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(54) Title: METHODS FOR PRODUCING 3-HYDROXYPROPIONIC ACID AND COMPOUNDS THEREOF

(57) Abstract: The present invention provides methods for producing 3-hydroxypropionic acid (3HP), comprising cultivating a metabolically engineered E. coli strain derived from a strain previously engineered for high lactic acid or high L-alanine production, wherein the metabolically engineered E. coli strain produces 3-hydroxypropionic acid (3HP).

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METHODS FOR PRODUCING 3-HYDROXYPROPIONIC ACID AND COMPOUNDS THEREOF

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Background of the Invention

Field of the Invention

The present invention relates to methods for producing 3-hydroxypropionic acid
10 and compounds thereof.

Description of Related Art

Organic acids have long been used in the food, pharmaceutical, and textile
industries, and more recently in the production of biodegradable plastics. Several
15 organic acids such as citric acid and lactic acid (also known as 2-hydroxypropanoic acid)
are produced in large-scale today by microbial fermentation processes. Other organic
acids, like fumaric acid and malic acid, can also be produced by microbial processes,
but currently are not produced commercially in this manner because chemical synthesis
from petroleum-derived precursors is more economical.

20 Some organic acids are also recognized as potential platform chemicals, or
building blocks, which can be produced by fermentation from renewable feedstocks, and
then further modified by chemical or enzymatic means to produce an array of other
valuable industrial chemicals. One prominent example is lactic acid, which is produced
by fermentation and then converted to ethyl lactate or polylactic acid. Polylactic acid is a
25 biodegradable plastic that provides an alternative to petroleum-based plastics and is
expanding into many areas including textiles, medical implants, drug carriers, food
packaging, and cosmetics. Other examples of organic acids that can be produced by
fermentation and converted to other industrial compounds are succinic acid and 3-
hydroxypropionic acid (also known as 3-hydroxypropanoic acid or beta-lactic acid and
30 hereinafter referred to as 3HP). Succinic acid can be converted, for example, to
1,4-butanediol or tetrahydrofuran. 3HP can be converted, for example, to acrylic acid or
1,3-propanediol.

Several microorganisms have been developed for industrial production of lactic
acid by fermentation, including *Lactobacillus* species, *Kluyveromyces* species,
35 *Saccharomyces cerevisiae*, *Rhizopus oryzae*, and *Escherichia coli*. Due to its
amenability to genetic engineering, known genome sequence, ability to metabolize
hexose and pentose sugars, and proven industrial utility, *E. coli* has been the focus of

recent efforts to develop improved and more economical lactic acid producing strains and fermentation processes (Zhou *et al.*, 2003, *Appl. Environ. Microbiol.* 69: 399-407; Zhou *et al.*, 2005, *Biotechnology Letters* 27: 1891-1896; Zhou *et al.*, 2006a, *Biotechnology Letters* 28: 671-676; Zhou *et al.*, 2006b, *Biotechnology Letters* 28: 663-670; U.S. Published Patent Application No. 2007/0037265; Shukla *et al.*, 2004, *Biotechnology Letters* 26: 689-693; Purvis *et al.*, 2005, *Appl. Environ. Microbiol.* 71: 3761-3769; Grabar *et al.*, 2006, *Biotechnology Letters* 28: 1527-1535; Miller and Ingram, 2007, *Biotechnology Letters* 29: 213-217).

Two of the key challenges in creating improved, commercially competitive lactic acid-producing strains of *E. coli* were overcoming osmotic stress to the organism caused by high concentrations of substrate and product, and overcoming the toxicity of lactic acid (Zhou *et al.*, 2006a, *supra*; Warnecke and Gill, 2005, *Microbial Cell Factories* 4: 1-8; van Maris *et al.*, 2004, *Metabolic Engineering* 6: 245-255). To overcome these obstacles, Ingram and co-workers used a combination of traditional metabolic pathway engineering, process optimization, and metabolic evolution to develop very high lactic acid producing *E. coli* strains and fermentation processes (Zhou *et al.*, 2003, *supra*; Zhou *et al.*, 2005, *supra*; Zhou *et al.*, 2006a, *supra*; Zhou *et al.*, 2006b, *supra*; U.S. Published Patent Application No. 2007/0037265; Shukla *et al.*, 2004, *supra*; Purvis *et al.*, 2005, *supra*; Grabar *et al.*, 2006, *supra*; Miller and Ingram, 2007, *supra*). Metabolic evolution, as it pertains to the creation of improved lactic acid-producing strains, is a co-selection process for strains with improved growth and lactic acid production (U.S. Published Patent Application No. 2007/0037265).

Commercial interest in producing 3HP by fermentation has increased due to its potential to serve as a chemical building block for the manufacture of acrylic acid, acrylamide, 1,3-propanediol, and other compounds. Several potential metabolic routes from the renewable carbon source D-glucose to 3HP have been proposed (WO 2002/042418; WO 2003/062173, WO 2007/042494), and nearly all of the proposed pathways proceed through pyruvate as the central intermediate (Straathof *et al.*, 2005, *Appl. Microbiol. Biotechnol.* 67:727-734; WO 2002/042418; WO 2003/062173). In fact, some metabolic pathways to 3HP proceed through both pyruvate and lactic acid as metabolic intermediates (WO 2002/042418). However, high level production of 3HP *de novo* from glucose or other renewable feedstocks by metabolically engineered microorganisms has not yet been achieved.

The present invention relates to improved methods for producing 3HP and compounds derived from 3HP in commercially relevant quantities using metabolically engineered *E. coli* strains.

Brief Summary of the Invention

The present invention relates to methods for producing 3HP, comprising:
cultivating a metabolically engineered *E. coli* strain derived from a strain previously
5 engineered for high lactic acid or L-alanine production in a cultivation medium.

Brief Description of the Figures

- Figure 1 shows a metabolic pathway for the production of 3-HP via lactate.
10 Figure 2 shows a metabolic pathway for the production of poly-3HP.
Figure 3 shows a metabolic pathway for the production of 3HP esters via lactate.
Figure 4 shows a metabolic pathway for the production of 3-HP via acetyl-CoA.
Figure 5 shows a metabolic pathway for the production of 3-HP, 3HP esters,
poly-3HP, acrylate, acrylate esters, and polyacrylate.
15 Figure 6 shows a metabolic pathway for the production of 3-HP via beta-alanine.
Figure 7 shows a metabolic pathway for the production of 3-HP via beta-alanine
and malonate semialdehyde.

Detailed Description of the Invention

20 The present invention relates to methods for producing 3HP utilizing *E. coli* strains that have been engineered by redirecting metabolic pathways toward 3HP for high 3HP production. In one aspect of the present invention, *E. coli* strains are engineered to increase 3HP biosynthesis from glucose, sucrose, or other sugars via
25 pyruvate through lactate in the presence of a functional *ldhA* gene by incorporating one or more (several) mutations and/or deletions in the genes *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, *alaD*, and *atpFH* (Causey *et al.*, 2004, *Proc. Natl. Acad. Sci. USA* 101: 2235-2240; Zhou *et al.*, 2003, *supra*; Zhou *et al.*, 2005, *supra*), and engineering the metabolic steps leading from pyruvate to 3HP to allow for unimpeded flow of intermediates from pyruvate
30 to 3HP. In another aspect, *E. coli* strains are engineered to increase 3HP biosynthesis via pyruvate through acetyl-CoA by incorporating one or more (several) mutations and/or deletions in the genes *ldhA*, *alaD*, *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, and *atpFH*, and engineering the metabolic steps leading from pyruvate to 3HP to allow for unimpeded flow of intermediates from pyruvate to 3HP. In another aspect, *E. coli* strains are
35 engineered to increase 3HP biosynthesis from propionate through propionyl-CoA and acrylyl-CoA by engineering the metabolic steps leading from propionate to 3HP to allow for unimpeded flow of intermediates from propionate to 3HP. In another aspect, *E. coli*

strains are engineered to increase 3HP biosynthesis via phosphoenolpyruvate or pyruvate through beta-alanine and acrylyl-CoA by incorporating one or more (several) mutations and/or deletions in the genes *ldhA*, *alaD*, *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, and *atpFH*, and engineering the metabolic steps leading from phosphoenolpyruvate or pyruvate to 3HP to allow for unimpeded flow of intermediates from phosphoenolpyruvate or pyruvate to 3HP. In another aspect, *E. coli* strains are engineered to increase 3HP biosynthesis via phosphoenolpyruvate or pyruvate through beta-alanine and malonate semialdehyde by incorporating one or more (several) mutations and/or deletions in the genes *ldhA*, *alaD*, *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, and *atpFH*, and engineering the metabolic steps leading from phosphoenolpyruvate or pyruvate to 3HP to allow for unimpeded flow of intermediates from phosphoenolpyruvate to 3HP.

The methods of the present invention can also be used to produce other compounds derived from 3HP, such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3HP, and esters of 3HP.

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Metabolic Pathways of Producing 3HP

In practicing the methods of the present invention, several metabolic pathways can be engineered for producing 3HP and compounds derived from 3HP by constructing various metabolic pathways involving, for example, lactate, malonyl-CoA, or beta-alanine as an intermediate. Such metabolic pathways useful for production of 3HP are shown in Figures 1-7.

As shown in Figure 1, lactate can be converted into lactyl-CoA with a polypeptide having CoA transferase activity (EC 2.8.3.1); the resulting lactyl-CoA can then be converted into acrylyl-CoA with a polypeptide (or multiple polypeptide complex such as an activated E2 alpha and E2 beta complex) having lactyl-CoA dehydratase activity (EC 4.2.1.54); the resulting acrylyl-CoA can then be converted into 3-hydroxypropionyl-CoA (3HP-CoA) with a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (EC 4.2.1.-); and finally, the resulting 3HP-CoA can be converted into 3HP with a polypeptide having CoA transferase activity or a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity (EC 3.1.2.-), which may be a polypeptide having 3-hydroxyisobutryl-CoA hydrolase activity (EC 3.1.2.4).

As shown in Figure 2, lactate can be converted into lactyl-CoA with a polypeptide having CoA synthetase activity (EC 6.2.1.-); the resulting lactyl-CoA can then be converted into acrylyl-CoA with a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase activity; the resulting acrylyl-CoA can then be converted into 3HP-CoA with a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity; and

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finally, the resulting 3HP-CoA can be converted into polymerized 3HP with a polypeptide having polyhydroxyacid synthase activity (EC 2.3.1.-).

As shown in Figure 3, lactate can be converted into lactyl-CoA with a polypeptide having CoA transferase activity or a polypeptide having lactyl-CoA synthetase activity; the resulting lactyl-CoA can then be converted into acrylyl-CoA with a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase activity; the resulting acrylyl-CoA can then be converted into 3HP-CoA with a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity; the resulting 3HP-CoA can then be converted into 3HP with a polypeptide having CoA transferase activity or a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity, which may be a polypeptide having 3-hydroxyisobutryl-CoA hydrolase activity; and finally the resulting 3HP can be converted into an ester of 3HP with a polypeptide having lipase activity (EC 3.1.1.-).

As shown in Figure 4, acetyl-CoA can be converted into malonyl-CoA with a polypeptide having acetyl-CoA carboxylase activity (EC 6.4.1.2); and the resulting malonyl-CoA can then be converted into 3HP with a polypeptide or polypeptides having malonyl-CoA reductase activity (EC 1.1.1 and 1.2.1).

Polypeptides having malonyl-CoA reductase activity can use NADPH as a co-factor. Likewise, polypeptides having malonyl-CoA reductase activity can use NADH as a co-factor. Such polypeptides can be obtained by converting a polypeptide that has malonyl-CoA reductase activity and uses NADPH as a cofactor into a polypeptide that has malonyl-CoA reductase activity and uses NADH as a cofactor. Any method can be used to convert a polypeptide that uses NADPH as a cofactor into a polypeptide that uses NADH as a cofactor such as those described Eppink *et al.*, 1999, *J. Mol. Biol.* 292: 87-96; Hall and Tomsett, 2000, *Microbiology* 146(Pt 6): 1399-406; and Dohr *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 81-86.

As shown in Figure 5, propionate can be converted into propionyl-CoA with a polypeptide having CoA synthetase activity; the resulting propionyl-CoA can then be converted into acrylyl-CoA with a polypeptide having dehydrogenase activity and finally the resulting acrylyl-CoA can be converted into (1) acrylate with a polypeptide having CoA transferase activity or CoA hydrolase activity, (2) 3HP-CoA with a polypeptide having 3HP dehydratase activity (also referred to as acrylyl-CoA hydratase or simply hydratase), or (3) polymerized acrylate with a polypeptide having polyhydroxyacid synthase activity. The resulting acrylate can be converted into an ester of acrylate with a polypeptide having lipase activity. The resulting 3HP-CoA can be converted into (1) 3HP with a polypeptide having CoA transferase activity or a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity (EC 3.1.2.-), which may be a polypeptide

having 3-hydroxyisobutyryl-CoA hydrolase activity (EC 3.1.2.4), or (2) polymerized 3HP with a polypeptide having polyhydroxyacid synthase activity (EC 2.3.1.-).

Other pathways can be constructed leading to the production of 3HP through a beta-alanine intermediate. In general, prokaryotes and eukaryotes metabolize glucose via the Embden-Meyerhof-Parnas pathway to phosphoenolpyruvate (PEP), a central metabolite in carbon metabolism. The PEP generated from glucose can be carboxylated to oxaloacetate or converted to pyruvate. Carboxylation of PEP to oxaloacetate can be catalyzed by a polypeptide having PEP carboxylase activity (EC 4.1.1.31), a polypeptide having PEP carboxykinase activity (EC 4.1.1.49), or a polypeptide having PEP transcarboxylase activity (EC 4.1.1.-). Pyruvate generated from PEP by a polypeptide having pyruvate kinase activity (EC 2.7.1.40) can also be converted to oxaloacetate by a polypeptide having pyruvate carboxylase activity (EC 6.4.1.1).

Oxaloacetate generated either from PEP or pyruvate can act as a precursor for production of aspartic acid. This conversion can be carried out by a polypeptide having aspartate aminotransferase activity (EC 2.6.1.1), which transfers an amino group from an amino donor such as glutamate to oxaloacetate. The decarboxylation of aspartate to beta-alanine is catalyzed by a polypeptide having aspartate decarboxylase activity (EC 4.1.1.11).

Pyruvate generated from PEP can be converted to alpha-alanine by a polypeptide having alanine dehydrogenase activity (EC 1.4.1.1) (Zhang *et al.*, 2007, *Appl Microbiol Biotechnol.* 77:355-366; WO 2003/062173) or a polypeptide having alanine aminotransferase activity (EC 2.6.1.2). Alpha-alanine produced by alanine dehydrogenase, alanine aminotransferase, or another polypeptide capable of converting pyruvate to alpha-alanine can then be converted to beta-alanine by a polypeptide having alanine 2,3-aminomutase activity (WO 2003/062173).

The biosynthesis of beta-alanine can be accomplished by polypeptides having enzyme activity native (endogenous) to the *E. coli* strain that convert PEP or pyruvate to beta-alanine, or these conversions can be accomplished recombinantly using known polypeptides such as polypeptides having PEP carboxylase activity (EC 4.1.1.31), aspartate aminotransferase activity (EC 2.6.1.1), aspartate decarboxylase activity (EC 4.1.1.11), alanine dehydrogenase activity (EC 1.4.1.1), alanine aminotransferase activity (EC 2.6.1.2), and alanine 2,3-aminomutase activity.

The beta-alanine produced by any of the biosynthetic routes described above can be converted to 3HP by two possible pathways. In the first pathway (Figure 6), beta-alanine can be converted into beta-alanyl-CoA with a polypeptide having CoA transferase activity (EC 2.8.3.-); the resulting beta-alanyl-CoA can then be converted

into acrylyl-CoA with a polypeptide having beta-alanyl-CoA ammonia lyase activity; the resulting acrylyl-CoA can then be converted into 3HP-CoA with a polypeptide having 3HP-CoA dehydratase activity; and finally the resulting 3HP-CoA can be converted into 3HP with a polypeptide having CoA transferase activity (EC 2.8.3.-).

5 In the second pathway (Figure 7), 3HP can be made from beta-alanine by first converting beta-alanine to malonate semialdehyde with a polypeptide having beta-alanine aminotransferase activity, which may be a polypeptide having 4-aminobutyrate aminotransferase activity (EC 2.6.1.19); and the resulting malonate semialdehyde can then be converted into 3HP with a polypeptide having 3HP dehydrogenase activity,
10 which may be a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity (EC 1.1.1.31).

Each step provided in the pathways shown in Figures 1-7 can be performed *in vivo* (within a cell) or *in vitro* (outside a cell). Additionally, the organic acid products can be generated through a combination of *in vivo* synthesis and *in vitro* synthesis.
15 Moreover, the *in vitro* synthesis step, or steps, can be via chemical reaction or enzymatic reaction.

For example, a cellular extract of an engineered *E. coli* strain described herein comprising polypeptides having the indicated enzyme activities, or a combination thereof, can be used to perform the steps described in Figure 1-7. In addition, chemical
20 treatments can be used to perform one or more conversions provided in Figures 1-7. For example, acrylyl-CoA can be converted into acrylate by hydrolysis. Other chemical treatments include, without limitation, transesterification to convert acrylate into an acrylate ester.

Additionally, intermediate chemical products can be starting points. For
25 example, beta alanine can be introduced into a fermentation broth. The exogenously supplied beta-alanine can be imported into the *E. coli* cells and converted to 3HP by either of the pathways shown in Figures 6 and 7. Other useful intermediate chemical starting points can include propionic acid, acrylic acid, lactic acid, and pyruvic acid.

30 Polypeptides Having Enzyme Activity and Polynucleotides Thereof

In practicing the methods of the present invention, the polypeptides having enzyme activity described herein can be produced individually in an *E. coli* strain, in combination in an *E. coli* strain, or individually and in combination in two or more *E. coli* strains. The polypeptides having enzyme activity and polynucleotides thereof may be
35 native or foreign (heterologous) to the *E. coli* strain. Moreover, the polypeptides having a particular enzyme activity can be a polypeptide that is either naturally-occurring or non-naturally-occurring. A naturally-occurring polypeptide is any polypeptide having an

amino acid sequence as found in nature, including wild-type and polymorphic polypeptides. Such naturally-occurring polypeptides can be obtained from any species including, without limitation, animal (e.g., mammalian), plant, fungal, and bacterial species. A non-naturally-occurring polypeptide is any polypeptide having an amino acid sequence that is not found in nature. Thus, a non-naturally-occurring polypeptide can be a mutated version of a naturally-occurring polypeptide, or an engineered polypeptide. For example, a non-naturally-occurring polypeptide having alanine 2,3-aminomutase activity can be a mutated version of a naturally-occurring polypeptide having lysine 2,3-aminomutase activity (WO 2003/062173). A polypeptide can be mutated by, for example, one or more (several) amino acid additions, deletions, substitutions, or combinations thereof.

The term "polypeptide" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "polypeptide" also encompasses hybrid polypeptides, which comprise a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides wherein one or more may be heterologous to the *E. coli* strain. Polypeptides further include naturally occurring allelic and engineered variations of a polypeptide. The term "foreign polypeptide" is defined herein as a polypeptide that is not native to the host cell; a native polypeptide in which structural modifications have been made to alter the native polypeptide, e.g., the protein sequence of the native polypeptide; or a native polypeptide whose expression is quantitatively altered as a result of a manipulation of the DNA encoding the polypeptide by recombinant DNA techniques, e.g., a stronger promoter. The polypeptide may be an engineered variant of any polypeptide.

Polypeptides having enzyme activity useful for practicing the present invention include, but are not limited to, 3-hydroxypropionyl-CoA dehydratase, 3-hydroxypropionyl-CoA hydratase, CoA transferases, lactyl-CoA dehydratase, 3-hydroxypropionyl-CoA hydrolase, 3-hydroxyisobutryl-CoA hydrolase, polyhydroxyacid synthase, CoA synthetase, malonyl-CoA reductase, beta-alanine ammonia lyase, lipase, PEP carboxylase, PEP carboxykinase, PEP transcarboxylase, pyruvate kinase, pyruvate carboxylase, beta-alanyl-CoA ammonia lyase, 3HP-CoA dehydratase, beta-alanine aminotransferase, 4-aminobutyrate aminotransferase, 3HP dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, PEP carboxykinase, aspartate aminotransferase, alanine dehydrogenase, alanine aminotransferase, alanine 2,3-aminomutase, and aspartate decarboxylase.

The polypeptides having enzyme activity as well as polynucleotides thereof can be obtained from various species including, without limitation, *Acidianus brierleyi*.

Candida rugosa, *Candida tropicalis*, *Chloroflexus aurantiacus*, *Clostridium propionicum*, *Clostridium kluyveri*, *Comamonas acidovorans*, *Escherichia coli*, *Chloroflexus aurantiacus*, *Candida rugosa*, *Megasphaera elsdenii*, *Pseudomonas fluorescens*, *Pseudomonas oleovorans*, *Ralstonia eutropha*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Saccharomyces cerevisiae*, *Salmonella enterica*, and *Sulfolobus metacillus*. See, for example, WO 2002/42418.

In a preferred aspect, polypeptides having CoA transferase activity as well as polynucleotides thereof are obtained from *Megasphaera elsdenii*, *Clostridium propionicum*, *Clostridium kluyveri*, and *Escherichia coli*. For example, a polynucleotide that encodes a polypeptide having CoA transferase activity can be obtained from *Megasphaera elsdenii* as described in Example 1 of WO 2002/42418.

In another preferred aspect, polypeptides (or the polypeptides of a multiple polypeptide complex such as an activated E2 alpha and E2 beta complex) having lactyl-CoA dehydratase activity as well as polynucleotides thereof can be obtained from *Megasphaera elsdenii* and *Clostridium propionicum*. For example, a polynucleotide encoding an E1 activator, an E2 alpha subunit, and an E2 beta subunit that can form a multiple polypeptide complex having lactyl-CoA dehydratase activity can be obtained from *Megasphaera elsdenii*.

In another preferred aspect, polypeptides having 3-hydroxypropionyl-CoA dehydratase activity as well as polynucleotides thereof can be obtained from *Chloroflexus aurantiacus*, *Candida rugosa*, *Rhodospirillum rubrum*, and *Rhodobacter capsulatus*.

In another preferred aspect, polypeptides having 3-hydroxypropionyl-CoA hydrolase activity as well as polynucleotides thereof can be obtained from *Candida rugosa*.

In another preferred aspect, polypeptides having 3-hydroxyisobutryl-CoA hydrolase activity as well as polynucleotides thereof can be obtained from *Pseudomonas fluorescens*, *rattus*, and *homo sapiens*.

In another preferred aspect, polypeptides having CoA synthetase activity as well as polynucleotides thereof can be obtained from *Escherichia coli*, *Rhodobacter sphaeroides*, *Saccharomyces cerevisiae*, and *Salmonella enterica*.

In another preferred aspect, polypeptides having polyhydroxyacid synthase activity as well as polynucleotides thereof can be obtained from *Comamonas acidovorans*, *Pseudomonas oleovorans*, *Ralstonia eutropha*, and *Rhodobacter sphaeroides*.

In another preferred aspect, polypeptides having lipase activity as well as polynucleotides thereof can be obtained from *Candida albicans*, *Candida rugosa*, and *Candida tropicalis*.

5 In another preferred aspect, polypeptides having acetyl-CoA carboxylase activity as well as polynucleotides thereof can be obtained from *Escherichia coli* and *Chloroflexus aurantiacus*.

In another preferred aspect, polypeptides having malonyl-CoA reductase activity as well as polynucleotides thereof can be obtained from *Acidianus brierleyi*, *Chloroflexus aurantiacus*, and *Sulfolobus metacillus*.

10 In another preferred aspect, polypeptides having propionyl-CoA hydrolase activity as well as polynucleotides thereof can be obtained from *Chloroflexus aurantiacus*. For example, *Chloroflexus aurantiacus* cells (ATCC 29365) can be used as a source of genomic DNA for cloning a polynucleotide encoding a polypeptide having CoA synthetase activity, dehydratase activity, and dehydrogenase activity (propionyl-
15 CoA synthetase) and a polynucleotide encoding a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (also referred to as acrylyl-CoA hydratase activity).

In another preferred aspect, polypeptides having acrylyl-CoA hydrolase activity as well as polynucleotides thereof can be obtained from *Chloroflexus aurantiacus*. For
20 example, *Chloroflexus aurantiacus* cells (ATCC 29365) can be used as a source of genomic DNA for cloning a polynucleotide encoding a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (also referred to as acrylyl-CoA hydratase activity).

Techniques used to isolate or clone a polynucleotide encoding a polypeptide
25 having enzyme activity are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the polynucleotide of interest from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR). See, for example, Innis *et al.*, 1990, *PCR Protocols: A Guide to Methods and Application*, Academic Press, New York. The cloning
30 procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into the mutant *E. coli* cell where multiple copies or clones of the nucleic acid sequence will be replicated. The polynucleotide may be of genomic, cDNA, semisynthetic, synthetic
35 origin, or any combinations thereof.

The nucleotide sequence or a subsequence thereof, as well as the amino acid sequence or a fragment thereof, of a known enzyme may be used to design nucleic acid

probes to identify and clone DNA encoding polypeptides having the same or similar enzyme activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, preferably at least 25, more preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may be used, e.g., nucleic acid probes that are preferably at least 600 nucleotides, more preferably at least 700 nucleotides, even more preferably at least 800 nucleotides, or most preferably at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P , ^3H , ^{35}S , biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may, therefore, be screened for DNA that hybridizes with the probes and encodes a polypeptide having enzyme activity. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA; the carrier material is preferably used in a Southern blot.

For purposes of the present invention, hybridization indicates that the nucleotide sequence hybridizes to a labeled nucleic acid probe under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at 45°C (very low stringency), more preferably at 50°C (low stringency), more preferably at 55°C

(medium stringency), more preferably at 60°C (medium-high stringency), even more preferably at 65°C (high stringency), and most preferably at 70°C (very high stringency).

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally.

For short probes of about 15 nucleotides to about 70 nucleotides in length, the carrier material is washed once in 6X SSC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m .

Polypeptide sequencing techniques can also be used to identify and obtain a polynucleotide that encodes a polypeptide having enzyme activity. For example, a purified polypeptide can be separated by gel electrophoresis, and its amino acid sequence determined by, for example, amino acid microsequencing techniques. Once determined, the amino acid sequence can be used to design degenerate oligonucleotide primers. The degenerate oligonucleotide primers can then be used to obtain the polynucleotide encoding the polypeptide by PCR.

Expression cloning techniques also can be used to identify and obtain such a polynucleotide. For example, a substrate known to interact with a particular enzymatic polypeptide can be used to screen a phage display library containing that enzymatic polypeptide. Phage display libraries can be generated as described elsewhere (Burritt *et al.*, 1990, *Anal. Biochem.* 238: 1-13), or can be obtained from commercial suppliers such as Novagen (Madison, WI, USA).

A polynucleotide encoding a polypeptide having enzyme activity can be mutated using common molecular cloning techniques (e.g., site-directed mutagenesis). Possible mutations include, without limitation, a substitution, deletion, and/or insertion of one or more (or several) amino acids of the polypeptide such that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, increase specific activity, improve or alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in the parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and

the resultant mutant molecules are tested for enzyme activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by
5 such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides
10 that are related to a polypeptide.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci.*
15 *USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman *et al.*, 1991, *Biochem.* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput,
20 automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a
25 polypeptide of interest, and can be applied to polypeptides of unknown structure.

For example, a polypeptide can have three functional domains: a domain having CoA synthetase activity, a domain having 3HP-CoA dehydratase activity, and a domain having CoA-reductase activity. Such polypeptides can be selectively modified by
30 mutating and/or deleting domains such that one or two of the enzyme activities are reduced. Reducing the dehydratase activity can cause acrylyl-CoA to be obtained from propionyl-CoA. The acrylyl-CoA then can be contacted with a polypeptide having CoA hydrolase activity to produce acrylate from propionate (Figure 5). Similarly, acrylyl-CoA can be obtained from 3HP by using, for example, such a polypeptide having reduced CoA reductase activity.

35 A polynucleotide encoding a polypeptide having enzyme activity may be manipulated in a variety of ways to provide for expression of the polynucleotide in an *E. coli* host cell. The construction of nucleic acid constructs and recombinant expression

vectors for the polynucleotide encoding a polypeptide of interest can be accomplished as described herein.

Nucleic Acid Constructs

5 Nucleic acid constructs can be prepared comprising an isolated polynucleotide encoding a polypeptide having enzyme activity operably linked to one or more (several) control sequences that direct the expression of the coding sequence in *E. coli* under conditions compatible with the control sequences. The isolated polynucleotide may be manipulated to provide for expression of the polypeptide. Manipulation of the
10 polynucleotide's sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotide sequences utilizing recombinant DNA methods are well known in the art.

The control sequence may be an appropriate promoter sequence, a nucleotide sequence that is recognized by a host cell for expression of a polynucleotide encoding a
15 polypeptide of the present invention. The promoter sequence contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any nucleotide sequence that shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to
20 the host cell.

Examples of suitable promoters for directing transcription of the nucleic acid constructs, especially in an *E. coli* host cell, are the *lac* promoter (Gronenborn, 1976, *Mol. Gen. Genet.* 148: 243-250), *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80: 21-25), *trc* promoter (Brosius *et al.*, 1985, *J. Biol. Chem.* 260: 3539-3541), T7 RNA polymerase promoter (Studier and Moffatt, 1986, *J. Mol. Biol.* 189: 113-130), phage promoter *p_L* (Elvin *et al.*, 1990, *Gene* 87: 123-126), *tetA* promoter (Skerra, 1994, *Gene* 151: 131-135), *araBAD* promoter (Guzman *et al.*, 1995, *J. Bacteriol.* 177: 4121-4130), and *rhaP_{BAD}* promoter (Haldimann *et al.*, 1998, *J. Bacteriol.* 180: 1277-1286). Other promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242: 74-94; and in Sambrook *et al.*,
30 Molecular cloning: a laboratory manual, Cold Spring Harbour Laboratory Press, New York, USA, second edition (1989).

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator
35 sequence is operably linked to the 3' terminus of the nucleotide sequence encoding the polypeptide. Any terminator that is functional in an *E. coli* cell may be used in the present invention.

It may also be desirable to add regulatory sequences that allow regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems.

Expression Vectors

Recombinant expression vectors can be prepared comprising a polynucleotide described herein, a promoter, and transcriptional and translational stop signals. The various nucleic acids and control sequences described herein may be joined together to produce a recombinant expression vector that may include one or more (several) convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the polypeptide at such sites. Alternatively, a polynucleotide sequence may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or bacteriophage) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the nucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the *E. coli* into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

The vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

The vectors preferably contain one or more (several) selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of bacterial selectable markers are markers that confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, or tetracycline resistance.

The vectors preferably contain an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell
5 independent of the genome.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing
10 integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which have a high degree of identity to
15 the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous
20 recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator"
25 is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate *in vivo*.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*.

More than one copy of a polynucleotide encoding a polypeptide having enzyme
30 activity may be inserted into a host cell to increase production of the gene product. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the
35 polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent. For example, the *E. coli* cells described herein can

contain a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular polynucleotide.

The procedures used to ligate the elements described herein to construct the recombinant expression vectors are well known to one skilled in the art (see, e.g.,
5 Sambrook *et al.*, 1989, *supra*).

Host Cells

In practicing the methods of the present invention, the host cells are *E. coli* strains that have been engineered to redirect a metabolic pathway to production of 3HP
10 or a compound derived from 3HP in commercial relevant quantities. In addition, the metabolically engineered *E. coli* strains preferably have overcome (1) osmotic stress caused by high concentrations of substrates and/or products and (2) the toxicity of high concentrations of products.

In a preferred aspect, a metabolically engineered *E. coli* strain contains one or
15 more foreign polynucleotides that encode polypeptides necessary to perform one or more of the steps shown in any of Figures 1-7. Such cells can contain any number of foreign polynucleotides. The genetically modified *E. coli* cells are preferably capable of producing 3HP when grown under a variety of fermentation conditions, so that a high yield of 3HP results from relatively inexpensive raw products such as glucose or
20 sucrose.

The metabolically engineered *E. coli* strains may also have reduced polypeptide activity. The term "reduced" as used herein with respect to a cell and a particular polypeptide's activity refers to a lower level of activity than that measured in a comparable cell of the same species. For example, a particular *E. coli* strain lacking an
25 enzyme activity is considered to have reduced enzyme activity if a comparable *E. coli* strain has at least some enzyme activity. It is noted that a cell can have the activity of any type of polypeptide reduced including, without limitation, enzymes, transcription factors, transporters, receptors, signal molecules, and the like. For example, a cell can contain a foreign polynucleotide that disrupts a regulatory and/or coding sequence of a
30 polypeptide having pyruvate decarboxylase activity or alcohol dehydrogenase activity. Disrupting pyruvate decarboxylase and/or alcohol dehydrogenase expression can lead to the accumulation of lactate as well as products produced from lactate such as 3HP, 1,3-propanediol, acrylic acid, polyacrylate, acrylate esters, 3HP esters, and poly-3HP. Reduced polypeptide activities can be the result of lower polypeptide concentration,
35 lower specific activity of a polypeptide, or combinations thereof. Any method known in the art can be used to make a cell having reduced polypeptide activity.

A cell having reduced activity of a polypeptide can be identified using any method known in the art. For example, enzyme activity assays can be used to identify cells having a reduced enzyme activity. See *Enzyme Nomenclature*, Academic Press, Inc., New York, 2007.

5 *Escherichia coli* strains useful in the present invention include, but are not limited to, SZ32, SZ37, SZ40, SZ58, SZ63, LY52, SZ110, SZ132, SZ136, SZ162, SZ186, SZ194, TG102, TG103, TG105, TG106, TG107, TG108, TG112, TG113, TG114, TG128, TG129, TG130, XZ103, XZ104, XZ105, XZ106, XZ107, XZ108, XZ109, XZ110, XZ111, XZ112, XZ113, XZ115, XZ121, XZ123, XZ126, XZ129, XZ130, XZ131 and
10 XZ132 (Zhou *et al.*, 2003, 2005, 2006b, 2007; Shukla *et al.* 2004, Grabar *et al.*, 2006; Zhang *et al.*, 2007). Strains SZ32, SZ37, SZ40, SZ58, SZ63, SZ110, SZ132, SZ136, SZ162, SZ186, SZ194, TG102, TG103, TG105, TG106, TG107, TG108, TG112, TG113, TG114, TG128, TG129, TG130 were engineered to produce increased levels of lactic acid (Zhou *et al.*, 2003, 2005, 2006b, 2007, *supra*; Shukla *et al.*, 2004, Grabar *et al.*,
15 2006). Strains XZ103, XZ104, XZ105, XZ106, XZ107, XZ108, XZ109 and XZ110 were obtained from the high lactic acid-producing strain SZ194 by replacing the native D-lactate dehydrogenase gene with the *alaD* (alanine dehydrogenase) gene from *Geobacillus stearothermophilus*, thereby replacing lactic acid production with L-alanine production (Zhang *et al.*, 2007). Strains XZ111, XZ112, XZ113, XZ115, XZ121, XZ123,
20 XZ126, XZ129, XZ130, XZ131 and XZ132 were obtained from strain XZ105 for improved alanine production through additional metabolic engineering and metabolic evolution as described by Zhang *et al.*, 2007, *supra*.

E. coli hosts useful in the present invention are available from the Agricultural Research Service Culture Collection, 1815 N. University Street, Peoria, IL, USA. The
25 accession numbers are as follows: NRRL B-30861 (SZ132), NRRL B-30862 (SZ186), NRRL B-30863 (SZ194), NRRL B-30864 (TG103), NRRL B-30921 (TG102), NRRL B-30922 (TG105), NRRL B-30923 (TG106), NRRL B-30924 (TG107), NRRL B-30925 (TG108), NRRL B-30926 (TG112), NRRL B-30927 (TG113), NRRL B-30928 (TG114), NRRL B-30962 (TG128), NRRL B-30963 (TG129), and NRRL B-30964 (TG130).

30 In one aspect of the present invention, the metabolically engineered *E. coli* strain comprises one or more (several) mutations and/or deletions in the genes *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, and *atpFH*.

In another aspect, the native lactate dehydrogenase gene is inactivated.

In another aspect, the native lactate dehydrogenase gene is replaced with the
35 *alaD* (alanine dehydrogenase) gene from *Geobacillus stearothermophilus* to replace lactate production with alanine production.

In another aspect, the *E. coli* strain is metabolically engineered to increase 3HP biosynthesis from glucose, sucrose, or other sugars via pyruvate through lactate in the presence of a functional *ldhA* gene by incorporating one or more (several) mutations and/or deletions in the genes *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, *alaD*, and *atpFH* (Causey *et al.*, 2004, *Proc. Natl. Acad. Sci. USA* 101: 2235-2240; Zhou *et al.*, 2003, *supra*; Zhou *et al.*, 2005, *supra*), and engineering the metabolic steps leading from pyruvate to 3HP to allow for unimpeded flow of intermediates from pyruvate to 3HP.

In another aspect, the *E. coli* strain is metabolically engineered to increase 3HP biosynthesis via pyruvate through acetyl-CoA by incorporating one or more (several) mutations and/or deletions in the genes *ldhA*, *alaD*, *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, and *atpFH*, and engineering the metabolic steps leading from pyruvate to 3HP to allow for unimpeded flow of intermediates from pyruvate to 3HP.

In another aspect, the *E. coli* strain is metabolically engineered to increase 3HP biosynthesis via propionate through propionyl-CoA and acrylyl-CoA by engineering the metabolic steps leading from propionate to 3HP to allow for unimpeded flow of intermediates from propionate to 3HP.

In another aspect, the *E. coli* strain is metabolically engineered to increase 3HP biosynthesis via phosphoenolpyruvate or pyruvate through beta-alanine and acrylyl-CoA by incorporating one or more (several) mutations and/or deletions in the genes *ldhA*, *alaD*, *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, and *atpFH*, and engineering the metabolic steps leading from phosphoenolpyruvate or pyruvate to 3HP to allow for unimpeded flow of intermediates from phosphoenolpyruvate or pyruvate to 3HP.

In another aspect, the *E. coli* strain is metabolically engineered to increase 3HP biosynthesis via phosphoenolpyruvate or pyruvate through beta-alanine and malonate semialdehyde by incorporating one or more (several) mutations and/or deletions in the genes *ldhA*, *alaD*, *adhE*, *ackA*, *frdA*, *pflB*, *poxB*, and *atpFH*, and engineering the metabolic steps leading from phosphoenolpyruvate or pyruvate to 3HP to allow for unimpeded flow of intermediates from phosphoenolpyruvate or pyruvate to 3HP.

In another aspect, the *E. coli* strain comprises lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity. In another aspect, the *E. coli* strain produces one or more of the following polypeptides: a polypeptide having E1 activator activity; an E2 alpha polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 beta polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; and a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity. Additionally, the strain can have CoA transferase activity, CoA synthetase activity, polyhydroxyacid synthase activity, 3-hydroxypropionyl-CoA hydrolase activity, 3-hydroxyisobutryl-CoA hydrolase activity, alanine dehydrogenase,

alanine aminotransferase, alanine 2,3-aminomutase, and/or lipase activity. Moreover, the *E. coli* strain can contain at least one foreign polynucleotide that encodes one or more polypeptides that have CoA transferase activity, 3-hydroxypropionyl-CoA hydrolase activity, 3-hydroxyisobutryl-CoA hydrolase activity, CoA synthetase activity, polyhydroxyacid synthase activity, alanine dehydrogenase, alanine aminotransferase, alanine 2,3-aminomutase, and/or lipase activity.

In another aspect, the *E. coli* strain comprising, for example, lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity produces a product, for example, 3HP, polymerized 3HP, and/or an ester of 3HP, such as methyl hydroxypropionate, ethyl hydroxypropionate, propyl hydroxypropionate, and/or butyl hydroxypropionate. Accordingly, the present invention also provides methods of producing one or more of these products. These methods involve culturing the *E. coli* strain comprising, for example, lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity under conditions that allow the product to be produced. These strains also can have, for example, CoA synthetase activity and/or polyhydroxyacid synthase activity.

In another aspect, the *E. coli* strain comprises CoA synthetase activity, lactyl-CoA dehydratase activity, and polyhydroxyacid synthase activity for production of polymerized acrylate. In some aspects, these cells also can comprise a foreign polynucleotide that encodes one or more of the following polypeptides: a polypeptide having E1 activator activity; an E2 alpha polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 beta polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; a polypeptide having CoA synthetase activity; and a polypeptide having polyhydroxyacid synthase activity.

In another aspect of the present invention, the *E. coli* strain comprises CoA transferase activity, lactyl-CoA dehydratase activity, and lipase activity. In some aspects, the strain also can comprise a foreign polynucleotide that encodes one or more of the following polypeptides: a polypeptide having CoA transferase activity; a polypeptide having E1 activator activity; an E2 alpha polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 beta polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; and a polypeptide having lipase activity. Such a strain can be used, for example, to produce products such as esters of acrylate (e.g., methyl acrylate, ethyl acrylate, propyl acrylate, and butyl acrylate).

A foreign polynucleotide can be introduced into an *E. coli* cell using any method known in the art such as protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145).

Methods of Producing 3HP and Compounds Derived from 3HP

The *E. coli* strains and polypeptides having enzyme activity described herein can be used to produce 3HP and/or other organic compounds derived from 3HP such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3HP, and polymerized 3HP. Consequently, the present invention also relates to methods for producing 3HP and compounds derived from 3HP via lactate, phosphoenolpyruvate, pyruvate, acetyl-CoA, propionyl-CoA, or beta-alanine as intermediates. These methods involve culturing an *E. coli* strain comprising at least one foreign polynucleotide that encodes at least one polypeptide such that 3HP is produced.

Typically, 3HP is produced by culturing the *E. coli* strain in a culture medium such that 3HP is produced. In general, the culture media and/or culture conditions can be such that the microorganisms grow to an adequate density and produce 3HP efficiently. For large-scale production processes, any method can be used such as those described elsewhere (Manual of Industrial Microbiology and Biotechnology, 2nd Edition, Editors: A. L. Demain and J. E. Davies, ASM Press; and Principles of Fermentation Technology, P. F. Stanbury and A. Whitaker, Pergamon). Briefly, a large tank (e.g., a 100 gallon, 200 gallon, 500 gallon, or more fermentation tank) containing appropriate culture medium with, for example, glucose as a carbon source is inoculated with a particular microorganism. After inoculation, the microorganisms are incubated to allow biomass to be produced. Once a desired biomass is reached, the broth containing the microorganisms can be transferred to a second tank. This second tank can be any size. For example, the second tank can be larger, smaller, or the same size as the first tank. Typically, the second tank is larger than the first such that additional culture medium can be added to the broth from the first tank. In addition, the culture medium within this second tank can be the same as, or different from, that used in the first tank. For example, the first tank can contain medium with xylose, while the second tank contains medium with glucose.

Once transferred, the microorganisms can be incubated to allow for the production of 3HP. Once produced, any method can be used to isolate the 3HP. For example, common separation techniques can be used to remove the biomass from the broth, and common isolation procedures (e.g., extraction, distillation, and ion-exchange procedures) can be used to obtain the 3HP from the microorganism-free broth. In addition, 3HP can be isolated while it is being produced, or it can be isolated from the broth after the product production phase has been terminated. If 3HP and/or the other organic compounds derived from 3HP are secreted into the nutrient medium, the

products can be recovered directly from the medium. If the products are not secreted into the medium, they can be recovered from cell lysates.

In other aspects of the present invention, cells are separated and collected from a culture of transformed cells; processed transformed cells are subjected to acetone treatment or lyophilization; a cell-free extract is prepared from such transformed microorganism cells or processed cells; fractions such as membrane fractions are fractionated from such cell-free extract; or immobilized materials can be produced by immobilizing transformed microorganism cells, processed cells, cell-free extract and fractions, any of which independently or combined contacted with sucrose and/or glucose to produce 3HP. The microorganism can consist of one kind of microorganism, or can be used as an arbitrary mixture of two or more kinds of microorganisms.

Growth and production of 3HP can be performed in normal batch fermentations, fed-batch fermentations or continuous fermentations. In certain aspects, it is desirable to perform fermentations under reduced oxygen or anaerobic conditions for certain hosts. In other aspects, 3HP production can be performed with oxygen; and, optionally with the use of air-lift or equivalent fermentors.

Fermentation parameters are dependent on the *E. coli* strain used for production of the recombinant enzyme. Cultivation of the metabolically engineered *E. coli* strains of the present invention is preferably performed under aerobic or anaerobic conditions for about 0.5 to 240 hours. The cultivation temperature is preferably controlled at about 25°C to 45°C, and pH is preferably controlled at 5-8 during cultivation. Inorganic or organic, acidic, or alkaline substances as well as ammonia gas or the like can be used for pH adjustment. The pH of the fermentation should be sufficiently high enough to allow growth and 3HP production by the host. Adjusting the pH of the fermentation broth may be performed using neutralizing agents such as calcium carbonate or hydroxides. Alternatively, 3HP can be removed continuously during the fermentation using methods such as membrane technology, electro-dialysis, solvent extraction, and absorbent resins. The selection and incorporation of any of the above fermentation methods is highly dependent on the *E. coli* strain and the preferred downstream process.

Growth medium may be minimal/defined or complete/complex. Fermentable carbon sources could include hexose and pentose sugars (e.g., ribose, arabinose, xylose, and lyxose), starch, cellulose, xylan, oligosaccharides, and combinations thereof. Examples of carbohydrates that cells are capable of metabolizing to pyruvate include sugars such as dextrose, triglycerides, and fatty acids. One form of growth media that can be used in accordance with the subject invention includes modified Luria-Bertani (LB) broth (with 10 g Difco tryptone, 5 g Difco yeast extract, and 5 g

sodium chloride per liter) as described by Miller, 1992, *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Press. In other aspects of the present invention, cultures of metabolically engineered *E. coli* strains of the present invention can be grown in NBS mineral salts medium (as described by Causey *et al.*, 2004, *Proc. Natl. Acad. Sci. USA* 101: 2235-2240) and supplemented with 2% to 20% sugar (w/v) or either 5% or 10% sugar (glucose or sucrose). The microorganisms can be grown in or on NBS mineral salts medium. NBS mineral salts medium comprises, consists essentially of, or consists of the following components (per liter): 3.5 g of KH_2PO_4 ; 5.0 g of K_2HPO_4 ; 3.5 g of $(\text{NH}_4)_2\text{HPO}_4$; 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 15 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 0.5 mg of thiamine; and 1 ml of trace metal stock, glucose (e.g., 2% in plates or 3% in broth), and 1.5% agar (for plates). The trace metal stock is prepared in 0.1 M HCl and comprises, consists essentially of, or consists of (per liter): 1.6 g of FeCl_3 ; 0.2 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; 0.1 g of CuCl_2 ; 0.2 g of $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$; 0.2 g of NaMoO_4 ; and 0.05 g of H_3BO_3 . 4-Morpholinopropanesulfonic acid (0.1 M, pH 7.1) can be added to both liquid and solid media (filter-sterilized) when needed for pH control (and is optionally included in medium used for 10-liter fermentations). Minimal medium can also be prepared by using succinate (1 g/liter) as a sole source of carbon (nonfermentable substrate) and can be added as a supplement to glucose-minimal medium when needed. In certain aspects, antibiotics can be included as needed.

In one aspect of the present invention, the methods produce 3HP via pyruvate through lactate by engineering the metabolic steps leading from pyruvate to 3HP to allow for unimpeded flow of intermediates from pyruvate to 3HP as shown in Figure 1.

In another aspect, the methods produce 3HP via pyruvate through acetyl-CoA by engineering the metabolic steps leading from pyruvate to 3HP to allow for unimpeded flow of intermediates from pyruvate to 3HP as shown in Figure 4.

In another aspect, the methods produce 3HP from propionate through propionyl-CoA and acrylyl-CoA by engineering the metabolic steps leading from pyruvate to 3HP to allow for unimpeded flow of intermediates from propionate to 3HP as shown in Figure 5.

In another aspect, the methods produce 3HP via phosphoenolpyruvate or pyruvate through beta-alanine and acrylyl-CoA by engineering the metabolic steps leading from phosphoenolpyruvate or pyruvate to 3HP to allow for unimpeded flow of intermediates from phosphoenolpyruvate or pyruvate to 3HP as shown in Figure 6.

In another aspect, the methods produce 3HP via phosphoenolpyruvate or pyruvate through beta-alanine and malonate semialdehyde by engineering the metabolic

steps leading from phosphoenolpyruvate or pyruvate to 3HP to allow for unimpeded flow of intermediates from phosphoenolpyruvate or pyruvate to 3HP as shown in Figure 7.

In another aspect, the methods produce 3HP wherein lactate is contacted with a first polypeptide having CoA transferase activity or CoA synthetase activity such that lactyl-CoA is formed, then contacting lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA, then contacting acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-hydroxypropionic acid-CoA, and then contacting 3-hydroxypropionic acid-CoA with the first polypeptide to form 3HP or with a fourth polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity to form 3HP.

In another aspect, the methods produce polymerized 3HP. These methods involve producing 3-hydroxypropionic acid-CoA as described herein, and then contacting the 3-hydroxypropionic acid-CoA with a polypeptide having polyhydroxyacid synthase activity to form polymerized 3HP.

In another aspect, the methods produce an ester of 3HP. These methods involve producing 3HP as described herein, and then additionally contacting 3HP with a fifth polypeptide having lipase activity to form an ester.

The organic compounds produced from any of the steps provided in Figures 1-7 can be chemically converted into other organic compounds. For example, 3HP can be hydrogenated to form 1,3-propanediol, a valuable polyester monomer. Hydrogenating an organic acid such as 3HP can be performed using any method such as those used to hydrogenate succinic acid and/or lactic acid. For example, 3HP can be hydrogenated using a metal catalyst. In another example, 3HP can be dehydrated to form acrylic acid. Any method can be used to perform a dehydration reaction. For example, 3HP can be heated in the presence of a catalyst (e.g., a metal or mineral acid catalyst) to form acrylic acid. Propanediol also can be obtained using polypeptides having oxidoreductase activity (e.g., enzymes is the 1.1.1.- class of enzymes) *in vitro* or *in vivo*.

In another aspect, the methods produce polymerized acrylate. These methods involve culturing a cell that has both CoA synthetase activity, lactyl-CoA dehydratase activity, and polyhydroxyacid synthase activity such that polymerized acrylate is produced. Accordingly, the present invention also provides methods of producing polymerized acrylate wherein lactate is contacted with a first polypeptide having CoA synthetase activity to form lactyl-CoA, then contacting lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA, and then contacting acrylyl-CoA with a third polypeptide having polyhydroxyacid synthase activity to form polymerized acrylate.

In another aspect, the methods produce an ester of acrylate. These methods involve culturing an *E. coli* cell that has CoA transferase activity, lipase activity, and lactyl-CoA dehydratase activity under conditions that allow the cell to produce an ester.

In another aspect, the methods produce an ester of acrylate, wherein acrylyl-CoA is formed as described herein, and then acrylyl-CoA is contacted with a polypeptide having CoA transferase activity to form acrylate, and acrylate is contacted with a polypeptide having lipase activity to form the ester.

In another aspect, the methods produce 1,3-propanediol, which can be obtained from either 3HP-CoA or 3HP via the use of polypeptides having enzyme activity described herein. These polypeptides can be used either *in vitro* or *in vivo*. When converting 3HP-CoA to 1,3-propanediol, polypeptides having oxidoreductase activity or reductase activity (e.g., enzymes from the 1.1.1.-class of enzymes) can be used. Alternatively, when creating 1,3-propanediol from 3HP, a combination of (1) a polypeptide having aldehyde dehydrogenase activity (e.g., an enzyme from the 1.1.1.34 class) and (2) a polypeptide having alcohol dehydrogenase activity (e.g., an enzyme from the 1.1.1.32 class) can be used.

In another aspect, the methods produce 3HP or a compound derived from 3HP in a concentration of at least about 100 mg per L (e.g., at least about 1 g/L, 5 g/L, 10 g/L, 25 g/L, 50 g/L, 75 g/L, 80 g/L, 90 g/L, 100 g/L, 120 g/L or 130 g/L).

When determining the yield of an organic compound, such as 3HP, for a particular cell, any method can be used. See, e.g., *Applied Environmental Microbiology* 59: 4261-4265 (1993) and Sullivan and Clarke, 1955, *J. Assoc. Offic. Agr. Chemists*, 38: 514-518.

25 Production of 3HP and Related Products Via *in vitro* Techniques

The purified polypeptides having enzyme activity described herein can be used alone or in combination with cells to produce 3HP or other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3HP, and polymerized 3HP. For example, a preparation containing a substantially pure polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can be used to catalyze the formation of 3HP-CoA, a precursor to 3HP. Further, cell-free extracts containing a polypeptide having enzyme activity can be used alone or in combination with purified polypeptides and/or cells to produce 3HP. For example, a cell-free extract containing a polypeptide having CoA transferase activity can be used to form lactyl-CoA, while a microorganism containing polypeptides have the enzyme activities necessary to catalyze the reactions needed to form 3HP from lactyl-CoA can be used to produce 3HP. Any method can be used to produce a cell-free extract. For example, osmotic

shock, sonication, and/or a repeated freeze-thaw cycle followed by filtration and/or centrifugation can be used to produce a cell-free extract from intact cells.

3HP produced by a metabolically engineered *E. coli* strain, purified polypeptide, and/or cell-free extract can be treated chemically to produce another compound. For example, a microorganism can be used to produce 3HP, while a chemical process is used to modify 3HP into a derivative such as polymerized 3HP or an ester of 3HP. Likewise, a chemical process can be used to produce a particular compound that is converted into 3HP or other organic compound (e.g., 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3HP, and polymerized 3HP) using a cell, substantially pure polypeptide, and/or cell-free extract described herein. For example, a chemical process can be used to produce acrylyl-CoA, while a microorganism can be used convert acrylyl-CoA into 3HP.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Examples

All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1: Inactivation of the *ldhA* gene or the cloned *Geobacillus stearothermophilus alaD* gene in high lactic acid-producing or high alanine-producing strains of *E. coli*

The starting *Escherichia coli* strains for further genetic manipulation include, but are not limited to, SZ32, SZ37, SZ40, SZ58, SZ63, LY52, SZ110, SZ132, SZ136, SZ162, SZ186, SZ194, TG102, TG103, TG105, TG106, TG107, TG108, TG112, TG113, TG114, TG128, TG129, TG130, XZ103, XZ104, XZ105, XZ106, XZ107, XZ108, XZ109, XZ110, XZ111, XZ112, XZ113, XZ115, XZ121, XZ123, XZ126, XZ129, XZ130, XZ131 and XZ132.

To convert the high lactic acid- or alanine-producing *E. coli* strains listed above to a high 3HP-producing strain, genetic modification at the *ldhA* gene locus is required to eliminate the lactate dehydrogenase activity, thereby increasing the availability of pyruvate for 3HP biosynthesis. The modifications to the *ldhA* gene locus are one or more mutations, deletions, insertions, or substitutions and are achieved using any of a number of established *E. coli* genetic modification methods that are well known in the art. For example, deletion of the *ldhA* gene is achieved using the method of Datsenko

and Wanner (2000, *Proc. Natl. Acad. Sci.* 97: 6640-6645), while insertion or gene substitution is achieved using essentially the same procedure used by Zhang *et al.*, 2007, *Appl Microbiol Biotechnol.* 77: 355-366, to integrate the *Geobacillus stearothermophilus alaD* gene into the *ldhA* gene. In the case of the alanine-producing strains, the *Geobacillus stearothermophilus alaD* gene, which is inserted into the *E. coli* native *ldhA* gene in a way that results in the *alaD* gene being expressed from the native *ldhA* promoter sequence, is inactivated using the same well-known *E. coli* genetic methods as used for inactivation of the *ldhA* gene (Datsenko and Wanner, 2000, *supra*).

The resulting strains in which the *ldhA* gene or *Geobacillus stearothermophilus alaD* gene is inactivated retain all other properties that contribute to high lactic acid or alanine production when the *ldhA* or *Geobacillus stearothermophilus alaD* gene are functional.

Example 2: Cloning of genes encoding enzymes involved in conversion of pyruvate to 3HP

Multiple metabolic routes have been described for the *in vivo* conversion of pyruvate to 3HP (WO 02/042418; WO 03/062173, WO 2007/042494, Straathof *et al.*, 2005, *supra*). Any combination of genes that enable biosynthesis of 3HP (WO 02/042418; WO 03/062173, WO 2007/042494, Straathof *et al.*, 2005, *supra*) is introduced into any of the *E. coli* strains listed in Example 1 or strains derived from the strains listed in Example 1 by inactivating the *ldhA* gene or the *Geobacillus stearothermophilus alaD* gene. The resulting 3HP-producing strains retain all other properties that contributed to high lactic acid or alanine production when the *ldhA* or *Geobacillus stearothermophilus alaD* gene are functional and the genes required for 3HP production are not introduced.

Cloning of genes required for 3HP production and introduction of the cloned DNA into any of the *E. coli* strains listed in Example 1 is performed using standard techniques known to one skilled in the art (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). Cloned genes may be introduced into any of the *E. coli* strains listed in Example 1 in the form of replicating plasmids or may be integrated at any site in the *E. coli* genome (Datsenko and Wanner, 2000, *supra*), which does not result in a non-viable strain. Expression of the cloned genes required for 3HP production in any of the *E. coli* strains listed in Example 1 is achieved using any DNA sequence that promotes expression of the cloned gene in any of the *E. coli* strains listed in Example 1. For example, expression of the desired cloned genes may be achieved by cloning the genes under genetic regulatory control of promoters that effect gene expression in *E. coli* such as, but

not limited to, the *lac*, *tac*, *araB*, *trp*, *trc*, *amyE*, *spac*, or lambda phage PL or PR promoters. Cloned genes required for 3HP production can also be cloned under control of native or genetically modified *E. coli* chromosomal promoter sequences such as the *ldhA* promoter, as was done for cloning the *alaD* gene from *Geobacillus*
5 *stearothermophilus* under control of the *ldhA* gene promoter in the high alanine producing strains listed in Example 1.

Example 3: Improving 3HP-producing *E. coli* strains by random or targeted mutagenesis procedures

10 3HP-Producing *E. coli* strains obtained as described in Example 2 are further improved by random or targeted mutagenesis procedures (Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; WO 95/22625; Lowman *et al.*, 1991, *Biochem.* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204; Derbyshire *et al.*, 1986, *Gene* 46:
15 145; Ner *et al.*, 1988, *DNA* 7: 127; Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896; Cunningham and Wells, 1989, *Science* 244: 1081-1085; Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708). Any of the high lactic acid- or alanine-producing strains listed in Example 1 and modified to produce 3HP by any of the methods described in Example 2 is treated with chemicals such as N-methyl-N'-nitro-N-nitrosoguanidine or ethyl
20 methane sulfonate, or treated with ultraviolet radiation, to cause mutations in the strain's genetic material. Alternatively, the *E. coli* strains are subjected to, for example, transposon mutagenesis using methods well known to one skilled in the art. Following the mutagenic treatment, the population of *E. coli* cells is screened for cells having improved 3HP production compared to the parental *E. coli* strain (*i.e.*, the
25 unmutagenized strain).

The resulting 3HP-producing mutants retain all properties that contribute to high lactic acid or alanine production when the *ldhA* or *Geobacillus stearothermophilus alaD* gene is functional compared to when the genes required for 3HP production are not introduced and/or there is no mutagenesis.

Example 4: Growth of 3HP-producing *E. coli* strains

3HP production using any of the *E. coli* strains described in Example 2 or 3 is achieved by cultivating the cells in any medium and under any cultivation conditions that allow growth of the *E. coli* strains described in Examples 1, 2 or 3. For example, any of
35 the *E. coli* strains described in Examples 1, 2 or 3 are cultivated in a nutrient-rich medium such as Luria-Bertani (LB) broth or nutrient broth, or in a chemically defined medium such as (but not limited to) NBS mineral salts medium (Causey *et al.*, 2004,

supra). The carbon source used for growth in any mineral salts medium is glucose, sucrose, pentoses or any other carbon source that supports the growth of *E. coli*. The carbon sources may be well established commercially available compositions such as dextrose syrup, or may be present in mixtures obtained following physical, chemical and/or enzymatic degradation of lignocellulosic biomass.

The cultivation conditions, including but not limited to, time, temperature, pH, oxygen supply (anaerobic, aerobic or minimally aerobic conditions) and stirring, may be varied in any way provided that the conditions allow growth of the 3HP-producing *E. coli* strains. Growth and production of 3HP using any of the *E. coli* strains described in Examples 2 or 3 may be performed in batch, fed-batch, or continuous cultivation processes, and may be performed with or without addition of osmoprotectants such as, but not limited to, betaine, to the cultivation medium.

Under suitable cultivation conditions, the *E. coli* strains described in Examples 2 and 3 are able produce 3HP in the cultivation medium at concentrations and yields similar to the lactic acid or alanine production in the lactic acid- or alanine-producing strains from which they were derived. For example, 3HP concentration in the culture broth ranges from 0.5 M to 1.3 M, and the yield of 3HP (based on substrate, e.g., glucose, consumed) ranges from 80% to 99%. Co-products such as succinic acid, acetate and ethanol, if produced, are produced at concentrations below 100 mM.

Example 5: Analysis of cell mass, organic acids, sugars and other compounds present in cultivation broths

Cell mass of any of the *E. coli* strains described in Example 1, 2 or 3 are measured using any of a number of methods well known in the art, such as measurement of optical density and measurement of cell dry weight. Organic acids, sugars, and other compounds are measured in the cultures of any of the *E. coli* strains described in Example 1, 2 or 3, grown as described in Example 4, using analytical techniques that are well known in the art. For example, organic acids, and sugars are measured by high performance liquid chromatography (Zhou *et al.*, 2003, *supra*) or by ultraperformance liquid chromatography-tandem mass spectrometry (Ross *et al.*, 2007, *Anal. Chem.* 79: 4840-4844) while ethanol is measured by gas chromatography, as described, for example, by Zhou *et al.*, 2003, *supra*.

Example 6: Recovery of 3HP from culture broth

The 3HP present in the culture broth is recovered by any method known in the art including, but not limited to, acidification of the broth with a mineral acid, such as sulfuric acid, to allow recovery of the 3HP with calcium sulfate as a byproduct (WO

2002/090312), extraction of 3HP using organic solvents (WO 2002/090312, WO 2005/003074, WO 2005/021470), or electrodilaysis.

5 The invention described and claimed herein is not to be limited in scope by the
specific aspects herein disclosed, since these aspects are intended as illustrations of
several aspects of the invention. Any equivalent aspects are intended to be within the
scope of this invention. Indeed, various modifications of the invention in addition to
those shown and described herein will become apparent to those skilled in the art from
the foregoing description. Such modifications are also intended to fall within the scope
10 of the appended claims. In the case of conflict, the present disclosure including
definitions will control.

Claims

What is claimed:

- 5 1. A method for producing 3-hydroxypropionic acid (3HP), comprising cultivating a metabolically engineered *E. coli* strain derived from a strain previously engineered for high lactic acid or high L-alanine production in a cultivation medium, wherein the genetically modified *E. coli* strain produces 3-hydroxypropionic acid (3HP).

- 10 2. The method of claim 1, wherein the previously engineered *E. coli* strain is selected from the group consisting of SZ32, SZ37, SZ40, SZ58, SZ63, LY52, SZ110, SZ132 (NRRL B30861), SZ136, SZ162, SZ186 (NRRL B-30862), SZ194 (NRRL B-30863), TG102 (NRRL B-30921), TG103, (NRRL B-30864), TG105 (NRRL B-30922), TG106 (NRRL B-30923), TG107 (NRRL B-30924), TG108 (NRRL B-30925), TG112
 15 (NRRL B-30926), TG113 (NRRL B-30927), TG114 (NRRL B-30928), TG128 (NRRL B-30962), TG129 (NRRL B-30963), TG130 (NRRL B-30964), XZ103, XZ104, XZ105, XZ106, XZ107, XZ108, XZ109, XZ110, XZ111, XZ112, XZ113, XZ115, XZ121, XZ123, XZ126, XZ129, XZ130, XZ131 and XZ132.

- 20 3. The method of claim 1, wherein the metabolically engineered *E. coli* strain comprises (a) CoA transferase activity, (b) lactyl-CoA dehydratase activity, (c) 3-hydroxypropionyl-CoA dehydratase activity, and (d) 3-hydroxypropionyl-CoA hydrolase activity.

- 25 4. The method of claim 1, wherein the metabolically engineered *E. coli* strain comprises acetyl-CoA carboxylase activity and malonyl-CoA reductase activity.

- 30 5. The method of claim 1, wherein the metabolically engineered *E. coli* strain comprises activities selected from the group consisting of PEP carboxylase activity, pyruvate carboxylase activity, aspartate aminotransferase activity, aspartate decarboxylase activity, CoA transferase activity, beta-alanyl-CoA ammonia lyase activity, 3HP-CoA dehydratase activity, alanine dehydrogenase activity, alanine aminotransferase activity, and alanine 2,3-aminomutase activity.

- 35 6. The method of claim 1, wherein the metabolically engineered *E. coli* strain comprises activities selected from the group consisting of PEP carboxylase activity, pyruvate carboxylase activity, aspartate aminotransferase activity, aspartate

decarboxylase activity, alanine dehydrogenase activity, alanine aminotransferase activity, alanine 2,3-aminomutase activity, beta-alanine aminotransferase activity, and 3HP dehydrogenase activity.

5 7. The method of claim 1, wherein the metabolically engineered *E. coli* strain comprises one or more (several) mutations and/or deletions in the genes *ldhA*, *alaD*, *adhE*, *ackA*, *frdA*, *pflB*, *poxB* and *atpFH*.

10 8. The method of claim 1, wherein the native lactate dehydrogenase gene in the metabolically engineered *E. coli* strain is inactivated.

9. The method of claim 1, wherein the metabolically engineered *E. coli* strain does not produce lactic acid.

15 10. The method of claim 1, wherein the native lactate dehydrogenase gene in the metabolically engineered *E. coli* strain is replaced with the *alaD* (alanine dehydrogenase) gene from *Geobacillus stearothermophilus* to replace lactate production with alanine production, and the *Geobacillus stearothermophilus alaD* (alanine dehydrogenase) gene is inactivated.

20 11. The method of claim 1, wherein the metabolically engineered *E. coli* strain does not produce alanine via the alanine dehydrogenase encoded by the cloned *Geobacillus stearothermophilus alaD* (alanine dehydrogenase) gene.

25 12. The method of claim 1, wherein the metabolically engineered *E. coli* strain is cultured in a medium comprising a carbohydrate as a carbon source.

13. The method of claim 12, wherein the carbohydrate is glucose or sucrose.

30 14. The method of claim 1, further comprising recovering the 3HP.

15. The method of claim 1, wherein the 3HP is further converted to a compound selected from the group consisting of 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3HP, and esters of 3HP.

35

16. The method of claim 15, further comprising recovering the compound selected from the group consisting of 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3HP, and esters of 3HP.
- 5 17. The method of claim 1, wherein the metabolically engineered *E. coli* strain comprises one of the pathways described in Figures 1-7.

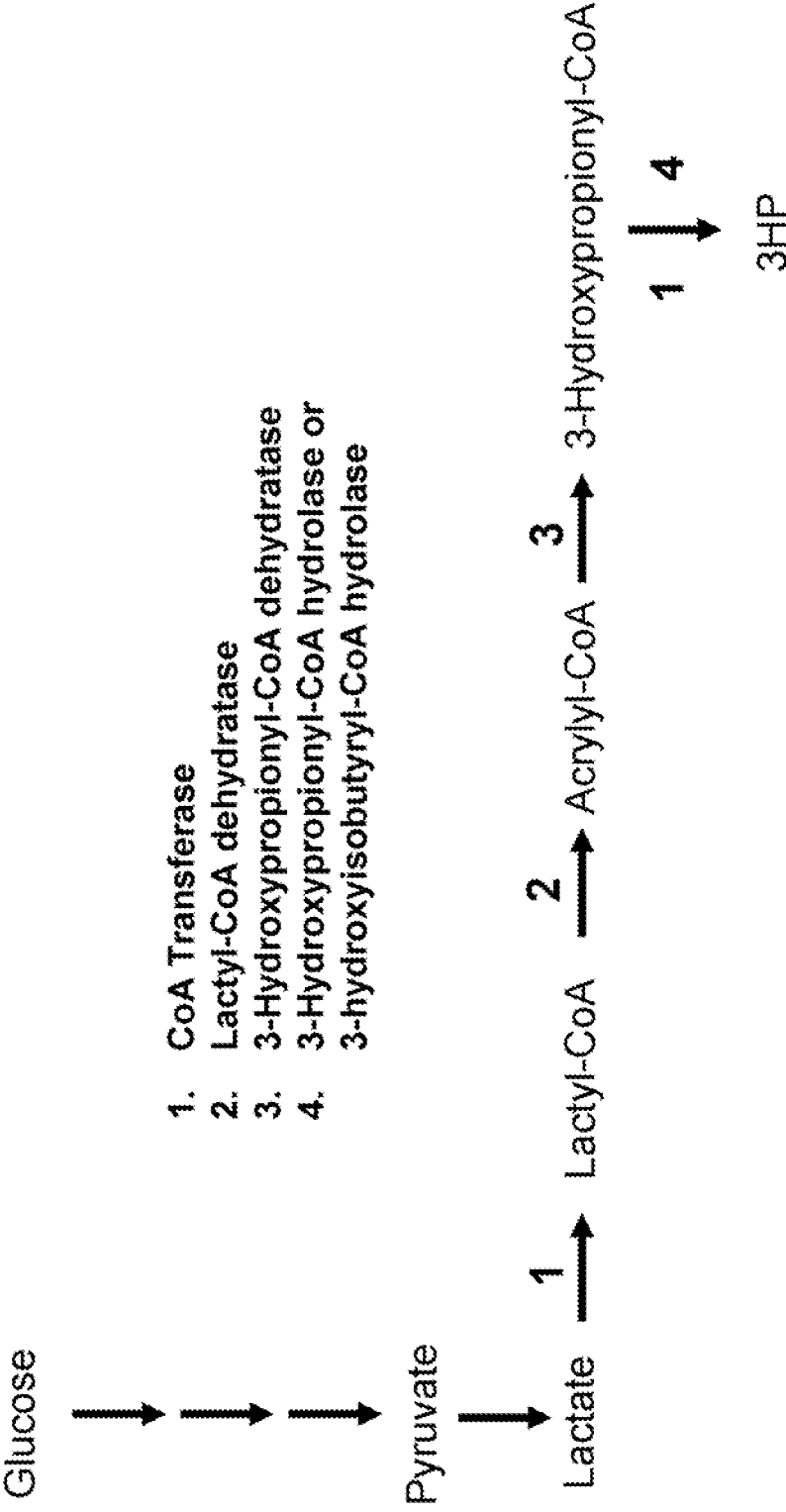


Fig. 1



Fig. 2

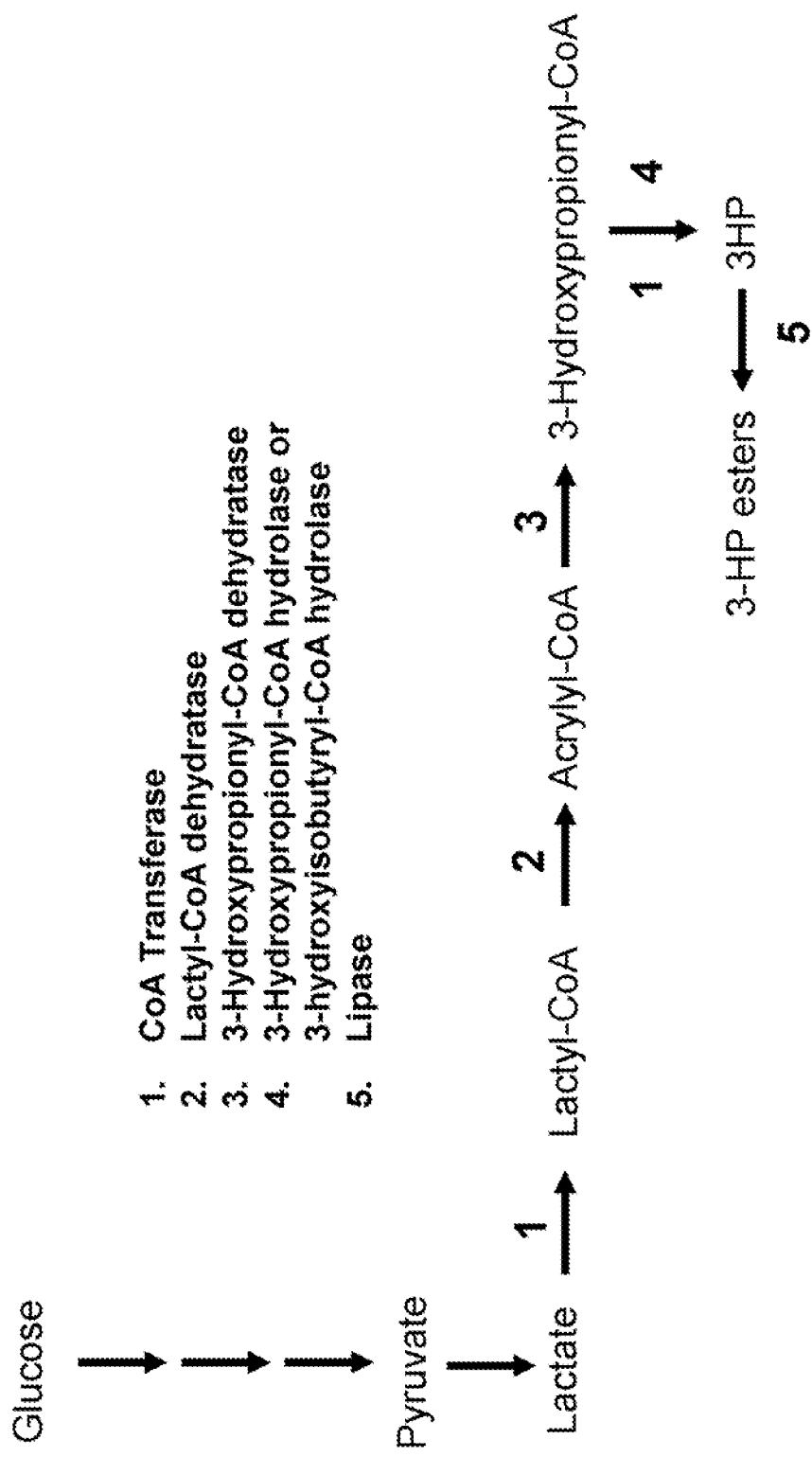


Fig. 3

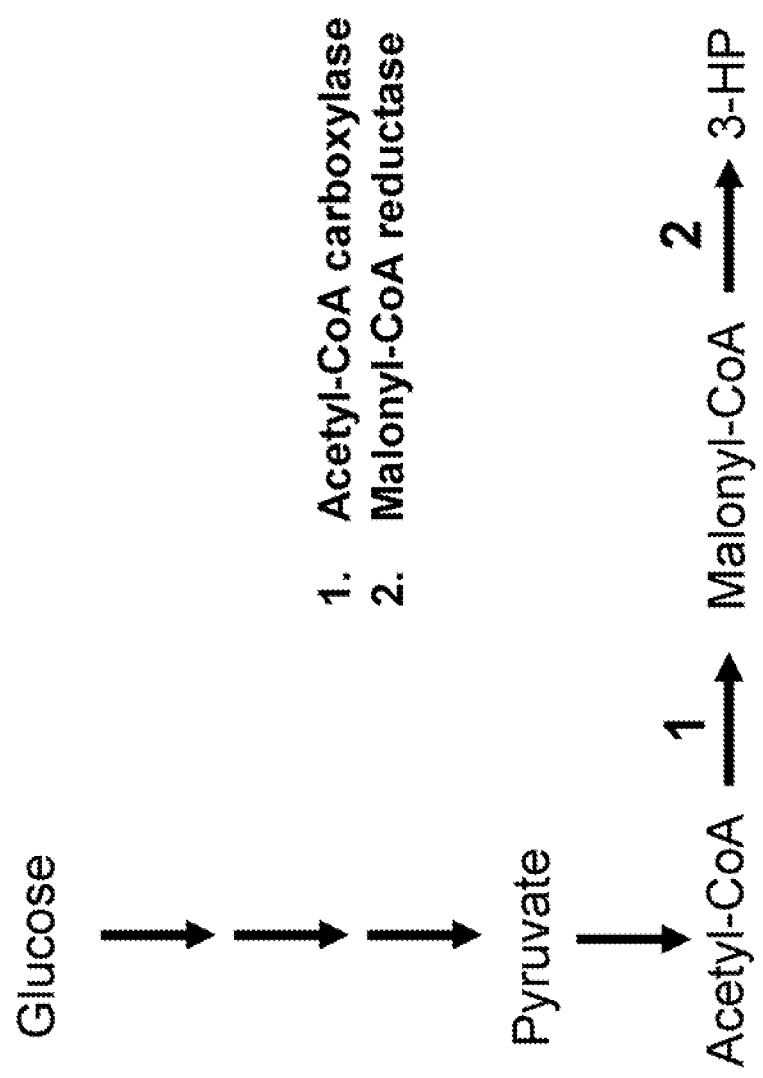


Fig. 4

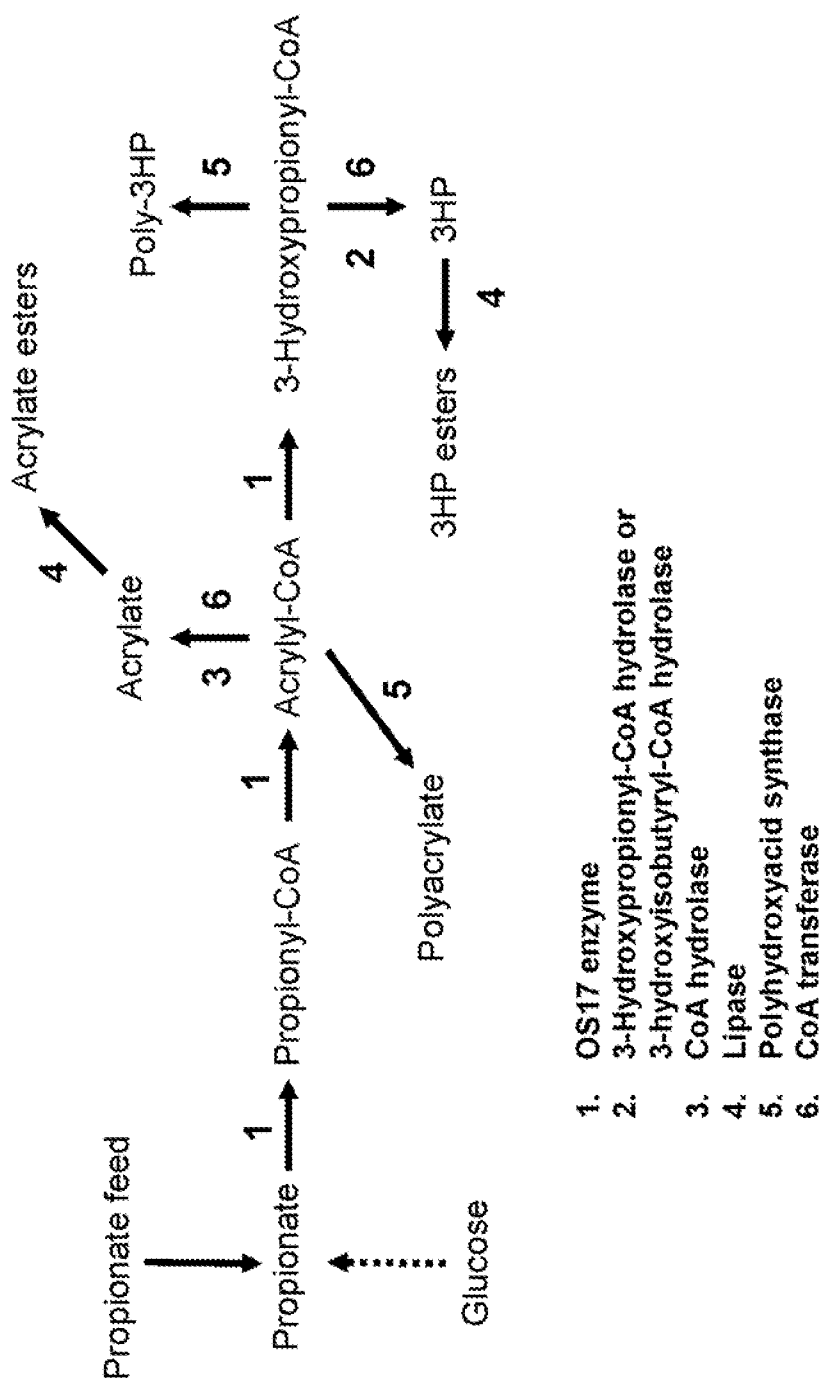


Fig. 5

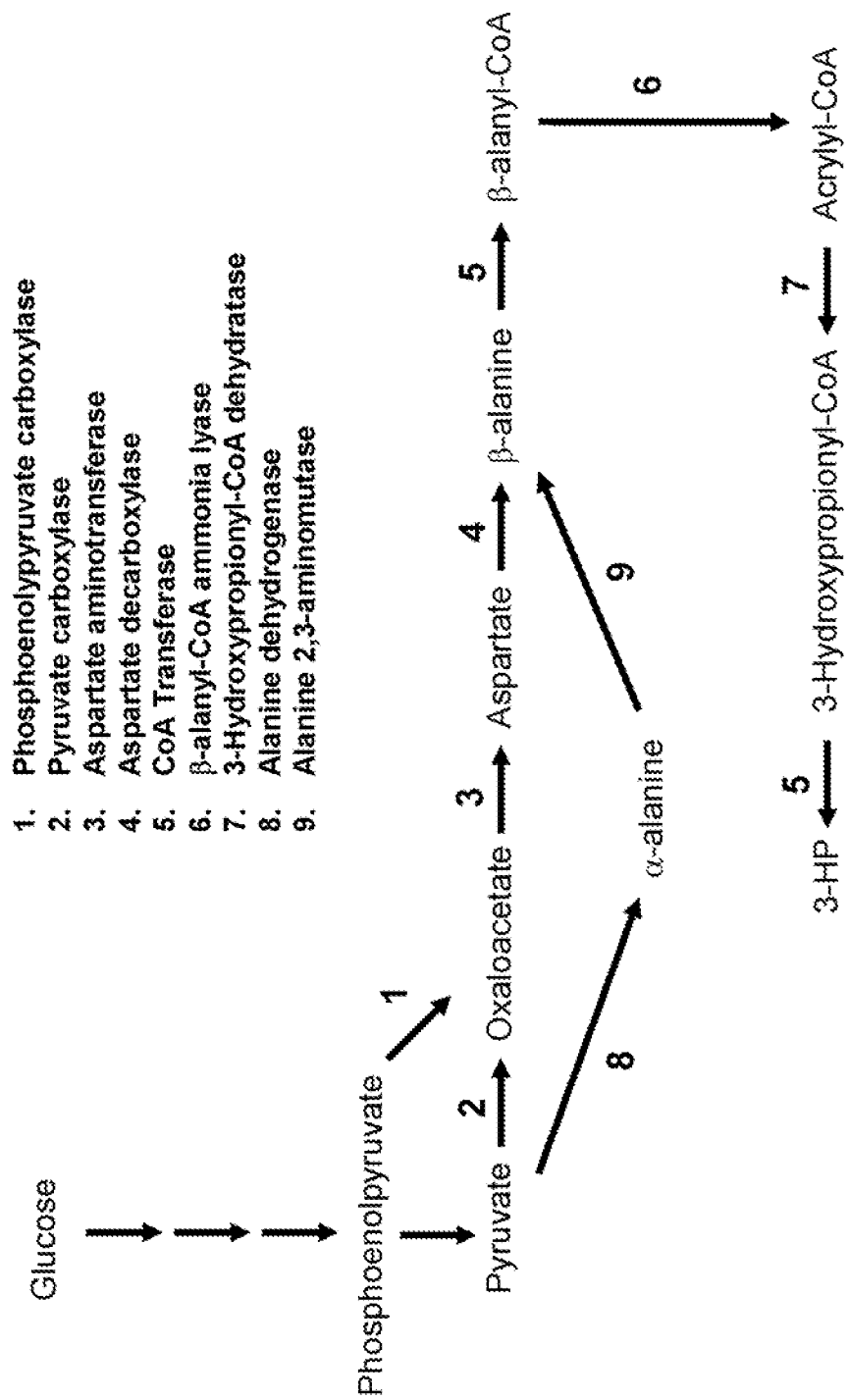


Fig. 6

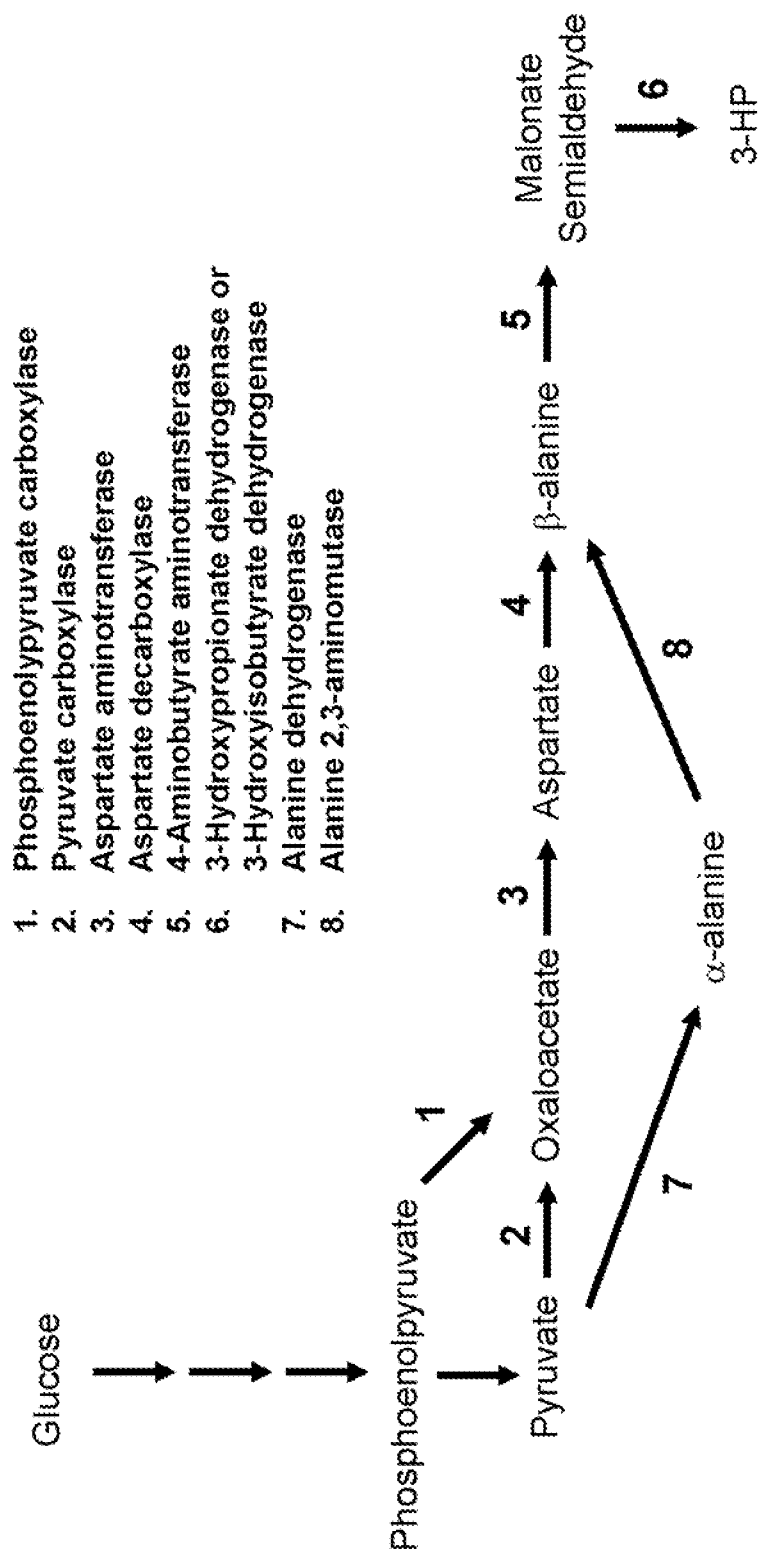


Fig. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/030607

A. CLASSIFICATION OF SUBJECT MATTER INV. C12P7/42 C12N15/52		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12P C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 10 2005 048818 A1 (DEGUSSA [DE]; FORSCHUNGSZENTRUM JUELICH GMBH [DE]) 12 April 2007 (2007-04-12) page 13, paragraph 77	1-17
X	WO 02/42418 A (CARGILL INC [US]; GOKARN RAVI R [US]; SELIFONOVA OLGA V [US]; JESSEN H) 30 May 2002 (2002-05-30) cited in the application page 44, line 6 - line 20 figures 1,3	1-17
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *Z* document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
4 May 2009	18/05/2009	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Niebuhr-Ebel, K	

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2009/030607

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ISHII M ET AL: "Occurrence, biochemistry and possible biotechnological application of the 3-hydroxypropionate cycle." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY JUN 2004, vol. 64, no. 5, June 2004 (2004-06), pages 605-610, XP002526381 ISSN: 0175-7598 the whole document	1-17
P, X	WO 2008/027742 A (CARGILL INC [US]; JESSEN HOLLY JEAN [US]; LIAO HANS H [US]; GORT STEVE) 6 March 2008 (2008-03-06) page 3, line 16 - line 25 figure 1	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2009/030607

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WO 0242418 A	30-05-2002	AR 031480 A1 AR 046846 A2 AU 1981802 A BR 0115877 A CA 2429039 A1 CN 1556855 A EP 1343874 A2 JP 2004514431 T MX PA03004324 A US 2009053783 A1 US 2004076982 A1 US 2007184524 A1	24-09-2003 28-12-2005 03-06-2002 07-10-2003 30-05-2002 22-12-2004 17-09-2003 20-05-2004 26-01-2004 26-02-2009 22-04-2004 09-08-2007
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