5B), and assayed for acetyl phosphatase activity (FIG. 5A). ACP, acetyl phosphate.

FIG. 6A provides results of anion exchange chromatography on fraction #10 of Y967. The most active fraction from this purification, # 14, was analyzed by mass spectrometry to determine the identity of the proteins in the fraction (FIG. 6B). RHR2 was identified as a phosphatase in the active fraction.

FIG. 7 provides results of acetyl phosphatase activity assays on CFEs of a wild-type yeast strain (Y968) or recombinant yeast strains comprising a deletion of RHR2, HOR2 or both RHR2 and HOR2.

FIGS. 8A-8C provides acetate levels (A), glycerol levels (B) and optical densities (C) of recombinant yeast strain populations. Strain Y12746.ms63909.ms64472 comprises a deletion of the PDH-bypass (acslA acs2 A ald6A), and heterologously expresses acetaldehyde dehydrogenase acetylating (Dz.eutE), phosphoketolase (Lm.PK), phosphotransacetylase (Ck.PTA), and genes in the farnesene production pathway. Strain
Y12746.ms63909.ms64472 rhr2^ is isogenic to strain Y12746.ms63909.ms64472 but further comprises a deletion of RHR2 (rhr2^).

[0040] FIGS. 8D-8E provides acetate levels (D) and optical densities (E) of recombinant yeast strain populations. Strain Y12745 comprises a deletion of the PDH-bypass (acs1A acs2 A ald6A), and heterologously expresses acetaldehyde dehydrogenase acetylating (Dz.eutE), phosphoketolase (Lm.PK), and phosphotransacetylase (Ck.PTA). Strain Y12746 rhr2^ is isogenic to strain Y12746 but further comprises a deletion of RHR2 (rhr2^).

[0041] FIG. 9 provides relative farnesene levels (top) and relative optical densities (bottom) of recombinant yeast strain populations wherein the RHR2 gene is intact (RHR2+) or deleted (rhr2^). Y968 (right panel) is a wild-type yeast strain. Y12869.ms63907.ms64472 ("Y12869"; 2^nd from right panel) comprises a deletion of the PDH-bypass (acs1A acs2 A ald6A), and heterologously expresses acetaldehyde dehydrogenase acetylating (Dz.eutE) and genes in the farnesene production pathway, but does not express phosphoketolase or phosphotransacetylase. Y12746.ms63907.ms64472 ("Y12746"; 2^nd from left panel) comprises a deletion of the PDH-bypass (acs1A acs2 A ald6A), and heterologously expresses acetaldehyde dehydrogenase acetylating (Dz.eutE) and genes in the farnesene production pathway, and uses phosphoketolase and phosphotransacetylase as a pathway to produce cytosolic acetyl-CoA, which is used for synthesis of farnesene. Y12745.ms63907.ms64472 ("Y12745"; left panel) comprises a deletion of the PDH-bypass (acs1A acs2 A ald6A), and genes in the farnesene production pathway, and uses phosphoketolase and phosphotransacetylase as a pathway to produce cytosolic acetyl-CoA, which is used for synthesis of farnesene.

5. DETAILED DESCRIPTION OF THE EMBODIMENTS

5.1 Terminology

[0042] 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.

[0043] 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.

[0044] 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. In some embodiments the functional disruption of a target gene results in a
reduction by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%,
65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of the expression level of the target gene
compared to its expression when not functionally disrupted. Similarly, to "functionally
disrupt" or a "functional disruption" e.g., of a target protein, for example, a protein having
acetyl phosphatase activity, 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 functional
disruption of a target protein results in a reduction by at least 5%, 10%, 15%, 20%, 25%,
30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of
the activity or expression level of the target protein compared to its activity or expression
when not functionally disrupted. 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.
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 phosphotransacetylase, and heterologous expression of one or more enzymes of the mevalonate pathway.

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.

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).

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.

As used herein, the phrase "acetyl-CoA derived compound" refers to a compound which uses acetyl-CoA as a substrate in its biosynthesis. Exemplary acetyl-CoA derived compounds include, but are not limited to, isoprenoids, polyketides, fatty acids, and alcohols. In some embodiments, an acetyl-CoA derived compound is ethanol, for example, bioethanol produced from pentose substrates, as described in U.S. Patent No. 7,253,001, the contents of which are hereby incorporated by reference in their entirety.

As used herein, the term "variant" refers to a polypeptide differing from a specifically recited "reference" polypeptide (e.g., a wild-type sequence) by amino acid insertions, deletions, mutations, and substitutions, but retains an activity that is substantially similar to the reference polypeptide. In some embodiments, the variant is created by recombinant DNA techniques, such as mutagenesis. In some embodiments, a variant polypeptide differs from its reference polypeptide by the substitution of one basic residue for another (i.e. Arg for Lys), the substitution of one hydrophobic residue for another (i.e. Leu for Ile), or the substitution of one aromatic residue for another (i.e. Phe for Tyr), etc. In some
embodiments, variants include analogs wherein conservative substitutions resulting in a substantial structural analogy of the reference sequence are obtained. Examples of such conservative substitutions, without limitation, include glutamic acid for aspartic acid and vice-versa; glutamine for asparagine and vice-versa; serine for threonine and vice-versa; lysine for arginine and vice-versa; or any of isoleucine, valine or leucine for each other.

5.2 Host Cells

[0051] Host cells useful compositions and methods provided herein include archae, prokaryotic, or eukaryotic cells.

[0052] 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, Scenedesmus, Serratia, Shigella, Staphlococcus, Streptomyces, Synnecoccus, and Zymomonas. Examples of prokaryotic strains include, but are not limited to: Bacillus subtilis, Bacillus amyloliquefacines, Brevibacterium ammoniagenes, Brevibacterium immariophilum, Clostridium beigerincki, 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.

[0053] Suitable archae hosts include, but are not limited to, cells belonging to the genera: Aeropyrum, Archaeoglobus, 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.

[0054] 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,

In some embodiments, the host microbe is Saccharomyces cerevisiae, Pichia pastoris, Schizosaccharomyces pombe, Dekkara bruxellensis, Kluyveromyces lactis (previously called Saccharomyces lactis), Kluyveromyces 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.

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.
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.

5.3 The Phosphoketolase (PK) / Phosphotransacetylase (PTA) Pathway to
Acetyl-CoA

In some embodiments, the phosphoketolase pathway is activated in the
genetically modified host cells provided herein by engineering the cells to express polynucleotides and/or polypeptides encoding phosphoketolase and, optionally, phosphotransacetylase. Thus, in some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having phosphoketolase activity. In other embodiments, particularly where acetyl phosphate can be supplied as a metabolic intermediate independent of phosphoketolase activity, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having phosphotransacetylase activity. In other embodiments, the genetically modified host cells provided herein comprise both a heterologous polynucleotide encoding a polypeptide having phosphoketolase activity and a heterologous polynucleotide encoding a polypeptide having phosphotransacetylase activity.

5.3.1 Phosphoketolase (PK)

Phosphoketolase (EC 4.1.2.9) catalyzes the conversion of xylulose 5-
phosphate into glyceraldehyde 3-phosphate and acetyl phosphate; and/or the conversion of fructose-6-phosphate into erythrose-4-phosphate and acetyl phosphate. Phosphoketolase activity has been identified in several yeast strains growing with xylose as the sole carbon source but not in yeast strains grown with glucose (Evans and Ratledge, Arch. Microbiol. 139: 48-52; 1984). Inhibitors of phosphoketolase include, but are not limited to, erythrose 4-phosphate and glyceraldehyde 3-phosphate.

Numerous examples of polynucleotides, genes and polypeptides encoding phosphoketolase activity are known in the art and can be used in the genetically modified host cell provided herein. In some embodiments, such a polynucleotide, gene and/or polypeptide is the xylulose 5-phosphateketolase (XpkA) of Lactobacillus pentosus MD363 (Posthuma et al., Appl. Environ. Microbiol. 68: 831-7; 2002). XpkA is the central enzyme of
the phosphoketolase pathway (PKP) in lactic acid bacteria, and exhibits a specific activity of 4.455 µmol/min/mg (Posthuma et al., Appl. Environ. Microbiol. 68: 831-7; 2002). In other embodiments, such a polynucleotide, gene and/or polypeptide is the phosphoketolase of *Leuconostoc mesenteroides* (Lee et al., Biotechnol Lett. 27(12):853-858 (2005)), which exhibits a specific activity of 9.9 µmol/min/mg and is stable at pH above 4.5 (Goldberg et al., Methods Enzymol. 9: 515-520; 1966). This phosphoketolase exhibits a Km of 4.7 mM for D-xylulose 5-phosphate and a Km of 29 mM for fructose 6-phosphate (Goldberg et al., Methods Enzymol. 9: 515-520; 1966). Representative phosphoketolase nucleotide sequences of *Leuconostoc mesenteroides* includes accession number AY804190, and SEQ ID NO: 1 as provided herein. Representative phosphoketolase protein sequences of *Leuconostoc mesenteroides* include accession numbers YP_819405, AAV66077.1, and SEQ ID NO: 2 as provided herein. In other embodiments, such a polynucleotide, gene and/or polypeptide is the D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase gene xfp from *B. lactis*, as described, for example, in a pentose-metabolizing *S. cerevisiae* strain by Sonderegger et al. (Appl. Environ. Microbiol. 70: 2892-7; 2004).

[0061] Other useful phosphoketolases include, but are not limited to, those from *Bifidobacterium dentium* ATCC 27678 (ABIX02000002. 1:2350400..2352877; EDT46356.1); *Bifidobacterium animalis* (NC_017834.1:1 127580..1130057; YP_006280131.1); and *Bifidobacterium pseudolongum* (AY518216.1:988..3465; AAR98788.1); *Aspergillus nidulans* FGSC A4 (CBF76492.1); *Bifidobacterium longum* (AAR98787.1); *Bifidobacterium bifidum* NCIMB 41171 (ZP 03646196.1); *Bifidobacterium animalis* subsp. *lactis* HN019 (ZP 02962870.1); *Lactobacillus plantarum* WCFS1 (NP_786060.1); *Lactobacillus brevis* subsp. *gravesensis* ATCC 27305 (ZP_03940142.1); *Lactobacillus reuteri* 100-23 (ZP_03073172.1); and *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 (YP_818922.1).

[0062] Other useful phosphoketolases include those described in International Publication No. WO 201 1/15985, the contents of which are hereby incorporated by reference in their entirety. These phosphoketolases include: (YP_001601863.1; *Glucanacetobacter diazotrophicus* Pal 5), (YP_001093221.1; *Shewanella loithica* PV-4), (YP_926792.1; *Shewanella amazonensis* SB2B), (YP_735093.1; *Shewanella sp. MR-4*), (YP_001049439.1; *Shewanella baltica* OS155), (ZP_02157884.1; *Shewanella benthica* KT99), (YP_001472925.1; *Shewanella sediminis* HAW-EB3), (YP_001759669.1; *Shewanella woodyi* ATCC 51908), (YP_001673352.1; *Shewanella halifaxensis* HA W-EB4), (YP_563733.1;
Shewanella denitrificans OS217), (ZP_05111697.1; Legionella drancourtii LLAP 12),
(EEQ84307.1; Ajellomyces dermatitidis ER-3), (XP_002626734.1; Ajellomyces dermatitidis
SLH14081), (XP_001539009.1; Ajellomyces capsulatus NAml), (EEH04133.1; Ajellomyces
capsulatus G186AR), (EEH20258.1; Paracoccidioides brasiliensis Pb03), (EEH44652.1;
Paracoccidioides brasiliensis Pb 18), (XP_002582752.1; Uncinocarpus reessii 1704),
(EER26377.1; Coccioidioides posadasii C735 delta SOWgp), (EEQ28085.1; Microsporum
canis CBS 113480), (XP_001819785.1; Aspergillus oryzae RIB40), (XP_001399780.1;
Aspergillus niger), (XP_001263382.1; Neosartorya fischeri NRRL 181), (XP_001271080.1;
Aspergillus clavatus NRRL 1), (XP_001213784.1; Aspergillus terreus NIH2624),
(CBF76492.1; Aspergillus nidulans FGSC4A), (XP_002561913.1; Penicillium chrysogenum
Wisconsin 54-1255), (XP_002480391.1; Talaromyces stipitatus ATCC 10500),
(XP_002144014.1; Penicillium stipitatus ATCC 10500), (XP_002144014.1; Penicillium
mameffei ATCC 18224), (XP_755453.1; Aspergillus fumigatus Afl 93), (XP_001556635.1;
Botryotinia fuckeliana B05.1 0), (XP_001592549.1; Sclerotinia sclerotiorum 1980),
(XP_386729.1; Gibberella zeae PH-1), (EEU47171.1; Nectria haematococca mp VI 77-13-
4), (EEY16637.1; Verticillium alboatrum VaMs.1 02), (XP_956649.1; Neurospora crassa
OR74A), (XP_364271.2; Magnaporthe grisea 70-15), (XP_001904585.1; Podospora
anserine), (XP_001836159.1; Coprinopsis cinerea okay’ama?#130), (NP_595963.1;
Schizosaccharomyces pombe), (XP_002173441.1; Schizosaccharomyces exjaponicus yFS275),
(XP_570860.1; Cryptococcus neoformans var. neoformans JEC21), (XP_001556635.1;
Ustilago maydis 521), (ZP_05027078.1; Microcoleus chthonoplastes PCC 7420), (YP_003101114.1;
Actinosynnema mirum DSM 43827), (ZP_03568244.1; Atopobium rimae ATCC 49626),
(YP_003180237.1; Atopobium parvulum DSM 20469), (ZP_03946928.1; Atopobium vaginæ
DSM 15829), (ZP_03296299.1; Collinsella stercoris DSM 13279), (AAR98787.1;
Bifidobacterium longum), (ZP_03618909.1; Bifidobacterium breve DSM 20213),
(ZP_03646196.1; Bifidobacterium bifidum NCIMB 41171), (ZP_04448101.1;
Bifidobacterium angulatum DSM 20098), (ZP_03324204.1; Bifidobacterium catenulatum
DSM 16992), (AAR98790.1; Bifidobacterium sp. CFAR 172), (AAR98789.1;
Bifidobacterium pullorum), (ZP_03937610.1; Gardnerella vaginalis ATCC 14019),
(ZP_05965201.1; Bifidobacterium gallicum DSM 20093), (ZP_02962870.1; Bifidobacterium
animalis subsp. lactis HNO19), (AAR98788.1; Bifidobacterium pseudolongum subsp.
Globosum), (ZP_03946518.1; Atopobium vaginæ DSM 15829), (YP_001511171.1; Frankia
sp. EANlpec), (YP_713678.1; Frankia alniACNUa), (YP_002778395.1; Rhodococcus
opacus B4), (YP_701466.1; Rhodococcus jostii RHAl), (ZP_04383880.1; Rhodococcus erythropolis SKI 21), (YP 947598.1; Arthrobacter aurescens TC 1), (CAD48946.1; Propionibacterium freudenreichii subsp. Shermanii), (NP_79 1495.1; Pseudomonas syringae pv. Tomato str. DC3000), (YP_003 125992.1; Chitinophaga pinensis DSM 2588), (ABX56639.1; Verrucomicrobiae bacterium V4), (YP_00237 1883.1; Cyanothece sp. PCC 8801), (YP_00 1806596.1; Cyanothece sp. ATCC 51142), (ZP_01730652.1; Cyanothece sp. CXY0110), (CAQ48286.1; Planktothrix rubescens NIVA-CYA 98), (ZP_03276298.1; Arthrospira maxima CS-328), (ZP_03157277.1; Cyanothece sp. PCC 7822), (YP_002379031.1; Cyanothece sp. PCC 7424), (YP_001658501.1; Microcystis aeruginosa NIES-843), (ZP_01621774.1; Lyngbya sp. PCC 8106), (NP_485524.1; Nostoc sp. PCC 7120), (ZP_05036350.1; Synechococcus sp. PCC 7335), (YP_001514813.1; Acaryochloris marina MBIC 11 017), (ZP_05039537.1; Synechococcus sp. PCC 7335), (ZP_02886235.1; Burkholderia graminis C4DIM), (ZP_03264503.1; Burkholderia sp. H160), (ZP_01085819.1; Synechococcus sp. WH 5701), (ZP_05045603.1; Cyanobium sp. PCC 7001), (ZP_01123645.1; Synechococcus sp. WH 7805), (YP_001223932.1; Synechococcus sp. WH 7803), (ZP_01079038.1; Synechococcus sp. RS9917), (YP_001889002.1; Burkholderia phytofirmans PsJN), (YP_553967.1; Burkholderia xenovorans LB400), (ZP_02881709.1; Burkholderia graminis C4DIM), (ZP_03270532.1; Burkholderia sp. H160), (YP_00 1861620.1; Burkholderia phymatum ST815), (YP_002755285.1; Acidobacterium capsulatum ATCC 51196), (EDZ38884.1; Leptospiillum sp. Group I1 "5-way CO"), (EES53204.1; Leptospiillum ferrooxidotrophum), (YP_172723.1; Synechococcus elongatus PCC 6301), (NP_681976.1; Thermosynechococcus elongatus BP-1), (YP_00114037.1; Methyllococcus capsulatus str. Bath), (YP_002482577.1; Cyanothece sp. PCC 7425), (NP_442996.1; Synechocystis sp. PCC 6803), (YP_002482735.1; Cyanothece sp. PCC 7425), (ZP_04774866.1; Allochromatium vinosum DSM 180), (ZP_01453 148.1; Mariprofundus ferrooxydans PV-l), (ZP_04830548.1; Gallionella ferraruginea ES-2), (XP_001273863.1; Aspergillus clavatus NRRL 1), (XP_001258643.1; Neosartorya fischeri NRRL 181), (XP_001727680.1; Aspergillus oryzae RIB40), (XP_001396306.1; Aspergillus niger), (XP_001216075.1; Aspergillus terreus NIH2624), (XP_002567 130.1; Penicillium chrysogenum Wisconsin 54-1255), (XP_002143851.1; Penicillium marneffei ATCC 18224), (XP_002480216.1; Talaromyces stipitatus ATCC 10500), (XP_001559949.1; Botryotinia fuckeliana B05.10), (XP_001593100.1; Sclerotinia sclerotiorum 1980), (XP_001932192.1; Pyrenophora triticirepentis Pt-lC-BFP), (XP_001793729.1; Phaeosphaeria nodorum SN 15),
(XP_567776.1; Cryptococcus neoformans var. neoformans JEC21), (XP_386504.1; Oibberella zeae PH-1), (EEU46265.1; Nectria haematococca mp VII 77-13-4), (AC024516.1; Metarhizium anisopliae), (XP_959985.1; Neurospora crassa OR74A), (XP_001904686.1; Podospora anserine), (YP_002220141.1; Acidithiobacillus ferroxidans ATCC 53993), (YP_001220128.1; Acidiphilium cryptum JF-5), (YP_001471202.1; Thermotoga lettingiae TMO), (YP_002352287.1; Dictyoglomus turgidum DSM 6724), (YP_571790.1; Nitrobacter hamburgensis X14), (ZP_0109240.1; Blastopirellula marina DSM 3645), (YP_001340809.1; Marinomonas sp. MWYLJ), (NP_866384.1; Rhodopirellula baltica SH I), (ZP_05108502.1; Legionella drancourtii LLAP 12), (ZP_04995817.1; Streptomyces sp. Mgl), (ZP_04023055.1; Lactobacillus reuteri SD2112), (ZP_03960060.1; Lactobacillus vaginalis ATCC 49540), (ZP_03073172.1; Lactobacillus reuteri 100-23), (ZP_05553031.1; Lactobacillus coleohominis 101-4-CHN), (ZP_04021289.1; Lactobacillus acidophilus ATCC 4796), (ZP_03995194.1; Lactobacillus crispatus IV-VOL), (ZP_04010922.1; Lactobacillus alatumensis DSM 16047), (ZP_05549961.1; Lactobacillus crispatus 125-2-CRN), (ZP_03951361.1; Lactobacillus gasseri IV-V03), (ZP_05744515.1; Lactobacillus iners DSM 13335), (YP_618635.1; Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842), (ZP_03955917.1; Lactobacillus jensenii IV-VI6), (ZP_03942415.1; Lactobacillus buchneri ATCC 11577), (ZP_01544800.1; Oenococcus oeni ATCC BAA-1163), (NP_786060.1; Lactobacillus plantarum WCFSI), (Q937F6; XPKA_LACPE), (YP_394903.1; Lactobacillus sakei subsp. sakei 23K), (YP_803891.1; Pediococcus pentosaceus ATCC 25745), (BAI40729.1; Lactobacillus rhamnosus GG), (ZP_03940142.1; Lactobacillus brevis subsp. Gravessensis ATCC 27305), (ZP_04009273.1; Lactobacillus salivarius ATCC 11741), (ZP_03958643.1; Lactobacillus ruminis ATCC 25644), (ZP_04431433.1; Bacillus coagulans 36D1), (ZP_04601906.1; Kingella oralis ATCC 51147), (ZP_05736927.1; Granulicatella adiacens ATCC 49175), (YP_001449631.1; Streptococcus gordonii str. Challis substr. CHI), (NP_736274.1; Streptococcus agalactiae NEM316), (ZP_04442854.1; Listeria grayi DSM 20601), (ZP_05646360.1; Enterococcus casseliflavus EC30), (ZP_05650322.1; Enterococcus gallinarum EG2), (ZP_05675307.1; Enterococcus faecium Coml2), (BAH69929.1; Mycoplasma fermentans PG 18), (YP_000200006.1; Mycoplasma arthritidis 15 8L3-1), (YP_001256266.1; Mycoplasma agalactiae PG2), (YP_001988835.1; Lactobacillus casei BL23), (NP_786753.1; Lactobacillus plantarum WCFS I), (ZP_04009976.1; Lactobacillus salivarius ATCC 11741), (YP_818922.1; Leuconostoc mesenteroides subsp. Mesenteroides
ATCC 8293), (YP_794669.1; Lactobacillus brevis ATCC 367), (ZP_04782553.1; Weissella parames enteroides ATCC 33313), (YP_00 1727454.1; Leuconostoc citreum KM20), (YP_819405.1; Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293), (ABX75772.1; Lactococcus lactis subsp. Lactis), (YP_81 1314.1; Oenococcus oeni PSU-1), (ZP_0295 1191.1; Clostridium butyricum 5521), (ZP_05390294.1; Clostridium carboxidivorans P7), (NP_347971.1; Clostridium acetobutylicum ATCC 824), (ZP_03800296.1; Coprococcus comes ATCC 27758), (ZP_04857624.1; Ruminococcus sp. 5J_39B FAA), (ZP_04743029.2; Roseburia intestinalis L 1-82), (ZP_02038271.1; Bacteroides capillosus ATCC 29799), (YP_568630.1; Rhodopseudomonas palustris BisB5), (YP_487462.1; Rhodopseudomonas palustris HaA2), (YP_533660.1; Rhodopseudomonas palustris BisB18), (YP_973512.1; Polaromonas naphthalenivorans CJ2), (ZP_01464191.1; Stigmatella aurantiaca DW4/3-1), (YP_oo 1267778.1; Pseudomonas putida FT), (YP_829644.1; Arthrobacter sp. FB24), (YP_002486392.1; Arthrobacter chlorophenolicus A6), (ZP_05816651.1; Sanguibacter keddieii DSM 10542), (YP_002883053.1; Beutenbergia cavemae DSM 12333), (YP_003 161540.1; Jonesia denitrificans DSM 20603), (ZP_0391 1482.1; Xylanimonas cellulosilytica DSM 15894), (CAJ57850.1; Cellulomonas flavigena), (YP_001 134605.1; Mycobacterium gilvum PYR-GCK), (YP_953877.1; Mycobacterium vanbaalenii PYR-l), (YP_003 1556 11.1; Brachybacterium faecium DSM 4810), (YP_003148127.1; Kytococcus sedentarius DSM 20547), (YP_oo 1221 168.1; Clavibacter michiganensis subsp. michiganensis NCPPB 382), (YP_001 158426.1; Salinispora tropica CNB-440), (YP_oo 1536420.1; Salinispora arenicola CNS-205), (ZP_04608302.1; Micromonospora sp. ATCC 39149), (YP_887914.1; Mycobacterium smegmatis str. MC2 155), (YP_639956.1; Mycobacterium sp. MCS), (ZP_04749 157.1; Mycobacterium kansasi ATCC 12478), (YP_oo 185 1039.1; Mycobacterium marinum), (NP_960507.1; Mycobacterium avium subsp. paratuberculosis K-10), (ZP_05224330.1; Mycobacterium intracellulare ATCC 13950), (YP_oo 1703240.1; Mycobacterium abscessus), (ZP_00995133.1; Janibacter sp. HTCC2649), (YP_2991026.1; Thermobifida fusca YX), (ZP_0403 1845.1; Thermomonospora curvata DSM 43183), (ZP_04475514.1; Streptosporangium roseum DSM 43021), (ZP_04335641.1; Nocardiopsis dassonvillie subsp. dassonvillei DSM 43111), (ZP_04482201.1; Stackebrandtia nassauensis DSM 44728), (YP_0030997 12.1; Actinosynnema mirum DSM 43827), (NP_733508.1; Streptomyces coelicolor A3(2)).
Streptomyces ambofaciens ATCC 23877), (ZP_05536883.1; Streptomyces griseoflavus Tu4000), (ZP_05020421.1; Streptomyces sviceus ATCC 29083), (CBG67625.1; Streptomyces scabiei 87.22), (NP_822448.1; Streptomyces avermitilis MA-4680), (ZP_04689547.1; Streptomyces ghanaensis ATCC 14672), (ZP_05530021.1; Streptomyces viridochromogenes DSM 40736), (ZP_05512501.1; Streptomyces hygroscopicus ATCC 53653), (ZP_05800927.1; Streptomyces flavogriseus ATCC 33331), (YP_00 1828275.1; Streptomyces griseus subsp. griseus NBRC 13350), (ZP_04689547.1; Streptomyces avermitilis MA-4680), (NP_822448.1; Streptomyces avermitilis MA-4680), (ZP_04689547.1; Streptomyces ghanaensis ATCC 14672), (ZP_05530021.1; Streptomyces viridochromogenes DSM 40736), (ZP_05512501.1; Streptomyces hygroscopicus ATCC 53653), (ZP_05800927.1; Streptomyces flavogriseus ATCC 33331), (YP_00 1828275.1; Streptomyces griseus subsp. griseus NBRC 13350), (ZP_04705493.1; Streptomyces albus JI074), (ZP_04996963.1; Streptomyces sp. Mgl), (ZP_05485309.1; Streptomyces sp. SPB78), (ZP_03860882.1; Kribbellia flayida DSM 17836), (YP_117539.1; Frankia sp. EANlpec), (YP_482627.1; Frankia sp. CcI3), (YP_003116893.1; Catenulispora acidiphila DSM 44928), (YP_872280.1; Acidothermus lolyticus III), (YP_924807.1; Nocardioides sp. JS614), (YP_00104157.1; Saccharopolyspora erythraea NRRL 2338), (YP_002282673.1; Rhizobium leguminosarum by. trifolii WSM2304), (YP_002977256.1; Rhizobium leguminosarum by. trifolii WSM1325), (YP_001979796.1; Rhizobium etli CIAT 652), (YP_470926.1; Rhizobium etli CFN 42), (YP_002540633.1; Agrobacterium radiobacter K84), (ZP_05182366.1; Brucella sp. 83/13), (ZP_04683384.1; Ochrobactrum intermedium LMG 3301), (YP_001373254.1; Ochrobactrum anthropi ATCC 49188), (YP_001204109.1; Bradyrhizobium sp. ORS278), (YP_001238418.1; Bradyrhizobium sp. BTAil), (NP_769158.1; Bradyrhizobium japonicum USDA 110), (YP_577164.1; Nitrobacter hamburgensis XI4), (YP_002961612.1; Methylobacterium extorquens AM 1), (YP_674972.1; Mesorhizobium sp. BNCI), (ZP_05813617.1; Mesorhizobium opportunistum WSM2075), (YP_318559.1; Nitrobacter winogradskyi Nb-255), (YP_001755280.1; Methylobacterium radiotolerans JCM 2831), (YP_001753191.1; Methylobacterium radiotolerans JCM 2831), (YP_003066011.1; Methylobacterium extorquens DM4), (YP_002964777.1; Methylobacterium extorquens AM 1), (YP_002501292.1; Methylobacterium nodulans ORS 2060), (YP_002495265.1; Methylobacterium nodulans ORS 2060), (YP_001770387.1; Methylobacterium sp.4-46), (YP_002944712.1; Variovorax paradoxus SI10), (ZP_01156757.1; Oceanicola granulosus HTCC2516), (ZP_01628787.1; Nodularia spumigena CCY9414), (YP_001865546.1; Nostoc punctiforme PCC 73102), (YP_321015.1; Anabaena variabilis ATCC 29413), (ZP_03769140.1; Nostoc azollae’ 0708), (NP_923943.1; Gloeobacter violaceus PCC 7421), (YP_477385.1; Synechococcus sp. JA-2-3B’a(2-13)), (YP_001328659.1; Sinorhizobium medicae WSM419), (YP_765670.1; Rhizobium leguminosarum bv. viciae 3841),
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) and acetyl phosphate. 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.

5.3.2 Phosphotransacetylase (PTA)

In some embodiments, the genetically modified host cell provided herein comprises a heterologous nucleotide sequence encoding a phosphotransacetylase. Phosphotransacetylase (EC 2.3.1.8) converts acetyl phosphate into acetyl-CoA.
Numerous examples of polynucleotides, genes and polypeptides encoding phosphotransacetylase activity are known in the art and can be used in the genetically modified host cell provided herein. In some embodiments, such a polynucleotide, gene and/or polypeptide is the phosphotransacetylase from Clostridium klyuyeri. Representative phosphotransacetylase nucleotide sequences of Clostridium klyuyeri includes accession number NC_009706.1:1428554..1429555, and SEQ ID NO: 3 as provided herein. Representative phosphotransacetylase protein sequences of Clostridium klyuyeri include accession number YP_001394780 and SEQ ID NO: 4 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); Methanosarcina thermophila (L23147.1:207..1208; AAA72041.1); Lactobacillus sanfranciscensis (BAB19267.1); Lactobacillus plantarum WCFS1 (NP_784550.1); Lactobacillus fermentum ATCC 14931 (ZP_03944466.1); Bacillus subtilis subsp. subtilis str. 168 (NP_391646.1); Methanosarcina thermophila (AAA72041.1); Clostridium thermocellum DSM 4150 (ZP_03152606.1); Clostridium acetobutylicum ATCC 824 (NP_348368.1); Clostridium klyuyeri DSM 555 (YP_001394780.1); Veillonella parvula DSM 2008 (ZP_03855267.1); and Salmonella enterica subsp. enterica serovar Paratyphi A str. ATCC 9150 (YP_149725.1).

Other useful phosphotransacetylases include those described in International Publication No. WO 201 1/15985, the contents of which are hereby incorporated by reference in their entirety. These phosphotransacetylases include: (ZP_05427766.1; Eubacterium saphenum ATCC 49989), (ZP_03627696.1; bacterium Ellin514), (ZP_03131770.1; Chthonio bacterflavus Ellin428), (YP_00187803.1; Akkermansia muciniphila TCCBA-835), (ZP_04562924.1; Citrobacter sp.30_2), (YP_001451936.1; Citrobacter koseri ATCC BAA-895), (YP_149725.1; Salmonella enterica subsp. enterica serovar Paratyphi A str. ATCC 9150), (YP_001569496.1; Salmonella enterica subsp. anzaeae serovar 62:z4,z23:---), (NP_416953.1; Escherichia coli str. K-12 substr. MG1655), (YP_002920654.1; Klebsiella pneumoniae NTUH-K2044), (ZP_04637797.1; Yersinia intermedia ATCC 29909), (ZP_01222604.1; Photobacterium profundum 3TCK), (ZP_02156855.1; Shewanella benthica KT99), (YP_958508.1; Marinobacter aquaeolei VT8), (YP_066771.1; Desulfotalea psychrophila LSSv54), (YP_002780531.1; Rhodococcus opacus B4), (YP_703506.1; Rhodococcus jostii RHA1), (ZP_05479963.1; Streptomyces sp. AA4), (YP_002761398.1; Gemmatimonas aurantiaca T-27), (ZP_04670189.1; Clostridiales bacterium 1_7_47FAA).
(ZP_05493958.1; Clostridium papyrosolvens DSM 2782), (YP_003143506.1; Slackia heliotrinireducens DSM 20476), (ZP_05090822.1; Ruegeria sp. Rli), (ZP_01748021.1; Sagittula stellata E-37), (NP_604069.1; Fusobacterium nucleatum subsp. nucleatum ATCC 25586), (ZP_05814734.1; Fusobacterium sp. 3_1_33), (ZP_06026613.1; Fusobacterium periodonticum ATCC 33693), (ZP_05617632.1; Fusobacterium sp. 3_1_5R), (ZP_05628030.1; Fusobacterium sp. D12), (ZP_04860946.1; Fusobacterium vanum ATCC 27725), (ZP_04567441.1; Fusobacterium mortiferum ATCC 9817), (YP_001489437.1; Arcobacter butzleri RM4018), (YP_003163236.1; Leptotrichia buccalis C-1013-b), (ZP_05902420.1; Leptotrichia hofstadii F0254), (ZP_06011308.1; Leptotrichia goodfellowii F0264), (ZP_04479548.1; Streptobacillus moniliformis DSM 12112), (ZP_03855267.1; Veillonella parvula DSM 2008), (ZP_03928523.1; Acidaminococcus sp. D21), (NP_970659.1; Treponema denticola ATCC 35405), (ZP_05621510.1; Treponema vincentii ATCC 35580), (NP_218534.1; Treponema pallidum subsp. pallidum str. Nichols), (ZP_04047318.1; Brachyspira murdochii DSM 12563), (YP_002720478.1; Brachyspira hyodysenteriae WAT), (YP_001740706.1; Candidatus Cloacamonas acidaminovorans), (EER05013.1; Perkinsus manus ATCC 50983), (YP_945582.1; Borrelia turicatae 91El35), (YP_001884013.1; Borrelia hermsii DAH), (YP_002222233.1; Borrelia duttonii Ly), (ZP_03675306.1; Borrelia spielmanii A14S), (ZP_03435394.1; Borrelia afzelii ACA-l), (ZP_03540018.1; Borrelia garinii Far04), (ZP_03672928.1; Borrelia valaisiana VS116), (NP_212723.1; Borrelia burgdorferi B31), (YP_001956287.1; uncultured Termite group 1 bacterium phytype Rs-D17), (NP_975268.1; Mycoplasma mycoides subsp. mycoides SC str. PG1), (YP_424216.1; Mycoplasma capricolum subsp. capricolum ATCC 27343), (YP_053283.1; Mesoplasma florum LI), (CAK99540.1; Spiroplasma citri), (NP_072966.1; Mycoplasma genitalium G37), (NP_110116.1; Mycoplasma pneumonae M129), (NP_853403.1; Mycoplasma gallisepticum R), (NP_757889.1; Mycoplasma penetrans HF-2), (YP_116016.1; Mycoplasma hyopneumoniae 232), (YP_002960607.1; Mycoplasma conjunctivae), (YP_001256282.1; Mycoplasma agalactiae PG2), (BAH69503.1; Mycoplasma fermentans PG18), (YP_278771.1; Mycoplasma synoviae 53), (NP_326068.1; Mycoplasma pulmonis UAB CTIP), (YP_0515865.1; Mycoplasma mobile 163K), (YP_001256630.1; Mycoplasma agalactiae PG2), (YP_802685.1; Buchnera aphidicola str. Cc (Cinara cedri), (YP_001885432.1; Clostridium botulinum B str. Eklund 17B), (YP_001308302.1; Clostridium beijerinckii NCIMB 8052), (ZP_05131280.1; Clostridium sp. 7_2_43FAA), (ZP_02948604.1; Clostridium butyricum 5521), (NP_562641.1; Clostridium
Clostridium perfringens str. 13), (ZP_05391232.1; Clostridium carboxidivorans P7), (YP_001394780.1; Clostridium kluyveri DSM 555), (ZP_02995419.1; Clostridium sporogenes ATCC 15579), (YP_878298.1; Clostridium novyi NT), (ZP_04862192.1; Clostridium botulinum D str. 1873), (NP_781870.1; Clostridium tetani E88), (NP_04862192.1; Clostridium botulinum D str. 1873), (YP_878298.1; Clostridium novyi NT), (ZP_04804960.1; Clostridium cellulovorans 743B), (NP_348368.1; Clostridium acetobutylicum ATCC 824), (ACA51668.1; Thermoanaero bacterium saccharolyticum), (ZP_05336886.1; Thermoanaero bacterium thermosaccharolyticum SM 571), (NP_623097.1; Thermoanaero bacterr tengcongensis MB4), (YP_oo1663354.1; Thermoanaero bacter sp. X514), (YP_002508771.1; Halothermoth rix orenii H 168), (YP_003190679.1; Desulfotomaculum acetoxidans DSM 771), (YP_001917776.1; Natranaerobius thermophiles JWINM-WN-LF), (YP_360288.1; Carboxydothermus hydrogenoformans Z-2901), (EY83551.1; Bacteroides sp.2_1_33B), (ZP_02033408.1; Parabacteroides merdae ATCC 43184), (NP_905297.1; Porphyromonas gingivalis W83), (ZP_04056000.1; Porphyromonas uenonis 60-3), (ZP_04389884.1; Porphyromonas endodontalis ATCC 35406), (ZP_02068815.1; Bacteroides uniformis ATCC 8492), (ZP_03460749.1; Bacteroides eggerthii DSM 20697), (ZP_0367944.1; Bacteroides cellulosolyticum DSM 14838), (YP_097761.1; Bacteroides fragilis YCH46), (ZP_04545825.1; Bacteroides sp. Di), (ZP_03643544.1; Bacteroides coprophilus DSM 18228), (ZP_03207078.1; Bacteroides plebeius DSM 17135), (YP_001297855.1; Bacteroides vulgatus ATCC 8482), (ZP_05736702.1; Prevotella tannerae ATCC 51259), (ZP_06007587.1; Prevotella bergeensis DSM 17361), (ZP_05858935.1; Prevotella veroralis F0319), (ZP_05916997.1; Prevotella sp. oral taxon 472 str. F0295), (YP_002308782.1; Candidatus Azo bacteroides pseudotrichon ymphae genomovar. CFP2), (YP_753459.1; Syntrophomonas wolfei subs. wolfei str. Goettingen), (ZP_01771389.1; Collinsella aerofaciens ATCC 25986), (ZP_03296849.1; Collinsella stercoris DSM 13279), (ZP_04445308.1; Collinsella ntestinalis DSM 13280), (ZP_03567515.1; Atopobium rimaeATCC 49626), (YP_003179667.1; Atopobium parvulum DSM 20469), (ZP_03946133.1; Atopobium vaginae DSM 15829), (ZP_03990654.1; Oribacterium sinus F0268), (ZP_04450849.1; Abiotrophia defective ATCC 49176), (ZP_05797601.1; Oribacterium sp. oral taxon 078 str. F0262), (ZP_03730247.1; Clostridium sp. M62/l), (ZP_04856252.1; Ruminococcus sp. 5_1_39BFAA), (ZP_01966332.1; Ruminococcus obeum ATCC 29174), (ZP_05345616.1; Bryantella formategixens DSM 14469), (ZP_03780829.1; Blautia hydrogenotro phica DSM 10507), (ZP_03289360.1; Clostridium nexile DSM 1787), (ZP_02042092.1; Ruminococcus gnavus ATCC 29149), (ZP_031681 12.1; Ruminococcus
lactaris ATCC 291 76), (ZP_01968837.1; Ruminococcus torques ATCC 27756),
(ZP_02430426.1; Clostridium scindens ATCC 35704), (ZP_03779744.1; Clostridium
hylemonae DSM 15053), (ZP_02234595.1; Doreaformicigenerans ATCC 27755),
(ZP_01994673.1; Dorea longicatena DSM 13814), (YP_oo1558442.1; Clostridium
phytofermentans ISDg), (ZP_04667085.1; Clostridiales bacterium 1_7_47FAA),
(ZP_02085391.1; Clostridium bolteae ATCC BAA-613), (ZP_05790853.1; Butyribibrio
crossotus DSM 2876), (ZP_02026034.1; Eubacterium ventriosum ATCC 27560),
(YP_002930513.1; Eubacterium eligens ATCC 27750), (ZP_04808213.1; Helicobacter
pullorum MIT 98-5489), (ZP_03656120.1; Helicobacter Canadensis MIT 98-5491),
(ZP_04583217.1; Helicobacter winghamensis ATCCBAA-430), (NP_860840.1; Helicobacter
hepaticus ATCC 51449), (ZP_03657896.1; Helicobacter cinaedi CCUG 18818),
(ZP_02417779.1; Anaerostipes caccae DSM 14662), (ZP_02437622.1; Clostridium
sp. SS211), (ZP_02205430.1; Coprococcus eutactus ATCC 27759), (ZP_02692616.1;
Epulopiscium sp. ‘N.t. morphotype B’), (YP_003182082.1; Eggerthella lenta DSM 2243),
(YP_003151027.1; Cryptobacterium curium DSM 15641), (YP_003143601.1; Slackia
heliotrinireducens DSM 20476), (ZP_05498135.1; Clostridium papyrosolvens DSM 2782),
(ZP_03152606.1; Clostridium thermocellum JW20), (YP_001180817.1; Caldicellulosiruptor
saccharolyticus DSM 8903), (AAA72041.1; Methanosarcina thermophile), (NP_618482.1;
Methanosarcina acetivorans C2A), (YP_305342.1; Methanosarcina barkeri str. Fusaro),
(ZP_02142278.1; Roseobacter litoralis Och 149), (YP_681184.1; Roseobacter denitrificans
OCh 114), (YP_001533168.1; Dinoroseo bacter shibae DFL 12), (ZP_05124935.1;
Rhodobacteraceae bacterium KLI11), (ZP_05786337.1; Silicibacter lacuscaerulensis ITI-
1157), (YP_001313586.1; Sinorhizobium medicae WSM419), (NP_437512.1; Sinorhizobium
meliloti 1021), (ZP_04682129.1; Ochrobactrum intermedium LMG 3301),
(YP_oo1372036.1; Ochrobactrum anthropic ATCC 49188), (YP_00188815.1; Burkholderia
phytofirmans PsJN), (YP_554613.1; Burkholderia xenovorans LB400), (YP_oo1862297.1;
Burkholderia phymatum STM815), (YP_297974.1; Ralstonia eutropha JMP134),
(YP_02008219.1; Cupriavidus taiwanensis), (YP_001584488.1; Burkholderia multivorans
multivorans), (YP_002233797.1; Burkholderia cenocepacia J2315), (ZP_O1220235.1;
Photobacterium profundum 3TCK), (ZP_03698361.1; Lutiella nitroferrum 2002),
(ZP_01811515.1; Vibrionales bacterium SWATS), (ZP_00988349.1; Vibrio splendidus
12B01), (ZP_01866234.1; Vibrio shilonii AK1), (ZP_05885163.1; Vibrio coralliilyticus
ATCCBAA-450), (AAS78789.1; Paracoccus denitrificans), (YP_345196.1; Rhodobacter
sphaeroides 2.4.1), (AAN08490.1; Castellaniella defragrans), (ZP_00961345.1; Roseovarius nubinhibens ISM), (YP_168755.1; Ruegeria pomeroyi DSS-3), (ZP_01901193.1; Roseobacter sp. AzwK-3b), (ZP_01752570.1; Roseobacter sp. SK209-2-6), (ZP_02140073.1; Roseobacter litoralis Och 149), (YP_510789.1; Jannaschia sp. CCS1), (ZP_05073153.1; Rhodobacteral es bacterium HTCC2083), (YP_822367.1; Candidatus Solibacter usitatus Ellin6076), (ZP_01313101.1; Desulfuromonas as acetoxidans DSM 684), (YP_357950.1; Pelobacter carbinolicus DSM 2380), (YP_002537084.1; Geobacter sp. FRC-32), (YP_001232124.1; Geobacter uranireducens Rf4), (NP_953751.1; Geobacter sulfurreducens PCA), (YP_384000.1; Geobacter metallireducens GS-15), (YP_900968.1; Pelobacter propionicus DSM 2379), (YP_001910417.1; Helicobacter pylori Shii70), (NP_223559.1; Helicobacter pylori J99), (YP_665033.1; Helicobacter acinonychis str. Sheeba), (ZP_05311922.1; Geobacter sp. M18), (YP_003021758.1; Geobacter sp. M21), (YP_358255.1; Pelobacter carbinolicus DSM 2380), (ZP_03906856.1; Denitroviobrio acetophilus DSM 12809), (YP_001997093.1; Chloroherpeton thalassum ATCC 35110), (ZP_01924858.1; Victivallis vadensis ATCCBA-548), (ZP_03439825.1; Helicobacter pylori 98-10), (YP_003057614.1; Helicobacter pylori B38), (YP_001910417.1; Helicobacter pylori Shi470), (NP_223559.1; Helicobacter pylori J99), (YP_665033.1; Helicobacter acinonychis str. Sheeba), (ZP_01810337.1; Campylobacter jejuni subsp. jejuni CG8486), (ZP_00366840.1; Campylobacter coli RM2228), (ZP_00370527.1; Campylobacter upsaliensis RM3195), (YP_002575219.1; Campylobacter lari RM2100), (YP_001406718.1; Campylobacter hominis ATCCBA-381), (ZP_05624820.1; Campylobacter gracilis RM3268), (YP_891988.1; Campylobacter fetus subsp. fetus 82-40), (YP_001466901.1; Campylobacter concisus 13826), (YP_001408221.1; Campylobacter curvus 525.92), (ZP_05363348.1; Campylobacter showae RM3277), (ZP_03742933.1; Bifidobacterium pseudocatenulatum DSM 20438), (ZP_02918887.1; Bifidobacterium dentium ATCC 27678), (ZP_02028883.1; Bifidobacterium adolescentis L2-32), (ZP_04448100.1; Bifidobacterium angulatum DSM 20098), (ZP_03618886.1; Bifidobacterium breve DSM 20213), (ZP_03976084.1; Bifidobacterium longum subsp. infantis ATCC 55813), (YP_002323183.1; Bifidobacterium longum subsp. infantis ATCC 15697), (ZP_03646187.1; Bifidobacterium bifidum NCIMB 41171), (ZP_03937611.1; Gardnerella vaginalis ATCC 14019), (ZP_02962869.1; Bifidobacterium animalis subsp. lactis HNO19), (ZP_05965185.1; Bifidobacterium gallicum DSM 20093), (ZP_02043408.1; Actinomyces odontolyticus ATCC 17982), (ZP_03925176.1; Actinomyces coleocanis DSM 15436), (NP_601948.1; Corynebacterium glutamicum ATCC 13032), (NP_739201.1; Corynebacterium efficiens YS-
314), (NP_940379.1; Corynebacterium diphtheria NCTC 13129), (ZP_04835255.1; Corynebacterium glucuronolyticum ATCC 51867), (ZP_05708623.1; Corynebacterium genitalium ATCC 33030), (ZP_03977910.1; Corynebacterium lipophiloflavum DSM 44291), (ZP_03932064.1; Corynebacterium accolens ATCC 49725), (ZP_05366890.1; Corynebacterium aurimucosum ATCC 700975), (YP_250020.1; Corynebacterium jeikeium K411), (YP_oo1801132.1; Corynebacterium urealyticum DSM 7109), (YP_002906954.1; Corynebacterium kroppenstedtii DSM 44385), (ZP_03933297.1; Corynebacterium amycolatum SK46), (ZP_03718987.1; Neisseria flavescens NRL30031/H 210), (ZP_05318956.1; Neisseria sicca ATCC 29256), (YP_001598731.1; Neisseria meningitides 053442), (YP_04602977.1; Kingella oralis ATCC 51147), (YP_426466.1; Rhodospirillum rubrum ATCC 11170), (NP_871183.1; Wigglesworthia glossinidia endosymbiont of Glossina brevipalpis), (NP_777793.1; Buchnera aphidicola str. Bp (Baizongia pistaciae)), (ZP_03535302.1; Mycobacterium tuberculosis T17), (ZP_04056438.1; Capnocytophaga gingivalis ATCC 33624), (YP_003108500.1; Candidatus Sulcia muelleri SMDSEM), (P77844; Corynebacterium glutamicum), (ZP_03994160.1; Mobiluncus mulieris ATCC 35243), (ZP_03922640.1; Mobiluncus curtisi ATCC 43063), (ZP_03716209.1; Eubacterium hallii DSM 3353), (ZP_03718143.1; Eubacterium hallii DSM 3353), (ZP_05614434.1; Faecalibacterium prausnitzii A2-165), (ZP_02034852.1; Bacteroides capillosus ATCC 29799), (ZP_03753543.1; Roseburia inulinivorans DSM 16841), (ZP_04745275.2; Roseburia intestinalis Li-82), (YP_002937332.1; Eubacterium rectale ATCC 33656), (ZP_02074244.1; Clostridium sp. L2-50), (ZP_04455374.1; Shuttleworthia satelles DSM 14600), (ZP_03488480.1; Eubacterium biforme DSM 3989), (ZP_02078327.1; Eubacterium dolichum DSM 3991), (ZP_02077559.1; Eubacterium dolichum DSM 3991), (ZP_03305532.1; Anaerococcus hydrogenalis DSM 7454), (ZP_05473291.1; Anaerococcus vaginalis ATCC 51170), (ZP_03931050.1; Anaerococcus tetradius ATCC 35098), (YP_003153463.1; Anaerococcus prevotii DSM 20548), (YP_03916048.1; Anaerococcus lactolyticus ATCC 51172), (NP_607213.1; Streptococcus pyogenes MGAS8232), (AAK34003.1; Streptococcus pyogenes M1GAS), (YP_002562185.1; Streptococcus uberis 01401), (YP_002744451.1; Streptococcus equi subsp. Zooepidemicus), (BAH88016.1; Streptococcus mutans NN2025), (ZP_02920305.1; Streptococcus infantarius subsp. infantarius ATCCBAA-102), (YP_329798.1; Streptococcus agalactiae A909).
(ZP_04061789.1; Streptococcus salivarius SK126), (YP_139881.1; Streptococcus thermophiles LMG 18311), (ZP_04525024.1; Streptococcus pneumoniae CCRI 1974), (ZP_06060573.1; Streptococcus sp. 2JJ6FAA), (YP_001198423.1; Streptococcus suis 05ZHY33), (NP_964739.1; Lactobacillus johnsonii NCC 533), (YP_193610.1; Lactobacillus acidophilus NCFM), (ZP_04011019.1; Lactobacillus ultunensis DSM 16047), (ZP_03995297.1; Lactobacillus crispatus JV-VOI), (ZP_05752753.1; Lactobacillus helveticus DSM 20075), (ZP_03956024.1; Lactobacillus jensenii JV-VI6), (ZP_04645187.1; Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842), (ZP_05744366.1; Lactobacillus iners DSM 13335), (NP_391646.1; Bacillus amyloboliquefaciens FZB42), (YP_081073.1; Bacillus licheniformis ATCC 14580), (ZP_03055101.1; Bacillus pumilus ATCC 7061), (YP_002317098.1; Anoxybacillus flavithermus WKL), (YP_002951270.1; Geobacillus sp. WCH70), (YP_001127443.1; Geobacillus thermodinotrichicans NG80-2), (YP_149268.1; Geobacillus kaustophilus HTA426), (ZP_01861251.1; Exiguobacterium sp. SG-I), (ZP_03228169346.1; Bacillus coahuilensis m4-4), (ZP_01173945.1; Bacillus sp. NRRLB-14911), (NP_693944.1; Oceanobacillus iheyensis HTE831), (ZP_04314753.1; Bacillus cereus BGSC 6E1), (YP_014727.1; Listeria monocytogenes str. 4b F2365), (ZP_04443757.1; Listeria grayi DSM 20601), (NP_244690.1; Bacillus halodurans C-125), (YPJ77402.1; Bacillus clausii KSM-K1 6), (YP_002885816.1; Exiguobacterium sp. ATl6b), (YP_0011812721.1; Exiguobacterium sibiricum 255-15), (ZP_02169346.1; Bacillus selenitireducens MLS10), (ZP_04818386.1; Staphylococcus epidermidis M23864-WI), (ZP_03612973.1; Staphylococcus capitis SK14), (ZP_04677798.1; Staphylococcus haemolyticus JCSC1435), (ZP_04059818.1; Staphylococcus hominis SK119), (ABR57177.1; Staphylococcus xylosus), (YP_302214.1; Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305), (YP_002633340.1; Staphylococcus camosus subsp. camosus TM300), (YP_002561236.1; Macroccococcus caseolyticus JCSC5402), (ZP_03944466.1; Lactobacillus fermentum ATCC 14931), (ZP_05553502.1; Lactobacillus coleohominis 101-4-CHN), (ZP_0395629.1; Lactobacillus vaginalis ATCC 49540), (YP_001271004.1; Lactobacillus reuteri DSM 20016), (ZP_05745668.1; Lactobacillus antri DSM 16041), (YP_818931.1; Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293), (YP_00172783.1; Leuconostoc citreum KM20), (ZP_04782044.1; Weissella paramesenteroides ATCC 33313), (ZP_01544468.1;
Oenococcus oeni ATCC BAA-1 163), (ZP_05737294.1; Granulicatella adiacens ATCC 49175), (ZP_05851915.1; Granulicatella elegans ATCC 700633), (ZP_02183965.1; Camobacterium sp. ATT), (ZP_05649755.1; Enterococcus gallinarum EG2), (ZP_03947918.1; Enterococcus faecalis TX0104), (ZP_03982224.1; Enterococcus faecium TX1330), (YP_395954.1; Lactobacillus sakei subsp. sakei 23K), (ZP_04449762.1; Catonella morbi ATCC 51271), (YP_001032100.1; Lactococcus lactis subsp. cremoris MG1363), (YP_806234.1; Lactobacillus casei ATCC 334), (NP_784550.1; Lactobacillus plantarum WCFS1), (YP_794848.1; Lactobacillus brevis ATCC 367), (ZP_03954831.1; Lactobacillus hilgardii ATCC 8290), (BABI9267.1; Lactobacillus sanfranciscensis), (ZP_03958288.1; Lactobacillus ruminis ATCC 25644), (YP_536042.1; Lactobacillus salivarius UCC118), (ZP_05747635.1; Erysipelothrix rhusiopathiae ATCC 19414), (YP_803875.1; Pediococcus pentosaceus ATCC 25745), (ZP_02093784.1; Parvimonas micra ATCC 33270), (YP_001692923.1; Finegoldia magna ATCC 29328), (ZP_04431499.1; Bacillus coagulans 36DI), (ZP_04775813.1; Gemella haemoflyans ATCC 10379), (YP_001360609.1; Kineococcus radiotolerans SRS30216), (ZP_01115869.1; Reinekeia blandensis MED297), (YP_003074238.1; Teredinibac turturnear T7901), (YP_95841.1; Marinobacter quaeoei VT8), (YP_435580.1; Hahella chejuensis KCTC 2396), (YP_001189125.1; Pseudomonas mendocina ymp), (YP_792443.1; Pseudomonas aeruginosa UCBPP-PA14), (NP_791001.1; Pseudomonas synnagae pv. tomato str. DC3000), (YP_258069.1; Pseudomonas fluorescens Pf-5), (YP_606637.1; Pseudomonas entomophila L48), (YP_002800579.1; Azotobacter vinelandii DJ), (YP_001171663.1; Pseudomonas stutzeri A1501), (NP_840385.1; Nitrosomonas europaea ATCC 19718), (YP_002801221.1; Azotobacter vinelandii DJ), (YP_00278711.1; Deinococcus deserti VCD115), (YP_603523.1; Deinococcus geothermalis DSM 11300), (NP_293799.1; Deinococcus radiodurans R1), (YP_521550.1; Rhodoferax ferrireducens T118), (YP_530962.1; Rhodopseudo monas palustris BisB18), (YP_531882.1; Rhodopseudo monas palustris BisA53), (ZP_02367347.1; Burkholderia oklahomensis C6786), (YP_428079.1; Rhodospirillum rubrum ATCC 11170), (YP_530535.1; Rhodopseudo monas palustris BisB18), (NP_901200.1; Chromobacterium violaceum ATCC 12472), (ZP_03698345.1; Lutellia nitroferrum 2002), (YP_001279250.1; Psychrobacter sp. PRP-wf1), (YP_579484.1; Psychrobacter cryohalolentis K5), (ZP_05618978.1; Enhydrobacter aerosaccus SK60), (ZP_05362319.1; Acinetobacter radioresistens SK82), (YP_045288.1; Acinetobacter sp. ADP1), (ZP_05823314.1; Acinetobacter sp. RUH2624), (ZP_03824416.1; Acinetobacter sp. ATCC 27244), (YP_001380280.1; Anaeromyxobacter sp.
Fwl09-5 (YP_466103.1; Anaeromyxobacter dehalogenans 2CP-C), (YP_088190.1; Mannheimia succiniciproducens MBEL55E), (YP_001344949.1; Actinobacillus succinogenes 130X), (YP_00300741.1; Aggregatibacter aphrophilus NJ8700), (ZP_01788798.1; Haemophilus influenzae 3655), (YP_719012.1; Haemophilus somnus 129PT), (NP_245642.1; Pasteurella multocida subsp. multocida sir. Pm70), (ZP_05920444.1; Pasteurella dagmatis ATCC 43325), (ZP_00133992.2; Actinobacillus pleuropneumoniae serovar 1 str. 4074), (ZP_04753547.1; Actinobacillus minor NM305), (NP_873873.1; Haemophilus ducreyi 35000HP), (ZP_04978908.1; Mannheimia haemolytica PHL213), (YP_002475022.1; Haemophilus parasuis SH0165), (ZP_05730581.1; Pantoea sp. At-9b), (YP_001907133.1; Erwinia tasmaniensis Etl99), (YP_455287.1; Sodalis glossinidius str. 'morsitans'), (ZP_05723922.1; Dickeya dadantii Ech586), (YP_003258889.1; Pectobacterium wasabiae WPP163), (YP_002988159.1; Dickeya dadantii Ech703), (NP_668938.1; Yersinia pestis KIM 10), (YP_001479543.1; Serratia proteamaculans 568), (YP_002934098.1; Edwardsiella ictaluri 93-146), (YP_0002151502.1; Proteus mirabilis HI4320), (NP_930328.1; Photobacterium luminescens subsp. laundromii TTO1), (YP_002920553.1; Klebsiella pneumoniae NTUH-K2044), (YP_001177557.1; Enterobacter sp.638), (YP_003211286.1; Cronobacter turicensis), (BAA04663.1; Escherichia coli). (YP_002924403.1; Candidatus Hamiltonella defensa 5AT (Acyrthosiphon pisum)), (ZP_03827735.1; Pectobacterium carotovorum subsp. brasilensis PBR1692), (ZP_011159282.1; Photobacterium sp. SKA34), (YP_130973.1; Photobacterium profundum SS9), (ZP_06052481.1; Grimontia hollisae CIP 101886), (ZP_05877035.1; Vibrio fumissii CIP 102972), (ZP_05881960.1; Vibrio metschnikoyii CIP 69.14), (ZP_05881960.1; Vibrio metschnikoyii CIP 69.14), (ZP_02196748.1; Vibrio sp. AND4), (NP_934927.1; Vibrio vulnificus YJ016), (ZP_01866446.1; Vibrio shilonii AKI), (YP_002416612.1; Vibrio splendidus LGP32), (YP_002263486.1; Aliivibrio salmonicida LFI1238), (ZP_0441514.1; Vibrio cholerae by. albensis VL426), (YP_00143125.1; Aeromonas salmonicida subsp. salmonicida A449), (YP_002892091.1; Tolumonas auensis DSM 9187), (ZP_01215350.1; Psychromonas sp. CNPT3), (YP_944598.1; Psychromonas ingrahamii 37), (YP_001473443.1; Shewanella sediminis HAW-EB3), (YP_001761257.1; Shewanella woodyi ATCC 51908), (YP_001094519.1; Shewanella loihica PV-4), (YP_00167481.1; Shewanella halifaxensis HAW-EB4), (YP_869191.1; Shewanella sp. ANAS), (YP_927371.1; Shewanella amazonensis SB2B), (YP_751 160.1; Shewanella frigidimarina NCIMB 400), (YP_563413.1; Shewanella denitrificans OS217), (YP_001475272.1; Shewanella sediminis HAW-EB3).
(YP_001674949.1; Shewanella halifaxensis HAW-EB4), (ZP_04716660.1; Alteromonas macleodii ATCC 27126), (YP_0662160.1; Pseudoalteromonas atlantica T6c), (ZP_01612225.1; Alteromonadales bacterium TW-7), (ZP_01134640.1; Pseudoalteromonas tunicateDI), (YP_269873.1; Colwellia psychrerythrae a 34H), (YP_001341167.1; Marinomonas sp. MWYLI), (ZP_01077352.1; Marinomonas sp. MED121), (YP_001209362.1; Dichelobacter nodosus VCS1703A), (ZP_05705193.1; Cardiobacterium hominis ATCC 15826), (EEY62817.1; Phytophthora infestans T30-4), (EEY62816.1; Phytophthora infestans T30-4), (XP_001694504.1; Chlamydomonas reinhardtii), (XP_001753120.1; Physcomitrella patens subsp. Patens), (YP_001804510.1; Cyanothece sp. ATCC 51142), (ZP_01729220.1; Cyanothece sp. CCY0110), (YP_003138337.1; Cyanothece sp. PCC 8802), (YP_002380034.1; Cyanothece sp. PCC 7424), (NP_441027.1; Synechocystis sp. PCC 6803), (ZP_01061711.1; Leeuwenhoekiella blandensis MED217), (YP_001195862.1; Flavobacterium johnsoniae UWIOI), (YP_003194927.1; Robiginitalea biformalia HTCC2501), (ZP_01107792.1; Flavobacteriales bacterium HTCC2170), (ZP_01051731.1; Polaribacter sp. MED152), (ZP_01119204.1; Polaribacter erginsii 23-P), (ZP_003390929.1; Capnocytophaga sputigena ATCC 33612), (YP_003141977.1; Capnocytophaga ochracea DSM 7271), (YP_0012240.1; Desulfovibrio vulgaris str. Hildenborough), (YP_002436276.1; Desulfovibrio vulgaris str. 'Miyazaki F'), (YP_389730.1; Desulfovibrio desulfuricans subsp. desulfuricans str. G20), (YP_002992165.1; Desulfovibrio salexigens DSM 2638), (YP_003197901.1; Desulfohalobium retbaense DSM 5692), (YP_003157577.1; Desulfomicrobium baculatum DSM 4028), (ZP_03737911.1; Desulphonatronospira thiodismutans AS03-1), (YP_002990332.1; Desulfovibrio salexigens DSM 2638), (ZP_03312237.1; Desulfovibrio piger ATCC 29098), (YP_002478890.1; Desulfovibrio desulfuricans subsp. desulfuricans str. ATCC 27774), (YP_064294.1; Desulffotalea psychrophila Lsv54), (YP_5094656.1; Lawsonia intracellulars PHE/MNL-00), (ZP_01621820.1; Lyngbya sp. PCC 8106), (ZP_03272899.1; Arthrosira maxima CS-328), (YP_845596.1; Syntrophobacter fumaroxidans MPOB), (ZP_04773932.1; Allochromatium vinosum DSM 180), (NP_869002.1; Rhodopirellula baltica SH 1), (YP_392571.1; Sulfurimonas denitrificans DSM 1251), (ZP_05071717.1; Campylobacterales bacterium GD 1), (ZP_04421899.1; Sulfurospirillum deleyianum DSM 6946), (YP_001359295.1; Sulfurovum sp. NBC37-1), (YP_951544.1; Mycobacterium vanbaalenii PYR-1), (YP_001131488.1; Mycobacterium gilvum PYR-GCK), (YP_637714.1;
Mycobacterium sp. (YP_885 188.1; Mycobacterium smegmatis str. MC2 155),
YP_001704953.1; Mycobacterium abscessus, (ZP_04747529.1; Mycobacterium kansasii
ATCC 12478), (YP_001849024.1; Mycobacterium marinum), (NP_214922.1;
Mycobacterium tuberculosis H37Rv), (NP_962819.1; Mycobacterium avium subsp.
paratuberculosis K-10), (ZP_05223872.1; Mycobacterium intracellulare ATCC 13950),
YP_002764919.1; Rhodococcus erythropolis PR4), (YP_702162.1; Rhodococcus jostii
RHA1), (YP_121562.1; Nocardia farcinica IFM 10152), (ZP_04025361.1; Tsukamurella
paurometabola DSM 20162), (YP_003275431.1; Gordonia bronchialis DSM 43247),
YP_003160610.1; Jonesia denitrificans DSM 20603), (ZP_05816650.1; Sanguibacter
keddieii DSM 10542), (ZP_05223872.1; Mycobacterium intracellulare ATCC 13950),
YP_001849024.1; Mycobacterium marinum), (NP_214922.1; Mycobacterium tuberculosis
H37Rv), (NP_962819.1; Mycobacterium avium subsp. paratuberculosis K-10),
YP_05223872.1; Mycobacterium intracellulare ATCC 13950), (YP_002764919.1;
Rhodococcus erythropolis PR4), (YP_702162.1; Rhodococcus jostii RHA1), (YP_121562.1;
Nocardia farcinica IFM 10152), (ZP_04025361.1; Tsukamurella paurometabola
DSM 20162), (YP_003275431.1; Gordonia bronchialis DSM 43247), (YP_003160610.1;
Jonesia denitrificans DSM 20603), (ZP_05816650.1; Sanguibacter keddii DSM 10542),
(ZP_04368027.1; Cellulomonas flavigena DSM 20109), (YP_002883054.1;
Beutenbergia cavemae DSM 12333), (ZP_03911481.1; Xylanimonas cellulosilytica
DSM 15894), (YP_924143.1; Nocardia farcinica IFM 10152), (ZP_03864789.1;
Kribbella flavida DSM 17836), (ZP_00121057.1; marine actinobacterium PHSC20C1),
YP_001708941.1; Clavibacter michiganensis subsp. Sepedonicus), (YP_061462.1;
Leifsonia xyli subsp. xyli str. CTCB07), (YP_748183.1; Nitrosomonas eutropha C91),
YP_003116892.1; Catenulispora acidiphila DSM 44928), (YP_003199983.1; Nakamurella
multipartita DSM 44233), (YP_003154321.1; Brachybacterium faecium DSM 4810),
(ZP_03927492.1; Actinomyces urogenitalis DSM 15434), (YP_003148931.1; Kytococcus
sedentarius DSM 20547), (ZP_05803950.1; Streptomyces griseoflavus ATCC 33331),
YP_001823623.1; Streptomyces griseus subsp. griseus NBRC 13350), (ZP_05002693.1;
Streptomyces clavuligerus ATCC 27064), (ZP_05015493.1; Streptomyces sp. ATCC
29083), (ZP_05538660.1; Streptomyces griseoflavus Tu4000), (ZP_04685789.1;
Streptomyces ghanaensis ATCC 14672), (ZP_05534308.1; Streptomyces viridochromogenes
DSM 40736), (ZP_05523554.1; Streptomyces lividans TK24), (NP_823999.1; Streptomyces
avermitilis MA-4680), (CBG69921.1; Streptomyces scabiei 87.22), (ZP_04704905.1;
Streptomyces albus 11074), (ZP_04997745.1; Streptomyces sp. Mgl), (ZP_05509147.1;
Streptomyces sp. C), (ZP_05514718.1; Streptomyces hygroscopicus ATCC 53653),
(ZP_04994290.1; Streptomyces sp. SBP74), (ZP_04474082.1; Streptosporangium roseum
DSM 43021), (YP_001160501.1; Salinispora tropica CNB-440), (YP_001538853.1;
Salinispora arenicola CNS-205), (ZP_04605575.1; Micromonospora sp. ATCC 39149),
YP_832716.1; Arthrobacter sp. FB24), (ABR13603.1; Arthrobacter oxydans),
YP_002956296.1; Micrococcus luteus NCTC 2665), (ZP_05367249.1; Rothia mucilaginosa
ATCC 25296), (YP_001854004.1; Kocuria rhizophila DC2201), (ZP_04984463.1;
Francisella tularensis subsp. holarctica FSC022), (YP_001677422.1; Francisella philomiragia subsp. philomiragia ATCC 25017), (YP_588827.1; Baumannia cicadellinicola sir. He (Homalodisca oagulata)), (NP_240007.1; Buchnera aphidicola sir. APS (Acyrthosiphonpisum)), (ZP_05057494.1; Verrucomicrobiae bacterium DG1235), (ZP_02930252.1; Verrucomicrobium spinosum DSM4136), (ZP_01452386.1; Mariprofundus ferrooxydans PV-l), and (ZP_01307392.1; Bermanella marisrubri).

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.

5.4 Functional Disruption of Acetyl Phosphatase Activity

In some embodiments, the genetically modified host cell provided herein comprises a functional disruption in an enzyme that converts acetyl phosphate to acetate. In some embodiments, the enzyme is native to the host cell.

In some embodiments, the enzyme that converts acetyl phosphate to acetate is a glycerol-1-phosphatase (EC 3.1.3.21). In some embodiments, the enzyme having glycerol-1-phosphatase activity is RHR2 (GPP1/RHR2; systematic name: YIL053W), or a homolog or variant thereof. GPP1/RHR2 is a constitutively expressed glycerol-1-phosphatase involved in glycerol biosynthesis, and is induced in response to both anaerobic and osmotic stress. See, e.g., Norbeck et al, J Biol Chem 271(23): 13875-13881 (1996); Norbeck et al, J Biol Chem 272(9): 13875-13881 (1996); Pahlman et al, J Biol Chem 276(5): 3555-3563 (2001); Nevoigt and Stahl, FEMS Microbiol Rev 21(3):231-41 (1997); Byrne and Wolf, Genome Res 15(10): 1456-61; and Hirayama et al., Mol Gen Genet 249(2): 127-38, the contents of each of which are hereby incorporated by reference in their entireties. The sequence of the GPP1/RHR2 gene of S. cerevisiae has been previously described. See, e.g., Norbeck et al., J Biol Chem 271(23): 13875-13881 (1996); and Pahlman et al., J Biol Chem 276(5): 3555-3563 (2001). Gppl/Rhr2 has been previously described as catalyzing the following reaction:

\[ \text{glycerol-1-phosphate} + \text{H}_2\text{O} \rightleftharpoons \text{glycerol} + \text{phosphate}. \]
[0071] Representative GPP1/RHR2 nucleotide sequences of Saccharomyces cerevisiae include accession number NM_001179403.1, and SEQ ID NO: 5 as provided herein. Representative Gppl/Rhr2 protein sequences of Saccharomyces cerevisiae include accession number NP_012211.1, and SEQ ID NO: 6 as provided herein.

[0072] A closely related homolog of GPP1/RHR2 which also catalyzes the hydrolysis of acetyl phosphate to acetate is HOR2 (GPP2/HOR2; systematic name: YER062C). Gpp2/Hor2 has also been previously described as a glycerol-1-phosphatase capable of catalyzing the following reaction: glycerol-1-phosphate + H2O ⇌ glycerol + phosphate. Accordingly, functional disruption of GPP2/HOR2 also finds use in the compositions and methods provided herein. The sequence of the GPP2/HOR2 gene of S. cerevisiae has been previously described. See, e.g., Norbeck et al., J. of Biological Chemistry 271(23): 13875-13881 (1996); and Pahlman et al., J. of Biological Chemistry 276(5): 3555-3563 (2001).

Representative GPP2/HOR2 nucleotide sequences of Saccharomyces cerevisiae include accession number NM_001178953.3, and SEQ ID NO: 7 as provided herein. Representative Gppl/Rhr2 protein sequences of Saccharomyces cerevisiae include accession number NP_010984, and SEQ ID NO: 8 as provided herein.

[0073] As would be understood in the art, naturally occurring homologs of GPP1/RHR2 and/or GPP2/HOR2 in yeast other than S. cerevisiae can similarly be inactivated using the methods described herein. Moreover, a polynucleotide, gene and/or polypeptide encoding acetyl-phosphatase activity (e.g., RHR2 and/or HOR2) can be used to identify other polynucleotide, gene and/or polypeptide sequences or to identify homologs having acetyl-phosphatase activity in other host cells. Such sequences can be identified, for example, in the literature and/or in bioinformatics databases well known to the skilled person. For example, the identification of sequences encoding acetyl-phosphatase activity in other cell types using bioinformatics can be accomplished through BLAST (as described above) searching of publicly available databases with known DNA and polypeptide sequences encoding acetyl-phosphatase and/or glycerol-1-phosphatase activity, such as those provided herein. Identities can be based on the Clustal W method of alignment using the default parameters of GAP PENALTY = 10, GAP LENGTH PENALTY = 0.1, and Gonnet 250 series of protein weight matrix.

[0074] In some embodiments, the activity or expression of an endogenous enzyme that converts acetyl phosphate to acetate (e.g., RHR2 or HOR2) is reduced by at least about 50%. In another embodiment, the activity or expression of an endogenous enzyme that
converts acetyl phosphate to acetate 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 an endogenous enzyme that converts acetyl phosphate to acetate. In some embodiments, the endogenous enzyme that converts acetyl phosphate to acetate is RHR2, or homologues thereof. In some embodiments, the endogenous enzyme that converts acetyl phosphate to acetate is HOR2, or homologues thereof.

As is understood by those skilled in the art, there are several mechanisms available for reducing or disrupting the activity of a protein that converts acetyl phosphate to acetate, such as a glycerol-1-phosphatase (e.g., RHR2 and/or HOR2), 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.

In some embodiments, the genetically modified host cell comprises a mutation in at least one gene encoding acetyl-phosphatase activity (e.g., RHR2, HOR2 or a homolog or variant thereof), 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 a gene encoding acetyl-phosphatase activity (e.g., RHR2, HOR2 or a homolog or variant thereof), 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 acetyl-phosphatase activity (e.g., RHR2, HOR2 or a homolog or variant thereof), 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 acetyl-phosphatase activity (e.g., RHR2, HOR2 or a homolog or variant thereof), 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 acetyl-phosphatase activity (e.g., RHR2, HOR2 or a homolog or variant thereof).
In some embodiments, disruption of one or more genes encoding a protein capable of catalyzing the conversion of acetyl phosphate to acetate is achieved by using a "disruption construct" that is capable of specifically disrupting such a gene (e.g., RHR2 or HOR2) 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 encoding a protein capable of converting acetyl phosphate to acetate 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.

Disruption constructs capable of disrupting a gene 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. In 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 acetyl-phosphatase 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.

[0079] 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.


5.5 Additional Modifications to Improve Acetyl-CoA Production

5.5.1 ADA

[0081] In some embodiments, the genetically modified host cells provided herein further comprise one or more heterologous nucleotide sequences encoding acylating acetaldehyde dehydrogenase (alternately referred to as "acetaldehyde dehydrogenase, acetylating," "acetaldehyde dehydrogenase, acylating," or ADA (EC 1.2.1.10)).

[0082] Proteins capable of catalyzing this reaction that are useful for the compositions and methods provided herein include the following four types of proteins:

[0083] (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 NH2-terminal region of

(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: EDK331 16). 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 aid gene product in Clostridium beijerinckii NRRL B593 (Toth et al. (1999) Appl. Environ. Microbiol. 65: 4973-4980, accession no: AAD31841 ).

(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. III: 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.

(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

[0087] 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 (EDK331 16), Lactobacillus plantarum acdH, and Pseudomonas putida (YP 001268189), as described in International Publication No. WO 2009/013159, the contents of which are incorporated by reference in their entirety. 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 (ABQ445 11.2), Caldithrix abyssi eutE (ZP_09549576), and Halorubrum latusprofundi ATCC 49239 (YP_002565337.1).

[0088] 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: 9 as provided herein. A representative eutE protein sequence of Dickeya zeae includes accession number YP_003003316, and SEQ ID NO: 10 as provided herein.

[0089] 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.

5.5.2 Functional Disruption of the PDH-bypass

[0090] 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** (YOPv374w) 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.

[0091] In some embodiments, the genetically modified host cell provided herein further 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.

[0092] 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.

[0093] 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.

### 5.5.2.1 ALD4 and ALD6

[0094] 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.
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: 11 as provided herein. Representative Ald4 protein sequences of Saccharomyces cerevisiae include accession number NP_O15019.1, and SEQ ID NO: 12 as provided herein.

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: 13 as provided herein. Representative Ald6 protein sequences of Saccharomyces cerevisiae include accession number AAB01219, and SEQ ID NO: 14 as provided herein.

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.

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.

5.5.2.2 ACS1 and ACS2

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

In some embodiments, one or more genes encoding acetyl-CoA synthetase (ACS) activity is functionally disrupted in the host cell. ACS1 and ACS2 are both acetyl-CoA synthetases that can 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).

[00101] 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: 15 as provided herein. Representative Acsl protein sequences of *Saccharomyces cerevisiae* include accession number AAC04979, and SEQ ID NO: 16 as provided herein.

[00102] 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: 17 as provided herein. Representative Acs2 protein sequences of *Saccharomyces cerevisiae* include accession number CAA97725, and SEQ ID NO: 18 as provided herein.

[00103] As would be understood in the art, naturally occurring homologs of acetyl-CoA synthetase in yeast other than *S. cerevisiae* can similarly be inactivated using the methods described herein.

[00104] In some embodiments, the host cell comprises a cytosolic acetyl-coA synthetase 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 synthetase 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.

[00105] In some embodiments, the host cell comprises a cytosolic acetyl-coA synthetase 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 synthetase 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.

[00106] In some embodiments, the host cell comprises a heterologous PK and a cytosolic acetyl-coA synthetase activity (e.g. ACS1 and/or ACS2). In such embodiments, PK produces acetyl phosphate in the host cell. The intact cytosolic ACS activity can convert acetate that accumulates as a result of RHR2 and/or HOR2-catalyzed acetyl phosphate hydrolysis into acetyl-CoA.

5.6 MEV Pathway for Isoprenoid Production

[00107] In some embodiments, the genetically modified host cell provided herein 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.

[00108] In some embodiments, the one or more enzymes of the MEV pathway are selected from the group consisting of acetyl-CoA thiolase, acetoacetyl-CoA synthetase, 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.

[00109] 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.

[00110] 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.

### 5.6.1 Conversion of Acetyl-CoA to Acetoacetyl-CoA

[00111] 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*).
[0012] 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.

[0013] In host cells comprising acetyl-CoA thiolase and a heterologous ADA and/or phosphotransacetylase (PTA), the reversible reaction catalyzed 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. Similarly, the activity of PTA is reversible, and thus, a large acetyl-CoA pool may drive PTA towards the reverse reaction of converting acetyl-CoA to acetyl phosphate. Therefore, in some embodiments, in order to provide a strong pull on acetyl-CoA to drive the forward reaction of ADA and PTA, 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.

[0014] 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:19 as provided herein. Representative AACS protein sequences of *Streptomyces* sp. strain CL190 include accession numbers D7URV0, BAJ 10048, and SEQ ID NO:20 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 (AB1 13568; BAD07381); *Streptomyces* sp. C (NZ_ACEW0 10000640; ZP_0551 1702); *Nocardiopsis dassonvillei* DSM 431 11 (NZ_ABU10 1000023; ZP_04335288); *Mycobacterium ulcerans* Agy99 (NC_008611; YP_907152); *Mycobacterium marinum* M (NC_010612; YP_001851502); *Streptomyces* sp. Mgl (NZ_DS570501; ZP_05002626); *Streptomyces* sp. AA4 (NZ_ACEV0 1000037; ZP_05478992); *S. roseosporus* NRRL 15998
Additional suitable acetoacetyl-CoA synthases include those described in U.S. Patent Application Publication Nos. 2010/0285549 and 2011/0281315, the contents of which are incorporated by reference in their entireties.

[0015] 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 synthase is 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.

5.6.2 Conversion of Acetoacetyl-CoA to HMG-CoA

[0016] 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, GenelID 1122571; Staphylococcus aureus).

5.6.3 Conversion of HMG-CoA to Mevalonate

[0017] 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.

<table>
<thead>
<tr>
<th>Source</th>
<th>Coenzyme specificity</th>
<th>$K_m^{\text{NADPH}}$ (µM)</th>
<th>$K_m^{\text{NADH}}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. mevalonii</em></td>
<td>NADH</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td><em>A. fulgidus</em></td>
<td>NAD(P)H</td>
<td>500</td>
<td>160</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>NAD(P)H</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>NADPH</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

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* or *D. acidovorans*.

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 (EC 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: 21 as provided herein. Representative HMG-CoA reductase protein sequences of *Pseudomonas mevalonii* include accession numbers AAA25837, P13702, MVAA_PSEMV, and SEQ ID NO: 22 as provided herein.

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: 23 as provided herein. Representative HMG-CoA reductase protein sequences of *Silicibacter pomeroyi* include accession number YP_164994, and SEQ ID NO: 24 as provided herein.
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: 25 as provided herein. Representative HMG-CoA reductase protein sequences of *Delftia acidovorans* include accession number YP_001561318, and SEQ ID NO: 26 as provided herein.

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

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.

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 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*, 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), the contents of which are hereby incorporated in their entireties.
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), the contents of which are hereby incorporated by reference in their entireties.

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. 8:1 10-113 (1962); Siddiqi et al., J. Bacteriol. 93:207-214 (1967); and Takatsuji et al., Biochem. Biophys. Res. Commun. 110:187-193 (1983), the contents of which are hereby incorporated by reference in their entireties.

In some embodiments of the compositions and methods provided herein, the host cell comprises both a NADH-using HMGGr 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, GenelD 1122570; Staphylococcus aureus), (AB015627; Streptomyces sp. KO 3988), (AX128213, providing the sequence encoding a truncated HMG-CoA reductase; Saccharomyces cerevisiae), and (NC_001 145: complement (115734.1 18898; Saccharomyces cerevisiae).

**5.6.4 Conversion of Mevalonate to Mevalonate-5-Phosphate**

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

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).

5.6.6 Conversion of Mevalonate-5-Pyrophosphate to IPP

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).

5.6.7 Conversion of IPP to DMAPP

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 pyrophosphate (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).

5.6.8 Polyprenyl Synthases

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.

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;
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 thalian), (ATHFPS2R; Arabidopsis thalian), (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), (AFO 19892; Helianthus annuus), (HUMFAPS; Homo sapiens), (KLPFPSQCR; Kluyveromyces lactis), (LAU15777; Lupinus albus), (LAU20771; Lupinus albus), (AF309508; Mus musculus), (NCFFPSGEN; Neurospora crassa), (PAFPS1; Parthenium argentatum), (PAFPS2; Parthenium argentatum), (RATFPS; Rattus norvegicus), (YSCFP; Saccharomyces cerevisiae), (D89104; Schizosaccharomyces pombe), (CP000003, Locus AAT87386; Streptococcus pyogenes), (NC_008022, Locus YP_598856; Streptococcus pyogenes MGAS 10270), (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 thalian), (D84432, Locus BAA12575; Bacillus subtilis), (U12678, Locus AAC28894; Bradyrhizobium japonicum USDA 110), (BACFDPS; Geobacillus stearothermophilus), (NC_002940, Locus NP_873754; Haemophilus ducreyi 35000HP), (L42023, Locus AAC23087; Haemophilus influenzae Rd KW20), (J05262; Homo sapiens), (YP_395294; Lactobacillus sakei subsp. sakei 23K), (NC_005823, Locus YP_000273; Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130), (AB003187; Micrococcus luteus), (NC_002946, Locus YP_208768; Neisseria gonorrhoeae FA 1090), (U00090, Locus AAB91752; Rhizobium sp. NGR234), (J05091; Saccharomyces cerevisiae), (CP000031, Locus AAV93568; Silicibacter pomeroyi...
DSS-3), (AE008481, Locus AAK99890; *Streptococcus pneumoniae* R6), and (NC_004556, Locus NP 779706; *Xylella fastidiosa* Temecula).  

[00136] 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_1 19845; *Arabidopsis thaliana*), (NZ_AAIME 1000380, Locus ZP_008743052; *Bacillus thuringiensis* serovar *israelensis*, ATCC 35646 sq1S63), (CRGGPPS; *Catharanthus roseus*), (NZ_AABFO2000074, Locus ZP_00144509; *Fusobacterium nucleatum* subsp. *vincentii*, ATCC 49256), (GFGPPSGN; *Gibberella fujikuroi*), (AY371321; *Ginkgo biloba*), (AB055496; *Hevea brasiliensis*), (AB017971; *Homo sapiens*), (MCI276129; *Mucor circinelloides* f. *lusanicicus*), (ABO16044; *Mus musculus*), (AAXB0100298, Locus NCU01427; *Neurospora crassa*), (NCU20940; *Neurospora crassa*), (NZ_AAKL0 1000008, Locus ZP_00843566; *Ralstonia solanacearum* UW551), (ABI 18238; *Rattus norvegicus*), (SCU31632; *Saccharomyces cerevisiae*), (ABO16095; *Synechococcus* elongates), (SAGGPPS; *Sinapis alba*), (SSOGDS; *Sulfolobus acidocaldarius*), (NC_007759, Locus YP_461832; *Syntrophus aciditrophicus* SB), (NC_006840, Locus YP_204095; *Vibrio Fischeri* ESI 14), (NM_1 12315; *Arabidopsis thaliana*), (ERWCRTF; *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).

### 5.6.9 Terpene Synthases

[00137] In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding an enzyme that can modify a polypreenyl to form a hemiterpene, a monoterpen, a sesquiterpene, a diterpene, a triterpene, a tetraterpene, a polyterpene, a steroid compound, a carotenoid, or a modified isoprenoid compound.  

[00138] 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*).  

[00139] 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).

[00140] 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; Perillafrutescens), (AY473623; Picea abies), (DQ195274; Picea sitchensis), and (AF444798; Perillafrutescens var. crispa cultivar No. 79).

[00141] 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).

[00142] 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_1 13485; Arabidopsis thaliana ATTPS-CIN), (NM_1 13483; Arabidopsis thaliana ATTPS-CIN), (AF271259; Perilla frutescens), (AY473626; Picea abies), (AF369919; Picea abies), and (AJ304839; Quercus ilex).

[00143] 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_1 13485; Arabidopsis thaliana ATTPS-CIN), (NM_1 13483; Arabidopsis thaliana ATTPS-CIN), (NM_1 17775; Arabidopsis thaliana ATTPS03), (NM_00 1036574; Arabidopsis thaliana ATTPS03), (NMJ27982;
Arabidopsis thaliana TPS 10), (AB 110642; Citrus unshiu CitMTSL4), and (AY575970; Lotus corniculatus var. japonicus).

[00144] In some embodiments, the heterologous nucleotide encodes an a-pinene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to: (+) a-pinene synthase (AF543530, REGION: 1.1887; Pinus taeda), (-)a-pinene synthase (AF543527, REGION: 32.1921; Pinus taeda), and (+)/(-)a-pinene synthase (AGU87909, REGION: 6 111892; Abies grandis).

[00145] 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 (AF5 14288, REGION: 26.1834; Citrus Union).

[00146] 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.

[00147] In some embodiments, the heterologous nucleotide encodes a γ-terpinene synthase. Illustrative examples of suitable nucleotide sequences include: (AF514286, REGION: 30.1832 from Citrus Union) and (AB 110640, REGION 1.1803 from Citrus unshiu).

[00148] 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 Osmium basilicum) and (AY906866, REGION: 10.1887 from Pseudotsuga menziesii).

[00149] 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.

[00150] In some embodiments, the heterologous nucleotide encodes a a-farnesene synthase. Illustrative examples of suitable nucleotide sequences include, but are not limited to DQ309034 from Pyrus communis cultivar d’Anjou (pear; gene name AFSI) and AY182241 from Malus domestica (apple; gene AFSI). Pechouss et al., Planta 219(l):84-94 (2004).

[00151] 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 Tspal 1), and AY835398 from *Artemisia annua*. Picaud et al., *Phytochemistry* 66(9): 961-967 (2005).

[00152] 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).

[00153] 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 tpsl).

[00154] 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*.

[00155] 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*.

[00156] 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*).

[00157] In some embodiments, the host cell produces a C5 isoprenoid. These compounds are derived from one isoprene unit and are also called hemiterpenes. An illustrative example of a hemiterpene is isoprene. In other embodiments, the isoprenoid is a C10 isoprenoid. These compounds are derived from two isoprene units and are also called monoterpene. Illustrative examples of monoterpene are limonene, citral, geraniol, menthol, perillyl alcohol, linalool, thujone, and myrcene. In other embodiments, the isoprenoid is a C15 isoprenoid. These compounds are derived from three isoprene units and are also called sesquiterpene. Illustrative examples of sesquiterpenes are periplanone B, ginkgolide B, amorphadiene, artemisinin, artemisinic acid, valencene, nootkatone, epi-cedrol, epi-aristolochene, farnesol, gossypol, sanonin, periplanone, forskolin, and patchouliol (which is also known as patchouli alcohol). In other embodiments, the isoprenoid is a C20 isoprenoid. These compounds are derived from four isoprene units and also called diterpene. Illustrative examples of diterpene are casbene, eleutherobin, paclitaxel,
prostratin, pseudopterosin, and taxadiene. In yet other examples, the isoprenoid is a C\textsubscript{20}+ isoprenoid. These compounds are derived from more than four isoprene units and include: triterpene \textit{(C\textsubscript{30} isoprenoid compounds derived from 6 isoprene units)} such as arbusrideE, bruceantin, testosterone, progesterone, cortisone, digitoxin, and squalene; tetraterpene \textit{(C\textsubscript{40} isoprenoid compounds derived from 8 isoprenoids)} such as \(\beta\)-carotene; and polyterpene \textit{(C\textsubscript{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, \(\alpha\)-farnesene, \(\beta\)-farnesene, farnesol, geraniol, geranylgeraniol, isoprene, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, \(\beta\)-pinene, sabinene, \(\gamma\)-terpinene, terpinolene and valencene. Isoprenoid compounds also include, but are not limited to, carotenoids \textit{(such as lycopene, \(\alpha\)- and \(\beta\)-carotene, \(\alpha\)- and \(\beta\)-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.

\textbf{5.6.10 Methods of Producing Isoprenoids}

[00158] In another aspect, provided herein is a method for the production of an isoprenoid, the method comprising the steps of: \(\text{(a) culturing a population of any of the genetically modified host cells described herein that are capable of producing an isoprenoid in a medium with a carbon source under conditions suitable for making an isoprenoid compound; and (b) recovering said isoprenoid compound from the medium.}\)

[00159] In some embodiments, the genetically modified host cell comprises one or more modifications selected from the group consisting of: heterologous expression of a phosphoketolase, heterologous expression of a phosphotransacetylase, heterologous expression of one or more enzymes of the mevalonate pathway; and optionally, heterologous expression of an ADA, heterologous expression of an NADH-using HMG-CoA reductase, and heterologous expression of an AACS; 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.

[00160] 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.

[00161] 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.

[00162] 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.

[00163] 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.
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.

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.

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.

5.6.11 Culture Media and Conditions

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.
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. KDZ, Weinheim, Germany.

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.

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).

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, xylose, 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.

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 <0.1 g/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.

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.

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.
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.

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.

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.

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.

[00179] 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.

[00180] 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.

[00181] 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.

[00182] 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.

[00183] 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.

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.

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.

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.

5.6.12 Recovery of Isoprenoids

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 demulsifier and/or a nucleating agent into the fermentation reaction. Illustrative examples of demulsifiers 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.

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.
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.

5.7 Polyketides

In some embodiments, the genetically modified host cell provided herein is capable of producing a polyketide from acetyl-CoA. Polyketides are synthesized by sequential reactions catalyzed by a collection of enzyme activities called polyketide synthases (PKSs), which are large multi-enzyme protein complexes that contain a coordinated group of active sites. Polyketide biosynthesis proceeds stepwise starting from simple 2-, 3-, 4-carbon building blocks such as acetyl-CoA, propionyl-CoA, butyryl-CoA and their activated derivatives, malonyl-, methylmalonyl- and ethylmalonyl-CoA, primarily through decarboxylative condensation of malonyl-CoA-derived units via Claisen condensation reactions. The PKS genes are usually organized in one operon in bacteria and in gene clusters in eukaryotes. Three types of polyketide synthases have been characterized: Type I polyketide synthases are large, highly modular proteins subdivided into two classes: 1) iterative PKSs, which reuse domains in a cyclic fashion and 2) modular PKSs, which contain a sequence of separate modules and do not repeat domains. Type II polyketide synthases are aggregates of monofunctional proteins, and Type III polyketide synthases do not use acyl carrier protein domains.

Unlike fatty acid biosynthesis, in which each successive chain elongation step is followed by a fixed sequence of ketoreduction, dehydration and enoyl, reduction as described below, the individual chain elongation intermediates of polyketide biosynthesis undergo all, some, or no functional group modifications, resulting in a large number of chemically diverse products. Additional degrees of complexity arise from the use of different starter units and chain elongation units as well as the generation of new stereo-isomers.

The order of complete polyketide-synthesis as directed by a polyketide synthase follows (in the order N-terminus to C-terminus): starting or loading the initial carbon building blocks onto an acyl carrier protein, elongation modules which catalyze the extension of the growing macrolide chain and termination modules that catalyze the release of the synthesized macrolide. Component domains or separate enzyme functionalities active in this biosynthesis include acyl-transferases for the loading of starter, extender and intermediate acyl units; acyl carrier proteins which hold the growing macrolide as a thiol
ester; β-keto-acyl synthases which catalyze chain extension; β-keto reductases responsible for the first reduction to an alcohol functionality; dehydratases which eliminate water to give an unsaturated thiolester; enoyl reductases which catalyze the final reduction to full saturation; and thiolesterases which catalyze macrolide release and cyclization.

[00193] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can condense at least one of acetyl-CoA and malonyl-CoA with an acyl carrier protein, e.g. an acyltransferase.

[00194] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can condense a first reactant selected from the group consisting of acetyl-CoA and malonyl-CoA with a second reactant selected from the group consisting of malonyl-CoA or methylmalonyl-CoA to form a polyketide product, e.g. a β-keto-acyl synthase.

[00195] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can reduce a β-keto chemical group on a polyketide compound to a β-hydroxy group, e.g. a β-keto reductase.

[00196] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can dehydrate an alkane chemical group in a polyketide compound to produce an α-β-unsaturated alkene, e.g. a dehydratase.

[00197] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can reduce an α-β-double-bond in a polyketide compound to a saturated alkane, e.g. an enoyl-reductase.

[00198] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can hydrolyze a polyketide compound from an acyl carrier protein, e.g. a thioesterase.

[00199] In some embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising a KS catalytic region. In some embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising an AT catalytic region. In some embodiments, the polyketide producing cell comprises more than one heterologous nucleotide sequence encoding an enzyme comprising an AT catalytic region. In some
embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising a CLF catalytic region. In some embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising an ACP activity. In some embodiments, the polyketide producing cell comprises more than one heterologous nucleotide sequence encoding an enzyme comprising an ACP activity.

[00200] In a particular embodiment, the polyketide producing cell comprises a minimal aromatic PKS system, e.g., heterologous nucleotide sequences encoding an enzyme comprising a KS catalytic region, an enzyme comprising an AT catalytic region, an enzyme comprising a CLF catalytic region, and an enzyme comprising an ACP activity, respectively. In a particular embodiment, the polyketide producing cell comprises a minimal modular PKS system, e.g., heterologous nucleotide sequences encoding an enzyme comprising a KS catalytic region, an enzyme comprising an AT catalytic region, and an enzyme comprising an ACP activity, respectively. In yet another particular embodiment, the polyketide producing cell comprises a modular aromatic PKS system for de novo polyketide synthesis, e.g., heterologous nucleotide sequences encoding an enzyme comprising a KS catalytic region, one or more enzymes comprising an AT catalytic region, and one or more enzymes comprising an ACP activity, respectively.

[00201] In some embodiments, the polyketide producing cell comprising a minimal PKS system, e.g., a minimal aromatic PKS system or minimal modular PKS system, further comprises additional catalytic activities which can contribute to production of the endproduct polyketide. In some embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising a cyclase (CYC) catalytic region, which facilitates the cyclization of the nascent polyketide backbone. In some embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising a ketoreductase (KR) catalytic region. In some embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising an aromatase (ARO) catalytic region. In some embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising an enoylreductase (ER) catalytic region. In some embodiments, the polyketide producing cell comprises one or more heterologous nucleotide sequences encoding an enzyme comprising a thioesterase (TE) catalytic region. In some embodiments, the polyketide producing cell further comprises one
or more heterologous nucleotide sequences encoding an enzyme comprising a holo ACP synthase activity, which effects pantetheinylation of the ACP.

[00202] In some embodiments, the polyketide producing cell further comprises one or more heterologous nucleotide sequences conferring a postsynthesis polyketide modifying activity. In some embodiments, the polyketide producing cell further comprises one or more heterologous nucleotide sequences encoding an enzyme comprising a glycosylase activity, which effects postsynthesis modifications of polyketides, for example, where polyketides having antibiotic activity are desired. In some embodiments, the polyketide producing cell further comprises one or more heterologous nucleotide sequences encoding an enzyme comprising a hydroxylase activity. In some embodiments, the polyketide producing cell further comprises one or more heterologous nucleotide sequences encoding an enzyme comprising an epoxidase activity. In some embodiments, the polyketide producing cell further comprises one or more heterologous nucleotide sequences encoding an enzyme comprising a methylase activity.

[00203] In some embodiments, the polyketide producing cell further comprises one or more heterologous nucleotide sequences encoding a biosynthetic enzyme including, but not limited to, at least one polyketide synthesis pathway enzyme, and enzymes that can modify an acetyl-CoA compound to form a polyketide product such as a macrolide, an antibiotic, an antifungal, a cytostatic compound, an anticholesterolemic compound, an antiparasitic compound, a coccidiostatic compound, an animal growth promoter or an insecticide. In some embodiments, the HACD compound is a polyene. In some embodiments, the HACD compound is a cyclic lactone. In some embodiments, the HACD compound comprises a 14, 15, or 16-membered lactone ring. In some embodiments, the HACD compound is a polyketide selected from the group consisting of a polyketide macrolide, antibiotic, antifungal, cytostatic, anticholesterolemic, antiparasitic, a coccidiostatic, animal growth promoter and insecticide.

[00204] In some embodiments, the polyketide producing cell comprises heterologous nucleotide sequences, for example sequences encoding PKS enzymes and polyketide modification enzymes, capable of producing a polyketide selected from, but not limited to, the following polyketides: Avermectin (see, e.g., U.S. Pat. No. 5,252,474; U.S. Pat. No. 4,703,009; EP Pub. No. 118,367; MacNeil et al., 1993, "Industrial Microorganisms: Basic and Applied Molecular Genetics"; Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, "A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin,

5.8 Fatty Acids

[00205] In some embodiments, the genetically modified host cell provided herein is capable of producing a fatty acid from acetyl-CoA. Fatty acids are synthesized by a series of decarboxylative Claisen condensation reactions from acetyl-CoA and malonyl-CoA catalyzed by fatty acid synthases. Similar to polyketide synthases, fatty acid synthases are not a single enzyme but an enzymatic system composed of 272 kDa multifunctional polypeptide in which substrates are handed from one functional domain to the next. Two principal classes of fatty acid synthases have been characterized: Type I fatty acid synthases are single, multifunctional polypeptides common to mammals and fungi (although the structural arrangement of fungal and mammalian synthases differ) and the CMN group of bacteria (corynebacteria, mycobacteria, and nocardia). Type II synthases, found in archaeabacteria and eubacteria, are a series of discrete, monofunctional enzymes that participate in the synthesis of fatty acids. The mechanisms fatty acid elongation and reduction is the same in the two classes of synthases, as the enzyme domains responsible for these catalytic events are largely homologous amongst the two classes.

[00206] Following each round of elongation of the fatty acid chain in the decarboxylative Claisen condensation reactions, the β-keto group is reduced to a fully saturated carbon chain by the sequential action of a ketoreductase, a dehydratase, and an enol reductase. The growing fatty acid chain moves between these active sites attached to an acyl carrier protein and is ultimately released by the action of a thioesterase upon reaching a carbon chain length of 16 (palmitidic acid).

[00207] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding a biosynthetic enzyme including, but not limited to, at least one fatty acid synthesis pathway enzyme, and enzymes that can modify an acetyl-CoA compound to form a fatty acid product such as a palmitate, palmitoyl CoA, palmitoleic acid, sapienic acid, oleic acid, linoleic acid, α-linolenic acid, arachidonic acid, eicosapentaenoic acid, erucic acid, and docosahexaenoic acid. In some embodiments, the HACD compound is a fatty acid selected from the group consisting of
palmitate, palmitoyl CoA, palmitoleic acid, sapienic acid, oleic acid, linoleic acid, a-linolenic acid, arachidonic acid, eicosapentaenoic acid, erucic acid, and docosahexaenoic acid.

[00208] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can covalently link at least one of acetyl-CoA and malonyl-CoA with an acyl carrier protein, e.g. an acyl-transferase.

[00209] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can condense acetyl chemical moiety and a malonyl chemical moiety, each bound to an acyl carrier protein (ACP), to form acetoacetyl-ACP, e.g. a β-Ketoacyl-ACP synthase.

[00210] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can reduce the double bond in acetoacetyl-ACP with NADPH to form a hydroxyl group in D-3-hydroxybutyryl hydroxylase-ACP, e.g. a β-Ketoacyl-ACP reductase.

[00211] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can dehydrate D-3-Hydroxybutyryl hydroxylase-ACP to create a double bond between the beta- and gamma-carbons forming crotonyl-ACP, e.g. a β-hydroxyacyl-ACP dehydrase.

[00212] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can reduce crotonyl ACP with NADPH to form butyryl-ACP, e.g. an enoyl ACP reductase.

[00213] In some embodiments, the genetically modified microorganism disclosed herein comprises a heterologous nucleotide sequence encoding an enzyme that can hydrolyze a C16 acyl compound from an acyl carrier protein to form palmitate, e.g. a thioesterase.

[00214] In some embodiments, the fatty acid producing cell comprises one or more heterologous nucleotide sequences encoding acetyl-CoA synthase and/or malonyl-CoA synthase, to effect increased production of one or more fatty acids as compared to a genetically unmodified parent cell.

[00215] For example, to increase acetyl-CoA production, one or more of the following genes can be expressed in the cell: pdh, panK, aceEF (encoding the E1p dehydrogenase component and the E2p dihydrolipoamide acyltransferase component of the pyruvate and 2-oxoglutarate dehydrogenase complexes), fabH, fabD, fabG, acpP, and fabF. Illustrative examples of nucleotide sequences encoding such enzymes include, but are not limited to: pdh
(BAB34380, AAC73227, AAC73226), panK (also known as coa, AAC76952), aceEF (AAC73227, AAC73226), bH (AAC74175), fabD (AAC74176), fabG (AAC74177), acpP (AAC74178), fabF (AAC74179).

[00216] In some embodiments, increased fatty acid levels can be effected in the cell by attenuating or knocking out genes encoding proteins involved in fatty acid degradation. For example, the expression levels of fadE, gpsA, idhA, pflb, adhE, pta, poxB, ackA, and/or ackB can be attenuated or knocked-out in an engineered host cell using techniques known in the art. Illustrative examples of nucleotide sequences encoding such proteins include, but are not limited to: fadE (AAC73325), gpsA (AAC76632), IdhA (AAC74462), pflb (AAC73989), adhE (AAC74323), pta (AAC75357), poxB (AAC73958), ackA (AAC75356), and ackB (BAB81430). The resulting host cells will have increased acetyl-CoA production levels when grown in an appropriate environment.

[00217] In some embodiments, the fatty acid producing cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert acetyl-CoA into malonyl-CoA, e.g., the multisubunit AccABCD protein. An illustrative example of a suitable nucleotide sequence encoding AccABCD includes but is not limited to accession number AAC73296, EC 6.4.1.2.

[00218] In some embodiments, the fatty acid producing cell comprises a heterologous nucleotide sequence encoding a lipase. Illustrative examples of suitable nucleotide sequences encoding a lipase include, but are not limited to accession numbers CAA89087 and CAA98876.

[00219] In some embodiments, increased fatty acid levels can be effected in the cell by inhibiting PlsB, which can lead to an increase in the levels of long chain acyl-ACP, which will inhibit early steps in the fatty acid biosynthesis pathway (e.g., accABCD, fabH, and fabl). The expression level of PlsB can be attenuated or knocked-out in an engineered host cell using techniques known in the art. An illustrative example of a suitable nucleotide sequence encoding PlsB includes but is not limited to accession number AAC77011. In particular embodiments, the plsB D31IE mutation can be used to increase the amount of available acyl-CoA in the cell.

[00220] In some embodiments, increased production of monounsaturated fatty acids can be effected in the cell by overexpressing an sfa gene, which would result in suppression of fabA. An illustrative example of a suitable nucleotide sequence encoding sfa includes but is not limited to accession number AAN79592.
In some embodiments, increased fatty acid levels can be effected in the cell by modulating the expression of an enzyme which controls the chain length of a fatty acid substrate, e.g., a thioesterase. In some embodiments, the fatty acid producing cell has been modified to overexpress a tes or fat gene. Illustrative examples of suitable tes nucleotide sequences include but are not limited to accession numbers: (tesA: AAC73596, from E. coli, capable of producing C18:1 fatty acids) and (tesB: AAC73555 from E. coli). Illustrative examples of suitable fat nucleotide sequences include but are not limited to: (fatB: Q41635 and AAA34215, from Umbellularia California, capable of producing C12:0 fatty acids), (fatB2: Q39513 and AAC49269, from Cuphea hookeriana, capable of producing C8:0-C10:0 fatty acids), (fatB3: AAC49269 and AAC72881, from Cuphea hookeriana, capable of producing C4:0-C6:0 fatty acids), (fatB: Q39473 and AAC49151, from Cinnamomum camphorum, capable of producing C4:0 fatty acids), (fatB [M141TJ: CAA85388, from mArabidopsis thaliana, capable of producing C16:1 fatty acids), (fatA: NP 189147 and NP 193041, from Arabidopsis thaliana, capable of producing C18:1 fatty acids), (fatA: CAC39106, from Bradvriizobium japonicum, capable of preferentially producing C18:1 fatty acids), (fatA: AAC72883, from Cuphea hookeriana, capable of producing C18:1 fatty acids), and (fatA1, AAL79361 from Helianthus annus).

In some embodiments, increased levels of C10 fatty acids can be effected in the cell by attenuating the expression or activity of thioesterase C18 using techniques known in the art. Illustrative examples of suitable nucleotide sequences encoding thioesterase C18 include, but are not limited to accession numbers AAC73596 and P0ADA1. In other embodiments, increased levels of C10 fatty acids can be effected in the cell by increasing the expression or activity of thioesterase C10 using techniques known in the art. An illustrative example of a suitable nucleotide sequence encoding thioesterase C10 includes, but is not limited to accession number Q39513.

In some embodiments, increased levels of C14 fatty acids can be effected in the cell by attenuating the expression or activity of endogenous thioesterases that produce non-C14 fatty acids, using techniques known in the art. In other embodiments, increased levels of C14 fatty acids can be effected in the cell by increasing the expression or activity of thioesterases that use the substrate C14-ACP, using techniques known in the art. An illustrative example of a suitable nucleotide sequence encoding such a thioesterase includes, but is not limited to accession number Q39473.
In some embodiments, increased levels of C_{12} fatty acids can be effected in the cell by attenuating the expression or activity of endogenous thioesterases that produce non-
Co fatty acids, using techniques known in the art. In other embodiments, increased levels of C_{12} fatty acids can be effected in the cell by increasing the expression or activity of thioesterases that use the substrate C_{12}-ACP, using techniques known in the art. An illustrative example of a suitable nucleotide sequence encoding such a thioesterase includes, but is not limited to accession number Q41635.

5.9 PK/PTA for the Production of Other Compounds

In some embodiments, the genetically modified host cell provided herein (e.g., a host cell comprising PK/PTA and a functional disruption of a polypeptide encoding acetyl phosphatase activity, e.g., RHR2, HOR2, or homologues thereof) is engineered for the expression of biosynthetic pathways that initiate with cellular pyruvate to produce, for example, 2,3-butanediol, 2-butanol, 2-butanone, valine, leucine, lactic acid, malate, isoamyl alcohol, and isobutanol, as described in U.S. Patent Application Publication No. 20120156735. The disruption of the enzyme pyruvate decarboxylase (PDC) in recombinant host cells engineered to express a pyruvate-utilizing biosynthetic pathway has been used to increase the availability of pyruvate for product formation via the biosynthetic pathway. While PDC-KO recombinant host cells can be used to produce the products of pyruvate-utilizing biosynthetic pathways, PDC-KO recombinant host cells require exogenous carbon substrate supplementation (e.g., ethanol or acetate) for their growth. In particular, two exogenous carbon substrates are needed: one of which is converted to a desired product, the other fully or partly converted into acetyl-CoA by recombinant host cells requiring such supplementation for growth. However, expression of a heterologous phosphoketolase pathway reduces or eliminates the need for providing these exogenous carbon substrates for their growth compared to PDC-KO cells not heterologously PK/PTA. Thus, the additional functional disruption of RHR2, HOR2, or homologues thereof capable of catalyzing the hydrolysis of acetyl phosphate to acetate, is expected to further improve the ability of PK/PTA to increase the supply of acetyl-CoA available as a substrate for cellular growth in these cells.

5.10 Methods of Making Genetically Modified Cells

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 PK, PTA, and/or biosynthetic pathway enzymes,
e.g., for an acetyl-CoA derived compound. 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.


[00228] 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.

[00229] 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 \(K_{cat}\) or a lower or higher \(K_m\) for the substrate, or expressing an altered form of the enzyme that is more or less affected by feedback or feed-forward regulation by another molecule in the pathway.

[00230] 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.

[00231] 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 \(SHBLE\) 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 \(SHBLE\) gene product from \(Streptocolloteichus hindustanus\) confers resistance to Zeocin (bleomycin). In some embodiments, the antibiotic resistance marker is deleted after the genetically modified host cell disclosed herein is isolated.

[00232] 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 chromosommal 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-fluoroorotic 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.

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.

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.

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."
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).

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.

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.

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).

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).

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.

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.

[00243] 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, Issatchenka orientalis, Schizosaccharomyces spp., including S.
pombe, Cryptococcus spp., Aspergillus spp., Neurospora spp., or Ustilago spp. Sources of
genesis 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.

[00244] 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
PK, PTA, RHR2 or HOR2 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 a
gene/enzyme of interest, or by degenerate PCR using degenerate primers designed to amplify
a conserved region among a gene of interest. 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.

6. EXAMPLES

6.1 Example 1: Acetate Production in Host Cells Expressing PK and PTA

[00245] This example describes the production of acetate in yeast strains heterologously expressing phosphoketolase and phosphotransacetylase.

6.1.1 Materials and Methods

6.1.1.1 Strain Engineering

6.1.1.1.1 Y967 and Y968

[00246] Y967 and Y968 are wildtype prototrophic Saccharomyces cerevisiae CEN.PK2, Y967 is MatA, and Y968 is Matalpha. The starting strain for Y12869, Y12746, and all of their derivatives, was Saccharomyces cerevisiae strain Y003 (CEN.PK2, Mat alpha, ura3-52, trpl-289, leu2-3,122, his3^+)). 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.

6.1.1.2 Y12869

[00247] Y12869 was generated through three successive integrations into Y003. First, the gene ACS2 was deleted by introducing an integration construct (j2235; SEQ ID NO:27) 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.

[00248] Next, ALD6 was deleted and Dickeya zeae eutE was introduced in Y4940 with the integration construct (j74804; SEQ ID NO:28) pictured below.
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 yl2602.

Next, ACS1 was deleted in Y 12602 by introducing an integration construct (i76220; SEQ ID NO:29) 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 Y 12747.

Next, Y 12747 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.

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 *PGKl* terminator (259 bp downstream of the *PGKl* 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. The resulting strain was transformed with the construct (74810; SEQ ID NO:31) shown below.

<table>
<thead>
<tr>
<th>ALD6US</th>
<th>pTDH3</th>
<th>Lm.PK</th>
<th>tTDH3</th>
<th>TRP1</th>
<th>EHO3</th>
<th>Lm</th>
<th>EHO3p</th>
<th>ALD6DS</th>
</tr>
</thead>
</table>

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.

Next, ACS1 was deleted in by introducing an integration construct (76220; SEQ ID NO:29) 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.

6.1.1.1.4 Y12746

Y12746 was generated through three successive integrations into Y4940. First, Y4940 was transformed with the integration construct (73830; SEQ ID NO:30) pictured below.

<table>
<thead>
<tr>
<th>BUD9US</th>
<th>pTDH3</th>
<th>Lm.PK</th>
<th>m m</th>
<th>m m</th>
<th>m m</th>
<th>m m</th>
<th>BUD6DS</th>
</tr>
</thead>
</table>

- 86 -
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 PGKl terminator (259 bp downstream of the
PGKl 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.

The resulting strain was transformed with the construct (i74810; SEQ ID
NO:31) shown below.

<table>
<thead>
<tr>
<th>ALD6S</th>
<th>pTDH3</th>
<th>Lm.PK</th>
<th>tTDH3</th>
<th>TRP1</th>
<th>EHHQ13</th>
<th>Kpd1m</th>
<th>EHHQ1d</th>
<th>ALD6DS</th>
</tr>
</thead>
</table>

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.

Finally, the resulting strain was transformed with the construct (i76221; SEQ ID NO:32) shown below.

<table>
<thead>
<tr>
<th>ACS1US</th>
<th>pTDH3</th>
<th>Dz.eutE</th>
<th>tTEF2</th>
<th>HIS3</th>
<th>F2F113</th>
<th>HIS3</th>
<th>EHHQ1d</th>
<th>ACS1DS</th>
</tr>
</thead>
</table>

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 *ACSl* locus. Upon introduction into a host cell, this construct integrates by homologous recombination into the host cell genome, functionally disrupting *ACSl* by replacing the *ACSl* 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.

6.1.1.1.5 Y19390

Y19390 is a direct descendant of Y12869. A ura- auxotrophic derivative of Y12869 was transformed with the integration construct MS49253 (SEQ ID NO:36) shown below:

<table>
<thead>
<tr>
<th>BUD3US</th>
<th>pTDH3</th>
<th>LmPK</th>
<th>tTDH3</th>
<th>URA3</th>
<th>DTDH3</th>
<th>Kd'lam</th>
<th>DTDH3p</th>
<th>BUD3DS</th>
</tr>
</thead>
</table>

This integration construct comprises a selectable marker (URA3); two copies of 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); 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.

6.1.1.1.6 Y19391

Y19391 is a direct descendant of Y12869. A ura- auxotrophic derivative of Y12869 was transformed with the integration construct MS49298 (SEQ ID NO:37) shown below:

<table>
<thead>
<tr>
<th>BUD9US</th>
<th>pTDH3</th>
<th>CKPTA</th>
<th>IPGK1</th>
<th>URA3</th>
<th>IPGK1</th>
<th>Ta'prA</th>
<th>DTDH3p</th>
<th>BUD9DS</th>
</tr>
</thead>
</table>

This integration construct comprises a selectable marker (URA3); two copies of a yeast codon-optimized version of phosphotransacetylase from *Clostridium kluyveri* (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.

6.1.1.2 Culture conditions
[00266] Inoculum cultures of Y967, Y12869, Y12745, Y12746, Y19390 and Y19391 were grown from single colonies overnight in 5 ml of seed media at 30C and 200rpm (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 paraaminobenzoic acid) with 50 mM succinate pH 5.0, and 20 g/L sucrose. The precultures were then inoculated into a 125 ml flask carrying 25 ml of seed media with 50 mM succinate pH 5.0, and 40 g/L sucrose to an initial OD600 of 0.1, and grown at 30C and 200rpm.

6.1.1.3 Quantitation of acetate, fructose, glucose, and sucrose
[00267] Acetate and sugars (fructose, glucose, sucrose) were quantitated by transferring 1 ml of whole cell broth to a 1.5 ml eppendorf tubes, and spinning at 13,000 RPM for 1 minute using a tabletop centrifuge to clarify the supernatant. The supernatant was then diluted (1:1 v/v) in 8mM sulfuric acid, vortex ed, and recentrifuged before transferring to a 1.8ml vial. Samples were analyzed with an Agilent 1200 HPLC, with variable wavelength and refractive index detection, using a BioRad Aminex HPX-87H 300mm x 7.8mm column. The mobile phase was 4mM sulfuric acid, column temperature was 40C, and the flow rate was 0.5 ml/min.

6.1.1.4 Results
[00268] FIG. 3B shows that wildtype Cen.PK2, Y967, produces acetate during growth in batch defined sucrose shakeflask cultures. Y12869, comprising a deletion of the PDH-bypass (acs1A acs2 Δ ald6A) and heterologously expressing acetaldehyde dehydrogenase acylating (Dz.eutE), produces far less acetate than the wildtype control which uses the PDH-bypass, likely due to the deletion of ALD6, the cytosolic acetaldehyde dehydrogenase that converts acetaldehyde to acetate. In the strain Y12746, comprising a deletion of the PDH-
bypass \((\text{acsL A acs2 A ald6A})\) and heterologously expressing acetaldehyde dehydrogenase acylating \((Dz.eutE)\) as well as phosphoketolase \((\text{Lm.PK})\) and phosphotransacetylase \((\text{Ck.PTA})\), a large increase in acetate is observed, surpassing the amount produced by wildtype \(Y967\). The results with \(Y12869\) indicate that the baseline level of acetate is extremely low in a strain that is \(\text{acsL A acs2 A ald6A}\) and uses ADA to carry flux to cytosolic acetyl-CoA. In all cases, the rate of sugar consumption is comparable (sugars here are defined as the sum of sucrose, glucose, and fructose in the media), illustrating that the differences in acetate levels are not due to differential consumption of feedstock (FIG. 3A). These results suggest that the increase in acetate in \(Y12746\) is attributable to the presence of phosphoketolase and/or phosphotransacetylase. The catalytic activity of both phosphoketolase and phosphotransacetylase produces acetyl phosphate. Therefore, acetate accumulation may arise from spontaneous or catalyzed hydrolysis of acetyl phosphate in \(Y12746\).

[00269] To determine the source of acetate in the strain expressing ADA, PK and PTA \((Y12746)\), we transformed a strain which uses only ADA to provide cytosolic AcCoA \((Y12869,\) comprising a deletion of the PDH-bypass \((\text{acsL A acs2 A ald6A})\) and heterologously expressing acetaldehyde dehydrogenase acylating \((Dz.eutE))\) with either (1) an integration construct encoding two overexpressed copies of PK driven by the strong promoter \(P_{TDH3}\), resulting in \(Y19390\), or (2) an integration construct encoding two overexpressed copies of PTA driven by the strong promoter \(P_{TDH3}\), resulting in \(Y19391\). As shown in FIG. 3D, we observed an increase in acetate accumulation in strains that expressed either PK or PTA relative to the parent strain. Sugar consumption is shown in FIG. 3C to illustrate that acetate levels are not due to differential sugar consumption. PK converts X5P to Acetyl phosphate and G3P, whereas PTA can interconvert Acetyl CoA + Pi to Acetyl Phosphate + CoA. These observations suggest that acetyl phosphate, whether derived from X5P by PK, or derived from AcCoA by PTA, can be hydrolyzed to acetate as shown in FIG. 1.

6.2 Example 2: Identification of a Major Acetyl Phosphatase in \textit{Saccharomyces cerevisiae}

[00270] This example describes the identification of an enzyme capable of hydrolyzing acetyl phosphate in yeast.

6.2.1 Materials and Methods

6.2.1.1 Cell Culture

- 90 -
A single colony of a given yeast strain was cultured in 5 mL Yeast Extract Peptone media with 2% dextrose (YPD) as an overnight starter culture. The following day, 50 mL YPD was inoculated with this starter culture to an OD600 of 0.2. The flasks were incubated at 30 °C by shaking at 200 RPM for 24 hours unless otherwise specified.

6.2.1.2 Cell-Free Extract Preparation

Cell culture was divided into three 15 mL falcon tubes and harvested by centrifugation at 4000 x g for 5 minutes. The supernatant was then discarded and cells were washed by resuspending in 10 mL ice cold buffer W (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol) followed by centrifugation at 4000 x g for 5 minutes. Supernatant was discarded and cells were resuspended in 1 mL lysis buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM DTT, 1 EDTA free protease inhibitor tablet (Roche) per 10mL). 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. The tubes were then placed back in the bead beater again for 1 minute at 6 M/S and returned to the ice bath for 5 minutes. Tubes were spun at a minimum of 16000 x g for 20 minutes to pellet cell debris. 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)).

6.2.1.3 Acetyl phosphatase reaction and quantitation of acetyl phosphate

Acetyl phosphatase activity assays were carried out at 30 °C in reaction buffer consisting of 100 mM Tris-HCl pH 7.5, 150 mM NaCl, and 1 mM MgCl$_2$$. Acetyl phosphate was added to a starting concentration of either 5 mM or 10 mM as indicated. The reaction was initiated by the addition of cell free extract in the amounts indicated. To test for phosphatase inhibition, sodium fluoride was added to select wells at 30 mM concentration. The reactions were carried out in a sealed 96 well plate and total reaction volume of 250 μL. Acetylphosphate concentration was measured by the method developed by Lipmann and Tuttle (Lipmann F, Tuttle LC, J. Biol. Chem. 159, 21-28 (1945)). 50 μL reaction mixture was added to 50 μL 2M hydroxylamine pH 6.8, mixed well and incubated at room temperature for at least 10 minutes. 34 μL 15% trichloroacetic acid was then added and mixed followed by 34
µl 4N HCl and 34 µl 5% FeCl₃ mixing well after each addition. Plates were then centrifuged in a Beckman centrifuge J-E with swinging bucket rotor JS-5.3 for 5 minutes at 3000 rpm to pellet precipitated protein. 150 µl supernatant was then transferred to a fresh 96-well clear flat bottom plate (Greiner Bio-One Cat.-No.: 655 161). Plate was read by a Molecular Devices SpectraMax M5 plate reader at a wavelength of 505 nm.

6.2.1.4 Purification of Active Phosphatase Fraction

[00274] A single colony of a given yeast strain was cultured in 5 mL Yeast Extract Peptone media with 2% dextrose (YPD) as an overnight starter culture. The following day, two 2.8L Fermbach flasks with 500 ml YPD were inoculated with this starter culture to an OD600 of 0.2. The flasks were incubated at 30 °C by shaking at 160 RPM for 24 hours. The culture was harvested by centrifugation at 4000x g for 5 minutes. The cell pellet was washed with 500mL sterile water and centrifuged at 4000x g for 5 minutes. The cell pellet was then resuspended in 50 mL ice cold lysis buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM DTT, 1 EDTA free protease inhibitor tablet (Roche) per 10mL). Cell suspension was split into six 15 mL falcon tubes filled with 5 mL disruption beads (Disruption beads, 0.5Mm, Fisher). Tubes were then placed in a bead beater for 45 seconds at 6 M/S. The tubes were immediately placed in an ice water bath for at least 5 minutes.

Bead beating was repeated 3 additional times with at least 5 minutes in an ice water bath in between each disruption segment. Tubes were spun for 30 minutes at 16,000 rpm (30,966 x g) in a Beckman centrifuge J-E in a JA-20 rotor chilled to 4 °C to pellet cell debris. Cell lysate was additionally clarified by the selective flocculation method described by Salt et al. (Selective flocculation of cellular contaminants from soluble proteins using polyethyleneimine: A study of several organisms and polymer molecular weights. Enzyme and Microbial Technology 17, 107-1 13(1995)) as follows: cell free lysate was adjusted to pH 7.4 by addition of 5mM NaOH stock solution. Then equal volume of PEI/Borax solution (0.5M NaCl 0.25% PEI, 100mM Borax) was added to the cell lysate and mixed well. Mixture was then centrifuged for 30 minutes at 2,500 x g at 4 °C. Protein was then precipitated by slowly adding ammonium sulfate with constant stirring until 80% of saturation concentration was reached. Stirring continued for 10 more minutes, and then precipitated protein was harvested by centrifugation at 15,000 rpm at 4 °C in a Beckman JA-20 rotor for 10 minutes. Supernatant was removed and protein was gently resuspended in Buffer A (20 mM Tris-Cl, pH 7, 10% glycerol). Protein was then added to a 0.5-3mL 3,500 Da molecular weight cut off dialysis cassette (Pierce #66300) and dialyzed overnight at 4 °C.
in 1.5L buffer A. Dialyzed sample was centrifuged 16000 x g for 10 minutes to pellet precipitated protein. 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)). 20 mg protein was loaded onto a Source 15Q 4.6/100 PE anion exchange column on a GE ÄKTAexplorer FPLC. Protein was eluted with a 0-100% gradient of buffer B (20 mM Tris-Cl pH 7, 1M NaCl, 10% glycerol) over 30 column volumes at a flow rate of 0.5 mL/minute and 1 mL samples were collected. To assay activity of each fraction, 75 µL each fraction was added to 8 mM ACP in a 250 µL reaction containing 100mM Tris-Cl pH 7, 150 mM NaCl, 1 mM MgCl2 and assayed as described above. The active fraction from this separation was again dialyzed against buffer A overnight. The entire sample was then loaded onto the same a Source 15Q 4.6/100 PE anion exchange column and eluted with a gradient of 0-45% buffer B over 30 column volumes at a flow rate of 0.5 mL/minute and 1 mL samples were collected. Samples were assayed for activity as above.

### 6.2.1.5 Protein Gel Electrophoresis

[00275] Protein fractions were analyzed using a Criterion gel electrophoresis system. 10 µL of fraction was added to 10 µL of 2X Laemmli sample buffer (BioRad Cat # 161-0737) with 5% v/v 2-mercaptoethanol and boiled for 10 minutes. Samples were then briefly centrifuged and 15 µL was loaded on a 26 well 4-15% Criterion™ TGX™ Precast Gel and run in IX Tris-Glycine-SDS buffer (prepared from BioRad 10x Tris/Glycine/SDS #161-0732) for 50 minutes at 130 volts. The gel was rinsed in 200 mL deionized water three times for 5 minutes each. SimplyBlue™ SafeStain (Life Technologies Cat # LC6060) was then added to the gel to completely cover the gel and then incubated at room temperature for 1 hour with gentle rocking. The SafeStain was then discarded and the gel was washed with 200 mL deionized water for 1 hour with rocking.

### 6.2.1.6 Identification of Proteins in Active Phosphatase Fraction

[00276] Proteolytic digestion and separation of peptides

[00277] 100 µg of total protein was subjected to proteolysis by trypsin for subsequent identification by LC-MS/MS. 100 µg total protein was reduced with Tris-carboxyethylphosphine (4 mM) for 30 minutes at 37 °C, then alkylated with Iodoacetamide (15 mM) for 30 minutes at RT in the dark. 5 µg Trypsin was added to the digest mixture and the entire digestion was allowed to go for 12 hours at 37 °C. The reaction was quenched with 0.1% formic acid and injected onto an Ascentis Peptide express column (5cmx2.1mm ID, 2.1
um particle size), and separated over a 90 minute gradient from low acetonitrile to high acetonitrile, with 0.1% formic acid as a modifier. The LC pumps were two Shimadzu LC20AD's operated by a Shimadzu CBM20A LC Controller.

A QTP\textsuperscript{AP} 4000 hybrid triple-quadrupole linear ion trap mass spectrometer was used to identify peptides being eluted from the column. IDA parameters were as follows: Select ions from 350 to 1300 da; ER Scan used for charge state determination; 1+ ions rejected, unknowns allowed; Rolling collision energy: yes (AB SCIEX standard for qtrap 4000); Max fill time for each MS/MS: 950 ms.

Mascot, by Matrix Science was used to identify peptides from a CENPK2 sequence database with the following parameters. Fixed modifications: Carbamidomethyl. Variable modifications: deamidation (NQ), oxidation (MW). Precursor mass tolerance: 0.5 da. Product mass tolerance: 1.0 da. Missed cleavages allowed: 1.

6.2.1.7 Strain Engineering

A version of Y968 lacking a functional \textit{URA3} gene was transformed with either ms59858 to knock out \textit{RHR2} or ms59971 to knock out \textit{HOR2}. 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. The \textit{URA3} marker in this construct is flanked by direct repeats, facilitating its recycling. To recycle the \textit{URA3} marker, cells were grown in YPD overnight, then plated on 5'FOA. The loopout of \textit{URA3} was confirmed by PCR amplification and inability to grow on CSM-URA plates. The \textit{ura-} version of Y968.ms59858 was then transformed with ms59971 to generate a double \textit{RHR2} and \textit{HOR2} knockout strain Y968.ms59858.ms59971

6.2.2 Results

6.2.2.1 Hydrolysis of acetyl phosphate is enzyme-catalyzed and inhibited by heat and a broad spectrum phosphatase inhibitor

As shown in FIG 4, addition of cell free extract from wild type \textit{S. cerevisiae} strain Y967 catalyzes the hydrolysis of acetyl phosphate, and the rate of hydrolysis is dependent on the amount of cell free extract added. Increasing the amount of cell free extract increases the rate of hydrolysis. When the cell free extract is boiled, the addition of increasing amounts of cell free extract no longer has an effect on the hydrolysis rate of acetyl phosphate, indicating that the responsible component has been inactivated by heat. Similarly, the addition of 30 mM sodium fluoride, a broad spectrum phosphatase inhibitor, renders the
cell free extract ineffective at hydrolyzing acetyl phosphate. These results suggest that a phosphatase is likely responsible for the catalysis of acetyl phosphate hydrolysis.

6.2.2.2 Protein fractionation isolates a single enriched active fraction

Anion exchange chromatography was used to separate soluble protein in the cell free extracts. FIG. 5A shows that nearly all of the phosphatase activity was concentrated in one fraction, and the remaining activity in adjacent fractions. This indicates that the enzyme responsible for this activity in the cell free extract is either a single protein or proteins with similar ionic interactions which co-elute when separated by anion exchange chromatography.

The active fraction #10 from FPLC anion exchange purification was purified a second time using a more shallow gradient 0-45% buffer B. The most active fraction from this purification, #14, shown in FIG. 6A, was analyzed by mass spectrometry to determine the identity of the proteins in the fraction. Of the proteins identified in the active fraction (FIG. 6B), Rhr2 and its homolog Hor2, which cannot be distinguished by mass spectrometry due to significant sequence similarity, were the only proteins on the list identified as phosphatases by the SGD database. Rhr2 is a glycerol-1-phosphatase that is expressed constitutively at high levels. Hor2 catalyzes the identical reaction but is expressed only at low levels under normal conditions and is induced by osmotic stress (Norbeck et. al., Purification and Characterization of Two Isoenzymes of DL-Glycerol-3-phosphatase from Saccharomyces cerevisiae, J. Biol. Chem., 271, 13875-13881 (1996)). Acetyl phosphate is not a metabolite that is native to yeast, therefore it is expected that the hydrolysis is caused by a promiscuous reaction of an enzyme that targets a similar substrate. Rhr2/Hor2 were top candidates for this reaction since their native substrate, glycerol-1-phosphatase, is also a low molecular weight phosphorylated compound similar to acetyl phosphate, as shown below.

6.2.2.3 Deletion of RHR2 and/or HOR2 reduces phosphatase activity

In order to determine whether Rhr2 and/or Hor2 were responsible for the phosphatase activity observed in S. cerevisiae, new strains were created lacking either RHR2
or HOR2 and one strain lacking both RHR2 and HOR2. These strains were cultured as described previously, and cell free extract was prepared and tested for acetyl phosphatase activity. As shown in FIG. 7, deletion of RHR2 dramatically reduces phosphatase activity, while deletion of HOR2 has no effect on the rate of hydrolysis of acetyl phosphate. Deletion of HOR2 does however reduce hydrolysis of acetyl phosphate in a strain that already has RHR2 deleted. This is consistent with published work that indicates that expression of Hor2 is upregulated following deletion of RHR2 (DeLuna et al., Need-Based Up-Regulation of Protein Levels in Response to Deletion of Their Duplicate Genes, PLOS Biol., 8, el0000347 (2010)). Elimination of both of these phosphatases results in near background levels of acetyl phosphate hydrolysis as shown in FIG. 7. These results confirm that glycerol-1-phosphatases Rhr2 and Hor2 are responsible for the majority of the acetyl phosphatase activity in S. cerevisiae.

6.3 Example 3:  
Deletion of the acetyl phosphate phosphatase reduces acetate secretion and improves production of a compound derived from Acetyl-CoA

6.3.1 Materials and Methods

6.3.1.1 Strain construction

[00287] Versions of Y968, Y12869, and Y12746, lacking a functional URA3 gene, were transformed with either ms63907 or ms63909, and with ms64472, to convert them to farnesene producers.

[00288] The ms63907 integration construct (i84022; SEQ ID NO:33) is shown below.

| HOUS | GAL4 | dWHA | LSAL | pGAL10 | ERG10 | URA3 | FI4 | JO10 | pGAL1 | Sp.HMG | HO DS |

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. The URA3 marker in this construct is flanked by direct repeats, facilitating its recycling. To recycle the URA3 marker, cells were grown in YPD overnight, then plated on 5TOA. The loopout of URA3 was confirmed by PCR amplification and inability to grow on CSM-URA plates. The ms63909 integration construct (i84026; SEQ ID NO:34) is identical to ms63907, with one exception: the sequences encoding S. pomeroyi HMG-CoA reductase are replaced by tHMG(r), the truncated HMG1 coding sequence which encodes the native S. cerevisiae HMG-CoA reductase.

The ms64472 integration construct (i85207; SEQ ID NO:35) is shown below.

<table>
<thead>
<tr>
<th>GAL80</th>
<th>µGAL7</th>
<th>IDI1</th>
<th>SFAV</th>
<th>TITYPD5</th>
<th>µGAL10</th>
<th>ERG20</th>
<th>URA3</th>
<th>SJKI3</th>
<th>ZTVG</th>
<th>GLED3</th>
<th>GETAGD</th>
<th>µGAL1</th>
<th>ERG12</th>
<th>GAL80</th>
</tr>
</thead>
</table>

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. The URA3 marker in this construct is flanked by direct repeats, facilitating its recycling. To recycle the URA3 marker, cells were grown in YPD overnight, then plated on 5TOA. The loopout of URA3 was confirmed by PCR amplification and inability to grow on CSM-URA plates.

Next, ura- versions of Y968.ms63907.ms64472, Y12869.ms63907.ms64472, and Y12747.ms63907.ms64472, were transformed with ms59858 to knock out the RHR2 ORF. This integration construct consists of the upstream and downstream nucleotide sequences of RHR2, flanking the native S. cerevisiae URA3 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.
6.3.1.2 Culture conditions

[00291] Single colonies were inoculated in wells of a 96-well plate in 360 μΙ of seed media (described in Example 1), and grown at 34°C for three days by shaking at 1000 rpm. Then, 14.4 μΙ of culture was subcultured into 360 μΙ of seed media with 50 mM succinate pH 5.0 and 40 g/L galactose, and grown at 34°C for two days by shaking at 1000 rpm.

6.3.1.3 Quantitation of acetate and glycerol

[00292] Acetate and glycerol were quantitated by transferring 1 ml of whole cell broth to a 1.5 ml eppendorf tubes, and spinning at 13,000 RPM for 1 minute using a tabletop centrifuge to clarify the supernatant. The supernatant was then diluted (1:1 v/v) in 8mM sulfuric acid, vortexed, and recentrifuged before transferring to a 1.8ml vial. Samples were analyzed with an Agilent 1200 HPLC, with variable wavelength and refractive index detection, using a BioRad Aminex HPX-87H 300mm x 7.8mm column. The mobile phase was 4mM sulfuric acid, column temperature was 40°C, and the flow rate was 0.5 ml/min.

6.3.1.4 Quantitation of farnesene

[00293] At the end of two days incubation at 34°C, 98 μΙ of whole cell broth was mixed with 2 μΙ of Nile Red solution (100 μg/ml in DMSO) in a flat-bottom 96-well assay plate (Costar 3916), and mixed for 30 seconds on a 96-well plate shaker. The plates were then read on a Beckman M5 plate reader with excitation at 500nm and emission at 550nm.

6.3.1.5 Quantitation of optical density

[00294] In a 96-well assay plate, 8 μΙ of culture was mixed with 92 μΙ of diluent (20% PEG 200, 20% Ethanol, 2% Triton X-1 14) and incubated for 30 minutes at room temperature. The assay plate was vortexed before measuring ODω on a Beckman M5 plate reader.

6.3.2 Results

[00295] FIG. 8A shows that strain Y12746.ms63909.ms64472, comprising a deletion of the PDH-bypass (acslA acs2 Δ ald6A), heterologously expressing acetaldehyde dehydrogenase acylating (Dz.eutE) as well as phosphoketolase (Lm.PK) and phosphotransacetylase (Ck.PTA) and overexpressing genes in the farnesene production pathway, secretes more acetate than a version of Y 12746.ms63909.ms64472 in which the RHR2 gene has been deleted. As shown in FIG. 8B, deletion of RHR2 does not impact glycerol production, as glycerol levels of Y 12746.ms63909.ms64472 rhr2Δ are largely unchanged compared to Y12746.ms63909.ms64472. As shown in FIG. 8C, the substantially reduced levels of acetate in Y12746.ms63909.ms64472 rhr2Δ are not due to reduced cell growth, as cell densities are similar for both RHR2+ and rhr2Δ populations. These results...
demonstrate that Rhr2, which was responsible for the acetyl phosphate phosphatase activity in cell free extract, is also the primary cause behind the hydrolysis of acetyl phosphate to acetate in vivo.

To determine whether the reduction of acetate observed upon deletion of RHR2 occurs independent of farnesene production, acetate production was measured in versions of strain 12746 with an intact or deleted RHR2 gene, but not expressing genes in the farnesene production pathway. **FIG. 8D** shows that strain Y 12746, comprising a deletion of the PDH-bypass (acslA acs2 Δ ald6A), heterologously expressing acetaldehyde dehydrogenase acetylating (Dz.eutE) as well as phosphoketolase (Lm.PK) and phosphotransacetylase (Ck.PTA), secretes more acetate than a version of Y 12746 in which the RHR2 gene has been deleted. As shown in **FIG. 8E**, the substantially reduced levels of acetate in Y 12746.ms63907.ms64472 rhr2\(^{+}\) are not due to reduced cell growth, as cell densities are similar for both RHR2\(^{+}\) and rhr2\(^{-}\) populations. These data illustrate that the reduction in acetate occurs regardless of the presence of an overexpressed farnesene production pathway.

**FIG. 9** shows that the deletion of rhr2 improves farnesene production in Y 12746.ms63907.ms64472 by 2.1-fold, and in Y 12745.ms63907.ms64472 by 1.4-fold (In each strain background, the RHR2\(^{+}\) parent is normalized to 1). Moreover, deletion of rhr2 improves the final optical density of Y 12746.ms63907.ms64472 at carbon exhaustion. Both Y 12745.ms63907.64472 and Y 12746.ms63907.ms64472 use phosphoketolase and phosphotransacetylase, and thus acetyl phosphate as a pathway intermediate, to produce cytosolic acetyl-CoA, which is used for synthesis of farnesene. Strains Y968.ms63907.ms64472 and Y12869.ms63907.ms64472 do not express phosphoketolase or phosphotransacetylase, and do not use acetyl phosphate as a pathway intermediate. Deletion of rhr2 in these strain backgrounds has no effect on farnesene production or optical density in either strain background. This indicates that the benefit of knocking out rhr2 specifically applies to strains which use acetyl phosphate as an intermediate metabolite, e.g., strains comprising heterologous PK and/or PTA.

All publications, patents and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. 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.
WHAT IS CLAIMED:

1. A genetically modified host cell comprising:
   (a) a heterologous nucleic acid encoding a phosphoketolase (PK; EC 4.1.2.9); and
   (b) a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate.

2. The genetically modified host cell of claim 1, wherein the genetically modified host cell further comprises a heterologous nucleic acid encoding a phosphotransacetylase (PTA; EC 2.3.1.8).

3. A genetically modified host cell comprising:
   (a) a heterologous nucleic acid encoding a phosphotransacetylase (PTA; EC 2.3.1.8); and
   (b) a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate.

4. The genetically modified host cell of claim 3, wherein the genetically modified host cell further comprises a heterologous nucleic acid encoding a phosphoketolase (PK; EC 4.1.2.9).

5. The genetically modified host cell of any one of claims 1 to 4, wherein the enzyme that converts acetyl phosphate to acetate is a glycerol-1-phosphatase (EC 3.1.3.21).

6. The genetically modified host cell of claim 5, wherein the glycerol-1-phosphatase is selected from GPP1/RHR2, GPP2/HOR2, and homologues and variants thereof.

7. The genetically modified host cell of claim 6, wherein GPP1/RHR2, or a homologue or variant thereof, is functionally disrupted.

8. The genetically modified host cell of claim 6, wherein GPP2/HOR2, or a homologue or variant thereof, is functionally disrupted.
9. The genetically modified host cell of claim 6, wherein both GPP1/RHR2 and GPP2/HOR2, or both a homologue or variant of GPP1/RHR2 and a homologue or variant of GPP2/HOR2, are functionally disrupted.

10. The genetically modified host cell of any one of claims 1 to 9, wherein the genetically modified host cell is capable of producing an isoprenoid.

11. The genetically modified host cell of claim 10, wherein the genetically modified host cell comprises one or more heterologous nucleic acids encoding one or more enzymes of a mevalonate (MEV) pathway for making isopentenyl pyrophosphate.

12. The genetically modified host cell of claim 11, wherein the one or more enzymes of the MEV pathway comprise an NADH-using HMG-CoA reductase.

13. The genetically modified host cell of claim 11, wherein the one or more enzymes of the MEV pathway comprise an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA.

14. The genetically modified host cell of claim 11, wherein the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetoacetyl-CoA with acetyl-CoA to form HMG-CoA.

15. The genetically modified host cell of claim 11, wherein the one or more enzymes of the MEV pathway comprise an enzyme that converts HMG-CoA to mevalonate.

16. The genetically modified host cell of claim 11, wherein the one or more enzymes of the MEV pathway comprise an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate.

17. The genetically modified host cell of claim 11, wherein the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate.

18. The genetically modified host cell of claim 11, wherein the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate.
19. The genetically modified host cell of claim 11, wherein the one or more enzymes of the MEV pathway are selected from HMG-CoA synthase, mevalonate kinase, phosphomevalonate kinase and mevalonate pyrophosphate decarboxylase.

20. The genetically modified host cell of claim 11, wherein the host cell comprises a plurality of heterologous nucleic acids encoding all of the enzymes of the MEV pathway.

21. The genetically modified host cell of claim 11, 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.

22. The genetically modified host cell of claim 11, 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.

23. The genetically modified host cell of claim 11, further comprising a heterologous nucleic acid encoding an enzyme that can convert isopentenyl pyrophosphate (IPP) into dimethylallyl pyrophosphate (DMAPP).

24. The genetically modified host cell of claim 11, further comprising a heterologous nucleic acid encoding an enzyme that can condense IPP and/or DMAPP molecules to form a polyprenyl compound.

25. The genetically modified host cell of claim 11, further comprising a heterologous nucleic acid encoding an enzyme that can modify IPP or a polyprenyl to form an isoprenoid compound.

26. The genetically modified host cell of claim 25, 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, a-pinene synthase, β-pinene synthase, γ-terpinene synthase, terpinolene synthase, amorphadiene synthase, a-farnesene synthase, β-farnesene synthase, farnesol synthase, nerolidol synthase, patchouliol synthase, nootkatone synthase, and abietadiene synthase.
27. The genetically modified host cell of claim 10, wherein the isoprenoid is selected from the group consisting of a hemiterpene, monoterpene, diterpene, triterpene, tetraterpene, sesquiterpene, and polyterpene.

28. The genetically modified host cell of claim 10, wherein the isoprenoid is a C₅-C₂₀ isoprenoid.

29. The genetically modified host cell of claim 10, 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.

30. The genetically modified host cell of any one of claims 1 to 29, wherein the cell is selected from the group consisting of a bacterial cell, a fungal cell, an algal cell, an insect cell, and a plant cell.

31. The genetically modified host cell of any one of claims 1 to 29, wherein the cell is a yeast cell.

32. The genetically modified host cell of claim 31, wherein the yeast is *Saccharomyces cerevisiae*.

33. The genetically modified host cell of any one of claims 1 to 32, wherein the genetically modified host cell produces an increased amount of an acetyl-CoA derived compound compared to a yeast cell not comprising a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate.

34. A method for producing an isoprenoid comprising:

   (a) culturing a population of the genetically modified host cells of any one of claims 10 to 33 in a medium with a carbon source under conditions suitable for making said isoprenoid compound; and

   (b) recovering said isoprenoid compound from the medium.

35. A method for increasing the production of acetyl-CoA or an acetyl-CoA derived compound in a host cell, the method comprising:
(a) expressing in the host cell a heterologous nucleic acid encoding a phosphoketolase (PK; EC 4.1.2.9); and

(b) functionally disrupting an endogenous enzyme that converts acetyl phosphate to acetate.

36. The method of claim 35, further comprising expressing in the host cell a heterologous nucleic acid encoding a phosphotransacetylase (PTA; EC 2.3.1.8).

37. A method for increasing the production of acetyl-CoA in a host cell, the method comprising:

(a) expressing in the host cell a heterologous nucleic acid encoding a phosphotransacetylase (PTA; EC 2.3.1.8); and

(b) functionally disrupting an endogenous enzyme that converts acetyl phosphate to acetate.

38. The method of claim 37, further comprising expressing in the host cell a heterologous nucleic acid encoding a phosphoketolase (PK; EC 4.1.2.9).

39. The method of any one of claims 35 to 38, wherein the enzyme that converts acetyl phosphate to acetate is a glycerol-1-phosphatase (EC 3.1.3.21).

40. The method of claim 39, wherein the glycerol-1-phosphatase is selected from GPP1/RHR2, GPP2/HOR2, and homologues and variants thereof.

41. The method of claim 40, wherein GPP1/RHR2, or a homologue or variant thereof, is functionally disrupted.

42. The method of claim 40, wherein GPP2/HOR2, or a homologue or variant thereof, is functionally disrupted.

43. The method of claim 40, wherein both GPP1/RHR2 and GPP2/HOR2, or both a homologue or variant of GPP1/RHR2 and a homologue or variant of GPP2/HOR2, are functionally disrupted.
44. The method of any one of claims 35 to 43, wherein the host cell is selected from the group consisting of a bacterial cell, a fungal cell, an algal cell, an insect cell, and a plant cell.

45. The method of any one of claims 35 to 43, wherein the host cell is a yeast cell.

46. The method of claim 45, wherein the yeast is \textit{Saccharomyces cerevisiae}.

47. The method of any one of claims 35 to 46, wherein the host cell produces an increased amount of acetyl-CoA or an acetyl-CoA derived compound compared to a yeast cell not comprising a functional disruption of an endogenous enzyme that converts acetyl phosphate to acetate.
Figure 3A & 3B
FIG. 6

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