The invention provides a non-naturally occurring microbial organism having a muconate pathway having at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate. The muconate pathway including an enzyme selected from the group consisting of a beta-ketothiolase, a beta-ketoadipyl-CoA hydrolase, a beta-ketoadipyl-CoA transferase, a beta-ketoadipyl-CoA ligase, a 2-fumarilacetaete reductase, a 2-fumarilacetate dehydrogenase, a trans-3-hydroxy-4-hexendioate dehydratase, a 2-fumarilacetaete aminotransferase, a 2-fumarilacetaete aminotransferase, a trans-3-amino-4-hexenolate deaminase, a beta-ketoadipate enol-lactone hydrolase, a muconolactone isomerase, a muconate cycloisomerase, a beta-ketoadipyl-CoA dehydrogenase, a 3-hydroxyadipyl-CoA dehydratase, a 2,3-dehydroadipyl-CoA transferase, a 2,3-dehydroadipyl-CoA hydrolase, a 2,3-dehydroadipyl-CoA ligase, a muconate reductase, a 2-maleylacetate reductase, a 2-maleylacetate dehydrogenase, a cis-3-hydroxy-4-hexendioate dehydratase, a 2-maleylacetate aminotransferase, a 2-maleylacetate aminotransferase, a cis-3-amino-4-hexenolate deaminase, and a muconate cis/trans isomerase. Other muconate pathway enzymes also are provided. Additionally provided are methods of producing muconate.
FIG. 5
This application claims the benefit of priority of U.S. Provisional Application No. 61/231,637, filed Aug. 5, 2009, the entire contents of which are incorporated herein by this reference.

BACKGROUND OF THE INVENTION

The present disclosure relates generally to the design of engineered organisms and, more specifically to organisms having selected genotypes for the production of muconic acid.

Terephthalate (also known as terephthalic acid and PTA) is the immediate precursor of polyethylene terephthalate (PET), used to make clothing, resins, plastic bottles and even as a poultry feed additive. Nearly all PTA is produced from para-xylene by the oxidation in air in a process known as the Mid Century Process (Roffia et al., Ind. Eng. Chem. Prod. Res. Dev. 23:629-634 (1984)). This oxidation is conducted at high temperature in an acetic acid solvent with a catalyst composed of cobalt and/or manganese salts. Para-xylene is derived from petrochemical sources, and is formed by high severity catalytic re-forming of naphtha. Xylene is also obtained from the pyrolysis gasoline stream in a naphtha steam cracker and by toluene disproportion.

PTA, toluene and other aromatic precursors are naturally degraded by some bacteria. However, these degradation pathways typically involve monoxygenases that operate irreversibly in the degradative direction. Hence, biocatalytic pathways for PTA are severely limited by the properties of known enzymes to date.

Muconate (also known as muconic acid, MA) is an unsaturated dicarboxylic acid used as a raw material for resins, pharmaceuticals and agrochemicals. Muconate can be converted to adipic acid, a precursor of Nylon-6,6, via hydrogenation (Draths and Frost, J. Am. Chem. Soc. 116; 399-400 (1994)). Alternately, muconate can be hydrogenated using biometallic nanocatalysts (Thomas et al., Chem. Commun. 10:1126-1127 (2003)).

Muconate is a common degradation product of diverse aromatic compounds in microbes. Several biocata-lytic strategies for making cis,cis-muconate have been developed. Engineered E. coli strains producing muconate from glucose via shikimate pathway enzymes have been developed in the Frost lab (U.S. Pat. No. 5,487,987 (1996); Niu et al., Biotecnol. Prog. 18:201-211 (2002)). These strains are able to produce 36.8 g/L of cis,cis-muconate after 48 hours of culturing under fed-batch fermenter conditions (22% of the maximum theoretical yield from glucose). Muconate has also been produced biocatalytically from aromatic starting materials such as toluene, benzoic acid and catechol. Strains producing muconate from benzene achieved titers of 13.5 g/L and productivity of 5.5 g/L/hr (Choi et al., J. Ferment. Bioeng. 84;70-76 (1997)). Muconate has also been generated from the effluents of a styrene monomer production plant (Wu et al., Enzyme and Microbiology Technology 55:598-604 (2004)).

All biocatalytic pathways identified to date proceed through enzymes in the shikimate pathway, or degradation enzymes from catechol. Consequently, they are limited to producing the cis, cis isomer of muconate, since these pathways involve ring-opening chemistry. The development of pathways for producing trans,trans-muconate and cis,trans-muconate would be useful because these isomers can serve as direct synthetic intermediates for producing renewable PTA via the inverse electron demand Diels-Alder reaction with acetylene. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF INVENTION

The invention provides a non-naturally occurring microbial organism having a muconate pathway having at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate. The muconate pathway including an enzyme selected from the group consisting of a beta-ketothiolase, a beta-ketoacyl-CoA hydrolase, a beta-ketoacyl-CoA transferase, a beta-ketoacyl-CoA ligase, a 2-fumarylacetate dehydratase, a 2-fumarylacetate dehydrogenase, a trans-3-hydroxy-4-hexenoate dehydratase, a 2-fumarylacetate aminotransferase, a 2-fumarylacetate aminating oxidoreductase, a 2,3-amino-4-hexenoate deaminase, a beta-ketoacyl- enol-lactone hydrolase, a muconolactone isomerase, a muconate cycloisomerase, a beta-ketoacyl-CoA dehydrogenase, a 3-hydroxyacyl-CoA dehydratase, a 2,3-dehydroadipyl-CoA transferase, a 2,3-dehydroadipyl-CoA hydrolase, a 2,3-dehydroadipyl-CoA ligase, a muconate reductase, a 2-maleylacetate reductase, a 2-maleylacetate dehydrogenase, a cis-3-hydroxy-4-hexenoate dehydrogenase, a 2-maleylacetate aminating oxidoreductase, a 2-maleylacetate aminating oxidoreductase, a 2,3-amino-4-hexenoate deaminase, and a muconate cis/trans isomerase. Other muconate pathway enzymes also are provided. Additionally provided are methods of producing muconate.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the synthesis of terephthalate from muconate and acetylene via Diels-Alder chemistry. PI is cyclohexa-2,5-diene-1,4-dicarboxylate.

FIG. 2 shows pathways to trans-trans muconate from succinyl CoA. Enzymes are A) beta-ketothiolase, B) beta-ketoacyl-CoA hydrolase, transferase and/or ligase, C) 2-fumarylacetate reductase, D) 2-fumarylacetate dehydrogenase, E) trans-3-hydroxy-4-hexenoate dehydrogenase, F) 2-fumarylacetate aminotransferase and/or 2-fumarylacetate aminating oxidoreductase, G) trans-3-amino-4-hexenoate deaminase, H) beta-ketoacyl- enol-lactone hydrolase, I) muconolactone isomerase, J) muconate cycloisomerase, K) beta-ketoacyl-CoA dehydrogenase, L) 3-hydroxyacyl-CoA dehydratase, M) 2,3-dehydroadipyl-CoA transferase, hydrolase or ligase, N) muconate reductase, O) 2-maleylacetate reductase, P) 2-maleylacetate dehydrogenase, Q) cis-3-hydroxy-4-hexenoate dehydratase, R) 2-maleylacetate aminating oxidoreductase and/or 2-maleylacetate aminating oxidoreductase, S) cis-3-amino-4-hexenoate deaminase, T) muconate cis/trans isomerase, W) muconate cis/trans isomerase.

FIG. 3 shows pathways to muconate from pyruvate and malonate semialdehyde. Enzymes are A) 4-hydroxy-2-ketovalerate aldolase, B) 2-oxopentenoate hydratase, C) 4-oxocrotonate dehydrogenase, D) 2-hydroxy-4-hexenoate dehydrogenase, E) 4-hydroxy-2-oxohexanldioate oxidoreductase, F) 2,4-dihydroxyadipate dehydratase (acting on
2-hydroxy), G) 2,4-dihydroxyadipate dehydratase (acting on 4-hydroxyl group) and H) 3-hydroxy-4-hexenedioate dehydratase.

[0012] FIG. 4 shows pathways to muconate from pyruvate and succinic semialdehyde. Enzymes are A) HODH aldolase, B) OHED hydratase, C) OHED decarboxylase, D) HODH formate-lyase and/or HODH dehydrogenase, E) OHED formate-lyase and/or OHED dehydrogenase, F) 6-OHE dehydrogenase, G) 3-hydroxyadipyl-CoA dehydratase, H) 2,3-dehydroxyadipyl-CoA hydrolase, transferase or ligase, I) muconate reductase. Abbreviations are: HODH-4-hydroxy-2-oxoheptane-1,7-dioate, OHED-2-oxohept-4-ene-1,7-dioate, 6-OHE-6-oxo-2,3-dehydroxyanisole.

[0013] FIG. 5 shows pathways to muconate from lysine. Enzymes are A) lysine aminotransferase and/or aminating oxidoreductase, B) 2-aminoadipate semialdehyde dehydrogenase, C) 2-aminoadipate deaminase, D) muconate reductase, E) lysine-2,3-amino transferase, F) 3,6-diaminohexanase aminotransferase and/or aminating oxidoreductase, G) 3-aminoadipate semialdehyde dehydrogenase, H) 3-aminoadipate deaminase.

[0014] FIG. 6 shows 3 thiolases with demonstrated thiolase activity resulting in acetocadapril-CoA formation (left panel). FIG. 6 also shows that several enzymes demonstrated selective condensation of succinyl-CoA and acetyl-CoA to form β-ketoadipyl-CoA as the sole product (right panel).

DETAILED DESCRIPTION OF THE INVENTION

[0015] The present invention is directed, in part, to a biosynthetic pathway for synthesizing muconate from simple carbohydrate feedstocks, which in turn provides a viable synthetic route to PTA. In particular, pathways disclosed herein provide trans, trans-muconate or cis, trans-muconate biocatalytically from simple sugars. The all trans or cis, trans isomer of muconate is then converted to PTA in a two step process via inverse electron demand Diels-Alder reaction with acetylene followed by oxidation in air or oxygen. The Diels-Alder reaction between muconate and acetylene proceeds to form cyclohexa-2,5-diene-1,4-dicarboxylic (P1), as shown in FIG. 1. Subsequent exposure to air or oxygen rapidly converts P1 to PTA.

[0016] This invention is also directed, in part, to non-naturally occurring microorganisms that express genes encoding enzymes that catalyze muconate production. Pathways for the production of muconate disclosed herein are derived from central metabolic precursors. Successful engineering these pathways entails identifying an appropriate set of enzymes with sufficient activity and specificity, cloning their corresponding genes into a production host, optimizing the expression of these genes in the production host, optimizing fermentation conditions, and assaying for product formation following fermentation.

[0017] The maximum theoretical yield of muconic acid is 1.09 moles per mole glucose utilized. Achieving this yield involves assimilation of CO₂ as shown in equation 1 below:

\[ \text{C}_6\text{H}_{12}\text{O}_6 + \text{CO}_2 \rightarrow 12\text{C}_2\text{H}_4\text{O}_7 + 3\text{H}_2\text{O} \]  

(equation 1)

[0018] As used herein, the term “muconate” is used interchangably with muconic acid. Muconate is also used to refer to any of the possible isomeric forms: trans, trans, cis, trans, and cis, cis. However, the present invention provides pathways to the useful trans, trans and cis, cis forms, in particular.

[0019] As used herein, the term “non-naturally occurring” when used in reference to a microbial organism or microorganism of the invention is intended to mean that the microbial organism has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microbial genetic material. Such modifications include, for example, coding regions and functional fragments thereof, for heterologous, homologous or both heterologous and homologous polypeptides for the referenced species. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. Exemplary metabolic polypeptides include enzymes or proteins within a muconate biosynthetic pathway.

[0020] A metabolic modification refers to a biochemical reaction that is altered from its naturally occurring state. Therefore, non-naturally occurring microorganisms can have genetic modifications to nucleic acids encoding metabolic polypeptides or functional fragments thereof. Exemplary metabolic modifications are disclosed herein.

[0021] As used herein, the term “isolated” when used in reference to a microbial organism is intended to mean an organism that is substantially free of at least one component as the referenced microbial organism is found in nature. The term includes a microbial organism that is removed from some or all components as it is found in its natural environment. The term also includes a microbial organism that is removed from some or all components as the microbial organism is found in non-naturally occurring environments. Therefore, an isolated microbial organism is partly or completely separated from other substances as it is found in nature or as it is grown, stored or subsisted in non-naturally occurring environments. Specific examples of isolated microbial organisms include partially pure microbes, substantially pure microbes and microbes cultured in a medium that is non-naturally occurring.

[0022] As used herein, the terms “microbial,” “microbial organism” or “microorganism” is intended to mean any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea and eukarya of all species as well as eukaryotic microorganisms such as yeast and fungi. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

[0023] As used herein, the term “CoA” or “coenzyme A” is intended to mean an organic cofactor or prosthetic group (nonprotein portion of an enzyme) whose presence is required for the activity of many enzymes (the apoenzyme) to form an active enzyme system. Coenzyme A functions in certain condensing enzymes, acts in acetyl or other acyl group transfer and in fatty acid synthesis and oxidation, pyruvate oxidation and in other acetylation.

[0024] As used herein, the term “substantially anaerobic” when used in reference to a culture or growth condition is intended to mean that the amount of oxygen is less than about 10% of saturation for dissolved oxygen in liquid media. The
term also is intended to include sealed chambers of liquid or solid medium maintained with an atmosphere of less than about 1% oxygen.

[0025] "Exogenous" as it is used herein is intended to mean that the referenced molecule or the referenced activity is introduced into the host microbial organism. The molecule can be introduced, for example, by introduction of an encoding nucleic acid into the host genetic material such as by integration into a host chromosome or as non-chromosomal genetic material such as a plasmid. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the microbial organism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into the host reference organism. The source can be, for example, a homologous or heterologous encoding nucleic acid that expresses the referenced activity following introduction into the host microbial organism. Therefore, the term "endogenous" refers to a referenced molecule or activity that is present in the host. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the microbial organism. The term "heterologous" refers to a molecule or activity derived from a source other than the referenced species whereas "homologous" refers to a molecule or activity derived from the host microbial organism. Accordingly, exogenous expression of an encoding nucleic acid of the invention can utilize either or both a heterologous or homologous encoding nucleic acid.

[0026] The non-naturally occurring microbial organisms of the invention can contain stable genetic alterations, which refers to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

[0027] Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein, are described with reference to a suitable host organism such as E. coli and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes for a desired metabolic pathway. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the E. coli metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

[0028] An ortholog is a gene or genes that are related by vertical descent and are responsible for substantially the same or identical functions in different organisms. For example, mouse epoxide hydrolase and human epoxide hydrolase can be considered orthologs for the biological function of hydrolysis of epoxides. Genes are related by vertical descent when, for example, they share sequence similarity of sufficient amount to indicate they are homologous, or related by evolution from a common ancestor. Genes can also be considered orthologs if they share three-dimensional structure but not necessarily sequence similarity, of a sufficient amount to indicate that they have evolved from a common ancestor to the extent that the primary sequence similarity is not identifiable. Genes that are orthologous can encode proteins with sequence similarity of about 25% to 100% amino acid sequence identity. Genes encoding proteins sharing an amino acid similarity less than 25% can also be considered to have arisen by vertical descent if their three-dimensional structure also shows similarities. Members of the serine protease family of enzymes, including tissue plasminogen activator and elastase, are considered to have arisen by vertical descent from a common ancestor.

[0029] Orthologs include genes or their encoded gene products that through, for example, evolution, have diverged in structure or overall activity. For example, where one species encodes a gene product exhibiting two functions and where such functions have been separated into distinct genes in a second species, the three genes and their corresponding products are considered to be orthologs. For the production of a biochemical product, those skilled in the art will understand that the orthologous gene harboring the metabolic activity to be introduced or disrupted is to be chosen for construction of the non-naturally occurring microorganism. An example of orthologs exhibiting separable activities is where distinct activities have been separated into distinct gene products between two or more species or within a single species. A specific example is the separation of elastase proteolysis and plasminogen proteolysis, two types of serine protease activity, into distinct molecules as plasminogen activator and elastase. A second example is the separation of mycoplasma 5'-3' exonuclease and Drosophila DNA polymerase III activity. The DNA polymerase from the first species can be considered an ortholog of either or both of the exonuclease or the polymerase from the second species and vice versa.

[0030] In contrast, paralogs are homologs related by, for example, duplication followed by evolutionary divergence and have similar or common, but not identical functions. Paralogs can originate or derive from, for example, the same species or from a different species. For example, microsomal epoxide hydrolase (epoxide hydrolase I) and soluble epoxide hydrolase (epoxide hydrolase II) can be considered paralogs because they represent two distinct enzymes, co-evolved from a common ancestor, that catalyze distinct reactions and have distinct functions in the same species. Paralogs are proteins from the same species with significant sequence similarity to each other suggesting that they are homologous, or related through co-evolution from a common ancestor. Groups of paralogous protein families include HipA homologs, luciferase genes, peptidases, and others.

[0031] A nonorthologous gene displacement is a nonorthologous gene from one species that can substitute for a referenced gene function in a different species. Substitution includes, for example, being able to perform substantially the same or a similar function in the species of origin compared to the referenced function in the different species. Although generally, a nonorthologous gene displacement will be identifiable as structurally related to a known gene encoding the referenced function, less structurally related but functionally similar genes and their corresponding gene products nevertheless will still fall within the meaning of the term as it is used herein. Functional similarity requires, for example, at least some structural similarity in the active site or binding region of a nonorthologous gene product compared to a gene
encoding the function sought to be substituted. Therefore, a
nonorthologous gene includes, for example, a paralog or an
unrelated gene.

Therefore, in identifying and constructing the non-
naturally occurring microbial organisms of the invention hav-
ing muconate biosynthetic capability, those skilled in the art
will understand with applying the teaching and guidance
provided herein to a particular species that the identification of
metabolic modifications can include identification and
inclusion or inactivation of orthologs. To the extent that para-
logs and/or nonorthologous gene displacements are present in
the referenced microorganism that encode an enzyme cata-
lizing a similar or substantially similar metabolic reaction,
those skilled in the art also can utilize these evolutionally
related genes.

Orthologs, paralogs and nonorthologous gene dis-
placements can be determined by methods well known to
those skilled in the art. For example, inspection of nucleic
acid or amino acid sequences for two polypeptides will reveal
sequence identity and similarities between the compared
sequences. Based on such similarities, one skilled in the art
can determine if the similarity is sufficiently high to indicate
the proteins are related through evolution from a common
ancestor. Algorithms well known to those skilled in the art,
such as Align, BLAST, Clustal W and others compare and
determine a raw sequence similarity or identity, and also
determine the presence or significance of gaps in the sequence
which can be assigned a weight or score. Such algorithms also
are known in the art and are similarly applicable for deter-
mining nucleotide sequence similarity or identity. Parameters
for sufficient similarity to determine relatedness are com-
puted based on well known methods for calculating statistical
similarity, or the chance of finding a similar match in a ran-
dom polypeptide, and the significance of the match deter-
mined. A computer comparison of two or more sequences
can, if desired, also be optimized visually by those skilled in
the art. Related gene products or proteins can be expected to
have a high similarity, for example, 25% to 100% sequence
identity. Proteins that are unrelated can have an identity which
is essentially the same as would be expected to occur by chance,
if a database of sufficient size is scanned (about 5%).

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

In some embodiments, the invention provides a non-
naturally occurring microbial organism having a muconate
pathway that includes at least one exogenous nucleic acid
encoding a muconate pathway enzyme expressed in a suffi-
cient amount to produce muconate. The muconate pathway
includes an enzyme selected from the group consisting of a
beta-ketothiolase, a beta-ketoadipyl-CoA hydrolase, a beta-
ketoadipyl-CoA transferase, a beta-ketoadipyl-CoA ligase, a
2-fumarylacetate reductase, a 2-fumarylacetate dehydroge-
nase, a trans-3-hydroxy-4-hexendioate dehydratase, a 2-fu-
marylacetate aminotransferase, a 2-fumarylacetate aminating
oxidoreductase, a trans-3-amino-4-hexenoate deaminase, a
beta-ketoadipate enol-lactone hydrolase, a muconolactone
isomerase, a muconate cycloisomerase, a beta-ketoadipyl-
CoA dehydrogenase, a 3-hydroxyadipyl-CoA dehydrogenase,
a 2,3-dehydroadipyl-CoA transferase, a 2,3-dehydroadipyl-
CoA hydrolase, a 2,3-dehydroadipyl-CoA ligase, a muconate
reductase, a 2-maleylacetate reductase, a 2-maleylacetate
dehydrogenase, a cis-3-hydroxy-4-hexendioate dehydratase,
a 2-maleylacetate aminotransferase, a 2-maleylacetate amin-
ating oxidoreductase, a cis-3-amino-4-hexendioate deami-

In particular embodiments, the muconate pathway
includes a set of muconate pathway enzymes shown in FIG. 2
and selected from the group consisting of:

A) (1) beta-ketothiolase, (2) an enzyme selected from
beta-ketoadipyl-CoA hydrolase, beta-ketoadipyl-CoA
transferase, and beta-ketoadipyl-CoA ligase, (3) beta-keto-
adipate enol-lactone hydrolase, (4) muconolactone
isomerase, (5) muconate cycloisomerase, and (6) a muconate cis/trans
isomerase;

B) (1) beta-ketothiolase, (2) an enzyme selected from
beta-ketoadipyl-CoA hydrolase, beta-ketoadipyl-CoA
transferase and beta-ketoadipyl-CoA ligase, (3) 2-maleyl-
acetate reductase, (4) 2-maleylacetate dehydrogenase,
(5) cis-3-hydroxy-4-hexendioate dehydratase, and (6) muconate cis/
trans isomerase;

C) (1) beta-ketothiolase, (2) an enzyme selected from
beta-ketoadipyl-CoA hydrolase, beta-ketoadipyl-CoA
transferase and beta-ketoadipyl-CoA ligase, (3) 2-maleyl-
acetate reductase, (4) an enzyme selected from 2-maleylacetate
aminotransferase and 2-maleylacetate aminating oxi-

doreductase, (5) cis-3-amino-4-hexenoate deaminase, and (6)
uconate cis/trans isomerase;

D) (1) beta-ketothiolase, (2) beta-ketoadipyl-CoA

dehydrogenase, (3) 3-hydroxyadipyl-CoA dehydratase, (4)
an enzyme selected from 2,3-dehydroadipyl-CoA trans-
ferase, 2,3-dehydroadipyl-CoA hydrolase and 2,3-dehydro-
adipyl-CoA ligase, and (5) muconate reductase;

E) (1) beta-ketothiolase, (2) an enzyme selected from
beta-ketoadipyl-CoA hydrolase, beta-ketoadipyl-CoA
transferase and beta-ketoadipyl-CoA ligase, (3) 2-fumaryl-
acetate reductase, (4) 2-fumarylacetate dehydrogenase, and (5)
trans-3-hydroxy-4-hexendioate dehydratase;

F) (1) beta-ketothiolase, (2) an enzyme selected from
beta-ketoadipyl-CoA hydrolase, beta-ketoadipyl-CoA
transferase and beta-ketoadipyl-CoA ligase, (3) 2-fumaryl-
acetate reductase, (4) an enzyme selected from 2-fumaryl-
acetate aminotransferase and 2-fumarylacetate aminating oxi-
doreductase, and (5) trans-3-amino-4-hexenoate deaminase.

In some embodiments, a microbial organism having
a pathway exemplified by those shown in FIG. 2 can include
two or more exogenous nucleic acids each encoding a mucona-
tate pathway enzyme, including three, four, five, six, that is up
to all of the of enzymes in a muconate pathway. The non-
naturally occurring microbial organism having at least one
exogenous nucleic acid can include a heterologous nucleic
acid. A non-naturally occurring microbial organism having a pathway exemplified by those shown in FIG. 2 can be cultured in a substantially anaerobic culture medium.

[0044] In some embodiments, the invention provides a non-naturally occurring microbial organism having a muconate pathway that includes at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate. The muconate pathway includes an enzyme selected from the group consisting of a 4-hydroxy-2-ketovlactate aldolase, a 2-oxopentenoate hydratase, a 4-oxalocrotonate dehydrogenase, a 2-hydroxy-4-hexenedioate dehydratase, a 4-hydroxy-2-oxohexenedioate oxidoreductase, a 2,4-dihydroxyadipate dehydratase (acting on 2-hydroxy), a 2,4-dihydroxyadipate dehydratase (acting on 4-hydroxyl group) and a 3-hydroxy-4-hexenedioate dehydratase.

[0045] In particular embodiments, the muconate pathway includes a set of muconate pathway enzymes shown in FIG. 3 and selected from the group consisting of:

- A) (1) 4-hydroxy-2-ketovlactate aldolase, (2) 2-oxopentenoate hydratase, (3) 4-oxalocrotonate dehydrogenase, (4) 2-hydroxy-4-hexenedioate dehydratase;
- B) (1) 4-hydroxy-2-ketovlactate aldolase, (2) 4-hydroxy-2-oxohexenedioate oxidoreductase, (3) 2,4-dihydroxyadipate dehydratase (acting on 2-hydroxy), (4) 3-hydroxy-4-hexenedioate dehydratase; and
- C) (1) 4-hydroxy-2-ketovlactate aldolase, (2) 4-hydroxy-2-oxohexenedioate oxidoreductase, (3) 2,4-dihydroxyadipate dehydratase (acting on 4-hydroxyl group), (4) 2-hydroxy-4-hexenedioate dehydratase.

[0049] In some embodiments, a microbial organism having a pathway exemplified by those shown in FIG. 3 can include two or more exogenous nucleic acids each encoding a muconate pathway enzyme, including three, four, that is up to all of the of enzymes in a muconate pathway. The non-naturally occurring microbial organism having at least one exogenous nucleic acid can include a heterologous nucleic acid. A non-naturally occurring microbial organism having a pathway exemplified by those shown in FIG. 3 can be cultured in a substantially anaerobic culture medium.

[0050] In some embodiments, the invention provides a non-naturally occurring microbial organism having a muconate pathway that includes at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate. The muconate pathway includes an enzyme selected from the group consisting of an HODH aldolase, an OHED hydratase, an OHED decarboxylase, an HODH formate-lyase, an HODH dehydrogenase, an OHED formate-lyase, an OHED dehydrogenase, a 6-OHE dehydrogenase, a 3-hydroxydipyl-CoA dehydratase, a 2,3-dehydrodipyl-CoA hydratase, a 2,3-dehydrodipyl-CoA transferase, a 2,3-dehydrodipyl-CoA ligase, and a muconate reductase.

[0051] In particular embodiments, the muconate pathway includes a set of muconate pathway enzymes shown in FIG. 4 and selected from the group consisting of:

- A) (1) HODH aldolase, (2) OHED hydratase, (3) OHED decarboxylase, (4) 6-OHE dehydrogenase, and (5) muconate reductase;
- B) (1) HODH aldolase, (2) OHED hydratase, (3) an enzyme selected from OHED formate-lyase and OHED dehydrogenase, (4) an enzyme selected from 2,3-dehydrodipyl-CoA hydratase, 2,3-dehydrodipyl-CoA transferase and 2,3-dehydrodipyl-CoA ligase, and (5) muconate reductase.

[0054] C) (1) HODH aldolase, (2) an enzyme selected from HODH formate-lyase and HODH dehydrogenase, (3) 3-hydroxydipyl-CoA dehydratase, (4) an enzyme selected from 2,3-dehydrodipyl-CoA hydratase, 2,3-dehydrodipyl-CoA transferase and 2,3-dehydrodipyl-CoA ligase, and (5) muconate reductase.

[0055] In some embodiments, a microbial organism having a pathway exemplified by those shown in FIG. 4 can include two or more exogenous nucleic acids each encoding a muconate pathway enzyme, including three, four, five, that is up to all of the of enzymes in a muconate pathway. The non-naturally occurring microbial organism having at least one exogenous nucleic acid can include a heterologous nucleic acid. A non-naturally occurring microbial organism having a pathway exemplified by those shown in FIG. 4 can be cultured in a substantially anaerobic culture medium.

[0056] In some embodiments, the invention provides a non-naturally occurring microbial organism having a muconate pathway that includes at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate. The muconate pathway includes an enzyme selected from the group consisting of:

- A) (1) lysine aminotransferase, (2) lysine aminating oxidoreductase, (3) 2-aminoaldipate semialdehyde dehydrogenase, (4) 2-aminoaldipate deaminase, a muconate reductase, a lysine-2,3-amino-al- mutase, a 3,6-diaminohexanone aminotransferase, a 3,6-diaminohexanone aminating oxidoreductase, a 3-aminoaldipate semialdehyde dehydrogenase, and a 3-aminoaldipate deaminase.

[0057] In particular embodiments, the muconate pathway includes a set of muconate pathway enzymes shown in FIG. 5 and selected from the group consisting of:

- A) (1) lysine aminotransferase, (2) lysine aminating oxidoreductase, (3) 2-aminoaldipate semialdehyde dehydrogenase, (4) 2-aminoaldipate deaminase, and (5) muconate reductase;
- B) (1) lysine-2,3-amino-al- mutase, (2) 3,6-diaminohexanone aminotransferase, (3) 3,6-diaminohexanone aminating oxidoreductase, (4) 3-aminoaldipate semialdehyde dehydrogenase, (5) 3-aminoaldipate deaminase, and (6) muconate reductase.

[0060] In some embodiments, a microbial organism having a pathway exemplified by those shown in FIG. 5 can include two or more exogenous nucleic acids each encoding a muconate pathway enzyme, including three, four, five, six, that is up to all of the enzymes in a muconate pathway. The non-naturally occurring microbial organism having at least one exogenous nucleic acid can include a heterologous nucleic acid. A non-naturally occurring microbial organism having a pathway exemplified by those shown in FIG. 5 can be cultured in a substantially anaerobic culture medium.

[0061] In an additional embodiment, the invention provides a non-naturally occurring microbial organism having a muconate pathway wherein the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate to a product selected from the group consisting of succinyl-CoA to beta-ketoaldipyl-CoA, beta-ketoaldipyl-CoA to 3-hydroxydipyl-CoA, 3-hydroxydipyl-CoA to 2,3-dehydrodipyl-CoA, 2,3-dehydrodipyl-CoA to 2,3-dehydrodipyl-CoA, and 2,3-dehydrodipyl-CoA to trans,trans-muconate. Altern-
tively, the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate to a product selected from the group consisting of succinyl-CoA to beta-ketoacyl-CoA, beta-ketoacyl-CoA to beta-ketoacyl-CoA, beta-ketoacyl-CoA to 2-maleylacetate, 2-maleylacetate to cis-3-hydroxy-4-hexendioate, cis-3-hydroxy-4-hexendioate to cis,trans-muconate, and cis,trans-muconate to trans,trans-muconate. Alternatively, the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate to a product selected from the group consisting of succinyl-CoA to betBKoA, beta-ketoacyl-CoA to beta-ketoacyl-CoA, beta-ketoacyl-CoA to 2-maleylacetate, 2-maleylacetate to cis-3-hydroxy-4-hexendioate, cis-3-hydroxy-4-hexendioate to cis,trans-muconate, and cis,trans-muconate to trans,trans-muconate. Alternatively, the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate to a product selected from the group consisting of succinyl-CoA to beta-ketoacyl-CoA, beta-ketoacyl-CoA to beta-ketoacyl-CoA, beta-ketoacyl-CoA to 2-maleylacetate, 2-maleylacetate to trans-3-hydroxy-4-hexendioate, trans-3-hydroxy-4-hexendioate to trans,trans-muconate. Alternatively, the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate to a product selected from the group consisting of succinyl-CoA to beta-ketoacyl-CoA, beta-ketoacyl-CoA to beta-ketoacyl-CoA, beta-ketoacyl-CoA to 2-maleylacetate, 2-maleylacetate to trans-3-hydroxy-4-hexendioate, trans-3-hydroxy-4-hexendioate to trans,trans-muconate. Thus, the invention provides a non-naturally occurring microbial organism containing at least one exogenous nucleic acid encoding an enzyme or protein, where the enzyme or protein converts the substrates and products of a muconate pathway, such as those shown in FIG. 2.

[0062] In an additional embodiment, the invention provides a non-naturally occurring microbial organism having a muconate pathway, wherein the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate to a product selected from the group consisting of pyruvate and malonate semialdehyde to 4-hydroxy-2-oxohexanoidioate, 4-hydroxy-2-oxohexanoidioate to 4-oxocrotonate, 4-oxocrotonate to 2-hydroxy-4-hexendioate, and 2-hydroxy-4-hexendioate to muconate. Alternatively, the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate to a product selected from the group consisting of pyruvate and malonate semialdehyde to 4-hydroxy-2-oxohexanoidioate, 4-hydroxy-2-oxohexanoidioate to 4-oxocrotonate, 4-oxocrotonate to 2-hydroxy-4-hexendioate, and 2-hydroxy-4-hexendioate to muconate. Alternatively, the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding an enzyme or protein that converts a substrate to a product selected from the group consisting of pyruvate and malonate semialdehyde to 4-hydroxy-2-oxohexanoidioate, 4-hydroxy-2-oxohexanoidioate to 4-oxocrotonate, 4-oxocrotonate to 2-hydroxy-4-hexendioate, and 2-hydroxy-4-hexendioate to muconate. Thus, the invention provides a non-naturally occurring microbial organism containing at least one exogenous nucleic acid encoding an enzyme or protein, where the enzyme or protein converts the substrates and products of a muconate pathway, such as those shown in FIG. 5.

[0065] The invention is described herein with general reference to the metabolic reaction, reactant or product thereof, or with specific reference to one or more nucleic acids or
genes encoding an enzyme associated with or catalyzing, or a protein associated with, the referenced metabolic reaction, reactant or product. Unless otherwise expressly stated herein, those skilled in the art will understand that reference to a reaction also constitutes reference to the reactants and products of the reaction. Similarly, unless otherwise expressly stated herein, reference to a reactant or product also references the reaction, and reference to any of these metabolic constituents also references the gene or genes encoding the enzymes that catalyze or proteins involved in the referenced reaction, reactant or product. Likewise, given the well known fields of metabolic biochemistry, enzymology and genomics, reference herein to a gene or encoding nucleic acid also constitutes a reference to the corresponding encoded enzyme and the reaction it catalyzes or a protein associated with the reaction as well as the reactants and products of the reaction.

[0066] Muconate can be produced from succinyl-CoA via beta-ketoaipate in a minimum of five enzymatic steps, shown in FIG. 2. In the first step of all pathways, succinyl-CoA is joined to acetyl-CoA by a beta-ketothiolase to form beta-ketoaipyl-CoA (Step A). In one embodiment, the beta-keto functional group is reduced and dehydrated to form 2,3-dehydroadipyl-CoA (Steps K and L). The CoA moiety is then removed by a CoA hydrolase, transferase or ligase to form 2,3-dehydroadipate (Step M). Finally, 2,3-dehydroadipate is oxidized to form the conjugated diene muconate by an enolate oxidoreductase (Step N).

[0067] In other embodiments, beta-ketoaipyl-CoA is converted to beta-ketoaipate by a CoA hydrolase, transferase or ligase (Step B). Beta-ketoaipate is then converted to 2-maleylacetate by maleylacetate reductase (Step O). The beta-ketone of 2-maleylacetate is then reduced to form cis-3-hydroxy-4-hexenoate (Step P). This product is further dehydrated to cis-trans-muconate in Step Q. Step W provides a muconate cis-trans-isomerase to provide trans-trans-muconate.

[0068] A similar route entails the conversion of 2-maleylacetate to cis-3-amino-4-hexenoate by an aminotransferase or aminating oxidoreductase (Step R). Deamination of cis-3-amino-4-hexenoate is subsequently carried out to form cis-trans-muconate (Step S).

[0069] Alternatively, beta-ketoaipate can be converted to 2-fumarylacetate by action of a fumarylacetate reductase (Step C). Such a reductase can be engineered by directed evolution, for example, of the corresponding maleylacetate reductase. Reduction of the keto group and dehydrogenation provides trans-trans-muconate (Steps D and E). Alternatively, reductive amination, followed by deamination also affords the trans-trans-muconate product (Steps F and G).

[0070] In yet another route, beta-ketoaipate can be cyclized to an enol-lactone by beta-ketoaipyl enol-lactone hydrolase (Step H). The double bond in the lactone ring is then shifted by muconolactone isomerase (Step I). Finally, muconolactone is converted to cis,cis-muconate by muconate cyclooxygenase (Step J). Muconate cyclooxygenase may selectively form the cis,cis isomer of muconate. Further addition of a cis/trans isomerase converts the cis,cis isomer to the favored trans, trans or cis, cis configurations (Steps T and W, which can be incorporated into a single isomerization step).

[0071] The pathways detailed in FIG. 2 can achieve a maximum theoretical yield of 1.09 moles muconate per mole glucose utilized under anaerobic and aerobic conditions. With and without aeration, the maximum ATP yield is 1 mole of ATP per glucose utilized at the maximum muconate yield. The first step of this pathway, the condensation of succinyl-CoA and acetyl-CoA by beta-ketothiolase, has been demonstrated by Applicants is shown below in Example 1.

[0072] Another pathway for muconate synthesis involves the condensation of pyruvate and malonate semialdehyde, as shown in FIG. 3. Malonate semialdehyde can be formed in the cell by several different pathways. Two example pathways are: 1) decarboxylation of oxaloacetate, and 2) conversion of 2-phosphoglycerate to glyceraldehyde which can then be dehydrated to malonate semialdehyde by a diol dehydratase. In one pathway, malonate semialdehyde and pyruvate are condensed to form 4-hydroxy-2-oxoheptanoic acid (Step A). This product is dehydrated to form 4-oxoheptonate (Step B). 4-Oxohexonate is then converted to muconate by reduction and dehydration of the 2-keto group (Steps C and D).

[0073] Alternately, the 2-keto group of 4-hydroxy-2-oxoheptanoic acid is reduced by an alcohol forming oxidoreductase (Step E). The product, 2,4-dihydroxysuccinate is then dehydrated at the 2- or 4-hydroxy position to form 2-hydroxy-4-hexenol (Step G) or 3-hydroxy-4-hexenoic acid (Step F). Subsequent dehydration yields the diene, muconate (Steps D or H). This pathway is energetically favorable and is useful because it does not require carboxylation steps. Also, the pathway is driven by the stability of the muconate end product.

[0074] Several pathways for producing muconate from pyruvate and succinic semialdehyde are detailed in FIG. 4. Such pathways entail aldol condensation of pyruvate with succinic semialdehyde to 4-hydroxy-2-oxoheptanoate-1,7-dioate (HODH) by HODH aldolase (Step A). In one route, HODH is dehydrated to form 2-oxohept-4-ene-1,7-dioate (OHED) by OHED hydrolase (Step B). OHED is then dehydrogenated to form 6-oxo-2,3-dehydroheptanoate (6-OHED) (Step C). This product is subsequently oxidized to the diacid and then further oxidized to muconate (Steps F, I).

[0075] Alternately, HODH is converted to 3-hydroxyadipyl-CoA by a formate-lyase or acylating dehydrogenating dehydrogenase (Step D). The 3-hydroxy group of 3-hydroxyadipyl-CoA is then dehydrated to form the enoyl-CoA (Step G). The CoA moiety of 2,3-dehydroadipyl-CoA is removed by a CoA hydrolase, ligase or transferase (Step H). Finally, 2,3-dehydroadipate is oxidized to muconate by muconate reductase (Step I).

[0076] In yet another route, OHED is converted to 2,3-dehydroadipyl-CoA by a formate-lyase or acylating dehydrogenating dehydrogenase (Step E). 2,3-Dehydroadipyl-CoA is then transformed to muconate.

[0077] Pathways for producing muconate from lysine are detailed in FIG. 5. In one embodiment, lysine is converted to 2-aminoadipate semialdehyde by an aminotransferase or aminating oxidoreductase (Step A). 2-Aminoadipate semialdehyde is then oxidized to form 2-aminoadipate (Step B). The 2-amin group is then deaminated by a 2-aminoadipate deaminase (Step C). The product, 2,3-dehydroadipate is further oxidized to muconate by muconate reductase (Step D).

[0078] In an alternate route, the 2-aminogroup of lysine is shifted to the 3-position by lysine-2,3-aminomutase (Step E). The product, 3,6-diaminohexanoate, is converted to 3-aminoadipate semialdehyde by an aminotransferase or aminating oxidoreductase (Step F). Oxidation of the aldehyde (Step G) and deamination (Step H) yields 2,3-dehydroadipate, which is then converted to muconate (Step D).
All transformations depicted in FIGS. 2-5 fall into the general categories of transformations shown in Table 1. Below is described a number of biochemically characterized genes in each category. Specifically listed are genes that can be applied to catalyze the appropriate transformations in FIGS. 2-5 when properly cloned and expressed. Table 1 shows the enzyme types useful to convert common central metabolic intermediates into muconate. The first three digits of each label correspond to the first three Enzyme Commission number digits which denote the general type of transformation independent of substrate specificity.

**Table 1**

<table>
<thead>
<tr>
<th>Label</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.a</td>
<td>Oxidoreductase (o xo to alcohol, and reverse)</td>
</tr>
<tr>
<td>1.2.1.a</td>
<td>Oxidoreductase (acid to o xo)</td>
</tr>
<tr>
<td>1.2.1.c</td>
<td>Oxidoreductase (2-ketocid to acyl-CoA)</td>
</tr>
<tr>
<td>1.3.a</td>
<td>Oxidoreductase (alkene to alkane, and reverse)</td>
</tr>
<tr>
<td>1.4.a</td>
<td>Oxidoreductase (aminating)</td>
</tr>
<tr>
<td>2.1.b</td>
<td>Acytransferase (beta-ketothiolase)</td>
</tr>
<tr>
<td>2.3.a</td>
<td>Acytransferase (formate C-acyltransferase)</td>
</tr>
<tr>
<td>2.5.a</td>
<td>Aminotransferase</td>
</tr>
<tr>
<td>2.8.a</td>
<td>CO₂ transferase</td>
</tr>
<tr>
<td>3.1.a</td>
<td>Enol-lyase (hydrolyase)</td>
</tr>
<tr>
<td>3.1.a</td>
<td>CoA hydroxylase</td>
</tr>
<tr>
<td>4.1.a</td>
<td>Carboxyl-lyase</td>
</tr>
<tr>
<td>4.1.a</td>
<td>Aldehyde-lyase</td>
</tr>
<tr>
<td>4.2.a</td>
<td>Hydro-lyase</td>
</tr>
<tr>
<td>4.3.a</td>
<td>Ammonia-lyase</td>
</tr>
<tr>
<td>5.2.a</td>
<td>Cis/trans isomerase</td>
</tr>
<tr>
<td>5.3.a</td>
<td>Lactone isomerase</td>
</tr>
<tr>
<td>5.4.a</td>
<td>Aminomutase</td>
</tr>
<tr>
<td>5.5.a</td>
<td>Lactone cycloisomerase</td>
</tr>
<tr>
<td>6.2.a</td>
<td>CoA synthetase</td>
</tr>
</tbody>
</table>

Several transformations depicted in FIGS. 2-5 require oxidoreductases to convert a ketone functionality to a hydroxy group. The conversion of beta-ketoacyl-CoA to 3-hydroxyacyl-CoA (FIG. 2, Step K) is catalyzed by a 3-oxoacyl-CoA dehydrogenase. The reduction of 2-fumarylacetocitrolox id-4-hydroxidodehydrogenase (FIG. 2, Step D) or 2-maleylacetocitrolox id-4-hydroxidodehydrogenase (FIG. 2, Step E) is catalyzed by an oxidoreductase that converts a 3-oxoacyl-CoA to a 3-hydroxyacyl-CoA. Reduction of the ketone group of 4-oxoalocarotinate and 4-hydroxy-2-oxoaldehydated to their corresponding hydroxy group is also catalyzed by enzymes in this family (FIG. 3, Steps C and E).

Exemplary enzymes for converting beta-ketoacyl-CoA to 3-hydroxyacyl-CoA (FIG. 2, Step K) include 3-hydroxyacyl-CoA dehydrogenases. Such enzymes convert 3-oxoacyl-CoA molecules into 3-hydroxyacyl-CoA molecules and are often involved in fatty acid beta-oxidation or phenylacetate catabolism. For example, subunits of two fatty acid oxidation complexes in E. coli encoded by fadB and fadD, function as 3-hydroxyacyl-CoA dehydrogenases (Bintoch and Bintoch, Methods Enzymol. 71:403-411 (1981)). Furthermore, the gene products encoded by paaC in Sphingomonas putida U (Oliviera et al., Proc. Natl. Acad. Sci. U.S.A. 95:6419-6424 (1998)) and paaC in Sphingomonas fluorac acid ST (Di et al., Arch. Microbiol. 188:117-125 (2007)) catalyze the reverse reaction of ste B in FIG. 10, that is, the oxidation of 3-hydroxyacyl-CoA to form 3-oxoacyl-CoA, during the catabolism of phenylacetate or styrene. Note that the reactions catalyzed by such enzymes are reversible. In addition, given the proximity in E. coli or paaC to other genes in the phenylacetate degradation operon (Nogales et al., Microbiology 153:357-365 (2007)) and the fact that paaC mutants cannot grow on phenylacetate (Ismail et al., J. Biochem. 270:3047-3054 (2003)), it is expected that the E. coli paaC gene encodes a 3-hydroxyacyl-CoA dehydrogenase. Genbank information related to these genes is summarized in Table 3 below.

**Table 2**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>fadB</td>
<td>11981</td>
<td>P21177.2</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>fadD</td>
<td>334437</td>
<td>P77392.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>paaC</td>
<td>16129356</td>
<td>NP_415913.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>paaC</td>
<td>26990000</td>
<td>NP_155425.1</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>paaC</td>
<td>10663609</td>
<td>ABF82235.1</td>
<td>Pseudomonas fluorescens</td>
</tr>
</tbody>
</table>

Additional exemplary oxidoreductases capable of converting 3-oxoacyl-CoA molecules to their corresponding 3-hydroxyacyl-CoA molecules include 3-hydroxybutyryl-CoA dehydrogenases. The enzyme from Clostridium acetobutylicum, encoded by hbd, has been cloned and functionally expressed in E. coli (Youngleson et al., J. Bacteriol. 171:6800-6807 (1989)). Additional genes include hbd1 (C-terminal domain) and hbd2 (N-terminal domain) in Clostridium kluyveri (Hillmer et al., FEMS Lett. 21:351-354 (1972)) and HBSD1B10 in Bos taurus (Wakai et al., J. Biol. Chem. 207:631-638 (1954)). Yet other genes demonstrated to reduce acetoacetyl-CoA to 3-hydroxybutyryl-CoA are paaB from Zoogloea ramigera (Ploux et al., Int. J. Biochem. 174:177-182 (1988)) and phaB from Rhodobacter sphaeroides (Alber et al., Mol. Microbiol. 61:297-309 (2006)). The former gene is NADPH-dependent, its nucleotide sequence has been determined (Peoples and Simony, Mol. Microbiol. 3:349-357 (1989)) and the gene has been expressed in E. coli. Substrate specificity studies on the gene led to the conclusion that it could accept 3-oxopropionyl-CoA as an alternate substrate (Ploux et al., J. Bacteriol. 174:177-182 (1988)). Genbank information related to these genes is summarized in Table 3 below.

**Table 3**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>hbd1</td>
<td>18285689</td>
<td>P52041.2</td>
<td>Clostridium acetobutylicum</td>
</tr>
<tr>
<td>hbd2</td>
<td>146348271</td>
<td>ESIK34867.1</td>
<td>Clostridium kluyveri</td>
</tr>
<tr>
<td>hbd1</td>
<td>146348276</td>
<td>ESIK34867.1</td>
<td>Clostridium kluyveri</td>
</tr>
<tr>
<td>hbd2</td>
<td>1380324</td>
<td>O02569.3</td>
<td>B. taurus</td>
</tr>
<tr>
<td>phaB</td>
<td>158399695</td>
<td>NP_343931.4</td>
<td>Rhodobacter sphaeroides</td>
</tr>
<tr>
<td>phaB</td>
<td>20162442</td>
<td>AAM14896.1</td>
<td>Zoogloea ramigera</td>
</tr>
</tbody>
</table>

A number of similar enzymes have been found in other species of Clostridia and in Metallophaga sedula (Berg et al., Science 318:1782-1786 (2007)) as shown in Table 4.

**Table 4**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>hbd1</td>
<td>158399695</td>
<td>NP_343931.4</td>
<td>Clostridium acetobutylicum</td>
</tr>
<tr>
<td>hbd2</td>
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<td>Clostridium beijerinckii</td>
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<tr>
<td>hbd2</td>
<td>146348279</td>
<td>ESIK34867.1</td>
<td>Metallophaga sedula</td>
</tr>
</tbody>
</table>
[0085] There are various alcohol dehydrogenases for converting 2-maleylacetate to cis-3-hydroxy-4-hexenoate (FIG. 2, Step P), 2-fumarylacetate to trans-3-hydroxy-4-hexenoate (FIG. 2, Step D), 4-oxalocrotonate to 5-hydroxyhex-2-ene- dioate (FIG. 3, Step C) and 4-hydroxy-2-oxohecanedioate to 2,4-dihydroxyadipate (FIG. 3, Step E). Two enzymes capable of converting an oxoacid to a hydroxycacid are encoded by the malate dehydrogenase (mdh) and lactate dehydrogenase (ldhA) genes in E. coli. In addition, lactate dehydrogenase from *Ralstonia eutropha* has been shown to demonstrate high activities on substrates of various chain lengths such as lactate, 2-oxobutyrate, 2-oxopentanoate and 2-oxoglutarate (Steinbuchel and Schlegel, *Eur. J. Biochem.* 130:329-334 (1983)). Conversion of alpha-ketoacid into alpha-hydroxy acid can be catalyzed by 2-ketoacid reductase, an enzyme reported to be found in rat and in human placenta (Suda et al., *Arch. Biochem. Biophys.* 176:610-620 (1976); Suda et al., *Biochem. Biophys. Res. Commun.* 77:586-591 (1977)). An additional gene for these steps is the mitochondrial 3-hydroxybutyrate dehydrogenase (bdh) from the human heart which has been cloned and characterized (Marks et al., *J. Biol. Chem.* 267:15459-15463 (1992)). This enzyme is a dehydrogenase that operates on a 3-hydroxy acid. Another exemplary alcohol dehydrogenase converts acetone to isopropanol as was shown in *C. beijerincki* (Ismail et al., *Eur. J. Biochem.* 270:3047-3054 (2003)) and *T. brockii* (Umed and Zeikus, *Biochem. J.* 195:183-190 (1981); Peretz and Burstein, *Biochemistry* 28:6549-6555 (1989)). Genbank information related to these genes is summarized in Table 5 below.

![TABLE 5](image)

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
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<td>Escherichia coli</td>
</tr>
<tr>
<td>ldhA</td>
<td>16129341</td>
<td>NP_441598.1</td>
<td>Homo sapiens</td>
</tr>
<tr>
<td>bdh</td>
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<td>AAAS3832.1</td>
<td>Homo sapiens</td>
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<td>Clostridium beijerinckii</td>
</tr>
<tr>
<td>adhE</td>
<td>113443</td>
<td>P14941.1</td>
<td>Thermoanaerobacter brockii</td>
</tr>
</tbody>
</table>

[0086] Enzymes in the 1.2.1 family are NAD(P)+-dependent oxidoreductases that convert aldehydes to acids. Reactions catalyzed by enzymes in this family include the oxidation of 6-OH (FIG. 4, Step F), 2-aminoacid semialdehyde (FIG. 5, Step B) and 3-aminoacid semialdehyde (FIG. 5, Step G) to their corresponding acids. An exemplary enzyme is the NAD+-dependent aldehyde dehydrogenases (EC 1.2.1.3). Two aldehyde dehydrogenases found in human liver, ALDH-1 and ALDH-2, have broad substrate range for a variety of aliphatic, aromatic and polycyclic aldehydes (Klyosov, A. A., *Biochemistry* 35:4457-4467 (1996)). Active ALDH-2 has been efficiently expressed in *E. coli* using the GroE3 proteins as chaperonins (Lee et al., *Biochem. Biophys. Res. Commun.* 298:216-224 (2002)). The rat mitochondrial aldehyde dehydrogenase also has a broad substrate range that includes the enoyl-aldehyde crotonaldehyde (Shev et al., *Arch. Biochem. Biophys.* 176:638-649 (1976)). The *E. coli* gene adhD also encodes an NAD+-dependent aldehyde dehydrogenase active on succinic semialdehyde (Koznetsova et al., *FEMS Microbiol. Rev.* 29:263-279 (2005)). Genbank information related to these genes is summarized in Table 5 below.

[0087] Two transformations in FIG. 4 require conversion of a 2-ketoacid to an acyl-CoA (FIG. 4, Steps D and E) by an enzyme in the EC class 1.2.1. Such reactions are catalyzed by multi-enzyme complexes that catalyze a series of partial reactions which result in acylating oxidative decarboxylation of 2-keto-acids. Exemplary enzymes that can be used include 1) branched-chain 2-keto-acid dehydrogenase, 2) alpha-keto glutarate dehydrogenase, and 3) the pyruvate dehydrogenase multienzyme complex (PDHC). Each of the 2-keto-acid dehydrogenase complexes occupies positions in intermediary metabolism, and enzyme activity is typically tightly regulated (Fries et al., *Biochemistry* 42:6996-7002 (2003)). The enzymes share a complex but common structure composed of multiple copies of three catalytic components: alpha-ketoacid decarboxylase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). The E3 component is shared among all 2-keto-acid dehydrogenase complexes in an organism, while the E1 and E2 components are encoded by different genes. The enzyme components are present in numerous copies in the complex and utilize multiple cofactors to catalyze a directed sequence of reactions via substrate channeling. The overall size of these dehydrogenase complexes is very large, with molecular masses between 4 and 10 million Da (i.e., larger than a ribosome).

[0088] Activity of enzymes in the 2-keto-acid dehydrogenase family is normally low or limited under anaerobic conditions in *E. coli*. Increased production of NADH (or NADPH) could lead to a redox-imbalance, and NADH itself serves as an inhibitor to enzyme function. Engineering efforts have increased the anaerobic activity of the *E. coli* pyruvate dehydrogenase complex (Kim et al., *Appl. Environ. Microbiol.* 73:1766-1771 (2001); Kim et al., *J. Bacteriol.* 190: 3851-3858 (2008); Zhou et al., *Biotechnol. Lett.* 30:335-342 (2008)). For example, the inhibitory effect of NADH can be overcome by engineering an F22Y mutation in the E3 component (Kim et al., *J. Bacteriol.* 190:3851-3858 (2008)). Structural studies of individual components and how they work together in complex provide insight into the catalytic mechanism and architecture of enzymes in this family (Avarsson et al., *Nat. Struct. Biol.* 6:785-792 (1999); Zhou et al., *Proc. Natl. Acad. Sci. U.S.A.* 98:14802-14807 (2001)). The substrate specificity of the dehydrogenase complexes varies in different organisms, but generally branched-chain keto-acid dehydrogenases have the broadest substrate range.

and E2) and pdhD (E3, shared domain), is regulated at the transcriptional level and is dependent on the carbon source and growth phase of the organism (Resnekov et al., Mol. Gen. Genet. 234:285-296 (1992)). In yeast, the LPI1 gene encoding the E3 component is regulated at the transcriptional level by glucose (Roy and Dawes, J. Gen. Microbiol. 133:925-933 (1987)). The E1 component, encoded by KGD1, is also regulated by glucose and activated by the products of HAP2 and HAP3 (Repetto and Tzagoloff, Moll. Cell. Biol. 9:2695-2705 (1989)). The AKGD enzyme complex, inhibited by products NADH and succinyl-CoA, is known in mammalian systems, as impaired function of has been linked to several neurological diseases (Tretter and dam-Vizi, Philos. Trans. R. Soc. Lond B Biol. Sci. 360:2335-2345 (2005)). Genbank information related to these genes is summarized in Table 7 below.

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<th>Organism</th>
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<td>Escherichia coli</td>
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<td>lpd</td>
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<td>NP_414855.1</td>
<td>Escherichia coli</td>
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<tr>
<td>pdhA</td>
<td>51764265</td>
<td>P21295.2</td>
<td>Bacillus subtilis</td>
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<tr>
<td>pdhB</td>
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<td>Bacillus subtilis</td>
</tr>
<tr>
<td>pdhD</td>
<td>118972</td>
<td>P21880.1</td>
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<td>KGD1</td>
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<td>Saccharomyces cerevisiae</td>
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<td>KGD2</td>
<td>6326312</td>
<td>NP_010431.1</td>
<td>Saccharomyces cerevisiae</td>
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<tr>
<td>LPQ1</td>
<td>143188501</td>
<td>NP_116635.1</td>
<td>Saccharomyces cerevisiae</td>
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</tbody>
</table>

**[0090]** Branched-chain 2-keto-acid dehydrogenase complex (BCKAD), also known as 2-oxoisovalerate dehydrogenase, participates in branched-chain amino acid degradation pathways, converting 2-keto-acids derivatives of valine, leucine and isoleucine to their acetyl-CoA derivatives and CO₂. The complex has been studied in many organisms including Bacillus subtilis (Wang et al., Eur. J. Biochem. 213:1091-1099 (1993), Rattus norvegicus (Namby et al., J. Biol. Chem. 244:4437-4447 (1969)) and Pseudomonas putida (Sokatch et al., J. Bacteriol. 148:647-652 (1981)). In Bacillus subtilis the enzyme is encoded by genes pdhD (E3 component), bfmBB (E2 component), bfmBAA and bfmBAB (E1 component) (Wang et al., Eur. J. Biochem. 213:1091-1099 (1993)). In mammals, the complex is regulated by phosphorylation by specific phosphatases and protein kinases. The complex has been studied in rat hepatocytes (Chicco et al., J. Biol. Chem. 269:19427-19434 (1994)) and is encoded by genes Bkdha (E1 alpha), Bkdhb (E1 beta), Bdh (E2), and Ddh (E3). The E1 and E3 components of the Pseudomonas putida BCKAD complex have been crystallized (Aevansson et al., Nat. Struct. Biol. 6:785-792 (1999); Mattei et al., Science 255:1544-1550 (1992)), and the enzyme complex has been studied (Sokatch et al., J. Bacteriol. 148:647-652 (1981)). Transcription of the P. putida BCKAD genes is activated by the gene product of bkdR (Hesslinger et al., Mol. Microbiol. 27:477-492 (1998)). In some organisms including Rattus norvegicus (Paxton et al., Biochem. J. 234:295-303 (1986)) and Saccharomyces cerevisiae (Sinclair et al., Biochem. Mol. Biol. Int. 31:911-9122 (1993)), this complex has been shown to have a broad substrate range that includes linear oxo-acids such as 2-oxobutanonoate and alpha-keto-glutarate, in addition to the branched-chain amino acid precursors. The active site of the bovine BCKAD was engineered to favor alternate substrate acetyl-CoA (Meng and Chuang, Biochemistry 33:12879-12885 (1994)). Genbank information related to these genes is summarized in Table 8 below.

<table>
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<td>liep</td>
<td>16128109</td>
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<td>Escherichia coli</td>
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<td>pdhA</td>
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<td>P21295.2</td>
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<td>pdhB</td>
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<td>pdhD</td>
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<td>KGD1</td>
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<tr>
<td>KGD2</td>
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<td>NP_010431.1</td>
<td>Saccharomyces cerevisiae</td>
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<td>LPQ1</td>
<td>143188501</td>
<td>NP_116635.1</td>
<td>Saccharomyces cerevisiae</td>
</tr>
</tbody>
</table>

**[0092]** As an alternative to the large multienzyme 2-keto-acid dehydrogenase complexes described above, some anaerobic organisms utilize enzymes in the 2-ketoacid oxidoreductase family (OFOR) to catalyze acylating oxidative
decarboxylation of 2-keto-acids. Unlike the dehydrogenase complexes, these enzymes contain iron-sulfur clusters, utilize different cofactors, and use ferredoxin or flavodoxin as electron acceptors in lieu of NAD(P)H. While most enzymes in this family are specific to pyruvate as a substrate (POR) some 2-keto-acid:ferredoxin oxidoreductases have been shown to accept a broad range of 2-ketoacids as substrates including alpha-ketoglutarate and 2-oxobutanoate (Fukuda and Wakagi, Biochem. Biophys. Acta. 1597:74-80 (2002); Zhang et al., J. Biobiochem. 120:587-599 (1996)). One such enzyme is the ORF from the thermoadophilic archaeon Sulfobulbus tokodaii 7, which contains an alpha and beta subunit encoded by gene ST2300 (Fukuda and Wakagi, supra; Zhang et al., supra). A plasmid-based expression system has been developed for efficiently expressing this protein in E. coli (Fukuda et al., Eur. J. Biochem. 268:5639-5646 (2001)) and residues involved in substrate specificity were determined (Fukuda and Wakagi, supra). Two ORFs from Aeropyrum pernix strain K1 have also been recently cloned into E. coli, characterized, and found to react with a broad range of 2-oxo acids (Nishizawa et al., FEBS Lett. 579:2319-2322 (2005)). The gene sequences of these ORFs are available, although they do not have GenBank identifiers assigned to date. There is bioinformatic evidence that similar enzymes are present in all archaea, some anaerobic bacteria and amitochondrial eukarya (Fukuda and Wakagi, supra). This class of enzyme is also interesting from an energetic standpoint, as reduced ferredoxin could be used to generate NADH by ferredoxin-NAD reductase (Petitdemange et al., Biochem. Biophys. Acta 421:334-337 (1976)). Also, since most of the enzymes are designed to operate under anaerobic conditions, less enzyme engineering may be required relative to enzymes in the 2-keto-acid dehydrogenase complex family for activity in an anaerobic environment. Genbank information related to these genes is summarized in Table 10 below.

### Table 10

<table>
<thead>
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<tr>
<td>ST2300</td>
<td>15922633</td>
<td>NP_008920.1</td>
<td><em>Sulfobulbus tokodaii</em> 7</td>
</tr>
</tbody>
</table>

**[0093]** Three modifications fall into the category of oxo-oxidoreductase that reduce an alkene to an alkane (EC 1.3.1.-). The conversion of beta-ketoadipate to 2-maleylacetate (FIG. 2, Step O) is also catalyzed by the 2-enoate oxidoreductase maleylacetate reductase (MAR). A similar enzyme converts beta-ketoadipate to 2-fumarylacetate (FIG. 2, Step C). The oxidation of 2,3-dehydrodiadipate to muconate (FIG. 2, Step N) is catalyzed by a 2-enoate oxidoreductase with muconate reductase functionally.

**[0094]** 2-Enoate oxidoreductase enzymes are known to catalyze the NAD(P)+-dependent reduction and oxidation of a wide variety of 2-unsaturated carboxylic acids and aldehydes (Rohdich et al., J. Biol. Chem. 276:5779-5787 (2001)). In the recently published genome sequence of *C. kluveri*, 9 coding sequences for enoate reductases were reported, of which one has been characterized (Seedorf et al., Proc. Natl. Acad. Sci. U.S.A. 105:2128-2133 (2008)). The 14 ORFs from both *C. tyrobutyricum* and *M. thermocauticum* have been cloned and sequenced and show 50% identity to each other. The former gene is also found to have approximately 75% similarity to the characterized gene in *C. kluveri* (Giesler and Simon, Arch. Microbiol. 135:51-57 (1983)). It has been reported based on these sequence results that enr is very similar to the dienoyl CoA reductase in *E. coli* (fadH) (Rohdich et al., J. Biol. Chem. 276:5779-5787 (2001)). The *C. thermocauticum* enr gene has also been expressed in a catalytically active form in *E. coli* (Rohdich et al., supra). Genbank information related to these genes is summarized in Table 11 below.

### Table 11

<table>
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<td><em>Clostridium botulinum</em> A3 str</td>
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<td>enr</td>
<td>2765041</td>
<td>CAAT1086.1</td>
<td><em>Clostridium thermosphactum</em></td>
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<tr>
<td>enr</td>
<td>346/2834</td>
<td>CAA76983.1</td>
<td><em>Clostridium kluveri</em></td>
</tr>
<tr>
<td>enr</td>
<td>835/0886</td>
<td>YP_430895.1</td>
<td><em>Moorilla thomascactica</em></td>
</tr>
<tr>
<td>fadH</td>
<td>1673976</td>
<td>NP_417552.1</td>
<td><em>Escherichia coli</em></td>
</tr>
</tbody>
</table>

**[0095]** MAR is a 2-enoate oxidoreductase catalyzing the reversible reduction of 2-maleylacetate (cis-4-oxohex-2-ene diolactate) to 3-oxoadipate (FIG. 2, Step O). MAR enzymes naturally participate in aromatic degradation pathways (Camaro et al., J. Bacteriol. 191:4915-4917 (2003); Huang et al., Appl. Environ. Microbiol. 72:7238-7245 (2006); Kaschabek and Reineke, J. Bacteriol. 177:320-325 (1995); Kaschabek and Reineke, J. Bacteriol. 175:6075-6081 (1993)). The enzyme activity was identified and characterized in *Pseudomonas sp. strain B13* (Kaschabek and Reineke, (1995) sup;) Kaschabek and Reineke, (1993) supra), and the coding gene was cloned and sequenced (Kasberg et al., J. Bacteriol. 179:3801-3803 (1997)). Additional MAR genes include clcE gene from *Pseudomonas sp. strain B13* (Kasberg et al., supra), macA gene from *Rhodococcus opacus* (Seibert et al., J. Bacteriol. 175:6745-6754 (1993)), the macA gene from *Ralstonia eutropha* (also known as *Caprivavidus necator*) (Seibert et al., Microbiology 150:463-472 (2004)), tfdFII from *Ralstonia eutropha* (Seibert et al., (1993) supra) and Ngcl1112 in *Corynebacterium glutamicum* (Huang et al., Appl. Environ Microbiol. 72:7238-7245 (2006)). A MAR in *Pseudomonas reinekei* MT1, encoded by ccaD, was recently identified and the nucleotide sequence is available under the DDBJ/EMBL Genbank accession number EF159980 (Camara et al., J. Bacteriol. 191:4905-4915 (2009). Genbank information related to these genes is summarized in Table 12 below.

### Table 12

<table>
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<td><em>Pseudomonas sp. strain B13</em></td>
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<tr>
<td>macA</td>
<td>7387876</td>
<td>O84992.1</td>
<td><em>Rhodococcus opacus</em></td>
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<tr>
<td>macA</td>
<td>5916089</td>
<td>AAD5886</td>
<td><em>Caprivavidus necator</em></td>
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<tr>
<td>tfdFII</td>
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<td><em>Ralstonia eutropha</em> (MT1)</td>
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<td>Ngcl1112</td>
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<td>ccaD</td>
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<td><em>Pseudomonas reinekei</em> MT1</td>
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</tbody>
</table>

**[0096]** In Step R of FIG. 2, 2-maleylacetate is transaminated to form 3-amino-4-hexanoate. The conversion of 2-fumarylacetate to trans-3-amino-4-hexenoate is a similar transformation (FIG. 2, Step F). These reactions are performed by aminotransferases in the EC class 1.4.1. Enzymes in this EC class catalyze the oxidative deamination of alpha-amino acids with NAD+ or NAP+ as acetop, and the reactions are typically reversible. Exemplary enzymes include glutamate dehydrogenase (deaminating), encoded by gdhA, leucine dehydrogenase (deaminating), encoded by lldH, and aspartate dehydrogenase (deaminating), encoded by nadX. The gdhA gene product from *Escherichia coli* (Korber

<table>
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<th>Gene</th>
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(0097) The conversions of lysine to 2-aminoacidic semialdehyde (FIG. 5, Step A) and 3-diaminohexanoate to 3-aminoacidic semialdehyde (FIG. 5, Step F) are catalyzed by aminodecarboxylases that transform primary amines to their corresponding aldehydes. The lysine 6-dehydrogenase (deaminating), encoded by the ldySH genes, catalyzes the oxidative deamination of the 6-amino group of L-lysine to form 2-aminoacidic-6-semialdehyde, which can spontaneously and reversibly cyclize to form ε-aminelactam 6-carboxylate (Misono and Nagasaki, J. Bacteriol. 150:398-401 (1982)). Exemplary enzymes are found in \textit{Geobacillus stearothermophilus} (Heydari et al., Appl. Environ. Microbiol. 70:937-942 (2004)), \textit{Agrobacterium tumefaciens} (Hashimoto et al., J. Biochem. 106:76-80 (1989), Misono and Nagasaki, supra), and \textit{Achromobacter denitrificans} (Rulke and Harmston et al., JMB, Rep. 41:790-795 (2008)). Such enzymes can convert 3-diaminohexanoate to 3-aminoacidic semialdehyde giving the structural similarity between 3-diaminohexanoate and lysine. Genbank information related to these genes is summarized in Table 14 below.

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<td>\textit{Achromobacter denitrificans}</td>
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</table>

(0098) FIG. 2, step A uses a 3-oxoadipyl-CoA thiolase, or equivalently, succinyl CoA:acetyl CoA acyl transferase (β-ketothiolase). The gene products encoded by pcaF in \textit{Pseudomonas strain B13} (Kaschabek et al., J. Bacteriol. 184:207-215 (2002)), pdH in \textit{Pseudomonas putida U} (Oliveira et al., Proc. Natl. Acad. Sci. U.S.A. 95:6419-6424 (1998)), pcaF in \textit{Pseudomonas fluorescens ST} (Dí et al., Arch. Microbiol. 188:117-125 (2007)), and pcaF from \textit{E. coli} (Nogales et al., Microbiology 153:357-365 (2007)) catalyze the conversion of 3-oxoadipyl-CoA into succinyl-CoA and acetyl-CoA during the degradation of aromatic compounds such as phenylacetic or styrene. Since beta-ketothiolase enzymes catalyze reversible transformations, these enzymes can also be employed for the synthesis of 3-oxoadipyl-CoA. Several beta-ketothiolase were shown to have significant and selective activities in the oxadipyl-CoA forming direction as shown in Example 1 below including bkt from \textit{Pseudomonas putida}, pcaF and bkt from \textit{Pseudomonas aeruginosa} PAO1, bkt from \textit{Bacillus subtilis} AMMD, pcaF from \textit{E. coli}, and phaD from \textit{P. putida}. Genbank information related to these genes is summarized in Table 15 below.

<table>
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</tbody>
</table>

(0099) The acetylation of ketoacidic KDOH and OHED to their corresponding CoA derivatives (FIG. 4, Steps D and E) and concurrent release of formate, is catalyzed by formate C-acetyltransferase enzymes in the EC class 2.3.1. Enzymes in this class include pyruvate formate-lyase and ketoacid formate-lyase. Pyruvate formate-lyase (PFL, EC 2.3.1.54), encoded by pflB in \textit{E. coli}, converts pyruvate into acetyl-CoA and formate. The active site of PFL contains a catalytically essential glycid radical that is posttranslationally activated under anaerobic conditions by PFL-activating enzyme (PFLAE, EC 1.97.1.4) encoded by pflA (Knapp et al., Proc. Natl. Acad. Sci. U.S.A. 84:1332-1335 (1984); Wong et al., Biochemistry 32:14102-14110 (1993)). A pyruvate formate-lyase from \textit{Archeaebolus fulgidus} encoded by pflB has been cloned, expressed in \textit{E. coli} and characterized (Lehtio and Goldman, Protein Eng Des Sel 17:545-552 (2004)). The crystal structures of the \textit{A. fulgidus} and \textit{E. coli} enzymes have been resolved (Lehtio et al., J. Mol. Biol. 357:221-235 (2006); Leppanen et al., Structure 7:733-744 (1999)). Additional PFL and PFLAE enzymes are found in \textit{Clostridium pasteurianum} (Weidner and Sawyer, J. Bacteriol. 178:2440-2444 (1996)) and the eukaryotic algal \textit{Chlamydomonas reinhardti} (Hemmescheier et al., Eukaryot. Cell 7:518._526 (2008)). Ketoacid formate-lyase (EC 2.3.1.-), also known as 2-ketobutyrate formate-lyase (KFL) and pyruvate formate-lyase 4, is the gene product of tdeC in \textit{E. coli}. This enzyme catalyzes the conversion of 2-ketobutyrate to propionyl-CoA and formate during anaerobic threonine degradation, and can also substitute for pyruvate formate-lyase in anaerobic catabolism (Simansu et al., J. Bacteriol. 32:1195-1206 (2007)). The enzyme is oxygen-sensitive and, like PFB, requires post-translational modification by PFL-PE to activate a glycid radical in the active site (Hesslinger et al., Mol. Microbiol. 27:477-492 (1998)). Genbank information related to these genes is summarized in Table 16 below.
Several reactions in FIGS. 2 and 5 are catalyzed by aminotransferases in the EC class 2.6.1 (FIG. 2, Steps F and R and FIG. 5, Steps A and F). Such enzymes reversibly transfer amino groups from aminated donors to acceptors such as pyruvate and alpha-ketoglutarate. The conversion of lysine to 2-aminoadipate (FIG. 5, Step A) is naturally catalyzed by lysine-6-aminotransferase (EC 2.6.1.36). This enzyme function has been demonstrated in yeast and bacteria. Enzymes from Candida willis (Hammar et al. J. Basic Microbiol. 32:21-27 (1992), Flavobacterium lutescens (Fujii et al. J. Biochem. 128:391-397 (2000)) and Streptomyces clavuligenus (Romero et al. J. Ind. Microbiol. Biotechnol. 18:241-246 (1997)) have been characterized. A recombinant lysine-6-aminotransferase from S. clavuligenus was functionally expressed in E. coli (Tobin et al. J. Bacteriol. 173:6223-6229 (1991)). The F. lutescens enzyme is specific to alpha-ketoglutarate as the amino acceptor (Soda et al. Biochemistry 7:4110-4119 (1968)). Lysine-6-aminotransferase is also an enzyme that can catalyze the transamination of 3,6-diaminohexanoate (FIG. 5, Step F), as this substrate is structurally similar to lysine. Genbank information related to these genes is summarized in Table 17 below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>lat</td>
<td>10336502</td>
<td>BAA13756.1</td>
<td>Flavobacterium lutescens</td>
</tr>
<tr>
<td>lat</td>
<td>153343</td>
<td>AAA26777.1</td>
<td>Streptomyces clavuligenus</td>
</tr>
</tbody>
</table>

In Steps R and F of FIG. 2 the beta-ketones of 2-maleylacetate and 2-fumaric acid, respectively, are converted to secondary amines. Beta-alanine-alpha-ketoglutarate aminotransferase (WO08027742) reacts with beta-alanine to form malonic semialdehyde, a 3-oxoacid similar in structure to 2-maleylacetate. The gene product of SKPYD4 in Saccharomyces kluveri was shown to preferentially use beta-alanine as the amino group donor (Andersen and Hansen, Gene 124:105-109 (1993)). SkUGA1 encodes a homologue of Saccharomyces cerevisiae GABA aminotransferase, UGA1 (Ramos et al., Eur. J. Biochem. 149:401-404 (1985)), whereas SKPYD4 encodes an enzyme involved in both -alanine and GABA transamination (Andersen and Hansen, supra). 3-Amino-2-methylpropionate transaminase catalyzes the transformation from methylmalonate semialdehyde to 3-amino-2-methylpropionate. The enzyme has been characterized in Rattus norvegicus and Sus scrofa and is encoded by Abat (Kakimoto et al., Biochim. Biophys. Acta 156:374-380 (1968); Tanuki et al., Methods Enzymol. 324:376-389 (2000)). Genbank information related to these genes is summarized in Table 18 below.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
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</thead>
<tbody>
<tr>
<td>SkyPYD4</td>
<td>98626772</td>
<td>ABF58893.1</td>
<td>Lactococcus kluyveri</td>
</tr>
<tr>
<td>SKUGA1</td>
<td>98626792</td>
<td>ABF58894.1</td>
<td>Lactococcus kluyveri</td>
</tr>
<tr>
<td>UGA1</td>
<td>6321456</td>
<td>NP_011533.1</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>Abat</td>
<td>122051901</td>
<td>P50554.3</td>
<td>Rattus norvegicus</td>
</tr>
<tr>
<td>Abat</td>
<td>120968</td>
<td>P80147.2</td>
<td>Sus scrofa</td>
</tr>
</tbody>
</table>

Another enzyme that can catalyze the aminotransferase reactions in FIGS. 2 and 5 is gamma-aminobutyrate transaminase (GABA transaminase), which naturally interconverts succinic semialdehyde and glutamate to 4-aminobutyrate and alpha-ketoglutarate and is known to have a broad substrate range (Liu et al., Biochemistry 43:10896-10905 (2004); Shigeoka and Nakano, Arch. Biochem. Biophys. 288:22-28 (1991); Schulz et al., Appl. Environ. Microbiol. 56:1-6 (1990)). E. coli has two GABA transaminases, encoded by gab1 (Bartsch and Schulz, J. Bacteriol. 172:7035-7042 (1990)) and gab2 (Kuribara et al., J. Biol. Chem. 280:4602-4608 (2005)). GABA transaminases in Mus musculus, Pseudomonas fluorescens, and Sus scrofa have been shown to react with alternate substrates (Cooper, A. I., Methods Enzymol. 113:80-82 (1985); Scott and Jakoby, J. Biol. Chem. 234:932-936 (1959)). Genbank information related to these genes is summarized in Table 19 below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
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</thead>
<tbody>
<tr>
<td>gab1</td>
<td>16130576</td>
<td>NP_017148.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>gab2</td>
<td>16129623</td>
<td>P_015818.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>musc</td>
<td>36202121</td>
<td>NP_016492.1</td>
<td>Mus musculus</td>
</tr>
<tr>
<td>fluo</td>
<td>70733692</td>
<td>YP_257332.1</td>
<td>Pseudomonas fluorescens</td>
</tr>
<tr>
<td>susc</td>
<td>47523600</td>
<td>NP_099428.1</td>
<td>Sus scrofa</td>
</tr>
</tbody>
</table>

CoA transferases catalyze the reversible transfer of a CoA moiety from one molecule to another. Conversion of beta-ketoadipyl-CoA to beta-ketoadipate (FIG. 2, Step B) is accompanied by the acylation of succinate by beta-ketoadipyl-CoA transferase. The de-acylation of 2,3-dehydroadipyl-CoA (FIG. 2, Step M and FIG. 4, Step H) can also be catalyzed by an enzyme in the 2,3.3 family.

Betao-ketoadipyl-CoA transferase (EC 2.8.3.6), also known as succinyl-CoA:3-oxoacid-CoA transferase, is encoded by gca and pcaI in Pseudomonas putida (Kaschak et al., J. Bacteriol. 184:207-215 (2002)). Similar enzymes based on homology exist in Acinetobacter sp. ADP1 (Kowalchuk et al., Gene 146:23-30 (1999)). Additional exemplary succinyl-CoA:3-oxoacid-CoA transferases are present in Helicobacter pylori (Corthesy-Thuillaz et al., J. Biol. Chem. 272:25659-25667 (1997) and Bacillus subtilis (Stols et al., Protein Expr. Purif. 53:396-403 (2007)). Genbank information related to these genes is summarized in Table 20 below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>pca1</td>
<td>24985644</td>
<td>AAAN09545.1</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>pca2</td>
<td>26999057</td>
<td>NP_746082.1</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>pca3</td>
<td>50084858</td>
<td>YP_340368.1</td>
<td>Acinetobacter sp. ADP1</td>
</tr>
<tr>
<td>pca4</td>
<td>141776</td>
<td>AAC17417.1</td>
<td>Acinetobacter sp. ADP1</td>
</tr>
</tbody>
</table>
TABLE 20-continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcaA</td>
<td>21224997</td>
<td>NP_630776.1</td>
<td>Streptomyces coelicolor</td>
</tr>
<tr>
<td>pcaB</td>
<td>21224996</td>
<td>NP_630775.1</td>
<td>Streptomyces coelicolor</td>
</tr>
<tr>
<td>HPAG1-007</td>
<td>108553101</td>
<td>YP_007417</td>
<td>Helicobacter pylori</td>
</tr>
<tr>
<td>SceA</td>
<td>16080950</td>
<td>NP_391777</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>SceB</td>
<td>16080949</td>
<td>NP_391777</td>
<td>Bacillus subtilis</td>
</tr>
</tbody>
</table>

[0105] The glutaryl-CoA-transferase (EC 2.8.3.12) enzyme from anaerobic bacterium Acidaminococcus fermentans reacts with glutaryl-CoA and 3-butenyl-CoA (Mack et al., Eur. J. Biochem. 226:41-51 (1994)), substrates similar in structure to 2,3-dehydroacipryl-CoA. The genes encoding this enzyme are gtaA and gtaB. This enzyme has reduced but detectable activity with other CoA derivatives including glutaryl-CoA, 2-hydroxyglutaryl-CoA, adipyl-CoA and acetyl-CoA (Buckel et al., Eur. J. Biochem. 118:315-321 (1981)). The enzyme has been cloned and expressed in E. coli (Mack, supra). Genbank information related to these genes is summarized in Table 21 below.

TABLE 21

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>gtaA</td>
<td>559392</td>
<td>CAA57199.1</td>
<td>Acidaminococcus fermentans</td>
</tr>
<tr>
<td>gtaB</td>
<td>559393</td>
<td>CAA57200.1</td>
<td>Acidaminococcus fermentans</td>
</tr>
</tbody>
</table>

[0106] Other exemplary CoA transferases are catalyzed by the gene products of cat1, cat2, and cat3 of Clostridium kluveri which have been shown to catalyze the formation of succinyl-CoA, 4-hydroxybutyryl-CoA, and butyryl-CoA transferase activity, respectively (Seedorf et al., Proc. Natl. Acad. Sci. U.S.A. 105:2128-2133 (2008); Sohling and Gottschalk, J. Bacteriol. 178:871-880 (1996)). Similar CoA transferase activities are also present in Trichomonas vaginalis (van Grinsven et al., J. Biol. Chem. 283:1411-1416 (2008)) and Trypanosoma brucei (Riviére et al., J. Biol. Chem. 279:45337-45346 (2004)). Genbank information related to these genes is summarized in Table 22 below.

TABLE 22

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>cat1</td>
<td>720048</td>
<td>P38946.1</td>
<td>Clostridium kluveri</td>
</tr>
<tr>
<td>cat2</td>
<td>17204066</td>
<td>P38942.2</td>
<td>Clostridium kluveri</td>
</tr>
<tr>
<td>cat3</td>
<td>14934050</td>
<td>E8KZ586.1</td>
<td>Clostridium kluveri</td>
</tr>
<tr>
<td>TVAG-395550</td>
<td>123979304</td>
<td>XP_001300176</td>
<td>Trichomonas vaginalis G3</td>
</tr>
<tr>
<td>Tbl1.02.0290</td>
<td>75154875</td>
<td>XP_882352</td>
<td>Trypanosoma brucei</td>
</tr>
</tbody>
</table>

[0107] A CoA transferase that can utilize acetyl-CoA as the CoA donor is acetoacetyl-CoA transferase, encoded by the E. coli atoA (alpha subunit) and atoD (beta subunit) genes (Ko-rolev et al., Acta Crystallogr. D. Biol. Crystallogr. 58:2116-2121 (2002); Vanderwinkel et al., Biochem. Biophys. Res. Commun. 33:902-908 (1988)). This enzyme has a broad substrate range (Sramek and Ferman, Arch. Biochem. Biophys. 171:14-26 (1975) and has been shown to transfer the CoA moiety acetyl from a variety of branched and linear acyl-CoA substrates, including isobutyrate (Mathias and Schnick, Appl. Environ. Microbiol. 58:1435-1439 (1992)), valerate (Vanderwinkel et al, supra) and butanoate (Vanderwinkel et al, supra). This enzyme is induced at the transcriptional level by acetocetate, so modification of regulatory control may be necessary for engineering this enzyme into a pathway (Pauli and Overath, Eur. J. Biochem. 29:553-562 (1972)). Similar enzymes exist in Corynebacterium glutamicum ATCC 13032 (Duncan et al., Appl. Environ. Microbiol. 68:5186-5190 (2002)), Clostridium acetobutylicum (Cary et al., Appl. Environ. Microbiol. 56:1576-1583 (1990); Weisenborn et al., Appl. Environ. Microbiol. 55:323-329 (1989)), and Clostridium saccharoperbutylacetonicum (Kosaka et al., Biosci. Biotechnol. Biochem. 71:58-58 (2007)). Genbank information related to these genes is summarized in Table 23 below.

TABLE 23

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>atoA</td>
<td>2492994</td>
<td>P76459.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>atoD</td>
<td>2492990</td>
<td>P76458.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>actA</td>
<td>6291407</td>
<td>YP_22889.1</td>
<td>Corynebacterium glutamicum</td>
</tr>
<tr>
<td>catD</td>
<td>62380939</td>
<td>YP_22480.1</td>
<td>Corynebacterium glutamicum</td>
</tr>
<tr>
<td>ctaA</td>
<td>1504865</td>
<td>NP_149326.1</td>
<td>Clostridium acetibutylicum</td>
</tr>
<tr>
<td>ctaxA</td>
<td>1504867</td>
<td>NP_149327.1</td>
<td>Clostridium acetibutylicum</td>
</tr>
<tr>
<td>ctaB</td>
<td>31075384</td>
<td>AAP42564.1</td>
<td>Clostridium acetobutylicum</td>
</tr>
<tr>
<td>ctaC</td>
<td>31075385</td>
<td>AAP42565.1</td>
<td>Clostridium acetobutylicum</td>
</tr>
</tbody>
</table>

[0108] In Step H of FIG. 2, the lactonization of beta-keto-acyl-CoA to form beta-ketoacyl-eneol-lactone is catalyzed by the beta-ketoacyl-CoA enol-lactonase (EC-3.1.1.24). Beta-ketoacyl-CoA enol-lactonase also participates in the catechol branch of the beta-ketoacyl-CoA pathway to degrade aromatic compounds, in the reverse direction of that required in Step H of FIG. 2. This enzyme is encoded by the pcaD gene in Pseudomonas putida (Hughes et al., J. Gen. Microbiol. 134: 2877-2887 (1988)), Rhodococcus opacus (Eulberg et al., J. Bacteriol. 180:1072-1081 (1998)) and Raistonia eutrophina. In Acinetobacter calcoaceticus, genes encoding two beta-ketoacyl-CoA enol-lactone hydrolases were identified (Patel et al., J. Biol. Chem. 250:6567 (1975)). Genbank information related to these genes is summarized in Table 24 below.

TABLE 24

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Organism</th>
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</thead>
<tbody>
<tr>
<td>ELH</td>
<td>6015088</td>
<td>Q50903</td>
<td>Acinetobacter calcoaceticus</td>
</tr>
<tr>
<td>ELH2</td>
<td>6016146</td>
<td>P00632</td>
<td>Acinetobacter calcoaceticus</td>
</tr>
<tr>
<td>pcaA</td>
<td>24982842</td>
<td>AAN70003</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>pcaD</td>
<td>75426718</td>
<td>O67982</td>
<td>Rhodococcus opacus</td>
</tr>
<tr>
<td>pcaD</td>
<td>75411823</td>
<td>javascript:</td>
<td>Ralstonia eutrophina</td>
</tr>
</tbody>
</table>

[0109] The hydrolysis of acyl-CoA molecules to their corresponding acids is carried out by acyl-CoA hydrolase enzymes in the 3.1.2 family, also called thioesterases. Several eukaryotic acyl-CoA hydrolases (EC 3.1.2.1) have broad substrate specificity and thus represent suitable enzymes for hydrolyzing beta-ketoacyl-CoA and 2,3-dehydroacyl-CoA (FIG. 2, Steps B and M and FIG. 4, Step H). For example, the enzyme from Rattus norvegicus brain (Robinson et al., Biochem. Biophys. Res. Commun. 71:599-606 (1976)) can react with butyryl-CoA, hexanoyl-CoA and malonyl-CoA. The enzyme from the mitochondrion of the
pea leaf also has a broad substrate specificity, with demonstrated activity on acetyl-CoA, propionyl-CoA, butyryl-CoA, palmitoyl-CoA, oleoyl-CoA, succinyl-CoA, and crotonyl-CoA (Zeher and Randell, *Plant Physiol.* 94:20-27 (1990)). The acetyl-CoA hydrolase, ACH1, from *S. cerevisiae* represents another hydrolase (Bui et al., *J. Biol. Chem.* 278:17203-17209 (2003)). Genbank information related to these genes is summarized in Table 25 below.

**TABLE 25**

<table>
<thead>
<tr>
<th>Gene</th>
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</thead>
<tbody>
<tr>
<td>acet12</td>
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<td><em>Rattus norvegicus</em></td>
</tr>
<tr>
<td>ACH1</td>
<td>6319456</td>
<td>NP_000958</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
</tbody>
</table>

[0110] Another hydrolase is the human dicarboxylic acid thioesterase, acot8, which exhibits activity on glutaryl-CoA, diapryl-CoA, suberyl-CoA, sebacyl-CoA, and dodecanediyloyl-CoA (Westin et al., *J. Biol. Chem.* 280:38125-38132 (2005)) and the closest *E. coli* homolog, tesB, which can also hydrolyze a broad range of CoA thioesters (Naggett et al., *J. Biol. Chem.* 266:11044-11050 (1991)). A similar enzyme has also been characterized in the rat liver (Deana, R., *Biochem. Int.* 26:767-773 (1992)). Genbank information related to these genes is summarized in Table 26 below.

**TABLE 26**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>tesB</td>
<td>16128437</td>
<td>NP_414986</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>acot8</td>
<td>3191970</td>
<td>CAAT5502</td>
<td><em>Homo sapiens</em></td>
</tr>
<tr>
<td>acet8</td>
<td>51036669</td>
<td>NP_570112</td>
<td><em>Rattus norvegicus</em></td>
</tr>
</tbody>
</table>


**TABLE 27**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
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</thead>
<tbody>
<tr>
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<td><em>Escherichia coli</em></td>
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<td>ybgC</td>
<td>16128711</td>
<td>NP_415026</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ybaul</td>
<td>16129357</td>
<td>NP_415914</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ybgB</td>
<td>16128580</td>
<td>NP_415129</td>
<td><em>Escherichia coli</em></td>
</tr>
</tbody>
</table>

[0112] Yet another hydrolase is the glutamate CoA-transferase from *Acidaminococcus fermentans*. This enzyme was transformed by site-directed mutagenesis into an acyl-CoA hydrolase with activity on glutaryl-CoA, acetyl-CoA and 3-butenoyl-CoA (Mack and Buckel, *FEBS Lett.* 405:209-212 (1997)), compounds similar in structure to 2,3-dehydroxy-3-ketoacid-CoA transferase from *Acidaminococcus fermentans* acetyl-CoA transferase can also serve as enzymes for this reaction step but would require certain mutations to change their function. Genbank information related to these genes is summarized in Table 28 below.

**TABLE 28**

<table>
<thead>
<tr>
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<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
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</thead>
<tbody>
<tr>
<td>getA</td>
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<td>CAAT57199</td>
<td><em>Acidaminococcus fermentans</em></td>
</tr>
<tr>
<td>getB</td>
<td>559393</td>
<td>CAAT57200</td>
<td><em>Acidaminococcus fermentans</em></td>
</tr>
</tbody>
</table>

[0113] Step C of FIG. 4 is catalyzed by a 2-ketoacyl-ACP synthase that generates 6-oxo-2,3-dehydrohexanoate (6-OH) from 2-oxohept-4-en-1-7-dione (OHED). The decarboxylation of keto-acids is catalyzed by a variety of enzymes with varied substrate specificities, including pyruvate decarboxylase (EC 4.1.1.1), benzoylformate decarboxylase (EC 4.1.1.7), alpha-ketoglutarate decarboxylase and branched-chain alpha-ketoacid decarboxylase. Pyruvate decarboxylase (PDC), also termed keto-acid decarboxylase, is a key enzyme in alcoholic fermentation, catalyzing the decarboxylation of pyruvate to acetaldehyde. The enzyme from *Saccharomyces cerevisiae* has a broad substrate range for aliphatic 2-keto acids including 2-ketobutyrate, 2-ketovalerate, 3-hydroxyproprionate and 2-phenylpyruvate (22). This enzyme has been extensively studied, engineered for altered activity, and functionally expressed in *E. coli* (Killenberg-Jabs et al., *Eur. J. Biochem.* 268:1698-1704 (2001); Li and Jordan, *Biochemistry* 38:10004-10012 (1999); ter Schure et al., *Appl. Environ. Microbiol.* 64:1303-1307 (1998)). The PDC from *Zymomonas mobilis*, encoded by pdc, also has a broad substrate range and has been a subject of directed engineering studies to alter the affinity for different substrates (Siegert et al., *Protein Eng Des Sel.* 18:345-357 (2005)). The crystal structure of this enzyme is available (Killenberg-Jabs, et al., supra). Other well-characterized PDC enzymes include the enzymes from *Acetobacter pasteurianus* (Chandra et al., *Arch. Microbiol.* 176:443-451 (2001)) and *Kluyveromyces lactis* (Krieger et al., *Eur. J. Biochem.* 269:3256-3263 (2002)). Genbank information related to these genes is summarized in Table 29 below.

**TABLE 29**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>pdc</td>
<td>118391</td>
<td>P65672.1</td>
<td><em>Zymomonas mobilis</em></td>
</tr>
<tr>
<td>pdc1</td>
<td>30923172</td>
<td>P65169</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>pdc2</td>
<td>30685191</td>
<td>AM21208</td>
<td><em>Acetobacter pasteurianus</em></td>
</tr>
<tr>
<td>pdc3</td>
<td>52788279</td>
<td>Q12629</td>
<td><em>Kluyveromyces lactis</em></td>
</tr>
</tbody>
</table>

[0114] Like PDC, benzoylformate decarboxylase (EC 4.1.1.7) has a broad substrate range and has been the target of enzyme engineering studies. The enzyme from *Pseudomonas putida* has been extensively studied and crystal structures of this enzyme are available (Hasson et al., *Biochemistry* 37:9918-9930 (1998); Polonnikova et al., *Biochemistry* 42:1820-1830 (2003)). Site-directed mutagenesis of two residues in the active site of the *Pseudomonas putida* enzyme altered the affinity (K_m) of naturally and non-naturally occurring substrates (Siegert et al., *Protein Eng Des Sel.* 18:345-357 (2005)). The properties of this enzyme have been further modified by directed engineering (Lingen et al., *Protein Eng. 15:585-593* (2002); Lingen et al., *ChemBiochem. 4:721-726* (2003)). The enzyme from *Pseudomonas aeruginosa*, encoded by mldC, has also been characterized experimentally (Barrowman et al., *FEMS Microbiology Letters* 34:57-60 (1986)). Additional genes from *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and other organisms can be
inferred by sequence homology or identified using a growth selection system developed in *Pseudomonas putida* (Henning et al., *Appl. Environ. Microbiol.* 72:7510-7517 (2006)). Genbank information related to these genes is summarized in Table 30 below.

### TABLE 30

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
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<td>3915757</td>
<td>P20906.2</td>
<td><em>Pseudomonas putida</em></td>
</tr>
<tr>
<td>mdcC</td>
<td>81539678</td>
<td>Q9FUR2.1</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>dpgB</td>
<td>12602187</td>
<td>ABN80423.1</td>
<td><em>Pseudomonas stutzeri</em></td>
</tr>
<tr>
<td>ilvB</td>
<td>70730840</td>
<td>YP_260581.1</td>
<td><em>Pseudomonas fluorescens</em></td>
</tr>
</tbody>
</table>

[0115] A third enzyme capable of decarboxylating 2-oxoacids is alpha-ketoglutarate decarboxylase (KGD). The substrate range of this class of enzymes has not been studied to date. The KDC from *Mycobacterium tuberculosis* (Tian et al., *Proc. Natl. Acad. Sci. U.S.A.* 102:10670-10675 (2005)) has been cloned and functionally expressed, although it is large (~130 kD) and GC-rich. The KDC enzyme activity has been detected in several species of rhizobia including *Bradyrhizobium japonicum* and *Mesorhizobium loti* (Green et al., *J. Bacteriol.* 182:2838-2844 (2000)). Although the KDC-encoding gene(s) have not been isolated in these organisms, the genome sequences are available and several genes in each genome are annotated as putative KDCs. A KDC from *Euglena gracilis* has also been characterized but the gene associated with this activity has not been identified to date (Shigeoka and Nakano, *Arch. Biochem. Biophys.* 288:22-28 (1991)). The first twenty amino acids starting from the N-terminus were sequenced MTYKAPVKDVKFLDKYFKV (Shigeoka and Nakano, supra). The gene could be identified by testing genes containing this N-terminal sequence for KDC activity. Genbank information related to these genes is summarized in Table 31 below.

### TABLE 31

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>kgc</td>
<td>160395583</td>
<td>Q05463.4</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>kgc</td>
<td>27355563</td>
<td>NP_767092.1</td>
<td><em>Bradyrhizobium japonicum</em></td>
</tr>
<tr>
<td>kgc</td>
<td>13478636</td>
<td>NP_105204.1</td>
<td><em>Mesorhizobium loti</em></td>
</tr>
</tbody>
</table>

[0116] A fourth enzyme for catalyzing this reaction is branched chain alpha-ketoacid decarboxylase (BCKA). This class of enzyme has been shown to act on a variety of compounds varying in chain length from 3 to 6 carbons (Oku and Kaneda, *J. Bio. Chem.* 263:18386-18396 (1988); Smit et al., *Appl. Environ. Microbiol.* 71:303-311 (2005)). The enzyme in *Lactobacillus lactis* has been characterized on a variety of branched and linear substrates including 2-oxobutanoate, 2-oxoheptanoate, 2-oxopentanoate, 3-methyl-2-oxobutanoate, 4-methyl-2-oxobutanoate and isocaproate (Smit et al., supra). The enzyme has been structurally characterized (Berg et al., *Science* 318:1782-1786 (2007)). Sequence alignments between the *Lactococcus lactis* enzyme and the pyruvate decarboxylase of *Zymomonas mobilis* indicate that the catalytic and substrate recognition residues are nearly identical (Siegert et al., *Protein Eng Des Sel* 18:345-357 (2005)), so this enzyme can be subject to directed engineering. Decarboxylation of alpha-ketoglutarate by a BCKA was detected in *Bacillus subtilis*; however, this activity was low (5%) relative to activity on other branched-chain substrates (Oku and Kaneda, supra) and the gene encoding this enzyme has not been identified to date. Additional BCKA genes can be identified by homology to the *Lactococcus lactis* protein sequence. Many of the high-scoring BLASTp hits to this enzyme are annotated as indolepyruvate decarboxylases (EC 4.1.1.74). Indolepyruvate decarboxylase (IPDA) is an enzyme that catalyzes the decarboxylation of indolepyruvate to indoleacetaldelyde in plants and plant bacteria. Genbank information related to these genes is summarized in Table 32 below.

### TABLE 32

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>kdcA</td>
<td>4492167</td>
<td>AAS49165.1</td>
<td><em>Lactococcus lactis</em></td>
</tr>
</tbody>
</table>

[0117] Recombinant branched chain alpha-keto acid decarboxylase enzymes derived from the E1 subunits of the mitochondrial branched-chain alpha-keto acid dehydrogenase complex from *Homo sapiens* and *Bos taurus* have been cloned and functionally expressed in *E. coli* (Davie et al., *J. Biol. Chem.* 267:16601-16606 (1992); Wynn et al., *J. Biol. Chem.* 267:1881-1887 (1992); Wynn et al., *J. Biol. Chem.* 267:12400-12403 (1992)). It was indicated that co-expression of chaperons GroEL and GroES enhanced the specific activity of the decarboxylase by 500-fold (Wynn (1992) supra). These enzymes are composed of two alpha and two beta subunits. Genbank information related to these genes is summarized in Table 33 below.

### TABLE 33

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCKDHA</td>
<td>3410172</td>
<td>NP_898971.1</td>
<td><em>Homo sapiens</em></td>
</tr>
<tr>
<td>BCKDHA</td>
<td>11386135</td>
<td>NP_000700.1</td>
<td><em>Homo sapiens</em></td>
</tr>
<tr>
<td>BCKDHA</td>
<td>115502434</td>
<td>NP_231893</td>
<td><em>Bos taurus</em></td>
</tr>
<tr>
<td>BCKDHA</td>
<td>129030</td>
<td>P11178</td>
<td><em>Bos taurus</em></td>
</tr>
</tbody>
</table>

[0118] Aldehyde lyases in EC class 4.1.2 catalyze two key reactions in the disclosed pathways to muconate (FIG. 3, Step A and FIG. 4, Step A). HOHD aldolase, also known as HHD aldolase, catalyzes the conversion of 4-hydroxy-2-oxo-heptane-1,7-dione (HOHD) into pyruvate and succinic semialdehyde (FIG. 4, Step A). HOHD aldolase is a divalent metal ion-dependent class II aldolase, catalyzing the final step of 4-hydroxyphenylacetic acid degradation in *E. coli* C, *E. coli* W, and other organisms. In the native context, the enzyme functions in the degradative direction. The reverse (condensation) reaction is thermodynamically unfavorable; however the equilibrium can be shifted through coupling HOHD aldolase with downstream pathway enzymes that work efficiently on reaction products. Such strategies have been effective for shifting the equilibrium of other aldolases in the condensation direction (Nagata et al., *Appl. Microbiol. Biotechnol.* 44:432-438 (1995); Pollard et al., *Appl. Environ. Microbiol.* 64:4093-4094 (1998)). The *E. coli* C enzyme, encoded by hpdF1, has been extensively studied and has recently been crystalized (Ren et al., *J. Mol. Biol.* 373:866-876 (2007); Stringfellow et al., *Gene* 166:73-76 (1995)). The *E. coli* W enzyme is encoded by hpdF (Fried et al., *J. Bacteriol.* 178:111-120 (1996)). Genbank information related to these genes is summarized in Table 34 below.
TABLE 34

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>hplF</td>
<td>633197</td>
<td>CA87759.1</td>
<td>Escherichia coli C</td>
</tr>
<tr>
<td>hplW</td>
<td>38112625</td>
<td>AAR11360.1</td>
<td>Escherichia coli W</td>
</tr>
</tbody>
</table>

[0119] In Step A of FIG. 3, pyruvate and malonate semialdehyde are joined by an aldehyde lyase to form 4-hydroxy-2-oxohexanedioate. An enzyme catalyzing this exact reaction has not been characterized to date. A similar reaction is catalyzed by 2-dehydro-3-deoxycyclaric acid aldolase (DDGA, EC 4.1.2.20), a type II aldolase that participates in the catabolic pathway for D-glucaric/galactaric utilization in E. coli. Tartronate semialdehyde, the natural substrate of DDGA, is similar in size and structure to malonate semialdehyde. This enzyme has a broad substrate specificity and has been shown to reversibly condense a wide range of aldehydes with pyruvate (Fish and Blumenthal, Methods Enzymol. 9:529-534 (1966)). The crystal structure of this enzyme has been determined and a catalytic mechanism indicated (Izard and Blackwell, EMBO J. 19:3849-3856 (2000)). Other DDGA enzymes are found in Leptospira interogans (I. et al., Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun. 62:1269-1270 (2006)) and Sulfobolus solfataricus (Buchanan et al., Biochem. J. 343 Pt 3:563-570 (1999)). The S. solfataricus enzyme is highly thermostable and was cloned and expressed in E. coli (Buchanan et al., supra). Genbank information related to these genes is summarized in Table 35 below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>gard</td>
<td>LA_1624</td>
<td>1176153 P23522.2</td>
<td>Escherichia coli Leptospira interogans</td>
</tr>
<tr>
<td>Gard</td>
<td>A224174.1:1...885</td>
<td>2879782 CAA1186.1</td>
<td>Sulfobolus solfataricus</td>
</tr>
</tbody>
</table>


<table>
<thead>
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<th>Accession No.</th>
<th>Organism</th>
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</thead>
<tbody>
<tr>
<td>genC</td>
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<td>P50402.1</td>
<td>Escherichia coli K12</td>
</tr>
<tr>
<td>genC</td>
<td>39931596</td>
<td>Q8NN88.1</td>
<td>Corynebacterium glutamicum</td>
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<tr>
<td>genC</td>
<td>9789758</td>
<td>O69294.1</td>
<td>Campylobacter jejuni</td>
</tr>
<tr>
<td>genC</td>
<td>75427690</td>
<td>P84127</td>
<td>Thermus thermophilus</td>
</tr>
<tr>
<td>genC</td>
<td>126091</td>
<td>P44408.1</td>
<td>Rattus norvegicus</td>
</tr>
</tbody>
</table>

[0121] Another enzyme for catalyzing these reactions is citramalate hydratase (EC 4.2.1.34), an enzyme that naturally dehydrates 2-methylmalate to mesaconate. This enzyme has been studied in Methanosocalicoccus jannaschii in the context of the pyruvate pathway to 2-oxobutanoate, where it has been shown to have a broad substrate specificity (Dreidland et al., J. Bacteriol. 189:4391-4400 (2007)). This enzyme activity was also detected in Clostridium tetanomorphum, Morganella morgani, Citrobacter amalonaticus where it is thought to participate in glutamate degradation (Kato and Asano, Arch. Microbiol. 168:457-463 (1997)). The M. jannaschii protein sequence does not bear significant homology to genes in these organisms. Genbank information related to these genes is summarized in Table 37 below.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>leuD</td>
<td>3122345</td>
<td>Q58675.1</td>
<td>Methanosocalicoccus jannaschii</td>
</tr>
</tbody>
</table>

[0122] The enzyme OHED hydratase (FIG. 4, Step B) participates in 4-hydroxyphenylacetic acid degradation, where it converts 2-oxo-hept-4-ene-1,7-dione (OHED) to 2-oxo-4-hydroxy-hept-1,7-dione (HODU) using magnesium as a cofactor (Burks et al., J. Am. Chem. Soc. 120 (1998)). OHED hydratase enzymes have been identified and characterized in E. coli C (Izumi et al., J. Mol. Biol. 370:899-911 (2007)); Roper et al., Gene 156:47-51 (1995) and E. coli W (Prieto et al., J. Bacteriol. 178:111-120 (1996)). Sequence comparison reveals homologs in a range of bacteria, plants and animals. Enzymes with highly similar sequences are contained in Klebsiella pneumonia (91% identity, evalue<2e-138) and Salmonella enterica (91% identity, evalue<4e-138), among others. Genbank information related to these genes is summarized in Table 38 below.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
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</thead>
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<tr>
<td>hpcG</td>
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</tr>
<tr>
<td>hpl127861</td>
<td>8A88044.1</td>
<td>CAA1865.1</td>
<td>Escherichia coli W</td>
</tr>
<tr>
<td>hplH</td>
<td>150695810</td>
<td>ABR85030.1</td>
<td>Klebsiella pneumonia</td>
</tr>
<tr>
<td>Sat_01086</td>
<td>160685158</td>
<td>ABR21779.1</td>
<td>Salmonella enterica</td>
</tr>
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</table>

[0123] Dehydration of 3-hydroxyadipyl-CoA to 2,3-dehydradipyl-CoA (FIG. 2, Step I and FIG. 4, Step G) is catalyzed by an enzyme with enoyl-CoA hydratase activity. 3-Hydroxybutyryl-CoA dehydratase (EC 4.2.1.55), also called crotonase, is an enoyl-CoA hydratase that dehydrates...
3-hydroxyisobutyryl-CoA to form crotonyl-CoA (FIG. 3, step 2). Crotonase enzymes are required for n-butanol formation in some organisms, particularly *Clostridial* species, and also comprise one step of the 3-hydroxypropionate/4-hydroxybutyric acid cycle in thermoacidophilic Archaea of the genera *Sulfolobus*, *Acidianus*, and *Metallosphaera*. Exemplary genes encoding crotonase enzymes can be found in *C. acetobutylicum* (Atsumi et al., Metab. Eng. 10:305-211 (2008); Boynton et al., J. Bacteriol. 178:3015-3024 (1996)), *C. kluyveri* (Fillmer and Gottschalk, FEBS Lett. 21:351-354 (1972)), and *Metallosphaera sedula* (Berg et al., Science 318:1782-1786 (2007)) though the sequence of the latter gene is not known. Genbank information related to these genes is summarized in Table 39 below.

### Table 39

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
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<tbody>
<tr>
<td>cre</td>
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<td>Clostridium acetobutylicum</td>
</tr>
<tr>
<td>cre1</td>
<td>153953091</td>
<td>YP_001393856.1</td>
<td>Clostridium kluyveri</td>
</tr>
</tbody>
</table>


### Table 40

<table>
<thead>
<tr>
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<th>Accession No.</th>
<th>Organism</th>
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</thead>
<tbody>
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<td>ecb</td>
<td>26990073</td>
<td>NP_745498.1</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>paaA</td>
<td>26990002</td>
<td>NP_74427.1</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>paaB</td>
<td>26990001</td>
<td>NP_74426.1</td>
<td>Pseudomonas putida</td>
</tr>
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<td>phaA</td>
<td>106636093</td>
<td>A6F8223.1</td>
<td>Pseudomonas fluorescens</td>
</tr>
<tr>
<td>phaB</td>
<td>106636094</td>
<td>A6F8223.4</td>
<td>Pseudomonas fluorescens</td>
</tr>
<tr>
<td>pimA</td>
<td>39506365</td>
<td>CAE92158</td>
<td>Rhodopseudomonas palustris</td>
</tr>
<tr>
<td>maoC</td>
<td>16123948</td>
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<td>Escherichia coli</td>
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<td>NP_415912.1</td>
<td>Escherichia coli</td>
</tr>
</tbody>
</table>

**[0125]** Alternatively, the *E. coli* gene products of *faaA* and *fadb* encode a multienzyme complex involved in fatty acid oxidation that exhibits enoyl-CoA hydratase activity (Nakahigashi and Inokuchi, Nucleic Acids Res. 18:4037 (1990); Yang, S. Y. J. Bacteriol. 173:405-406 (1991); Yang et al., Biochemistry 30:6788-6795 (1991)). Knocking out a negative regulator encoded by *fadR* can be utilized to activate the *fadB* gene product (Sato et al., J. Biosci. Bioeng. 103:38-44 (2007)). The *fadA* and *fadB* genes encode similar functions and are naturally expressed under anaerobic conditions (Campbell et al., Mol. Microbiol. 47:793-805 (2003)). Genbank information related to these genes is summarized in Table 41 below.

### Table 41

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
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</thead>
<tbody>
<tr>
<td>fadA</td>
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<td>Escherichia coli</td>
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<td>fadb</td>
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<td>NP_418288.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>fadl</td>
<td>16130275</td>
<td>NP_416844.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>fadJ</td>
<td>16130274</td>
<td>NP_416843.1</td>
<td>Escherichia coli</td>
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<tr>
<td>fadR</td>
<td>16129150</td>
<td>NP_415705.1</td>
<td>Escherichia coli</td>
</tr>
</tbody>
</table>

**[0126]** An enzyme in the ammonia-lyase family is required to deaminate 3-amino-4-hexene-1-one (FIG. 2, Steps G and S), 2-amino adipate (FIG. 5, Step C) and 3-amino adipate (FIG. 5, Step II). Enzymes catalyzing this exact transformation has not been identified. However the three substrates bear structural similarity to aspartate, the native substrate of aspartase (EC 4.3.1.1.). Aspartase is a widespread enzyme in microorganisms, and has been characterized extensively (Wakil et al., J. Biol. Chem. 207:631-638 (1954)). The *E. coli* enzyme has been shown to react with a variety of alternate substrates including aspartatephenylmethyl ester, asparagine, benzyl-aspartate and malate (Ma et al., Ann N.Y. Acad. Sci. 472:60-65 (1992)). In addition, directed evolution was employed on this enzyme to alter substrate specificity (Asano et al., Biomol. Eng. 22:95-101 (2005)). The crystal structure of the *E. coli* aspartase, encoded by *aspa*, has been solved (Shi et al., Biochemistry 36:9136-9144 (1997)). Enzymes with aspartase functionality have also been characterized in *Haemophilus influenzae* (Sjostrom et al., Biochim. Biophys. Acta 1324:182-190 (1997)), *Pseudomonas fluorescens* (Takagi and Kusumi, J. Bacteriol. 161:1-6 (1985)), *Bacillus subtilis* (Sjostrom et al., supra) and *Serratia marcescens* (Takagi and Kusumi supra). Genbank information related to these genes is summarized in Table 42 below.

### Table 42

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspA</td>
<td>90111690</td>
<td>YP_418252</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>aspB</td>
<td>1168534</td>
<td>P48342.1</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>aspA</td>
<td>114273</td>
<td>P07346.1</td>
<td>Pseudomonas fluorescens</td>
</tr>
<tr>
<td>aspB</td>
<td>251757243</td>
<td>P28699.1</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>aspA</td>
<td>416661</td>
<td>P33109.1</td>
<td>Serratia marcescens</td>
</tr>
</tbody>
</table>

**[0127]** Another deaminase enzyme is 3-methylaspartase (EC 4.3.1.2). This enzyme, also known as beta-methylaspartase and 3-methylaspcape ammonia-lyase, naturally catalyzes the deamination of threo-3-methylaspartate to mesaconate. The 3-methylaspartate from *Clostridium* tetanomorphum has been cloned, functionally expressed in *E. coli*, and crystallized (Asuncion et al., Acta Crystallogr. D. Biol. Crystallogr. 57:731-733 (2001); Asuncion et al., J. Biol. Chem. 277:8306-8311 (2002); Botting et al. Biochemistry 27:2953-2955 (1988); Goda et al., Biochemistry 31:10747-10756 (1992)). In *Citrobacter* amalonaticus, this enzyme is encoded by BAA28709 (Kato and Asano, Arch. Microbiol. 168:457-463 (1997)). 3-methylaspartase has also been crystallized from *E. coli* YG1002 (Asano and Kato, FEBS Micro-
Although the protein sequence is not listed in public databases such as GenBank. Sequence homology can be used to identify additional genes, including CTC_02563 in C. tetani and ECS0761 in Escherichia coli O157:H7. Genbank information related to these genes is summarized in Table 43 below.

### Table 43

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>maxi</td>
<td>259429</td>
<td>AAB34070.1</td>
<td>Clostridium tetanomorphum</td>
</tr>
<tr>
<td>BAA28709</td>
<td>3184397</td>
<td>BAA28709.1</td>
<td>Clostridium amalonaticus</td>
</tr>
<tr>
<td>CTC_02563</td>
<td>28212411</td>
<td>NP_783085.1</td>
<td>Clostridium tetani</td>
</tr>
<tr>
<td>ECO0761</td>
<td>13306202</td>
<td>BAB34184.1</td>
<td>Escherichia coli O157:H7</td>
</tr>
</tbody>
</table>

### Table 44

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>maxiA</td>
<td>257587</td>
<td>BAA23002</td>
<td>Alcaligenes faecalis</td>
</tr>
<tr>
<td>maxiA</td>
<td>113866948</td>
<td>YP_725437</td>
<td>Raibstonia eutropha H16</td>
</tr>
<tr>
<td>maxiA</td>
<td>4760466</td>
<td>BAA72796</td>
<td>Geobacillus steatorisomorphiticus</td>
</tr>
<tr>
<td>maxiA</td>
<td>8500038</td>
<td>BAA96747.1</td>
<td>Serratia marcescens</td>
</tr>
</tbody>
</table>

Maleate cis, trans-isomerase (EC 5.2.1.1) catalyzes the conversion of maleic acid in cis formation to fumarate in trans formation (Scher and Jakoby, 1971). The Alcaligenes faecalis maxiA gene product has been cloned and characterized (Hatakeyama et al., 1997). Other maleate cis, trans-isomerase are available in Serratia marcescens (Hatakeyama et al., 1997), Raibstonia eutropha and Geobacillus steatorthermophilus. Genbank information related to these genes is summarized in Table 44 below.

### Table 45

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>maxiA</td>
<td>15597203</td>
<td>NP_250697</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>maxiA</td>
<td>15641359</td>
<td>NP_230901</td>
<td>Vibrio cholera</td>
</tr>
<tr>
<td>maxiA</td>
<td>18935547</td>
<td>EDU73766</td>
<td>Escherichia coli O157</td>
</tr>
</tbody>
</table>

The cti gene product catalyzes the conversion of cis-unsaturated fatty acids (UFA) to trans-UFAs. The enzyme has been characterized in P. putida (Junker and Ramos, 1999). Similar enzymes are found in Shewanella sp. MR-4 and Vibrio cholerae. Genbank information related to these genes is summarized in Table 46 below.

### Table 46

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>cti</td>
<td>i5257178</td>
<td>AAD41252</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>cti</td>
<td>113968844</td>
<td>YP_732637</td>
<td>Shewanella sp. MR-4</td>
</tr>
<tr>
<td>cti</td>
<td>c229506276</td>
<td>ZP_04395785</td>
<td>Vibrio cholera</td>
</tr>
</tbody>
</table>

The endocyclic migration of the double bond in the structure of β-ketoadipate-enol-lactone to form muconolactone (Fig. 2, Step 1) is catalyzed by muconolactone isomerase (EC 5.3.3.4). Muconolactone isomerase also participates in the catechol branch of the β-ketoacid pathway to degrade aromatic compounds, at the reverse direction of Step G. Muconolactone isomerase is encoded by the catC gene. The Pseudomonas putida muconolactone isomerase was purified and partial amino acid sequences of cyanogen bromide fragments were determined (Meagher, R. B., Biochim. Biophys. Acta 494:33-47 (1977)). A DNA fragment carrying the catBCDE genes from Acinetobacter calcoaceticus was isolated by complementing P. putida mutants and the complemented activities were expressed constitutively in the recombinant P. putida strains (Stanley et al., 1986). The A. calcoaceticus catBCDE genes were also expressed at high levels in Escherichia coli under the control of a lac promoter (Stanley et al., 1986). The anaerobic-oxidizing bacterium Rhodococcus sp. AN-22 CatC was purified to homogeneity and characterized as a homocatalase with a molecular mass of 100 kDa. The crystal structure of P. putida muconolactone isomerase was solved (Katie et al., 2005). Genbank information related to these genes is summarized in Table 47 below.

### Table 47

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1LH1T1</td>
<td>122612792</td>
<td>catC</td>
<td>Rhodococcus sp. AN-22</td>
</tr>
<tr>
<td>Q6G3Z52</td>
<td>5915883</td>
<td>catC</td>
<td>Actinobacter calcoaceticus</td>
</tr>
<tr>
<td>Q6EY41</td>
<td>75484174</td>
<td>catC</td>
<td>Raibstonia eutropha</td>
</tr>
<tr>
<td>P60648</td>
<td>5921190</td>
<td>catC</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>Q6Z9Y3</td>
<td>75475019</td>
<td>catC</td>
<td>Fratetia species ANA-18</td>
</tr>
</tbody>
</table>

Lysine 2,3-ammonomutase (EC 5.4.3.2) converts lysine to (3S)-3,6-diaminohexanoate (Fig. 5, Step E), shifting an amine group from the 2- to the 3-position. The enzyme is found in bacteria that ferment lysine to acetate and butyrate, including as Fusobacterium nucleatum (kamA) (Barker et al., 1982) and Clostridium subterminale (kamA) (Chirich et al., 1979). The enzyme from Clostridium subterminale has been crystallized (Lepore et al., 1980). An enzyme encoding this function is also encoded by yodO in Bacillus subtilis (Chen et al., 2000). The enzyme utilizes pyridoxal 5'-phosphate as a cofactor, requires activation by S-Adenosylmethionine, and is stereospecific, reacting with
the only with L-lysine. Genbank information related to these genes is summarized in Table 48 below.

### TABLE 48

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>yedO</td>
<td>4023469</td>
<td>O346767.1</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>kamA</td>
<td>75423266</td>
<td>Q9DBQ8.1</td>
<td>Clostridium subterminale</td>
</tr>
<tr>
<td>kamA</td>
<td>81485301</td>
<td>Q8RDX4</td>
<td>Paenibacillus tuleatum subsp. rubeum</td>
</tr>
</tbody>
</table>

[0134] In Step H of FIG. 2, the ring opening reaction of muconolactone to form muconate is catalyzed by muconate cycloisomerase (EC 5.5.1.1). Muconate cycloisomerase naturally converts cis,cis-muconate to muconolactone in the catechol branch of the β-ketoadipate pathway to degrade aromatic compounds. This enzyme has not been shown to react with the trans,trans isomer. The muconate cycloisomerase reaction is irreversible and is encoded by the catB gene. The Pseudomonas putida catB gene was cloned and sequenced (Aldrich et al., Gene 52:185-195 (1987)), the catB gene product was studied (Neidhart et al., Nature 347:692-694 (1990)) and its crystal structures were resolved (Helin et al., J. Mol. Biol. 254:918-941 (1995)). A DNA fragment carrying the catBCDE genes from Acinetobacter calcoaceticus was isolated by complementing P. putida mutants and the complemented activities were expressed constitutively in the recombinant P. putida strains (Stanley et al., J. Bacteriol. 165:557-563 (1986)). The A. calcoaceticus catBCDE genes were also expressed at high levels in Escherichia coli under the control of a lac promoter (Stanley et al., supra). The Rhodococcus sp. AN-22 CatB was purified to homogeneity and characterized as a monomer with a molecular mass of 44 kDa. The enzyme was activated by Mn²⁺, CoCl₂ and MgCl₂ (Matsumura et al., Biochem. J. 393:219-226 (2006)). Muconate cycloisomerases from other species, such as Rhodococcus rhodochrous N75, Frateuria species ANA-18, and Trichosporon cutaneum were also purified and studied (Ch and Bruce, FEBS Microbiol. Lett. 224:29-34 (2003); Mazur et al., Biochem. J. 33:1961-1970 (1994); Murakami et al., Biosci. Biotechnol. Biochem. 62:1129-1133 (1998)). Genbank information related to these genes is summarized in Table 49 below.

### TABLE 49

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>catB</td>
<td>1209310</td>
<td>115713</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>catB</td>
<td>Q83931</td>
<td>81485301</td>
<td>Acinetobacter calcoaceticus</td>
</tr>
<tr>
<td>catB</td>
<td>Q3L122</td>
<td>15261793</td>
<td>Rhodococcus sp. AN-22</td>
</tr>
<tr>
<td>catB</td>
<td>Q69281</td>
<td>57454202</td>
<td>Frateuria species ANA-18</td>
</tr>
<tr>
<td>catB</td>
<td>P46057</td>
<td>1170867</td>
<td>Trichosporon cutaneum</td>
</tr>
</tbody>
</table>

[0135] The conversion of beta-ketoacyl-CoA to beta-keto adipate (FIG. 2, Step B) and 2,3-dehydroyacyl-CoA to 2,3-dehydrodipate (FIG. 2, Step M and FIG. 4, Step H) can be catalyzed by a CoA acid-thiol ligase or CoA synthetase in the 6.2.1 family of enzymes. Enzymes catalyzing these exact transformations have not been characterized to date; however, several enzymes with broad substrate specificities have been described in the literature. AdoP-forming acyl-CoA synthetase (ACD, EC 6.2.1.13) is an enzyme that couples the conversion of acyl-CoA esters to their corresponding acids with the concomitant synthesis of ATP. ACD I from Archaegolobus fulgidus, encoded by AF1211, was shown to operate on a variety of linear and branched-chain substrates including isobutyrate, isopentanoate, and fumarate (Musfeldt and Schonheit, J. Bacteriol. 184:636-644 (2002)). A second reversible ACD in Archaeoglobus fulgidus, encoded by AF1983, was also shown to have a broad substrate range with high activity on cyclic compounds phenylacetate and indoleacetate (Musfeldt and Schonheit, supra). The enzyme from Haloarcula marismortui (annotated as a succinyl-CoA synthetase) accepts propionate, butyrate, and branched-chain acids (isovalerate and isobutyrate) as substrates, and was shown to operate in the forward and reverse directions (Brausen and Schonheit, Arch. Microbiol. 182:277-287 (2004)). The ACD encoded by PAE3250 from hyperthermophilic archaeon Pyrobaculum aerophilum showed the broadest substrate range of all characterized ACDs, reacting with acetyl-CoA, isobutyryl-CoA (preferred substrate) and phenylacetyl-CoA (Brausen and Schonheit, supra). Directed evolution or engineering can be used to modify this enzyme to operate at the physiological temperature of the host organism. The enzymes from A. fulgidus, H. marismortui, and P. aerophilum have all been cloned, functionally expressed, and characterized in E. coli (Brausen and Schonheit, supra; Musfeldt and Schonheit, supra). An additional enzyme is encoded by succCD in E. coli, which catalyzes the formation of succinyl-CoA from succinate with the concomitant consumption of one ATP, a reaction which is reversible in vivo (Buck et al., Biochemistry 24:6245-6252 (1985)). Genbank information related to these genes is summarized in Table 50 below.

### TABLE 50

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF1211</td>
<td>114998810</td>
<td>NP_070309.1</td>
<td>Archaeoglobus fulgidus DSM 4304</td>
</tr>
<tr>
<td>AF1983</td>
<td>114995955</td>
<td>NP_070807.1</td>
<td>Archaeoglobus fulgidus DSM 4304</td>
</tr>
<tr>
<td>ssc</td>
<td>59377722</td>
<td>NP_135572.1</td>
<td>Haloarcula marismortui</td>
</tr>
<tr>
<td>P0E3250</td>
<td>18313937</td>
<td>NP_500604.1</td>
<td>Pyrobaculum aerophilum str. IM2</td>
</tr>
<tr>
<td>susC</td>
<td>16128703</td>
<td>NP_415256.1</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>susD</td>
<td>1786949</td>
<td>AAT38323.1</td>
<td>Escherichia coli</td>
</tr>
</tbody>
</table>

Another enzyme for this step is 6-carboxyhexanoate-CoA ligase, also known as pimeloyl-CoA ligase (EC 6.2.1.14), which naturally activates pimelate to pimeloyl-CoA during biotin biosynthesis in gram-positive bacteria. The enzyme from Psychromonas mendocina, cloned into E. coli, was shown to accept the alternate substrates hexanadate and nonanedioate (Bianeda et al., Biochem. J. 340 Pt 3:793-801 (1999)). Other enzymes are found in Bacillus subtilis (Bower et al., J. Bacteriol. 178:4122-4130 (1996)) and Lysinibacillus sphaericus (formerly Bacillus sphaericus) (Ploux et al., Biochem. J. 287 Pt 3:685-690 (1992)). Genbank information related to these genes is summarized in Table 51 below.

### TABLE 51

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>psuA</td>
<td>15596214</td>
<td>NP_249708.1</td>
<td>Psychromonas mendocina</td>
</tr>
<tr>
<td>bioW</td>
<td>50812281</td>
<td>NP_500902.2</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>bioW</td>
<td>115012</td>
<td>P22622.1</td>
<td>Lysinibacillus sphaericus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>GI #</th>
<th>Accession No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>phiB</td>
<td>31982927</td>
<td>NP_0764172</td>
<td>Homo sapiens</td>
</tr>
<tr>
<td>AACS</td>
<td>21313520</td>
<td>NP_0844486</td>
<td>Mus musculus</td>
</tr>
<tr>
<td>psaF</td>
<td>22711873</td>
<td>AAC24333.2</td>
<td>Pseudomonas putida</td>
</tr>
<tr>
<td>phiB</td>
<td>152002983</td>
<td>ABS9624.1</td>
<td>P. chrysogenum</td>
</tr>
<tr>
<td>phiB</td>
<td>77019264</td>
<td>CAJ5517.1</td>
<td>P. chrysogenum</td>
</tr>
</tbody>
</table>

In some embodiments, the present invention provides a semi-synthetic method for synthesizing terephthalate (PTA) that includes preparing muconic acid by culturing the above-described organisms, reacting the resultant muconic acid with acetylene to form a cyclohexadiene adduct (P1, FIG. 1), and oxidizing the cyclohexadiene adduct to form PTA. Semi-synthetic methods combine the biosynthetic preparation of advanced intermediates with conventional organic chemical reactions.

While the culturing of muconic acid is discussed further below, the Diels-Alder reaction conditions are detailed here. Diels-Alder reactions are widespread in the chemical industry and are known to those skilled in the art (Carruthers, W., Some Modern Methods of Organic Synthesis, Cambridge University Press (1986); Norton, J., *Chem. Review* 51:319-523 (1942); Sauer, J., *Angewandte Chemie* 6:16-33 (1967)). This class of pericyclic reactions is well-studied for its ability to generate cyclic compounds at low energetic cost. Diels-Alder reactions are thus an attractive and low-cost way of making a variety of pharmaceuticals and natural products.

In a Diels-Alder reaction, a conjugated diene or heterodiene reacts with an alkene, alkylene, or other unsaturated functional group, known as a dienophile, to form a six-membered ring. One aspect of the Diels-Alder reaction is that the two components usually have complementary electronic character, as determined by the energies of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of the diene and dienophile (Carruthers, W., Some Modern Methods of Organic Synthesis, Cambridge University Press (1986)). In normal mode, the diene is electron-rich and the dienophile is electron-poor, although this is not always the case. The method of the present invention provides the opposite electronic configuration with an electron poor diene and a relatively electron rich dienophile, in what is termed an inverse electron demand Diels-Alder reaction. The main physical constraint for this type of reaction is that the conjugated diene must be able to adopt a cisoid conformation for the reaction to proceed. A wide variety of substituted conjugated dienes and dienophiles are able to undergo this chemistry.

In the disclosed reaction of FIG. 1, muconate is the conjugated diene, and is beneficially in the trans,trans or cis,trans isomeric configuration for the reaction to proceed. The cis, cis isomer of muconate, prevalent in biological systems as a degradation product of catechol, is unlikely to adopt the required cisoid conformation due to steric hindrance of the carboxylic acid groups. The trans,trans isomer of muconate (shown in FIG. 1) is able to react in Diels-Alder reactions with a variety of dienes (Deno, N. C., *J. Am. Chem. Soc.* 72:4057-4059 (1950); Sauer, J., *Angewandte Chemie* 6:16-33 (1967)).


Increased temperature can be used to perform the Diels-Alder reaction in FIG. 1. For example, the Diels-Alder reaction of acetylene with 1,3-butadiene to form 1,4-cyclohexadiene is performed in the range of 80-300°C (U.S. Pat. No. 3,513,209, Clement, R. A. supra).

Other reaction conditions that have been shown to enhance the rate of Diels-Alder reactions include elevated pressure, the addition of a Lewis acid, and stoichiometric excess of acetylene. Elevated pressure up to 1000 atmospheres was shown to enhance the rate of 1,4-cyclohexadiene formation from butadiene and acetylene (U.S. Pat. No. 3,513,209, Clement, R. A.). Catalytic amounts of Lewis acids can also improve reaction rate (Nicolaou et al., *Angewandte Chemie* 41:1668-1698 (2002)). Some suitable Lewis acids include magnesium halides such as magnesium chloride, magnesium bromide or magnesium iodide or zinc halides such as zinc chloride, zinc bromide or zinc iodide. Stoichiometric excess of acetylene will aid in reducing formation of homopolymerization byproducts.

Oxidation of the Diels-Alder product, cyclohexa-2,5-diene-1,4-dicarboxylate (P1), to PTA can be accomplished in the presence or absence of catalyst under mild reaction conditions. The driving force for P1 oxidation is the formation of the aromatic ring of PTA. Precedence for the conversion of P1 to PTA in the absence of catalyst is the conversion of 1,4-cyclohexadiene to benzene in air (U.S. Pat. No. 3,513,209, Clement, R. A.). 1,4-Cyclohexadiene is also converted to benzene by catalysis, for example using transition metal complexes such as bis(aryl)molybdenum(0) and bis(aryl)chromium(0) (Fochi, G., *Organometalics* 7:225-2256 (1988)) or electroactive binuclear rhodium complexes (Smith and Gray, *Catalysis Letters* 6:195-199 (1990)).

In some embodiments, the method for synthesizing PTA includes isolating muconic acid from the culture broth prior to reacting with acetylene in the Diels-Alder reaction. This is particularly helpful since the Diels-Alder reaction, is...
frequently done in the absence of a solvent, especially under thermal conditions. Isolation of muconic acid can involve various filtration and centrifugation techniques. Cells of the culture and other insoluble materials can be filtered via ultrafiltration and certain salts can be removed by nanofiltration. Because muconic acid is a diacid, standard extraction techniques can be employed that involve adjusting the pH. After removal of substantially all solids and salts, the muconic acid can be separated from water by removal of water with heating in vacuo, or by extraction at low pH. For example, following the addition of sulfuric acid or phosphoric acid to the fermentation broth in sufficient amounts (pH 3 or lower), the free carboxylate acid form of muconic acid precipitates out of solution (U.S. Pat. No. 4,608,338). In this form, muconic acid is readily separated from the aqueous solution by filtration or other conventional means.

In some embodiments, the muconic acid need not be isolated. Instead, the Diels-Alder reaction between muconic acid and acetylene can be performed in the culture broth. In such a case, the culture broth can be optionally filtered prior to adding acetylene.

The non-naturally occurring microbial organisms of the invention can be produced by introducing expressible nucleic acids encoding one or more of the enzymes or proteins participating in one or more muconate biosynthetic pathways. Depending on the host microbial organism chosen for biosynthesis, nucleic acids for some or all of a particular muconate biosynthetic pathway can be expressed. For example, if a chosen host is deficient in one or more enzymes or proteins for a desired biosynthetic pathway, then expressible nucleic acids for the deficient enzyme(s) or protein(s) are introduced into the host for subsequent exogenous expression. Alternatively, if the chosen host exhibits endogenous expression of some pathway genes, but is deficient in others, then an encoding nucleic acid is needed for the deficient enzyme(s) or protein(s) to achieve muconate biosynthesis. Thus, a non-naturally occurring microbial organism of the invention can be produced by introducing exogenous enzyme or protein activities to obtain a desired biosynthetic pathway or a desired biosynthetic pathway can be obtained by introducing one or more exogenous enzyme or protein activities that, together with one or more endogenous enzymes or proteins, produces a desired product such as muconate.

Depending on the muconate biosynthetic pathway constituents of a selected host microbial organism, the non-naturally occurring microbial organisms of the invention will include at least one exogenously expressed muconate pathway-encoding nucleic acid and up to all encoding nucleic acids for one or more muconate biosynthetic pathways. For example, muconate biosynthesis can be established in a host deficient in a pathway enzyme or protein through exogenous expression of the corresponding encoding nucleic acid. In a host deficient in all enzymes or proteins of a muconate pathway, exogenous expression of all enzyme or proteins in the pathway can be included, although it is understood that all enzymes or proteins of a pathway can be expressed even if the host contains at least one of the pathway enzymes or proteins.

For example, exogenous expression of all enzymes or proteins in a pathway for production of muconate can be included, such as those shown in FIGS. 2-5.

Given the teachings and guidance provided herein, those skilled in the art will understand that the number of encoding nucleic acids to introduce in an expressible form will, at least, parallel the muconate pathway deficiencies of the selected host microbial organism. Therefore, a non-naturally occurring microbial organism of the invention can have one, two, three, four, six, etc., up to all nucleic acids encoding the enzymes or proteins constituting a muconate biosynthetic pathway disclosed herein. In some embodiments, the non-naturally occurring microbial organisms also can include other genetic modifications that facilitate or optimize muconate biosynthesis or that confer other useful functions onto the host microbial organism. One such other functionality can include, for example, augmentation of the synthesis of one or more of the muconate pathway precursors such as succinyl-CoA.

Generally, a host microbial organism is selected such that it produces the precursor of a muconate pathway, either as a naturally produced molecule or as an engineered product that either provides de novo production of a desired precursor or increased production of a precursor naturally produced by the host microbial organism. For example, succinyl-CoA is produced naturally in a host organism such as E. coli. A host organism can be engineered to increase production of a precursor, as disclosed herein. In addition, a microbial organism that has been engineered to produce a desired precursor can be used as a host organism and further engineered to express enzymes or proteins of a muconate pathway.

In some embodiments, a non-naturally occurring microbial organism of the invention is generated from a host that contains the enzymatic capability to synthesize muconate. In this specific embodiment it can be useful to increase the synthesis or accumulation of a muconate pathway product to, for example, drive muconate pathway reactions toward muconate production. Increased synthesis or accumulation can be accomplished by, for example, overexpression of nucleic acids encoding one or more of the above-described muconate pathway enzymes or proteins. Overexpression of the enzyme or enzymes and/or protein or proteins of the muconate pathway can occur, for example, through exogenous expression of the endogenous gene or genes, or through exogenous expression of the heterologous gene or genes. Therefore, naturally occurring organisms can be readily generated to be non-naturally occurring microbial organisms of the invention, for example, producing muconate, through overexpression of one, two, three, four, five, six, that is, up to all nucleic acids encoding muconate biosynthetic pathway enzymes or proteins. In addition, a non-naturally occurring organism can be generated by mutagenesis of an endogenous gene that results in an increase in activity of an enzyme in the muconate biosynthetic pathway.

In particularly useful embodiments, exogenous expression of the encoding nucleic acids is employed. Exogenous expression confers the ability to custom tailor the expression and/or regulatory elements to the host and application to achieve a desired expression level that is controlled by the user. However, endogenous expression also can be utilized in other embodiments such as by removing a negative regulatory effector or induction of the gene's promoter when linked to an inducible promoter or other regulatory element. Thus, an endogenous gene having a naturally occurring inducible promoter can be up-regulated by providing the appropriate inducing agent, or the regulatory region of an endogenous gene can be engineered to incorporate an inducible regulatory element, thereby allowing the regulation of increased expression of an endogenous gene at a desired time. Similarly, an inducible promoter can be included as a regula-
tory element for an exogenous gene introduced into a non-naturally occurring microbial organism.

[0154] It is understood that, in methods of the invention, any of the one or more exogenous nucleic acids can be introduced into a microbial organism to produce a non-naturally occurring microbial organism of the invention. The nucleic acids can be introduced so as to confer, for example, a mucenate biosynthetic pathway onto the microbial organism. Alternatively, encoding nucleic acids can be introduced to produce an intermediate microbial organism having the biosynthetic capability to catalyze some of the required reactions to confer mucenate biosynthetic capability. For example, a non-naturally occurring microbial organism having a mucenate biosynthetic pathway can comprise at least two exogenous nucleic acids encoding desired enzymes or proteins. Thus, it is understood that any combination of two or more enzymes or proteins of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention. Similarly, it is understood that any combination of three or more enzymes or proteins of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention and so forth, as desired, so long as the combination of enzymes and/or proteins of the desired biosynthetic pathway results in production of the corresponding desired product. Similarly, any combination of four, or more enzymes or proteins of a biosynthetic pathway as disclosed herein can be included in a non-naturally occurring microbial organism of the invention, as desired, so long as the combination of enzymes and/or proteins of the desired biosynthetic pathway results in production of the corresponding desired product.

[0155] In addition to the biosynthesis of mucenate as described herein, the non-naturally occurring microbial organisms and methods of the invention also can be utilized in various combinations with each other and with other microbial organisms and methods well known in the art to achieve product biosynthesis by other routes. For example, one alternative to produce mucenate other than use of the mucenate producers is through addition of another microbial organism capable of converting a mucenate pathway intermediate to mucenate. One such procedure includes, for example, the fermentation of a microbial organism that produces a mucenate pathway intermediate. The mucenate pathway intermediate can then be used as a substrate for a second microbial organism that converts the mucenate pathway intermediate to mucenate. The mucenate pathway intermediate can be added directly to another culture of the second organism or the original culture of the mucenate pathway intermediate producers can be depleted of these microbial organisms by, for example, cell separation, and then subsequent addition of the second organism to the fermentation broth can be utilized to produce the final product without intermediate purification steps.

[0156] In other embodiments, the non-naturally occurring microbial organisms and methods of the invention can be assembled in a wide variety of subpathways to achieve biosynthesis of, for example, mucenate. In these embodiments, biosynthetic pathways for a desired product of the invention can be segregated into different microbial organisms, and the different microbial organisms can be co-cultured to produce the final product. In such a biosynthetic scheme, the product of one microbial organism is the substrate for a second microbial organism until the final product is synthesized. For example, the biosynthesis of mucenate can be accomplished by constructing a microbial organism that contains biosynthetic pathways for conversion of one pathway intermediate to another pathway intermediate or the product. Alternatively, mucenate also can be biosynthetically produced from microbial organisms through co-culture or co-fermentation using two organisms in the same vessel, where the first microbial organism produces a mucenate intermediate and the second microbial organism converts the intermediate to mucenate.

[0157] Given the teachings and guidance provided herein, those skilled in the art will understand that a wide variety of combinations and permutations exist for the non-naturally occurring microbial organisms and methods of the invention together with other microbial organisms, with the co-culture of other non-naturally occurring microbial organisms having subpathways and with combinations of other chemical and/or biochemical procedures well known in the art to produce mucenate.

[0158] Sources of encoding nucleic acids for a mucenate pathway enzyme or protein can include, for example, any species where the encoded gene product is capable of catalyzing the reference reaction. Such species include both prokaryotic and eukaryotic organisms including, but not limited to, bacteria, including archaea and eubacteria, and eukaryotes, including yeast, plant, insect, animal, and mammal, including human. Exemplary species for such sources include, for example, *Escherichia coli*, as well as other exemplary species disclosed herein or available as source organisms for corresponding genes. However, with the complete genome sequence available for now more than 550 species (with more than half of these available on public databases such as the NCBI), including 395 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of genes encoding the requisite mucenate biosynthetic activity for one or more genes in related or distant species, including for example, homologues, orthologs and nonorthologous gene displacements of known genes, and the exchange of genetic alterations between organisms is routine and well known in the art. Accordingly, the metabolic alterations enabling biosynthesis of mucenate described herein with reference to a particular organism such as *E. coli* can be readily applied to other microorganisms, including prokaryotic and eukaryotic organisms alike. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

[0159] In some instances, such as when an alternative mucenate biosynthetic pathway exists in an unrelated species, mucenate biosynthesis can be conferred onto the host species by, for example, exogenous expression of a paralog or paralogs from the unrelated species that catalyzes a similar, yet non-identical metabolic reaction to replace the referenced reaction. Because certain differences among metabolic networks exist between different organisms, those skilled in the art will understand that the actual gene usage between different organisms may differ. However, given the teachings and guidance provided herein, those skilled in the art also will understand that the teachings and methods of the invention can be applied to all microbial organisms using the cognate metabolic alterations to those exemplified herein to construct a microbial organism in a species of interest that will synthesize mucenate.

[0160] Host microbial organisms can be selected from, and the non-naturally occurring microbial organisms generated in, for example, bacteria, yeast, fungus or any of a variety of
other microorganisms applicable to fermentation processes. Exemplary bacteria include species selected from *Escherichia coli*, *Klebsiella oxytoca*, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Rhizobium etli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Glucobacter oxydans*, *Zymomonas mobilis*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptomyces coelicolor*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. Exemplary yeasts or fungi include species selected from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Aspergillus terreus*, *Aspergillus niger*, and *Pichia pastoris*. *E. coli* is a particularly useful host organism since it is a well-characterized microbial organism suitable for genetic engineering. Other particularly useful host organisms include yeast such as *Saccharomyces cerevisiae*.

[0161] Methods for constructing and testing the expression levels of a non-naturally occurring mucanoce-producing host can be performed, for example, by recombinant and detection methods well known in the art. Such methods can be found described in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Third Ed., Cold Spring Harbor Laboratory, New York (2001); and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Md. (1999).

[0162] Exogenous nucleic acid sequences involved in a pathway for production of mucanoce can be introduced stably or transiently into a host cell using techniques well known in the art including, but not limited to, conjugation, electroporation, chemical transformation, transduction, transfection, and ultrasound transformation. For exogenous expression in *E. coli* or other prokaryotic cells, some nucleic acid sequences in the genes or cDNAs of eukaryotic nucleic acids can encode targeting signals such as an N-terminal mitochondrial or other targeting signal, which can be removed before transformation into prokaryotic host cells, if desired. For example, removal of a mitochondrial leader sequence led to increased expression in *E. coli* (Hofmeister et al., *J. Biol. Chem.* 280:4329-4338 (2005)). For exogenous expression in yeast or other eukaryotic cells, genes can be expressed in the cytosol without the addition of leader sequence, or can be targeted to mitochondrion or other organelles, or targeted for secretion, by the addition of a suitable targeting sequence such as a mitochondrial targeting or secretion signal suitable for the host cells. Thus, it is understood that appropriate modifications to a nucleic acid sequence to remove or include a targeting sequence can be incorporated into an exogenous nucleic acid sequence to impart desirable properties. Furthermore, genes can be subjected to codon optimization with techniques well known in the art to achieve optimized expression of the proteins.

[0163] An expression vector or vectors can be constructed to include one or more mucanocon biosynthetic pathway encoding nucleic acids as exemplified herein operably linked to expression control sequences functional in the host organism. Expression vectors applicable for use in the microbial host organisms of the invention include, for example, plasmids, phage vectors, viral vectors, episomes and artificial chromosomes, including vectors and selection sequences or markers operable for stable integration into a host chromosome. Additionally, the expression vectors can include one or more selectable marker genes and appropriate expression control sequences. Selectable marker genes also can be included that, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like which are well known in the art. When two or more exogenous encoding nucleic acids are to be co-expressed, both nucleic acids can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter. The transformation of exogenous nucleic acid sequences involved in a metabolic or synthetic pathway can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product. It is understood that those skilled in the art that the exogenous nucleic acid is expressed in a sufficient amount to produce the desired product, and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

[0164] Directed evolution is a powerful approach that involves the introduction of mutations targeted to a specific gene in order to improve and/or alter the properties of an enzyme. Improved and/or altered enzymes can be identified through the development and implementation of sensitive high-throughput screening assays that allow the automated screening of many enzyme variants (e.g., 10^5). Iterative rounds of mutagenesis and screening typically are performed to afford an enzyme with optimized properties. Computational algorithms that can help to identify areas of the gene for mutagenesis also have been developed and can significantly reduce the number of enzyme variants that need to be generated and screened.

[0165] Numerous directed evolution technologies have been developed (for reviews, see Hibbert et al., *Biomol. Eng.* 22:11-19 (2005); Huisman and Lalonde, In *Bioconversion in the pharmaceutical and biotechnology industries* pp. 717-742 (2007); Patel (ed.), CRC Press; Often and Quax, *Biomol. Eng.* 22:1-9 (2005); and Sen et al., *Appl Biochem. Biotechnol.* 143:212-223 (2007)) to be effective at creating diverse variant libraries and these methods have been successfully applied to the improvement of a wide range of properties across many enzyme classes.

[0166] Enzyme characteristics that have been improved and/or altered by directed evolution technologies include, for example, selectivity/specificity—for conversion of non-natural substrates; temperature stability—for robust high temperature processing; pH stability—for bioprocessing under lower or higher pH conditions; substrate or product tolerance—so that high product titers can be achieved; binding (K_a)—broadens substrate binding to include non-natural substrates; inhibition (K_i)—to remove inhibition by products, substrates, or key intermediates; activity (kat)—increases enzymatic reaction rates to achieve desired flux; expression levels—increases protein yields and overall pathway flux; oxygen stability—for operation of air sensitive enzymes
under aerobic conditions; and anaerobic activity—for operation of an aerobic enzyme in the absence of oxygen. [0167] The following exemplary methods have been developed for the mutagenesis and diversification of genes to target desired properties of specific enzymes. Any of these can be used to alter/optimise activity of a deacetylase enzyme.

[0168] EpPCR (Pritchard et al., J Theor. Biol. 234:497-509 (2005)) introduces random point mutations by reducing the fidelity of DNA polymerase in PCR reactions by the addition of Mg²⁺ ions, by biasing dNTP concentrations, or by other conditional variations. The five-step cloning process to confine the mutagenesis to the target gene of interest involves: 1) error-prone PCR amplification of the gene of interest; 2) restriction enzyme digestion; 3) gel purification of the desired DNA fragment; 4) ligation into a vector; 5) transformation of the gene variants into a suitable host and screening of the library for improved performance. This method can generate multiple mutations in a single gene simultaneously, which can be useful. A high number of mutants can be generated by EpPCR, so a high-throughput screening assay or a selection method (especially using robotics) is useful to identify those with desirable characteristics.

[0169] Error-prone Rolling Circle Amplification (epRCA) (Fujii et al., Nucleic Acids Res 32:e145 (2004); and Fujii et al., Nat. Protoc. 1:2493-2497 (2006)) has many of the same elements as epPCR except a whole circular plasmid is used as the template and random 6-mers with exonuclease resistant thio-phosphate linkages on the last 2 nucleotides are used to amplify the plasmid followed by transformation into cells in which the plasmid is re-circularized at tandem repeats. Adjusting the Mg²⁺ concentration can vary the mutation rate somewhat. This technique uses a simple error-prone, single-step method to create a full copy of the plasmid with 3-4 mutations/kbp. No restriction enzyme digestion or specific primers are required. Additionally, this method is typically available as a kit.

[0170] DNA or Family Shuffling (Stemmer, Proc. Natl. Acad. Sci. U.S.A. 91:10747-10751 (1994); and Stemmer, Nature 370:389-391 (1994)) typically involves digestion of two or more variant genes with nucleases such as Dnase I or EndoV to generate a pool of random fragments that are reassembled by cycles of annealing and extension in the presence of DNA polymerase to create a library of chimeric genes. Fragments prime each other and recombination occurs when one copy primes another copy (template switch). This method can be used with >1 kbp DNA sequences. In addition to mutational recombinants created by fragment reassembly, this method introduces point mutations in the extension steps at a rate similar to error-prone PCR. The method can be used to remove deleterious, random and neutral mutations that might confer antigenicity.

[0171] Staggered Extension (STEP) (Zhao et al., Nat. Biotechnol 16:258-261 (1998)) entails template priming followed by repeated cycles of 2 step PCR with denaturation and very short duration of annealing/extension (as short as 5 sec). Growing fragments anneal to different templates and extend further, which is repeated until full-length sequences are made. Template switching means most resulting fragments have multiple parents. Combinations of low-fidelity polymerases (Taq and Mutzyme) reduce error-prone biases because of opposite mutational spectra.

[0172] In Random Priming Recombination (RPR) random sequence primers are used to generate many short DNA fragments complementary to different segments of the template. (Shao et al., Nucleic Acids Res 26:681-683 (1998)) Base misincorporation and mispriming via epPCR give point mutations. Short DNA fragments prime one another based on homology and are recombined and reassembled into full-length by repeated thermocycling. Removal of templates prior to this step assures low parental recombinants. This method, like most others, can be performed over multiple iterations to evolve distinct properties. This technology avoids sequence bias, is independent of gene length, and requires very little parent DNA for the application.

[0173] In Heteroduplex Recombination linearized plasmid DNA is used to form heteroduplexes that are repaired by mismatch repair. (Volkov et al, Nucleic Acids Res 27:e18 (1999); and Volkov et al., Methods Enzymol. 328:456-463 (2000)) The mismatch repair step is at least somewhat mutagenic. Heteroduplexes transform more efficiently than linear homoduplexes. This method is suitable for large genes and whole operons.

[0174] Random Chimeragenesis on Transient Templates (RACHITT) (Coco et al., Nat. Biotechnol 19:354-359 (2001)) employs Dnase I fragmentation and size fractionation of ssDNA. Homologous fragments are hybridized in the absence of polymerase to a complementary ssDNA scaffold. Any overlapping unhybridized fragment ends are trimmed down by an exonuclease. Gaps between fragments are filled in, and then ligated to give a pool of full-length diverse strands hybridized to the scaffold (that contains U to preclude amplification). The scaffold then is destroyed and is replaced by a new strand complementary to the diverse strand by PCR amplification. The method involves one strand (scaffold) that is from only one parent while the priming fragments derive from other genes; the parent scaffold is selected against. Thus, no reannealing with parental fragments occurs. Overlapping fragments are trimmed with an exonuclease. Otherwise, this is conceptually similar to DNA shuffling and STEP. Therefore, there should be no siblings, few inactives, and no unshuffled parentals. This technique has advantages in that few or no parental genes are created and many more crossovers can result relative to standard DNA shuffling.

[0175] Recombined Extension on Truncated templates (RETT) entails template switching of unidirectionally growing strands from primers in the presence of unidirectional ssDNA fragments used as a pool of templates. (Lee et al., J. Molec. Catalysis 26:119-129 (2003) No DNA endonucleases are used. Unidirectional ssDNA is made by DNA polymerase with random primers or serial deletions with exonuclease. Unidirectional ssDNA are only templates and not primers. Random priming and exonucleases don’t introduce sequence bias as true of enzymatic cleavage of DNA shuffling/RACHITT. RETT can be easier to optimize than STEP because it uses normal PCR conditions instead of very short extensions. Recombination occurs as a component of the PCR steps—no direct shuffling. This method can also be more random than STEP due to the absence of pauses.

[0176] In Degenerate Oligonucleotide Gene Shuffling (DOGS) degenerate primers are used to control recombination between molecules; (Bergquist and Gibbs, Methods Mol. Biol. 352:191-204 (2007); Bergquist et al., Biomol. Eng 22:63-72 (2005); Gibbs et al., Gene 271:13-20 (2001)) this can be used to control the tendency of other methods such as DNA shuffling to regenerate parental genes. This method can be combined with random mutagenesis (epPCR) of selected gene segments. This can be a good method to block the reformation of parental sequences. No endonucleases are
needed. By adjusting input concentrations of segments made, one can bias towards a desired backbone. This method allows DNA shuffling from unrelated parents without restriction enzyme digests and allows a choice of random mutagenesis methods.

[0177] Incremental Truncation for the Creation of Hybrid Enzymes (ITCHY) creates a combinatorial library with 1 base pair deletions of a gene or gene fragment of interest. (Ostermeier et al., *Proc. Natl. Acad. Sci. U.S.A.* 96:3562-3567 (1999); and Ostermeier et al., *Nat. Biotechnol.* 17:1205-1209 (1999)) Truncations are introduced in opposite direction on pieces of 2 different genes. These are ligated together and the fusions are cloned. This technique does not require homology between the 2 parental genes. When ITCHY is combined with DNA shuffling, the system is called SCRATCHY (see below). A major advantage of both is no need for homology between parental genes; for example, functional fusions between an *E. coli* and a human gene were created via ITCHY. When ITCHY libraries are made, all possible crossovers are captured.

[0178] Thioc-Incremental Truncation for the Creation of Hybrid Enzymes (THIO-ITCHY) is similar to ITCHY except that phosphorothioate dNTPs are used to generate truncations. (Lutz et al., *Nucleic Acids Res* 29:E16 (2001)) Relative to ITCHY, THIO-ITCHY can be easier to optimize, provide more reproducibility, and adjustability.

[0179] SCRATCHY combines two methods for recombinating genes, ITCHY and DNA shuffling. (Lutz et al., *Proc. Natl. Acad. Sci. U.S.A.* 98:11248-11253 (2001)) SCRATCHY combines the best features of ITCHY and DNA shuffling. First, ITCHY is used to create a comprehensive set of fusions between segments of genes in a DNA homology-independent fashion. This artificial family is then subjected to a DNA-shuffling step to augment the number of crossovers. Computational predictions can be used in optimization. SCRATCHY is more effective than DNA shuffling when sequence identity is below 80%.

[0180] In Random Drift Mutagenesis (RNDM) mutations made via epPCR followed by screening/selection for those retaining usable activity. (Bergquist et al., *Biotechnol. Engr 22:63-72 (2005)) Then, these are used in DOGS to generate recombinants with fusions between multiple active mutants or between active mutants and some other desirable parent. Designed to promote isolation of neutral mutations; its purpose is to screen for retained catalytic activity whether or not this activity is higher or lower than in the original gene. RNDM is usable in high throughput assays when screening is capable of detecting activity above background. RNDM has been used as a front end to DOGS in generating diversity. The technique imposes a requirement for activity prior to shuffling or other subsequent steps; neutral drift libraries are indicated to result in higher quicker improvements in activity from smaller libraries. Though published using epPCR, this could be applied to other large-scale mutagenesis methods.

[0181] Sequence Saturation Mutagenesis (SeSaM) is a random mutagenesis method that: 1) generates pool of random length fragments using random incorporation of a phosphorothioate nucleotide and cleavage; this pool is used as a template to 2) extend in the presence of "universal" bases such as inosine; 3) replication of a inosine-containing complement gives random base incorporation and, consequently, mutagenesis. (Wong et al., *Biotechnol. J.* 3:74-82 (2008); Wong et al., *Nucleic Acids Res* 32:e26 (2004); and Wong et al., *Anal. Biochem.* 341:187-189 (2005)) Using this technique it can be possible to generate a large library of mutants within 2-3 days using simple methods. This technique is non-directed in comparison to the mutational bias of DNA polymerases. Differences in this approach makes this technique complementary (or an alternative) to epPCR.

[0182] In Synthetic Shuffling, overlapping oligonucleotides are designed to encode "all genetic diversity in targets" and allow a very high diversity for the shuffled progeny, (Ness et al., *Nat. Biotechnol.* 20:1251-1255 (2002)) In this technique, one can design the fragments to be shuffled. This aids in increasing the resulting diversity of the progeny. One can design sequence/codon biases to make more closely related sequences recombine at rates approaching those observed with more closely related sequences. Additionally, the technique does not require physically possessing the template genes.

[0183] Nucleotide Exchange and Excision Technology NextT exploits a combination of dUTP incorporation followed by treatment with uracil DNA glycosylase and then piperidine to perform endpoint DNA fragmentation. (Muller et al., *Nucleic Acids Res* 33:e117 (2005)) The gene is reassembled using internal PCR primer extension with proofreading polymerase. The sizes for shuffling are directly controllable using varying dUTP:dTTP ratios. This is an end point reaction using simple methods for uracil incorporation and cleavage. Other nucleotide analogs, such as 8-oxo-guanine, can be used with this method. Additionally, the technique works well with very short fragments (86-bp) and has a low error rate. The chemical cleavage of DNA used in this technique results in very few unshuffled clones.

[0184] In Sequence Homology-Independent Protein Recombination (SHIPREC) a linker is used to facilitate fusion between two distantly unrelated genes. Nuclease treatment is used to generate a range of chimeras between the two genes. These fusions result in libraries of single-crossover hybrids. (Sieber et al., *Nat. Biotechnol.* 19:456-460 (2001)) This produces a limited type of shuffling and a separate process is required for mutagenesis. In addition, since no homology is needed this technique can create a library of chimeral proteins with varying fractions of each of the two unrelated parent genes. SHIPREC was tested with a hemagglutinating domain of a bacterial CP450 fused to N-terminal regions of a mammalian CP450; this produced mammalian activity in a more soluble enzyme.

[0185] In Gene Site Saturation Mutagenesis (GSSM) the starting materials are a supercoiled dsDNA plasmid containing an insert and two primers which are degenerate at the desired site of mutations. (Kretz et al., *Methods Enzymol.* 383:3-11 (2004)) Primers carrying the mutation of interest, anneal to the same sequence on opposite strands of DNA. The mutation is typically in the middle of the primer and flanked on each side by ~20 nucleotides of correct sequence. The sequence in the primer is NNN or NNK (coding) and MNN (noncoding) (N= all 4, K=G, T, M=A, C). After extension, DpnI is used to digest dam-methylated DNA to eliminate the wild-type template. This technique explores all possible amino acid substitutions at a given locus (i.e., one codon). The technique facilitates the generation of all possible replacements at a single site with no nonsense codons and results in equal to near-equal representation of most possible alleles. This technique does not require prior knowledge of the structure, mechanism, or domains of the target enzyme. If followed by shuffling or Gene Reassembly, this technology creates a diverse library of recombinants containing all possible
Combinatorial Cassette Mutagenesis (CCM) involves the use of short oligonucleotide cassettes to replace limited regions with a large number of possible amino acid sequence alterations. (Reidhaar-Olson et al. Methods Enzymol. 208:564-586 (1991); and Reidhaar-Olson et al. Science 241:53-57 (1988)) Simultaneous substitutions at two or three sites are possible using this technique. Additionally, the method tests a large multiplicity of possible sequence changes at a limited range of sites. This technique has been used to explore the information content of the lambda repressor DNA-binding domain.

Combinatorial Multiple Cassette Mutagenesis (CMCM) is essentially similar to CCM except it is employed as part of a larger program: 1) Use of epPCR at high mutation rate to 2) ID hot spots and hot regions and then 3) extension by CMCM to cover a defined region of protein sequence space. (Reetz et al., Angew. Chem. Int. Ed Engl. 40:3589-3591 (2001)) As with CCM, this method can test virtually all possible alterations over a target region. If used along with methods to create random mutations and shuffled genes, it provides an excellent means of generating diverse, shuffled proteins. This approach was successful in increasing, by 51-fold, the enaminate selectivity of an enzyme.

In the Mutator Strains technique conditional ts mutant plasmids allow increases of 20- to 4000-X in random and natural mutation frequency during selection and block accumulation of deleterious mutations when selection is not required. (Selitrennikov et al., Appl Environ Microbiol 61:3670-3679 (1995)) This technology is based on a plasmid-derived mutD5 gene, which encodes a mutant subunit of DNA polymerase III. This subunit binds to endogenous DNA polymerase III and compromises the proofreading ability of polymerase III in any strain that harbors the plasmid. A broadband of base substitutions and frameshift mutations occur. In order for effective use, the mutant plasmid should be removed once the desired phenotype is achieved; this is accomplished through a temperature sensitive origin of replication, which allows for plasmid curing at 41°C. It should be noted that mutator strains have been explored for quite some time (e.g., see Low et al., J. Mol. Biol. 260:335-3670 (1996)). In this technique very high spontaneous mutation rates are observed. The conditional property minimizes non-desired background mutations. This technology could be combined with adaptive evolution to enhance mutagenesis rates and more rapidly achieve desired phenotypes.

Look-Through Mutagenesis (LTM) is a multidimensional mutagenesis method that assesses and optimizes combinatorial mutations of selected amino acids.” (Rajpal et al., Proc Natl Acad Sci U.S.A. 102:8466-8471 (2005)) Rather than saturating each site with all possible amino acid changes, a set of nine is chosen to cover the range of amino acid R-group chemistry. Fewer changes per site allows multiple sites to be subjected to this type of mutagenesis. A >800-fold increase in binding affinity for an antibody from low nanomolar to picomolar has been achieved through this method.

This is a rational approach to minimize the number of random combinations and can increase the ability to find improved traits by greatly decreasing the numbers of clones to be screened. This has been applied to antibody engineering, specifically to increase the binding affinity and/or reduce dissociation. The technique can be combined with either screens or selections.

Gene Reassembly is a DNA shuffling method that can be applied to multiple genes at one time or to creating a large library of chimeras (multiple mutations) of a single gene. (Tunable GeneReassembly™ (TGR™) Technology supplied by Vereneum Corporation) Typically this technology is used in combination with ultra-high-throughput screening to query the represented sequence space for desired improvements. This technique allows multiple gene recombination independent of homology. The exact number and position of cross-over events can be pre-determined using fragments designed via bioinformatic analysis. This technology leads to a very high level of diversity with virtually no parental gene reformation and a low level of inactive genes. Combined with GSSSTM, a large range of mutations can be tested for improved activity. The method allows “blending” and “fine tuning” of DNA shuffling, e.g. codon usage can be optimized.

In Silico Protein Design Automation (PDA) is an optimization algorithm that anchors the structurally defined protein backbone possessing a particular fold, and searches sequence space for amino acid substitutions that can stabilize the fold and overall protein energetics. (Hayes et al., Proc Natl Acad Sci U.S.A. 99:15926-15931 (2002)) This technology uses in silico structure-based entropy predictions in order to search for structural tolerance toward protein amino acid variations. Statistical mechanics is applied to calculate coupling interactions at each position. Structural tolerance toward amino acid substitution is a measure of coupling. Ultimately, this technology is designed to yield desired modifications of protein properties while maintaining the integrity of structural characteristics. The method computationally assesses and allows filtering of a very large number of possible sequence variants (10^6). The choice of sequence variants to test is related to predictions based on the most favorable thermodynamics. Ostensibly only stability or properties that are linked to stability can be effectively addressed with this technology. The method has been successfully used in some therapeutic proteins, especially in engineering immunoglobulins. In silico predictions avoid testing extraordinarily large numbers of potential variants. Predictions based on existing three-dimensional structures are more likely to succeed than predictions based on hypothetical structures. This technology can readily predict and allow targeted screening of multiple simultaneous mutations, something not possible with purely experimental technologies due to exponential increases in numbers.

Iterative Saturation Mutagenesis (ISM) involves: 1) use knowledge of structure/function to choose a likely site for enzyme improvement; 2) saturation mutagenesis at chosen site using Stratagene QuickChange (or other suitable means); 3) screen/select for desired properties; and 4) with improved clone(s), start over at another site and continue repeating. (Reetz et al., Nat. Protoc. 2:991-903 (2007); and Reetz et al., Angew. Chem. Int. Ed Engl. 45:7745-7751 (2006)) This is a proven methodology, which assures all possible replacements at a given position are made for screening/selection.

Any of the aforementioned methods for mutagenesis can be used alone or in any combination. Additionally, any one or combination of the directed evolution methods can be used in conjunction with adaptive evolution techniques.

The present invention provides a method for producing mucorates that includes culturing a non-naturally
occurring microbial organism having a muconate pathway. The pathway includes at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate, under conditions and for a sufficient period of time to produce muconate. The muconate pathway includes an enzyme selected from the group consisting of a beta-ketothiolase, a beta-ketoacyl-CoA hydrolase, a beta-ketoacyl-CoA transerase, a beta-ketoacyl-CoA ligase, a 2-fumarylacetate reductase, a 2-fumarylacetate dehydrogenase, a trans-3-hydroxy-4-hexenooate dehydratase, a 2-fumarylacetate aminotransferase, a 2-fumarylacetate aminating oxidoreductase, a trans-3-amino-4-hexenoate deaminase, a beta-ketoacylate enol-lactone hydrolase, a muconolactone isomerase, a muconate cycloisomerase, a beta-ketoacyl-CoA dehydrogenase, a 3-hydroxyacyl-CoA hydrolase, a 2,3-dehydroacyl-CoA transferase, and a beta-ketoacyl-CoA ligase, (3) beta-ketoacyl-CoA transferase, and beta-ketoacyl-CoA ligase, (3) beta-ketoacyl-CoA dehydrogenase, (4) muconolactone isomerase, (5) muconate cycloisomerase, and (6) muconate cis/trans isomerase.

[0195] In some embodiments, the muconate pathway includes: A) at least one muconate pathway enzymes as those exemplified in FIG. 2; the set of muconate pathway enzymes are selected from the group consisting of:

[0196] A) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase, and beta-ketoacyl-CoA ligase, (3) beta-ketoacyl-CoA dehydrogenase, (4) muconolactone isomerase, (5) muconate cycloisomerase, and (6) muconate cis/trans isomerase;

[0197] B) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase and beta-ketoacyl-CoA ligase, (3) 2-maleylacetate reductase, (4) 2-maleylacetate dehydrogenase, (5) cis-3-hydroxy-4-hexenoate dehydratase, and (6) muconate cis/trans isomerase;

[0198] C) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase and beta-ketoacyl-CoA ligase, (3) 2-maleylacetate reductase, (4) 2-maleylacetate dehydrogenase, (5) cis-3-hydroxy-4-hexenoate dehydratase, and (6) muconate cis/trans isomerase; and

[0199] D) (1) beta-ketothiolase, (2) beta-ketoacyl-CoA dehydrogenase, (3) 3-hydroxyacyl-CoA hydrolase, (4) an enzyme selected from 2,3-dehydroacyl-CoA transferase, 2,3-dehydroacyl-CoA hydrolase and 2,3-dehydroacetyl-CoA ligase, and (5) muconate reductase;

[0200] E) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase and beta-ketoacyl-CoA ligase, (3) 2-fumarylacetate reductase, (4) 2-fumarylacetate dehydrogenase, and (5) trans-3-hydroxy-4-hexenoate dehydratase;

[0201] F) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase and beta-ketoacyl-CoA ligase, (3) 2-fumarylacetate reductase, (4) an enzyme selected from 2-fumarylacetate aminotransferase and 2-fumarylacetate aminating oxidoreductase, and (5) trans-3-amino-4-hexenoate deaminase.

[0202] In some embodiments, the present invention provides a method for producing muconate that includes culturing a non-naturally occurring microbial organism having a muconate pathway. The pathway comprising at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate, under conditions and for a sufficient period of time to produce muconate. The muconate pathway includes a 4-hydroxy-2-ketovalerate aldolase, a 2-oxopentenoate hydratase, a 4-oxalocrotonate dehydrogenase, a 2-hydroxy-4-hexenooate dehydratase, a 4-hydroxy-2-oxohexanooate oxidoreductase, a 2,4-dihydroxydepradipe dehydratase (acting on 2-hydroxy), a 2,4-dihydroxydepradipe dehydratase (acting on 4-hydroxy group) and a 3-hydroxy-4-hexenooate dehydratase.

[0203] In some embodiments, the muconate pathway includes, a set of muconate pathway enzymes such as those exemplified in FIG. 3; the set of muconate pathway enzymes are selected from the group consisting of:

[0204] A) (1) 4-hydroxy-2-ketovalerate aldolase, (2) 2-oxopentenoate hydratase, (3) 4-oxalocrotonate dehydrogenase, (4) 2-hydroxy-4-hexenooate dehydratase;

[0205] B) (1) 4-hydroxy-2-ketovalerate aldolase, (2) 4-hydroxy-2-oxohexanooate oxidoreductase, (3) 2,4-dihydroxydepradipe dehydratase (acting on 2-hydroxy), (4) 3-hydroxy-4-hexenooate dehydratase; and

[0206] C) (1) 4-hydroxy-2-ketovalerate aldolase, (2) 4-hydroxy-2-oxohexanooate oxidoreductase, (3) 2,4-dihydroxydepradipe dehydratase (acting on 4-hydroxy group), (4) 2-hydroxy-4-hexenooate dehydratase.

[0207] In some embodiments, the present invention provides a method for producing muconate that includes culturing a non-naturally occurring microbial organism having a muconate pathway. The pathway includes at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate, under conditions and for a sufficient period of time to produce muconate. The muconate pathway includes an enzyme selected from the group consisting of an HODH aldolase, an OHED hydratase, an OHED decarboxylase, an HODH formate-lyase, an HODH dehydratase, an OHED dehydratase, 6-OHE dehydratase, a 3-hydroxyacyl-CoA dehydratase, a 2,3-dehydroacyl-CoA hydrolase, a 2,3-dehydroacyl-CoA transferase, a 2,3-dehydroacyl-CoA ligase, and a muconate reductase.

[0208] In some embodiments, the muconate pathway includes: the set of muconate pathway enzymes such as those exemplified in FIG. 4; the set of muconate pathway enzymes are selected from the group consisting of:

[0209] A) (1) HODH aldolase, (2) OHED hydratase, (3) OHED dehydratase, (4) 6-OHE hydratase, and (5) muconate reductase;

[0210] B) (1) HODH aldolase, (2) OHED hydratase, (3) an enzyme selected from OHED formate-lyase and OHED dehydratase, (4) an enzyme selected from 2,3-dehydroacyl-CoA hydrolase, 2,3-dehydroacyl-CoA transferase and 2,3-dehydroacyl-CoA ligase, and (5) muconate reductase; and

[0211] C) (1) HODH aldolase, (2) an enzyme selected from HODH formate-lyase and HODH dehydratase, (3) 3-hydroxyacyl-CoA dehydratase, (4) an enzyme selected from 2,3-dehydroacyl-CoA hydrolase, 2,3-dehydroacyl-CoA transferase and 2,3-dehydroacyl-CoA ligase, and (5) muconate reductase.

[0212] In some embodiments, the present invention provides a method for producing muconate that includes cultur-
ing a non-naturally occurring microbial organism having a muconate pathway. The pathway includes at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate, under conditions and for a sufficient period of time to produce muconate. The muconate pathway includes an enzyme selected from the group consisting of a lysine aminotransferase, a lysine aminating oxidoreductase, a 2-amino adipate semi-aldehyde dehydrogenase, a 2-amino adipate deaminase, a muconate reductase, a lysine-2,3-aminomutase, a 3,6-diaminohexanoate aminotransferase, a 3,6-diaminohexanoate aminating oxidoreductase, a 3-amino adipate semi-aldehyde dehydrogenase, and a 3-amino adipate deaminase.

In some embodiments, the muconate pathway includes, a set of muconate pathway enzymes such as those exemplified in FIG. 5; the set of muconate pathway enzymes are selected from the group consisting of:

- (1) lysine aminotransferase, (2) lysine aminating oxidoreductase, (3) 2-amino adipate semi-aldehyde dehydrogenase, (4) 2-amino adipate deaminase, and (5) muconate reductase
- (1) lysine-2,3-aminomutase, (2) 3,6-diaminohexanoate aminotransferase, (3) 3,6-diaminohexanoate aminating oxidoreductase, (4) 3-amino adipate semi-aldehyde dehydrogenase, (5) 3-amino adipate deaminase, and (6) muconate reductase.

In some embodiments, the foregoing non-naturally occurring microbial organism can be cultured in a substantially anaerobic culture medium.

Suitable purification and/or assays to test for the production of muconate can be performed using well known methods. Suitable replicates such as triplicate cultures can be grown for each engineered strain to be tested. For example, product and byproduct formation in the engineered production host can be monitored. The final product and intermediates, and other organic compounds, can be analyzed by methods such as HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography-Mass Spectroscopy) and LC-MS (Liquid Chromatography-Mass Spectroscopy) or other suitable analytical methods using routine procedures well known in the art. The release of product in the fermentation broth can also be tested with the culture supernatant. Byproducts and residual glucose can be quantified by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Li et al., Biotechnol. Bioeng. 90:775-779 (2005)), or other suitable assay and detection methods well known in the art. The individual enzyme or protein activities from the exogenous DNA sequences can also be assayed using methods well known in the art. For example, a spectrophotometric assay for succinyl-CoA:3-ketoacid-CoA transferase (FIG. 2, Step B) entails measuring the change in absorbance corresponding to the product CoA molecule (i.e., succinyl-CoA) in the presence of the enzyme extract when supplied with succinate and β-ketoacyl-CoA (Corbesey-Theulaz et al., J Biol. Chem., 272(41) (1997)). Succinyl-CoA can also be assayed in the presence of excess hydroxylamine by complexing the succinohydroxamic acid formed to ferric salts as referred to in (Corbesey-Theulaz et al., J. Biol. Chem. 272(41) (1997)). The specific activity of muconate reductase can be assayed in the reductive direction using a colorimetric assay adapted from the literature (Durrie et al., FEMS Microbiol. Rev. 17:251-262 (1995); Palosaari et al., J. Bacteriol. 170:2971-2976 (1988); Welch et al., Arch. Biochem. Biophys. 273:309-318 (1989)). In this assay, the substrates muconate and NADH are added to cell extracts in a buffered solution, and the oxidation of NADH is followed by reading absorbance at 340 nM at regular intervals. The resulting slope of the reduction in absorbance at 340 nM per minute, along with the molar extinction coefficient of NADH at 340 nM (6000) and the protein concentration of the extract, can be used to determine the specific activity of muconate reductase.

The muconate can be separated from other components in the culture using a variety of methods well known in the art, as briefly described above. Such separation methods include, for example, extraction procedures as well as methods that include continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration. All of the above methods are well known in the art.

Any of the non-naturally occurring microbial organisms described herein can be cultured to produce and/or secrete the biosynthetic products of the invention. For example, the muconate producers can be cultured for the biosynthetic production of muconate.

For the production of muconate, the recombinant strains are cultured in a medium with carbon source and other essential nutrients. It is highly desirable to maintain anaerobic conditions in the fermenter to reduce the cost of the overall process. Such conditions can be obtained, for example, by first sparging the medium with nitrogen and then sealing the flask with a septum and crimp-cap. For strains where growth is not observed anaerobically, microaerobic conditions can be applied by perforating the septum with a small hole for limited aeration. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary aerobic and anaerobic conditions are described, for example, in U.S. patent application Ser. No. 11/891,602, filed Aug. 10, 2007. Fermentations can be performed in a batch, fed-batch or continuous manner, as disclosed herein.

If desired, the pH of the medium can be maintained at a desired pH, in particular neutral pH, such as a pH of around 7 by addition of a base, such as NaOH or other bases, or acid, as needed to maintain the culture medium at a desirable pH. The growth rate can be determined by measuring optical density using a spectrophotometer (600 nm), and the glucose uptake rate by monitoring carbon source depletion over time. NOTE—Ideally this process would operate at low pH using an organism that tolerates pH levels in the range 2-4.

The growth medium can include, for example, any carbohydrate source which can supply a source of carbon to the non-naturally occurring microorganism. Such sources include, for example, sugars such as glucose, xylose, arabinose, galactose, mannose, fructose, sucrose and starch. Other sources of carbohydrate include, for example, renewable feedstocks and biomass. Exemplary types of biomasses that can be used as feedstocks in the methods of the invention include cellulose biomass, hemicellulose biomass and lignin feedstocks or portions of feedstocks. Such biomass feedstocks contain, for example, carbohydrate substrates useful as carbon sources such as glucose, xylose, arabinose, galactose, mannose, fructose and starch. Given the teachings and guidance provided herein, those skilled in the art will understand that renewable feedstocks and biomass other than those
exemplified above also can be used for culturing the microbial organisms of the invention for the production of muconate.

[0223] In addition to renewable feedstocks such as those exemplified above, the muconate microbial organisms of the invention also can be modified for growth on syngas as its source of carbon. In this specific embodiment, one or more proteins or enzymes are expressed in the muconate producing organisms to provide a metabolic pathway for utilization of syngas or other gaseous carbon source.

[0224] Synthesis gas, also known as syngas or producer gas, is the major product of gasification of coal and of carbonaceous materials such as biomass materials, including agricultural crops and residues. Syngas is a mixture primarily of H2 and CO and can be obtained from the gasification of any organic feedstock, including but not limited to coal, coal oil, natural gas, biomass, and waste organic matter. Gasification is generally carried out under a high fuel to oxygen ratio. Although largely H2 and CO, syngas can also include CO2 and other gases in smaller quantities. Thus, synthesis gas provides a cost effective source of gaseous carbon such as CO and, additionally, CO2.

[0225] The Wood-Ljungdahl pathway catalyzes the conversion of CO and H2 to acetyl-CoA and other products such as acetate. Organisms capable of utilizing CO and syngas also generally have the capability of utilizing CO2 and CO2/H2 mixtures through the same basic set of enzymes and transformations encompassed by the Wood-Ljungdahl pathway. H2-dependent conversion of CO2 to acetate by microorganisms was recognized long before it was revealed that CO also could be used by the same organisms and that the same pathways were involved. Many acetogens have been shown to grow in the presence of CO2 and produce compounds such as acetate as long as hydrogen is present to supply the necessary reducing equivalents (see for example, Drake, Acetogenesis, pp. 3-60 Chapman and Hall, New York, (1994)). This can be summarized by the following equation:

\[ 2\text{CO}_2 + 4\text{H}_2 + \text{ATP} \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} + \text{2ATP} \]

[0226] Hence, non-naturally occurring microorganisms possessing the Wood-Ljungdahl pathway can utilize CO2 and H2 mixtures as well for the production of acetyl-CoA and other desired products.

[0227] The Wood-Ljungdahl pathway is well known in the art and consists of 12 reactions which can be separated into two branches: (1) methyl branch and (2) carboxyl branch. The methyl branch converts syngas to methyl-tetrahydrofolate (methyl-THF) whereas the carboxyl branch converts methyl-THF to acetyl-CoA. The reactions in the methyl branch are catalyzed in order by the following enzymes or proteins: ferredoxin oxidoreductase, formate dehydrogenase, formyltetrahydrofolate synthetase, methylenetetrahydrofolate cyclodehydratase, methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate reductase. The reactions in the carboxyl branch are catalyzed in order by the following enzymes or proteins: methylenetetrahydrofolate corrinoid protein methyltransferase (for example, AcsE), corrinoid iron-sulfur protein, nickel-protein assembly protein (for example, AesF), ferredoxin, acetyl-CoA synthase, carbon monoxide dehydrogenase and nickel-protein assembly protein (for example, CooC). Following the teachings and guidance provided herein for introducing a sufficient number of encoding nucleic acids to generate a muconate pathway, those skilled in the art will understand that the same engineering design also can be performed with respect to introducing at least the nucleic acids encoding the Wood-Ljungdahl enzymes or proteins absent in the host organism. Therefore, introduction of one or more encoding nucleic acids into the microbial organisms of the invention such that the modified organism contains the complete Wood-Ljungdahl pathway will confer syngas utilization ability.

[0228] Accordingly, given the teachings and guidance provided herein, those skilled in the art will understand that a non-naturally occurring microbial organism can be produced that secretes the biosynthesized compounds of the invention when grown on a carbon source such as a carbohydrate. Such compounds include, for example, muconate and any of the intermediate metabolites in the muconate pathway. All that is required is to engineer in one or more of the required enzyme or protein activities to achieve biosynthesis of the desired compound or intermediate including, for example, inclusion of some or all of the muconate biosynthetic pathways. Accordingly, the invention provides a non-naturally occurring microbial organism that produces and/or secretes muconate when grown on a carbohydrate or other carbon source and produces and/or secretes any of the intermediate metabolites shown in the muconate pathway when grown on a carbohydrate or other carbon source. The muconate producing microbial organisms of the invention can initiate synthesis from an intermediate, such as any of the intermediates shown in FIGS. 2-5.

[0229] The non-naturally occurring microbial organisms of the invention are constructed using methods well known in the art as exemplified herein to exogenously express at least one nucleic acid encoding a muconate pathway enzyme or protein in sufficient amounts to produce muconate. It is understood that the microbial organisms of the invention are cultured under conditions sufficient to produce muconate. Following the teachings and guidance provided herein, the non-naturally occurring microbial organisms of the invention can achieve biosynthesis of muconate resulting in intracellular concentrations between about 0.1-200 mM or more. Generally, the intracellular concentration of muconate is between about 3-150 mM, particularly between about 5-200 mM and more particularly between about 8-150 mM, including about 10 mM, 50 mM, 75 mM, 100 mM, or more. Intracellular concentrations between and above each of these exemplary ranges also can be achieved from the non-naturally occurring microbial organisms of the invention.

[0230] In some embodiments, culture conditions include anaerobic or substantially anaerobic growth or maintenance conditions. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary anaerobic conditions for fermentation processes are described herein and are described, for example, in U.S. patent application Ser. No. 11/891,602, filed Aug. 10, 2007. Any of these conditions can be employed with the non-naturally occurring microbial organisms as well as other anaerobic conditions well known in the art. Under such anaerobic conditions, the muconate producers can synthesize muconate at intracellular concentrations of 5-10 mM or more as well as all other concentrations exemplified herein. It is understood that, even though the above description refers to intracellular concentrations, muconate producing microbial organisms can produce muconate intracellularly and/or secrete the product into the culture medium.

[0231] The culture conditions can include, for example, liquid culture procedures as well as fermentation and other
large scale culture procedures. As described herein, particularly useful yields of the biosynthetic products of the invention can be obtained under anaerobic or substantially anaerobic culture conditions.

[0232] As described herein, one exemplary growth condition for achieving biosynthesis of muconate includes anaerobic culture or fermentation conditions. In certain embodiments, the non-naturally occurring microbial organisms of the invention can be sustained, cultured or fermented under anaerobic or substantially anaerobic conditions. Briefly, anaerobic conditions refers to an environment devoid of oxygen. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains between 0 and 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N₂/CO₂ mixture or other suitable non-oxygen gas or gases.

[0233] The culture conditions described herein can be scaled up and grown continuously for manufacturing of muconate. Exemplary growth procedures include, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. All of these processes are well known in the art. Fermentation procedures are particularly useful for the biosynthetic production of commercial quantities of muconate. Generally, and as with non-continuous culture procedures, the continuous and/or near-continuous production of muconate will include culturing a non-naturally occurring muconate producing organism of the invention in sufficient nutrients and medium to sustain and/or nearly sustain growth in an exponential phase. Continuous culture under such conditions can include, for example, 1 day, 2, 3, 4, 5, 6 or 7 days or more. Additionally, continuous culture can include 1 week, 2, 3, 4 or 5 or more weeks and up to several months. Alternatively, organisms of the invention can be cultured for hours, if suitable for a particular application. It is to be understood that the continuous and/or near-continuous culture conditions also can include all time intervals in between these exemplary periods. It is further understood that the time of culturing the microbial organism of the invention is for a sufficient period of time to produce a sufficient amount of product for a desired purpose.

[0234] Fermentation procedures are well known in the art. Briefly, fermentation for the biosynthetic production of muconate can be utilized in, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. Examples of batch and continuous fermentation procedures are well known in the art.

[0235] In addition to the above fermentation procedures using the muconate producers of the invention for continuous production of substantial quantities of muconate, the muconate producers also can be, for example, simultaneously subjected to chemical synthesis procedures to convert the product to other compounds or the product can be separated from the fermentation culture and sequentially subjected to chemical conversion to convert the product to other compounds, if desired.

[0236] In addition to the above procedures, growth condition for achieving biosynthesis of muconate can include the addition of an osmoprotectant to the culturing conditions. In certain embodiments, the non-naturally occurring microbial organisms of the invention can be sustained, cultured or fermented as described above in the presence of an osmoprotectant. Briefly, an osmoprotectant means a compound that acts as an osmolyte and helps a microbial organism as described herein survive osmotic stress. Osmoprotectants include, but are not limited to, betaines, amino acids, and the sugar trehalose. Non-limiting examples of such are glycine betaine, praline betaine, dimethylthetain, dimethylsulfonio-propionate, 3-dimethylsulfonio-2-methylpropionate, piperolic acid, dimethylsulfonioacetate, choline, L-carnitine and ectoine. In one aspect, the osmoprotectant is glycine betaine. It is understood to one of ordinary skill in the art that the amount and type of osmoprotectant suitable for protecting a microbial organism described herein from osmotic stress will depend on the microbial organism used. The amount of osmoprotectant in the culturing conditions can be, for example, no more than about 0.1 mM, no more than about 0.5 mM, no more than about 1.0 mM, no more than about 1.5 mM, no more than about 2.0 mM, no more than about 2.5 mM, no more than about 3.0 mM, no more than about 5.0 mM, no more than about 7.0 mM, no more than about 10 mM, no more than about 50 mM, no more than about 100 mM or no more than about 500 mM.


[0238] One computational method for identifying and designing metabolic alterations favoring biosynthesis of a desired product is the OptKnock computational framework (Burgard et al., Biotechnol. Bioeng. 84:647-657 (2003)). OptKnock is a metabolic modeling and simulation program that suggests gene deletion or disruption strategies that result in genetically stable microorganisms which overproduce the target product. Specifically, the framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become an obligatory byproduct of cell growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production. Lastly, when gene deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome. Therefore, this computational methodology can be used to either identify alternative pathways that lead to biosynthesis of a desired product or used in connection with the non-naturally occurring microbial organisms for further optimization of biosynthesis of a desired product.

[0239] Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework
of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or deletions. OptKnock computational framework allows the construction of model formulations that enable an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. publication 2002/0168654, filed Jan. 10, 2002, in International Patent No. PCT/US02/00660, filed Jan. 10, 2002, and U.S. publication 2009/0047719, filed Aug. 10, 2007.

Another computational method for identifying and designing metabolic alterations favoring biosynthetic production of a product is a metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. publication 2003/0233218, filed Jan. 14, 2002, and in International Patent Application No. PCT/US03/18838, filed Jan. 13, 2003. SimPheny® is a computational system that can be used to produce a network model in silico and to simulate the flux of mass, energy or charge through the chemical reactions of a biological system to define a solution space that contains any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components.

These computational approaches are consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all living systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement biosynthesis of a desired compound in host microbial organisms. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For illustration of the invention, some methods are described herein with reference to the OptKnock computational framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

The methods described above will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in a desired product as an obligatory product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through correlation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

Once identified, the set of reactions that are to be disrupted in order to achieve production of a desired product are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. One particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic modifications including, for example, mutation, deletion of regulatory regions such as promoters or cis binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the coupling of a product are desired or when genetic reversal is less likely to occur.

To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the biosynthesis, including growth-coupled biosynthesis of a desired product, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that obligatorily couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions. The integer cut method is well known in the art and can be found described in, for example, Burgard et al., Biotechnol. Prog. 17:791-797 (2001). As with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny®.

The methods exemplified herein allow the construction of cells and organisms that biosynthetically produce a desired product, including the obligatory coupling of production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an in silico method selected from OptKnock or SimPheny®. The set of metabolic
modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

[0247] As discussed above, the OptKnock methodology was developed on the premise that mutant microbial networks can be evolved towards their computationally predicted maximum-growth phenotypes when subjected to long periods of growth selection. In other words, the approach leverages an organism's ability to self-optimize under selective pressures. The OptKnock framework allows for the exhaustive enumeration of gene deletion combinations that force a coupling between biochemical production and cell growth based on network stoichiometry. The identification of optimal gene/reaction knockouts requires the solution of a bilevel optimization problem that chooses the set of active reactions such that an optimal growth solution for the resulting network overproduces the biochemical of interest (Burgard et al., *Bio/tech. Bioeng.* 84:647-657 (2003)).

[0248] An in silico stoichiometric model of *E. coli* metabolism can be employed to identify essential genes for metabolic pathways as exemplified previously and described in, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/01865 and US 2004/0009466, and in U.S. Pat. No. 7,127,379. As disclosed herein, the OptKnock mathematical framework can be applied to pinpoint gene deletions leading to the growth-coupled production of a desired product. Further, the solution of the bilevel OptKnock problem provides only one set of deletions. To enumerate all meaningful solutions, that is, all sets of knockouts leading to growth-coupled production formation, an optimization technique, termed integer cuts, can be implemented. This entails iteratively solving the OptKnock problem with the incorporation of an additional constraint referred to as an integer cut at each iteration, as discussed above.

[0249] It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also provided within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

**Example I**

Demonstration of Enzyme Activity for Condensing Succinyl-CoA and Acetyl-CoA to Form β-ketoadipyl-CoA

[0250] This Example shows the identification of enzymes for the formation of beta-ketoadipyl-CoA from succinyl-CoA and acetyl-CoA.

[0251] Several β-ketothiolase enzymes have been shown to break β-ketoadipyl-CoA into acetyl-CoA and succinyl-CoA. For example, the gene products encoded by p5′F in *Pseudomonas* strain B13 (Kasahake et al., *J. Bacteriol.* 184 (1): 207-15 (2002)), plusD in *Pseudomonas putida* U (Oliver et al., *Proc Natl Acad Sci U.S.A.* 95 (11), 6419-24 (1998)), and paAD in *Pseudomonas fluorescens* ST (Di Gennero et al., *Arch Microbiol.* 188(2), 117-25 (2007)), and paAD from *E. coli* (Nogales et al., *Microbiology* 153(P2), 357-65 (2007)) catalyze the conversion of 3-oxoadipyl-CoA into succinyl-CoA and acetyl-CoA during the degradation of aromatic compounds such as phenylacetate or stirene. To confirm that β-ketothiolase enzymes exhibit condensation activity, several thiolases (Table 53) were cloned into a derivative of pZEr13 (Lutz et al., *Nucleic Acids Res.* 29(18), 3873-81 (2001)), which results in the clones having a carboxy-terminal 6xHis tag.

**Table 53**

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May 26, 2011
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The enzymes were expressed in E. coli and the proteins purified using Ni-NTA spin columns and quantified. To assay enzyme activity in vitro, a 5xCoA:DTNB (Ellman’s reagent or 5,5'-dithiobis-(2-nitrobenzoic acid)) mixture was prepared. The mixture consisted of 10 mM succinyl-CoA, 5 mM acetyl-CoA, 30 mM DTNB in 100 mM Tris buffer, pH 7.4. Five μL of the CoA:DTNB mixture was added to 0.5 μM purified thiolase enzyme in 100 mM Tris buffer, pH 7.8 in a final volume of 50 μL. The reaction was incubated at 30°C for 30 minutes, then quenched with 2.5 μL 10% formic acid and samples frozen at -20°C until ready for analysis by LC/MS. Because many thiolases can cleave two acyl-CoA molecules into acetoacetyl-CoA, production of acetoacetyl-CoA was examined. FIG. 6 shows that 3 thiolases demonstrated thiolase activity which resulted in acetoacetyl-CoA formation. These were fadA from Pseudomonas putida, thiA from Clostridium acetobutylicum and thiB also from Clostridium acetobutylicum. When enzyme assays were examined for condensation of succinyl-CoA and acetyl-CoA into β-ketoacyl-CoA, several enzymes demonstrated the desired activity; paaJ from Escherichia coli (Nogales et al., Microbiol. 153:357-365 (2007)), paaD from Pseudomonas putida (Olivera et al., Proc. Natl. Acad. Sci. USA 95:6419-6424 (1998)), bkt from Burkholderia ambifaria AMMD, pcaf from Pseudomonas putida KT2440 (Harwood et al., J. Bacteriol. 176:6479-6488 (1994)), and pcaf from Pseudomonas aeruginosa PA01. There was excellent specificity between the thiolases. Those that generated amounts of β-ketoacyl-CoA did not produce significant amounts of acetoacetyl-CoA and likewise those that made acetoacetyl-CoA did not make detectable amounts of β-ketoacyl-CoA.
Example II
Preparation of Terephthalate from Acetylene and Muconate

[0253] This Example provides conditions for the thermal inverse electron demand Diels-Alder reaction for the preparation of PTA from acetylene and muconate.

[0254] A lab-scale Parr reactor is flushed with nitrogen gas, evacuated and charged with (1 equivalent) trans, trans-muconic acid and (10 equivalents) acetylene. The reactor is then heated to 200°C and held at this temperature for 12 hours. An initial pressure of 500 p.s.i.g. is applied. The reactor is then vented, exposed to air and cooled. The contents of the reactor are distilled at room temperature and pressure to yield volatile and nonvolatile fractions. The contents of each fraction are evaluated qualitatively by gas chromatographic analysis (GC-MS).

[0255] For quantitative analysis, standards of the starting materials and the expected product, cyclohexa-2,5-diene-1, 4-dicarboxylic acid and terephthalate, are prepared. A known amount of cyclohexane is mixed with a known amount of the volatile fraction and the mixture is subjected to gas chromatography. The cyclohexane and terephthalate components are condensed from the effluent of the chromatogram into a single trap, the contents of which are diluted with carbon tetrachloride or CDCl3, and then examined by NMR spectroscopy. Comparison of the appropriate areas of the NMR spectrum permits calculation of yields.

Example III
Preparation of a Muconate Producing Microbial Organism, in which the Muconate is Derived from succinyl-CoA

[0256] This example describes the generation of a microbial organism that has been engineered to produce muconate from succinyl-CoA and acetyl-CoA via beta-ketoaapitate, as shown in FIG. 2. This example also provides a method for engineering a strain that overproduces muconate.

[0257] Escherichia coli is used as a target organism to engineer a muconate-producing pathway as shown in FIG. 5. E. coli provides a good host for generating a non-naturally occurring microorganism capable of producing muconate. E. coli is amenable to genetic manipulation and is known to be capable of producing various products, like ethanol, acetic acid, formic acid, lactic acid, and succinic acid, effectively under anaerobic, microaerobic or aerobic conditions.

[0258] First, an E. coli strain is engineered to produce muconate from succinyl-CoA via the route outlined in FIG. 2. For the first stage of pathway construction, genes encoding enzymes to transform central metabolites succinyl-CoA and acetyl-CoA to 2-maleylacetate (FIG. 2, Step A) is assembled onto vectors. In particular, the genes pcaf (AAA85138), pcaIF (AAN69545 and NP_746082) and cleE (O30847) genes encoding beta-ketothiolase, beta-ketoacyl-CoA transferase and 2-maleylacetate reductase, respectively, are cloned into the pZE13 vector (Expressys, Ruelzheim, Germany), under the control of the PAI/legO promoter. The genes bdh (AAA8352.1) and fumC (P05042.1), encoding 2-maleylacetate dehydrogenase and 3-hydroxy-4-hexenediole dehydratase, respectively, are cloned into the pZA3 vector (Expressys, Ruelzheim, Germany) under the PAI/legO promoter. The two sets of plasmids are transformed into E. coli strain MG1655 to express the proteins and enzymes required for muconate synthesis from succinyl-CoA.

[0259] The resulting genetically engineered organism is cultured in glucose containing medium following procedures well known in the art (see, for example, Sambrook et al., supra, 2001). The expression of muconate pathway genes is corroborated using methods well known in the art for determining polypeptide expression or enzymatic activity, including for example, Northern blots, PCR amplification of mRNA and immunoblotting. Enzymatic activities of the expressed enzymes are confirmed using assays specific for the individually activities. The ability of the engineered E. coli strain to produce muconate through this pathway is confirmed using HPLC, gas chromatography-mass spectrometry (GCMS) or liquid chromatography-mass spectrometry (LCMS).

[0260] Microbial strains engineered to have a functional muconate synthesis pathway from succinyl-CoA are further augmented by optimization for efficient utilization of the pathway. Briefly, the engineered strain is assessed to determine whether any of the exogenous genes are expressed at a rate limiting level. Expression is increased for any enzymes expressed at low levels that can limit the flux through the pathway by, for example, introduction of additional gene copy numbers.

[0261] After successful demonstration of enhanced muconate production via the activities of the exogenous enzymes, the genes encoding these enzymes are inserted into the chromosome of a wild type E. coli host using methods known in the art. Such methods include, for example, sequential single crossover (Gay et al., J. Bacterial. 153:1424-1431 (1983)) and Red/ET methods from GeneBridges (Zhang et al., Improved RecET or RecET cloning and subcloning method (WO/2003/010322)). Chromosomal insertion provides several advantages over a plasmid-based system, including greater stability and the ability to co-localize expression of pathway genes.

[0262] To generate better producers, metabolic modeling is utilized to optimize growth conditions. Modeling is also used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224563, US 2004/ 0029149, US 2004/0072723, US 2003/0059792, US 2002/ 0168654 and US 2004/009466, and in U.S. Pat. No. 7,127, 379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of muconate. One modeling method is the bilevel optimization approach. OptKnock (Burgard et al., Biotechnol. Bioengineer. 84:647-657 (2003)), which is applied to select gene knockouts that collectively result in better production of muconate. Adaptive evolution also can be used to generate better producers of, for example, the 4-acetylbutyrate intermediate or the muconate product. Adaptive evolution is performed to improve both growth and production characteristics (Fong and Palsson, Nat. Genet. 36:1056-1058 (2004); Alper et al., Science 314:1565-1568 (2006)). Based on the results, subsequent rounds of modeling, genetic engineering and adaptive evolution can be applied to the muconate producer to further increase production.

[0263] For large-scale production of muconate, the above muconate pathway-containing organism is cultured in a fermenter using a medium known in the art to support growth of the organism under anaerobic conditions. Fermentations are performed in either a batch, fed-batch or continuous manner. Anaerobic conditions are maintained by first sparging the
medium with nitrogen and then sealing culture vessel (e.g., flasks can be sealed with a septum and crimp-cap). Microaerobic conditions also can be utilized by providing a small hole for limited aeration. The pH of the medium is maintained at a pH of 7 by addition of an acid, such as H₂SO₄. The growth rate is determined by measuring optical density using a spectrophotometer (600 nm), and the glucose uptake rate by monitoring carbon source depletion over time. Byproducts such as undesirable alcohols, organic acids, and residual glucose can be quantified by HPLC (Shimadzu) with an HPX-087 column (BioRad), using a refractive index detector for glucose and alcohols, and a UV detector for organic acids, Lin et al., *Biotechnol. Bioeng.*, 775-779 (2005).

Throughout this application various publications have been referenced. The disclosures of these publications in their entirety are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains. Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 21
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<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 22
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer
<400> SEQUENCE: 25
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<210> SEQ ID NO 26
<211> LENGTH: 1179
<212> TYPE: DNA
<213> ORGANISM: Clostridium acetobutylicum
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<211> LENGTH: 1182
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<213> ORGANISM: Candida albicans

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**FEATURE:** OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

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**ORGANISM:** Artificial Sequence
**FEATURE:** OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

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<400> SEQUENCE: 37
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<212> TYPE: DNA
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1. A non-naturally occurring microbial organism, comprising a microbial organism having a muconate pathway comprising at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate, said muconate pathway comprising an enzyme selected from the group consisting of a beta-ketothiolase, a beta-ketoacyl-CoA hydrolase, a beta-ketoacyl-CoA transferase, a beta-ketoacyl-CoA ligase, a 2-fumarylacetate reductase, a 2-fumarylacetate dehydratase, a trans-3-hydroxy-4-hexenoate dehydrogenase, a 2-fumarylacetate aminotransferase, a 2-fumarylacetate aminating oxidoreductase, a trans-3-amino-4-hexenoate deaminase, a beta-ketoacyl-CoA dehydrogenase, a 3-hydroxyacyl-CoA dehydratase, a 2,3-dehydroacyl-CoA transferase, a 3-dehydroacyl-CoA ligase, a muconate reductase, a 2-maleylacetate reductase, a 2-maleylacetate dehydratase, a cis-3-hydroxy-4-hexenoate dehydrogenase, a 2-maleylacetate aminotransferase, a 2-maleylacetate aminating oxidoreductase, a cis-3-amino-4-hexenoate deaminase, and a muconate cis/trans isomerase.

2. The non-naturally occurring microbial organism of claim 1, wherein said muconate pathway comprises a set of muconate pathway enzymes, said set of muconate pathway enzymes selected from the group consisting of:

A) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase, and beta-ketoacyl-CoA ligase, (3) beta-ketoacyl-CoA dehydrogenase, (4) muconolactone isomerase, (5) muconate cycloisomerase, and (6) a muconate cis/trans isomerase;

B) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase and beta-ketoacyl-CoA ligase, (3) 2-maleylacetate reductase, (4) 2-maleylacetate dehydrogenase, (5) cis-3-hydroxy-4-hexenoate dehydratase, and (6) muconate cis/trans isomerase;

C) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase and beta-ketoacyl-CoA ligase, (3) 2-maleylacetate reductase, (4) an enzyme selected from 2-maleylacetate aminotransferase and 2-maleylacetate aminating oxidoreductase, (5) cis-3-amino-4-hexenoate deaminase, and (6) muconate cis/trans isomerase;

D) (1) beta-ketothiolase, (2) beta-ketoacyl-CoA dehydrogenase, (3) 3-hydroxyacyl-CoA dehydratase, (4) an enzyme selected from 2,3-dehydroacyl-CoA transferase, 2,3-dehydroacyl-CoA hydrolase, and 2,3-dehydroacyl-CoA ligase, and (5) muconate reductase;

E) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase and beta-ketoacyl-CoA ligase, (3) 2-fumarylacetate reductase, (4) 2-fumarylacetate dehydratase, and (5) trans-3-hydroxy-4-hexenoate dehydratase;

F) (1) beta-ketothiolase, (2) an enzyme selected from beta-ketoacyl-CoA hydrolase, beta-ketoacyl-CoA transferase and beta-ketoacyl-CoA ligase, (3) 2-fumarylacetate reductase, (4) an enzyme selected from 2-fumarylacetate aminotransferase and 2-fumarylacetate aminating oxidoreductase, and (5) trans-3-amino-4-hexenoate deaminase.

3. The non-naturally occurring microbial organism of claim 2, wherein said microbial organism comprises two exogenous nucleic acids each encoding a muconate pathway enzyme.
4. The non-naturally occurring microbial organism of claim 2, wherein said microbial organism comprises three exogenous nucleic acids each encoding a muconate pathway enzyme.

5. The non-naturally occurring microbial organism of claim 2, wherein said microbial organism comprises four exogenous nucleic acids each encoding a muconate pathway enzyme.

6. The non-naturally occurring microbial organism of claim 2, wherein said microbial organism comprises five exogenous nucleic acids each encoding a muconate pathway enzyme.

7. The non-naturally occurring microbial organism of claim 2, wherein said microbial organism comprises six exogenous nucleic acids each encoding a muconate pathway enzyme.

8. The non-naturally occurring microbial organism of claim 1, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

9. The non-naturally occurring microbial organism of claim 1, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

10. A method for producing muconate, comprising culturing the non-naturally occurring microbial organism according to claim 1, under conditions and for a sufficient period of time to produce muconate according to claim 1.

11-18. (canceled)

19. A non-naturally occurring microbial organism, comprising a microorganism having a muconate pathway comprising at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate, said muconate pathway comprising an enzyme selected from the group consisting of 4-hydroxy-2-ketovalerate aldolase, 2-oxopentenoate hydratase, 4-oxoalocrotonate dehydrogenase, 2-hydroxy-4-hexenedioate dehydrogenase, 4-hydroxy-2-oxo-4-hexenedioic acid oxidoreductase, 2,4-dihydroxydipate dehydratase (acting on 2-hydroxy), a 2,4-dihydroxydipate dehydratase (acting on 4-hydroxy group) and a 3-hydroxy-4-hexenolide dehydratase.

20. The non-naturally occurring microbial organism of claim 19, wherein said muconate pathway comprises a set of muconate pathway enzymes, said set of muconate pathway enzymes selected from the group consisting of:

A) (1) 4-hydroxy-2-ketovalerate aldolase, (2) 2-oxopentenoate hydratase, (3) 4-oxoalocrotonate dehydrogenase, (4) 2-hydroxy-4-hexenedioate dehydratase;

B) (1) 4-hydroxy-2-ketovalerate aldolase, (2) 4-hydroxy-2-oxo-4-hexenedioic acid oxidoreductase, (3) 2,4-dihydroxypate dehydratase (acting on 2-hydroxy), (4) 3-hydroxy-4-hexenolide dehydratase; and

C) (1) 4-hydroxy-2-ketovalerate aldolase, (2) 4-hydroxy-2-oxo-4-hexenedioic acid oxidoreductase, (3) 2,4-dihydroxypate dehydratase (acting on 4-hydroxy group), (4) 2-hydroxy-4-hexenolide dehydratase.

21. The non-naturally occurring microbial organism of claim 20, wherein said microbial organism comprises two exogenous nucleic acids each encoding a muconate pathway enzyme.

22. The non-naturally occurring microbial organism of claim 20, wherein said microbial organism comprises three exogenous nucleic acids each encoding a muconate pathway enzyme.

23. The non-naturally occurring microbial organism of claim 20, wherein said microbial organism comprises four exogenous nucleic acids each encoding a muconate pathway enzyme.

24. The non-naturally occurring microbial organism of claim 19, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

25. The non-naturally occurring microbial organism of claim 19, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

26. A method for producing muconate, comprising culturing the non-naturally occurring microbial organism according to claim 19, under conditions and for a sufficient period of time to produce muconate.

27-32. (canceled)

33. A non-naturally occurring microbial organism, comprising a microorganism having a muconate pathway comprising at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate, said muconate pathway comprising an enzyme selected from the group consisting of an HODH aldolase, an OHED hydratase, an OHED decarboxylase, an OHED formate-lyase, an HODH dehydrogenase, an OHED formate-lyase, an OHED dehydrogenase, a 6-OHE dehydrogenase, a 3-hydroxydipal-CO-A dehydratase, a 2,3-dehydroadipic-CoA hydrolase, a 2,3-dehydroadipyl-CoA transferase, a 2,3-dehydroadipyl-CoA ligase, and a muconate reductase.

34. The non-naturally occurring microbial organism of claim 33, wherein said muconate pathway comprises a set of muconate pathway enzymes, set of muconate pathway enzymes selected from the group consisting of:

A) (1) HODH aldolase, (2) OHED hydratase, (3) OHED decarboxylase, (4) 6-OHE dehydrogenase, and (5) muconate reductase;

B) (1) HODH aldolase, (2) OHED hydratase, (3) an enzyme selected from OHED formate-lyase and OHED dehydrogenase, (4) an enzyme selected from 2,3-dehydroadipic-CoA hydrolase, 2,3-dehydroadipyl-CoA transferase and 2,3-dehydroadipyl-CoA ligase, and (5) muconate reductase;

C) (1) HODH aldolase, (2) an enzyme selected from HODH formate-lyase and HODH dehydrogenase, (3) 3-hydroxyadipyl-CoA dehydratase, (4) an enzyme selected from 2,3-dehydroadipyl-CoA hydrolase, 2,3-dehydroadipyl-CoA transferase and 2,3-dehydroadipyl-CoA ligase, and (5) muconate reductase.

35. The non-naturally occurring microbial organism of claim 34, wherein said microbial organism comprises two exogenous nucleic acids each encoding a muconate pathway enzyme.

36. The non-naturally occurring microbial organism of claim 34, wherein said microbial organism comprises three exogenous nucleic acids each encoding a muconate pathway enzyme.

37. The non-naturally occurring microbial organism of claim 34, wherein said microbial organism comprises four exogenous nucleic acids each encoding a muconate pathway enzyme.

38. The non-naturally occurring microbial organism of claim 34, wherein said microbial organism comprises five exogenous nucleic acids each encoding a muconate pathway enzyme.
39. The non-naturally occurring microbial organism of claim 33, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

40. The non-naturally occurring microbial organism of claim 33, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

41. A method for producing muconate, comprising culturing the non-naturally occurring microbial organism according to claim 33, under conditions and for a sufficient period of time to produce muconate.

42.-47. (canceled)

48. A non-naturally occurring microbial organism, comprising a microbial organism having a muconate pathway comprising at least one exogenous nucleic acid encoding a muconate pathway enzyme expressed in a sufficient amount to produce muconate, said muconate pathway comprising an enzyme selected from the group consisting of a lysine aminotransferase, a lysine aminating oxidoreductase, a 2-aminoadipate semialdehyde dehydratase, a 2-aminoadipate deaminase, a muconate reductase, a lysine-2,3-aminomutase, a 3,6-diaminohexanoate aminotransferase, a 3,6-diamino- hexanoate aminating oxidoreductase, a 3-aminoadipate semi- aldehyde dehydratase, and a 3-aminoadipate deaminase.

49. The non-naturally occurring microbial organism of claim 48, wherein said muconate pathway comprises a set of muconate pathway enzymes, set of muconate pathway enzymes selected from the group consisting of:

A) (1) lysine aminotransferase, (2) lysine aminating oxidoreductase, (3) 2-aminoadipate semialdehyde dehydratase, (4) 2-aminoadipate deaminase, and (5) muconate reductase

B) (1) lysine-2,3-aminomutase, (2) 3,6-diaminohexanoate aminotransferase, (3) 3,6-diaminohexanoate aminating oxidoreductase, (4) 3-aminoadipate semialdehyde dehydratase, (5) 3-aminoadipate deaminase, and (6) muconate reductase.

50. The non-naturally occurring microbial organism of claim 49, wherein said microbial organism comprises two exogenous nucleic acids each encoding a muconate pathway enzyme.

51. The non-naturally occurring microbial organism of claim 49, wherein said microbial organism comprises three exogenous nucleic acids each encoding a muconate pathway enzyme.

52. The non-naturally occurring microbial organism of claim 49, wherein said microbial organism comprises four exogenous nucleic acids each encoding a muconate pathway enzyme.

53. The non-naturally occurring microbial organism of claim 49, wherein said microbial organism comprises five exogenous nucleic acids each encoding a muconate pathway enzyme.

54. The non-naturally occurring microbial organism of claim 49, wherein said microbial organism comprises six exogenous nucleic acids each encoding a muconate pathway enzyme.

55. The non-naturally occurring microbial organism of claim 48, wherein said at least one exogenous nucleic acid is a heterologous nucleic acid.

56. The non-naturally occurring microbial organism of claim 48, wherein said non-naturally occurring microbial organism is in a substantially anaerobic culture medium.

57. A method for producing muconate, comprising culturing the non-naturally occurring microbial organism according to claim 48, under conditions and for a sufficient period of time to produce muconate.

58.-65. (canceled)

66. A semi-synthetic method for synthesizing terephthalate (PTA) comprising preparing muconic acid by culturing an organism of any one of claims, 2, 20, 34, or 49, reacting said muconic acid with acetylene to form a cyclohexadiene adduct, and oxidizing said cyclohexadiene adduct to form PTA.

67. The method of claim 66, further comprising isolating muconic acid prior to reacting with acetylene.

68. The method of claim 66, wherein the step of reacting said muconic acid with acetylene comprises adding acetylene to the culture broth.

69. The method of claim 68, wherein the culture broth is filtered prior to adding acetylene.

70. The method of claim 68, wherein said step of oxidizing said cyclohexadiene adduct comprises adding air or oxygen to the culture broth.

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