Abstract:

Title: ISOPROPA NOL PRODUCTION BY BACTERIAL HOSTS

(57) Abstract: Provided herein are recombinant Lactobacillus host cells, comprising an active isopropanol pathway and a heterologous butyrate-acetoacetate CoA transferase gene. Also described are methods of using the recombinant Lactobacillus host cells to produce isopropanol and propylene.
ISOPROPANOL PRODUCTION BY BACTERIAL HOSTS

Cross-Reference to Related Applications

This application claims priority from U.S. provisional application Serial No. 61/720,832, filed on October 31, 2012, the content of which is fully incorporated herein by reference.

Reference to a Sequence Listing

This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

Background

Concerns related to future supply of oil have prompted research in the area of renewable energy and renewable sources of other raw materials. Biofuels, such as ethanol and bioplastics (e.g., particularly polylactic acid) are examples of products that can be made directly from agricultural sources using microorganisms. Additional desired products may then be derived using non-enzymatic chemical conversions, e.g., dehydration of ethanol to ethylene.

Polymerization of ethylene provides polyethylene, a type of plastic with a wide range of useful applications. Ethylene is traditionally produced by refined non-renewable fossil fuels, but dehydration of biologically-derived ethanol to ethylene offers an alternative route to ethylene from renewable carbon sources, i.e., ethanol from fermentation of fermentable sugars. This process has been utilized for the production of "Green Polyethylene" that - save for minute differences in the carbon isotope distribution - is identical to polyethylene produced from oil.

Similarly, isopropanol and n-propanol can be dehydrated to propylene, which in turn can be polymerized to polypropylene. As with polyethylene, using biologically-derived starting material (i.e., isopropanol or n-propanol) would result in "Green Polypropylene." However, unlike polyethylene, the production of the polypropylene starting material from renewable sources has proved challenging. Proposed efforts at propanol production have been reported in WO 2009/049274, WO 2009/103026, WO 2009/131286, WO 2010/071697, WO 201/031897, WO 201/029166, WO 201/022651, WO 2012/058603.

It is clear that successful biological production of propanol for a particular host requires careful selection of expressed genes of a chosen metabolic pathway.

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Lactobacillus host cells, such as Lactobacillus reuteri, are capable of tolerating propanol at concentrations of as much as 60 g/L. However, knowledge of the Lactobacillus metabolic network and availability of Lactobacillus genetic tools is significantly lower compared to other well-known metabolic hosts, making metabolic engineering of a propanol pathway in Lactobacillus less appealing.

It would be advantageous in the art to improve isopropanol production, as a result of genetic engineering using recombinant DNA techniques.

**Summary**

Applicants have surprisingly found that expression of a putative Eremococcus coleocola butyrate-acetoacetate CoA transferase gene in a Lactobacillus host cell having an active isopropanol pathway results in significantly improved isopropanol production.

Accordingly, in one aspect is a recombinant Lactobacillus host cell comprising an active isopropanol pathway, wherein the cell comprises a first heterologous polynucleotide encoding a polypeptide having at least 75% sequence identity to SEQ ID NO: 63, and a second heterologous polynucleotide encoding a polypeptide having at least 75% sequence identity to SEQ ID NO: 66; and wherein the cell is capable of producing isopropanol. In some aspects, the cell produces a greater amount of isopropanol compared to the cell without the first and second heterologous polynucleotides.

In some aspects, the recombinant Lactobacillus host cell comprises a disruption to an endogenous gene encoding an acetate kinase (e.g., an acetate kinase related to SEQ ID NO: 60).

In some aspects, the Lactobacillus host cell is a Lactobacillus reuteri host cell.

Also described are methods of producing isopropanol, comprising: (a) cultivating a recombinant Lactobacillus host cell described herein in a medium under suitable conditions to produce isopropanol; and (b) recovering the isopropanol.

Also described are methods of producing propylene, comprising: (a) cultivating the recombinant Lactobacillus host cell described herein in a medium under suitable conditions to produce isopropanol; (b) recovering the isopropanol; (c) dehydrating the isopropanol under suitable conditions to produce propylene; and (d) recovering the propylene.

**Brief Description of the Figures**

Figure 1 shows a metabolic isopropanol pathway for the production of isopropanol from glucose.
Figure 2 shows a plasmid map for pSJ 10600.

Figure 3 shows a plasmid map for pSJ10603.

Figure 4 shows two codon-optimized DNA sequences (SEQ ID NOs: 61 and 62; D1396V and D1396W, respectively) and the deduced amino acid sequence (SEQ ID NO: 63) of an *Eremococcus colecola* butyrate-acetoacetate CoA transferase subunit A.

Figure 5 shows two codon-optimized DNA sequences (SEQ ID NOs: 64 and 65; D1396X and D1396Y, respectively) and the deduced amino acid sequence (SEQ ID NO: 66) of an *Eremococcus colecola* butyrate-acetoacetate CoA transferase subunit B.

**Definitions**

**Acetate Kinase:** The term "acetate kinase" is defined herein as a transferase enzyme that catalyzes the chemical reaction of diphosphate and acetate phosphate and acetyl phosphate (e.g., EC 2.7.2.1). The acetate kinase may be monomeric or in the form of a protein complex comprising two or more subunits under cellular conditions. Acetate kinase activity may be determined from cell-free extracts as described in the art, e.g., as described in S. Mukhopadhyay et al., 2008, Bioorg Chem. 36: 65-69.

**Active isopropanol pathway:** As used herein, a host cell having an "active isopropanol pathway" produces active enzymes necessary to catalyze each reaction in a metabolic pathway from a fermentable sugar to isopropanol, and therefore is capable of producing isopropanol in measurable yields when cultivated under fermentation conditions in the presence of at least one fermentable sugar. A host cell having an active isopropanol pathway comprises one or more isopropanol pathway genes. An "isopropanol pathway gene" as used herein refers to a gene that encodes an enzyme involved in an active isopropanol pathway.

The active enzymes necessary to catalyze each reaction in active isopropanol pathway may result from activities of endogenous gene expression, activities of heterologous gene expression, or from a combination of activities of endogenous and heterologous gene expression.

**Thiolase:** The term "thiolase" is defined herein as an acyltransferase that catalyzes the chemical reaction of two molecules of acetyl-CoA to acetoacetyl-CoA and CoA (e.g., EC 2.3.1.9). The thiolase may be monomeric or in the form of a protein complex comprising two or more subunits (e.g., two heteromeric subunits) under cellular conditions. Thiolase activity may be determined from cell-free extracts as described in the art, e.g., as described in D. P. Wiesenborn et al., 1988, *Appl. Environ. Microbiol.* 54:2717-2722. For example, thiolase activity may be measured spectrophotometrically by monitoring the condensation
reaction coupled to the oxidation of NADH using 3-hydroxyacyl-CoA dehydrogenase in 100 mM Tris hydrochloride (pH 7.4), 1.0 mM acetyl-CoA, 0.2 mM NADH, 1 mM dithiothreitol, and 2 U of 3-hydroxyacyl-CoA dehydrogenase. After equilibration of the cuvette contents at 30°C for 2 min, the reaction is initiated by the addition of about 125 ng of thiolase in 10 µL. The absorbance decrease at 340 nm due to oxidation of NADH is measured, and an extinction coefficient of 6.22 mM⁻¹ cm⁻¹ used.

**CoA-transferase:** As used herein, the term "CoA-transferase" is defined as any enzyme that catalyzes the removal of coenzyme A from acetoacetyl-CoA to generate acetoacetate, such as an acetoacetyl-CoA:acetate/butyrate CoA transferase of EC 2.8.3.9 (e.g., the butyrate-acetoacetate CoA transferase described herein), an acetoacetyl-CoA hydrolase of EC 3.1.2.1, or a succinyl-CoA:acetoacetate transferase of EC 2.8.3.5. The CoA-transferase may be in the form of a protein complex comprising two or more subunits (e.g., two heteromeric subunits) as described herein. CoA-transferase activity may be determined from cell-free extracts as described in the art, e.g., as described in L. Stols et al., 1989, *Protein Expression and Purification* 53:396-403.

**Acetoacetate decarboxylase:** The term "acetoacetate decarboxylase" is defined herein as an enzyme that catalyzes the chemical reaction of acetoacetate to carbon dioxide and acetone (e.g., EC 4.1.1.4). The acetoacetate decarboxylase may be monomeric or in the form of a protein complex comprising two or more subunits (e.g., two heteromeric subunits) under cellular conditions. Acetoacetate decarboxylase activity may be determined from cell-free extracts as described in the art, e.g., as described in D.J. Petersen et al., 1990, *Appl. Environ. Microbiol.* 56, 3491-3498. For example, acetoacetate decarboxylase activity may be measured spectrophotometrically by monitoring the depletion of acetoacetate at 270 nm in 5 mM acetoacetate, 0.1 M K₂HPO₄, pH 5.9 at 26°C.

**Isopropanol dehydrogenase:** The term "isopropanol dehydrogenase" is defined herein as any suitable oxidoreductase that catalyzes the reduction of acetone to isopropanol (e.g., any suitable enzyme of EC 1.1.1.1 or EC 1.1.1.80). The isopropanol dehydrogenase may be monomeric or in the form of a protein complex comprising two or more subunits (e.g., two heteromeric subunits) under cellular conditions. Isopropanol dehydrogenase activity may be determined spectrophotometrically from cell-free extracts as described in the art, e.g., by decrease in absorbance at 340 nm in an assay containing 200 µM NADPH and 10 mM acetone in 25 mM potassium phosphate, pH 7.2 at 25°C.

**Disruption:** The term "disruption" means that a coding region and/or control sequence of a referenced gene is partially or entirely modified (such as by deletion, insertion, and/or substitution of one or more nucleotides, or by association with RNAi or
antisense technology) resulting in the absence (inactivation) or decrease in expression, and/or the absence or decrease of enzyme activity of the encoded polypeptide. The effects of disruption can be measured using techniques known in the art such as detecting the absence or decrease of enzyme activity using from cell-free extract measurements referenced herein; or by the absence or decrease of corresponding mRNA (e.g., at least 25% decrease, at least 50% decrease, at least 60% decrease, at least 70% decrease, at least 80% decrease, or at least 90% decrease); the absence or decrease in the amount of corresponding polypeptide having enzyme activity (e.g., at least 25% decrease, at least 50% decrease, at least 60% decrease, at least 70% decrease, at least 80% decrease, or at least 90% decrease); or the absence or decrease of the specific activity of the corresponding polypeptide having enzyme activity (e.g., at least 25% decrease, at least 50% decrease, at least 60% decrease, at least 70% decrease, at least 80% decrease, or at least 90% decrease). Disruptions of a particular gene of interest can be generated by methods known in the art, e.g., by directed homologous recombination (see Methods in Yeast Genetics (1997 edition), Adams, Gottschling, Kaiser, and Stems, Cold Spring Harbor Press (1998)).

Coding sequence: The term "coding sequence" or "coding region" means a polynucleotide sequence, which specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG and ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a sequence of genomic DNA, cDNA, a synthetic polynucleotide, and/or a recombinant polynucleotide.

Sequence Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes described herein, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, J. Mol. Biol. 1970, 48, 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., Trends Genet 2000, 16, 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment - Total Number of Gaps in Alignment)
For purposes described herein, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, supra) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, supra), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows: 
(Identical Deoxynucleotides x 100)/(Length of Alignment - Total Number of Gaps in Alignment)

**Heterologous polynucleotide:** The term "heterologous polynucleotide" is defined herein as a polynucleotide that is not native to the host cell; a native polynucleotide in which structural modifications have been made to the coding region; a native polynucleotide whose expression is quantitatively altered as a result of a manipulation of the DNA by recombinant DNA techniques, e.g., a different (foreign) promoter; or a native polynucleotide whose expression is quantitatively altered by the introduction of one or more (several) extra copies of the polynucleotide into the host cell.

**Heterologous gene:** The term "heterologous gene" is defined herein as a gene comprising a heterologous polynucleotide.

**Endogenous gene:** The term "endogenous gene" means a gene that is native to the parent *Lactobacillus* strain.

**Nucleic acid construct:** The term "nucleic acid construct" means a polynucleotide comprises one or more (e.g., two, several) control sequences. The polynucleotide may be single-stranded or double-stranded, and may be isolated from a naturally occurring gene, modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature, or synthetic.

**Control sequence:** The term "control sequence" means a nucleic acid sequence necessary for polypeptide expression. Control sequences may be native or foreign to the polynucleotide encoding the polypeptide, and native or foreign to each other. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter sequence, signal peptide sequence, and transcription terminator sequence. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a polypeptide.

**Operably linked:** The term "operably linked" means a configuration in which a
control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs the expression of the coding sequence.

**Expression:** The term "expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion. Expression can be measured—for example, to detect increased expression—by techniques known in the art, such as measuring levels of mRNA and/or translated polypeptide.

**Expression vector:** The term "expression vector" means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences, wherein the control sequences provide for expression of the polynucleotide encoding the polypeptide. At a minimum, the expression vector comprises a promoter sequence, and transcriptional and translational stop signal sequences.

**Host cell:** The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The term "recombinant host cell" is defined herein as a non-naturally occurring host cell comprising one or more (e.g., several) heterologous polynucleotides.

**Allelic variant:** The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

**Volumetric productivity:** The term "volumetric productivity" refers to the amount of referenced product produced (e.g., the amount of isopropanol produced) per volume of the system used (e.g., the total volume of media and contents therein) per unit of time.

**Fermentable medium:** The term "fermentable medium" or "fermentation medium" refers to a medium comprising one or more (e.g., several) sugars, such as glucose, fructose, sucrose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides, wherein the medium is capable, in part, of being converted (fermented) by a host cell into a desired product, such as isopropanol. In some instances, the fermentation medium is derived from a natural source, such as sugar cane, starch, or cellulose, and may be the result of pretreating the source by enzymatic hydrolysis.
Sugar cane juice: The term "sugar cane juice" refers to the liquid extract from pressed Saccharum grass (sugarcane), such as pressed Saccharum officinarum or Saccharum robustum.

High stringency conditions: The term "high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 65°C.

Low stringency conditions: The term "low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 50°C.

Medium stringency conditions: The term "medium stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 55°C.

Medium-high stringency conditions: The term "medium-high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 60°C.

Very high stringency conditions: The term "very high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 70°C.
Very low stringency conditions: The term "very low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 45°C.

Reference to "about" a value or parameter herein includes aspects that are directed to that value or parameter per se. For example, description referring to "about X" includes the aspect "X". When used in combination with measured values, "about" includes a range that encompasses at least the uncertainty associated with the method of measuring the particular value, and can include a range of plus or minus two standard deviations around the stated value.

As used herein and in the appended claims, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise. It is understood that the aspects described herein include "consisting" and/or "consisting essentially of" aspects.

Unless defined otherwise or clearly indicated by context, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

Detailed Description

Described herein, inter alia, are recombinant Lactobacillus host cells comprising an active isopropanol pathway and one or more heterologous polynucleotides encoding a Eremococcus coleocola butyrate-acetoacetate CoA transferase. In some aspects, the Lactobacillus host cells produce a greater amount of isopropanol compared to the cells without the one or more heterologous polynucleotides encoding the butyrate-acetoacetate CoA transferase when cultivated under identical conditions.

The butyrate-acetoacetate CoA transferase can be any butyrate-acetoacetate CoA transferase that is suitable for the host cells and their methods of use described herein, such as a naturally occurring Eremococcus coleocola butyrate-acetoacetate CoA transferase or a variant thereof that retains butyrate-acetoacetate CoA transferase activity. In one aspect, the butyrate-acetoacetate CoA transferase is present in the cytosol of the host cells. The butyrate-acetoacetate CoA transferase may be a protein complex comprising a first subunit and the second subunit wherein the subunits comprise different amino acid sequences.
In some aspects, the host cells comprise one or more (e.g., two, several) heterologous polynucleotides encoding a butyrate-acetoacetate CoA transferase described herein. In some aspects, the host cells comprising the one or more heterologous polynucleotides that encode the butyrate-acetoacetate CoA transferase have an increased level of CoA transferase activity compared to the host cells without the one or more polynucleotides, when cultivated under the same conditions. In some aspects, the host cells comprising the one or more heterologous polynucleotides that encode a butyrate-acetoacetate CoA transferase have an increased level of CoA transferase activity of at least 5%, e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at least 500% compared to the host cells without the one or more polynucleotides, when cultivated under the same conditions.

In one aspect, host cells comprise a first heterologous polynucleotide encoding a first butyrate-acetoacetate CoA transferase subunit and a second heterologous polynucleotide encoding a second butyrate-acetoacetate CoA transferase subunit, wherein the first subunit and second subunit form a protein complex having butyrate-acetoacetate CoA transferase activity.

In one aspect, the first heterologous polynucleotide encoding the first butyrate-acetoacetate CoA transferase subunit and the second heterologous polynucleotide encoding the second butyrate-acetoacetate CoA transferase subunit are contained in a single heterologous polynucleotide. In another aspect, the first heterologous polynucleotide encoding the first butyrate-acetoacetate CoA transferase subunit and the second heterologous polynucleotide encoding the second butyrate-acetoacetate CoA transferase subunit are each contained in separate unlinked heterologous polynucleotides. An expanded discussion of nucleic acid constructs and expression vectors is described herein.

In one aspect, the first heterologous polynucleotide: (a) encodes for a polypeptide having at least 65% sequence identity to SEQ ID NO: 63; (b) comprises a coding sequence that hybridizes under low stringency conditions with the full-length complementary strand of SEQ ID NO: 61 or 62; or (c) comprises a coding sequence having at least 65% sequence identity to SEQ ID NO: 61 or 62; and

the second heterologous polynucleotide: (a) encodes a polypeptide having at least 65% sequence identity to SEQ ID NO: 66; (b) comprises a coding sequence that hybridizes under low stringency conditions with the full-length complementary strand of SEQ ID NO: 64 or 65; or (c) comprises a coding sequence having at least 65% sequence identity to SEQ ID NO: 64 or 65. As can be appreciated by one of skill in the art, in some instances the first and second heterologous polynucleotides may qualify under more than one of the
respective selections (a), (b) and (c) noted above.

In one aspect, the first heterologous polynucleotide encodes a polypeptide having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 63; and the second heterologous polynucleotide encodes a polypeptide having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 66. In one aspect, the first heterologous polynucleotide encodes a polypeptide having a sequence that differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two amino acids, or by one amino acid from SEQ ID NO: 63; and the second heterologous polynucleotide encodes a polypeptide having a sequence that differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two amino acids, or by one amino acid from SEQ ID NO: 66.

In one aspect, the first heterologous polynucleotide encodes a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 63, an allelic variant thereof, or a fragment of the foregoing; and the second heterologous polynucleotide encodes a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 66, an allelic variant thereof, or a fragment of the foregoing.

In one aspect, the first heterologous polynucleotide encodes a polypeptide comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 63; and/or the first heterologous polynucleotide encodes a polypeptide comprising a substitution, deletion, and/or insertion of one or more amino acids of SEQ ID NO: 66. An amino acid substitution means that an amino acid corresponding to a position of the referenced sequence is different; an amino acid deletion means that an amino acid corresponding to a position of the referenced sequence is not present; and an amino acid insertion means that an amino acid is present that is not present at a corresponding position of the referenced sequence. In some aspects, the total number of amino acid substitutions, deletions and/or insertions of SEQ ID NO: 63 is not more than 10, e.g., not more than 9, 8, 7, 6, 5, 4, 3, 2, or 1. In some aspects, the total number of amino acid substitutions, deletions and/or insertions of SEQ ID NO: 66 is not more than 10, e.g., not more than 9, 8, 7, 6, 5, 4, 3, 2, or 1.
The amino acid changes are generally of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino-terminal or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, LeuA/Al, Ala/Glu, and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide or polypeptide complex, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scaning 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 butyrate-acetoacetate CoA transferase 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 butyrate-acetoacetate CoA transferase or other biological interaction can also be determined by physical analysis of structure, as determined by 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 other butyrate-acetoacetate CoA transferases that are related to the referenced butyrate-acetoacetate CoA transferase.
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. 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, Biochemistry 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, 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 butyrate-acetoacetate CoA transferases 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 polypeptide.

In one aspect, the first heterologous polynucleotide comprises a coding sequence that hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 61 or 62; and the second heterologous polynucleotide comprises a coding sequence that hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 64 or 65 (see, e.g., J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, supra).

In one aspect, the first heterologous polynucleotide comprises a coding sequence having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 61 or 62; and the second heterologous polynucleotide comprises a coding sequence having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 64 or 65.

In one aspect, the first heterologous polynucleotide comprises SEQ ID NO: 61 or 62, and the second heterologous polynucleotide comprises SEQ ID NO: 64 or 65.
In one aspect, the first heterologous polynucleotide comprises a subsequence of SEQ ID NO: 61 or 62; and/or the second heterologous polynucleotide comprises a subsequence of SEQ ID NO: 64 or 65; wherein the first and second polynucleotides encode a polypeptides that together form a protein complex having butyrate-acetoacetate CoA transferase activity. In one aspect, the number of nucleotides residues in the subsequence is at least 75%, e.g., at least 80%, 85%, 90%, or 95% of the number of nucleotide residues in SEQ ID NO: 61, 62, 64, or 65.

In one aspect, the first heterologous polynucleotide encodes a fragment of SEQ ID NO: 63, and/or the second heterologous polynucleotide encodes a fragment of SEQ ID NO: 66, wherein the first and second fragments together form a protein complex having butyrate-acetoacetate CoA transferase activity. In one aspect, the number of amino acid residues in the fragment is at least 75%, e.g., at least 80%, 85%, 90%, or 95% of the number of amino acid residues in SEQ ID NO: 63 or 66.

The polypeptides encoded by the first and second heterologous polynucleotides may fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus. A fused polypeptide may be produced by fusing a polynucleotide encoding another polypeptide to the first or second polynucleotide. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator. Fusion proteins may also be constructed using intein technology in which fusions are created post-translationally (Cooper et al., 1993, EMBO J. 12: 2575-2583; Dawson et al., 1994, Science 266: 776-779).


Techniques used to isolate or clone a polynucleotide —such as the coding sequence of the first and second polynucleotides, or a polynucleotide encoding any other polypeptide used in any of the aspects mentioned herein, are known in the art and include isolation
from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the polynucleotides from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shares structural features. See, e.g., Innis et al., 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleotide sequence-based amplification (NASBA) may be used. The polynucleotides may be cloned from a strain of *Aspergillus*, or another or related organism, and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleotide sequence.

The polynucleotide of SEQ ID NO: 61, 62, 64, 65, or a subsequence thereof; as well as the amino acid sequence of SEQ ID NO: 63, 66, or a fragment thereof; may be used to design nucleic acid probes to identify and clone a butyrate-acetoacetate CoA transferase 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, e.g., at least 14 nucleotides, at least 25 nucleotides, at least 35 nucleotides, at least 70 nucleotides in lengths. The probes may be longer, e.g., at least 100 nucleotides, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides in lengths. Even longer probes may be used, e.g., at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or 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, $^{3}$H, $^{35}$S, biotin, or avidin).

A genomic DNA or cDNA library prepared from such other strains may be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having butyrate-acetoacetate CoA transferase 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 that is homologous with SEQ ID NO: 61, 62, 64, or 65, or a subsequence thereof, the carrier material may be used in a Southern blot.

For purposes of the probes described above, hybridization indicates that the polynucleotide hybridizes to a labeled nucleic acid probe corresponding to SEQ ID NO: 61,
62, 64, or 65, or the full-length complementary strand thereof, or a subsequence of the foregoing; 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.

In one aspect, the nucleic acid probe is SEQ ID NO: 61, 62, 64, or 65. In another aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 63, 66, or a fragment thereof.

For long probes of at least 100 nucleotides in length, very low to very high stringency and washing conditions are defined as described supra. For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency and washing conditions are defined as described supra.

Polynucleotides encoding the butyrate-acetoacetate CoA transferase and subunits thereof may be obtained from microorganisms of any genus. In one aspect, the butyrate-acetoacetate CoA transferase may be a bacterial, a yeast, or a filamentous fungal butyrate-acetoacetate CoA transferase obtained from the microorganisms described herein. In another aspect, the butyrate-acetoacetate CoA transferase is a Eremococcus butyrate-acetoacetate CoA transferase, such as a Eremococcus coleocola butyrate-acetoacetate CoA transferase, e.g., the Eremococcus coleocola butyrate-acetoacetate CoA transferase complex comprising the polypeptide sequence of SEQ ID NO: 63 and the polypeptide sequence of SEQ ID NO: 66.

The butyrate-acetoacetate CoA transferase may be a bacterial butyrate-acetoacetate CoA transferase. For example, the butyrate-acetoacetate CoA transferase may be a Gram-positive bacterial butyrate-acetoacetate CoA transferase such as a Bacillus, Streptococcus, Streptomyces, Staphylococcus, Enterococcus, Lactobacillus, Lactococcus, Clostridium, Geobacillus, or Oceanobacillus butyrate-acetoacetate CoA transferase, or a Gram-negative bacterial butyrate-acetoacetate CoA transferase such as an E. coli, Pseudomonas, Salmonella, Campylobacter, Helicobacter, Flavobacterium, Fusobacterium, Ilyobacter, Neisseria, or Ureaplasma butyrate-acetoacetate CoA transferase.

In one aspect, the butyrate-acetoacetate CoA transferase is a Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus firmus, Bacillus laetus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus stearothermophilus, Bacillus subtilis, or Bacillus thuringiensis butyrate-acetoacetate CoA transferase.

In another aspect, the butyrate-acetoacetate CoA transferase is a Streptococcus
equisimilis, Streptococcus pyogenes, Streptococcus uberis, or Streptococcus equi subsp. Zooepidemicus butyrate-acetoacetate CoA transferase. In another aspect, the butyrate-acetoacetate CoA transferase is a Streptomyces achromogenes, Streptomyces avermitilis, Streptomyces coelicolor, Streptomyces griseus, or Streptomyces lividans butyrate-acetoacetate CoA transferase.

The butyrate-acetoacetate CoA transferase may be a fungal butyrate-acetoacetate CoA transferase. In one aspect, the fungal butyrate-acetoacetate CoA transferase is a yeast butyrate-acetoacetate CoA transferase such as a Candida, Kluyveromyces, Pichia, Saccharomyces, Schizosaccharomyces, or Yarrowia butyrate-acetoacetate CoA transferase.

In another aspect, the fungal butyrate-acetoacetate CoA transferase is a filamentous fungal butyrate-acetoacetate CoA transferase such as an Acremonium, Aspergillus, Alternaria, Aspergillus, Aureobasidium, Botryosphaeria, Ceriporiopsis, Chaetomium, Chrysosporium, Claviceps, Cochliobolus, Coprinopsis, Coptotermes, Corynascus, Cryptococcus, Dipodula, Exidia, Filibasidium, Fusarium, Gibberella, Holomastigotoides, Humicola, Irpex, Lentinula, Leptosphaeria, Magnaporthe, Melanocarpus, Meripilus, Mucor, Myceliophthora, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Phanerochaete, Piromyces, Poitrasia, Pseudoplectania, Pseudotrichonympha, Rhizomucor, Schizophyllum, Scytalidium, Talaromyces, Thermoascus, Thielavia, Tolypocladium, Trichoderma, Trichophae, Verticillium, Volvariella, or Xylaria butyrate-acetoacetate CoA transferase.

In another aspect, the butyrate-acetoacetate CoA transferase is a Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces diastaticus, Saccharomyces douglasii, Saccharomyces kluveri, Saccharomyces norbensis, or Saccharomyces oviformis butyrate-acetoacetate CoA transferase.

In another aspect, the butyrate-acetoacetate CoA transferase is an Acremonium cellulolyticus, Aspergillus aculeatus, Aspergillus awamori, Aspergillus flavus, Aspergillus fumigatus, Aspergillus foetidus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Aspergillus sojae, Chrysosporium keratinophilum, Chrysosporium lucknowense, Chrysosporium tropicum, Chrysosporium merdarium, Chrysosporium inops, Chrysosporium pannicola, Chrysosporium queenslandicum, Chrysosporium zonatum, Fusarium bactridioides, Fusarium cerealis, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminum, Fusarium heterosporum, Fusarium negundi, Fusarium oxysporum, Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium sarscochroum, Fusarium sporotrichioides, Fusarium sulphureum, Fusarium
torulosum, Fusarium trichothecioides, Fusarium venenatum, Humicola grisea, Humicola insolens, Humicola lanuginosa, Irpex lacteus, Mucor miehei, Myceliophthora thermophila, Neurospora crassa, Penicillium funiculosum, Penicillium purpurogenum, Phanerochaete chrysosporium, Thielavia achromatica, Thielavia albomyces, Thielavia albopilosa, Thielavia australensis, Thielavia fimeti, Thielavia microspora, Thielavia ovispora, Thielavia peruviana, Thielavia spenedonium, Thielavia setosa, Thielavia subthermophila, Thielavia terrestris, Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei, or Trichoderma viride butyrate-acetoacetate CoA transferase.

It will be understood that for the aforementioned species, both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, are encompassed regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

In some aspects, the butyrate-acetoacetate CoA transferase has at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the butyrate-acetoacetate CoA transferase activity of a protein complex comprising a first subunit having the polypeptide sequence of SEQ ID NO: 63, and a second subunit having the polypeptide sequence of SEQ ID NO: 66 under the same conditions.

The butyrate-acetoacetate CoA transferase may also be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, silage, etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, silage, etc.) using the above-mentioned probes. Techniques for isolating microorganisms and DNA directly from natural habitats are well known in the art. The polynucleotide encoding a butyrate-acetoacetate CoA transferase (or subunit) may then be derived by similarly screening a genomic or cDNA library of another microorganism or mixed DNA sample. Once a polynucleotide encoding a butyrate-acetoacetate CoA transferase (or subunit) has been detected with suitable probe(s) as described herein, the sequence may be isolated or cloned by utilizing techniques that are known to those of ordinary skill in the art (see, e.g., J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, New York).
The *Lactobacillus* host cells may be any suitable *Lactobacillus* strain capable of the recombinant production of one or more of the polypeptides described herein and capable of the recombinant production of isopropanol from an active isopropanol pathway. In some aspects, the *Lactobacillus* host cell is a *Lactobacillus plantarum*, *Lactobacillus fructivorans*, or *Lactobacillus reuteri* host cell. In one aspect, the *Lactobacillus* host cell is a *Lactobacillus plantarum* host cell. In another aspect, the *Lactobacillus* host cell is a *Lactobacillus fructivorans* host cell. In another aspect, the *Lactobacillus* host cell is a *Lactobacillus reuteri* host cell.

In some aspects, the *Lactobacillus* host cell secretes and/or is capable of secreting an increased level of isopropanol of at least 5%, e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at 500% compared to the host cell without the first and second heterologous polynucleotides, when cultivated under the same conditions. Examples of suitable cultivation conditions are described below and will be readily apparent to one of skill in the art based on the teachings herein.

In any of these aspects, the *Lactobacillus* host cell produces (and/or is capable of producing) isopropanol at a yield of at least 10%, e.g., at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%, of theoretical.

In any of these aspects, the *Lactobacillus* host has an isopropanol volumetric productivity greater than about 0.1 g/L per hour, e.g., greater than about 0.2 g/L per hour, 0.5 g/L per hour, 0.6 g/L per hour, 0.7 g/L per hour, 0.8 g/L per hour, 0.9 g/L per hour, 1.0 g/L per hour, 1.1 g/L per hour, 1.2 g/L per hour, 1.3 g/L per hour, 1.5 g/L per hour, 1.75 g/L per hour, 2.0 g/L per hour, 2.25 g/L per hour, 2.5 g/L per hour, or 3.0 g/L per hour; or between about 0.1 g/L per hour and about 2.0 g/L per hour, e.g., between about 0.3 g/L per hour and about 1.7 g/L per hour, about 0.5 g/L per hour and about 1.5 g/L per hour, about 0.7 g/L per hour and about 1.3 g/L per hour, about 0.8 g/L per hour and about 1.2 g/L per hour, or about 0.9 g/L per hour and about 1.1 g/L per hour.

The *Lactobacillus* host cells may be cultivated in a nutrient medium suitable for production of one or more of the polypeptides described herein and capable of the recombinant production of isopropanol from an active isopropanol pathway using methods well known in the art. For example, the cell may be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the desired polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, as described herein, using procedures known in the art. Suitable media are available from commercial suppliers, may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection), or may be prepared from commercially available ingredients.

The butyrate-acetocacetate CoA transferase, and activities thereof, can be detected using methods known in the art. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate.

**Isopropanol Pathway Genes**

The *Lactobacillus* host cells described herein can be used in the production of isopropanol when comprising an active isopropanol pathway. Isopropanol pathway genes and corresponding engineered *Lactobacillus* transformants for fermentation of isopropanol are known in the art (e.g., see PCT/US2011/58405, the content of which is hereby incorporated in its entirety). One exemplary isopropanol pathway for the production of isopropanol from glucose is depicted in Figure 1, wherein cellular acetyl-CoA is converted to acetoacetyl-CoA by a thiolase, acetoacetyl-CoA is converted to acetoacetate by a CoA-transferase (e.g., the butyrate-acetoacetate CoA transferase of the present invention), acetoacetate is converted to acetone by an acetoacetate decarboxylase, and acetone is converted to isopropanol by an isopropanol dehydrogenase. Any suitable isopropanol pathway gene, endogenous or heterologous, encoding a thiolase, acetoacetate decarboxylase, and/or isopropanol dehydrogenase, in addition to the butyrate-acetoacetate CoA transferase described herein, may be used to produce isopropanol. Thus, the host cells comprising an active isopropanol pathway may comprise thiolase activity, CoA transferase activity (e.g., provided by the *Eremococcus colecola* butyrate-acetoacetate CoA transferase of the present invention), acetoacetate decarboxylase activity and/or isopropanol dehydrogenase activity.

The *Lactobacillus* host cells may comprise any one or combination of a plurality of the heterologous isopropanol pathway genes described. For example, in one aspect, the recombinant host cell comprises the butyrate-acetoacetate CoA transferase described herein, and further comprises a heterologous thiolase gene, a heterologous acetoacetate decarboxylase gene, and/or a heterologous isopropanol dehydrogenase gene described herein. In some aspects, the host cell produces (or is capable of producing) a greater amount of isopropanol compared to the host cell without the heterologous polynucleotides when cultivated under the same conditions. In some of these aspects, the host cell lacks an endogenous thiolase gene, lacks an endogenous CoA-transferase gene, lacks an endogenous acetoacetate decarboxylase gene, and/or lacks an endogenous isopropanol dehydrogenase gene.

In one aspect, the *Lactobacillus* host cell comprises one or more (e.g., two, several)
heterologous polynucleotides encoding a thiolase described herein. In one aspect, the host cell comprises one or more heterologous polynucleotides encoding an acetoacetate decarboxylase described herein. In one aspect, the host cell comprises one or more heterologous polynucleotides encoding an isopropanol dehydrogenase described herein.

The thiolase, acetoacetate decarboxylase, and isopropanol dehydrogenase, and activities thereof, can be detected using methods known in the art or as described herein. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. See, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Third Ed., Cold Spring Harbor Laboratory, New York (2001); Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, MD (1999); and Hanai et al., *Appl. Environ. Microbiol.* 73:7814-7818 (2007).

**Thiolases**

In some aspects, the host cells comprise a heterologous gene that encodes a thiolase. The thiolase can be any thiolase that is suitable for the host cells and their methods of use described herein, such as a naturally occurring thiolase or a variant thereof that retains thiolase activity. In one aspect, the thiolase is present in the cytosol of the host cells.

In some aspects, the host cells comprising a heterologous thiolase gene have an increased level of thiolase activity compared to the host cells without the heterologous thiolase gene, when cultivated under the same conditions. In some aspects, the host cells have an increased level of thiolase activity of at least 5%, e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at 500% compared to the host cells without the heterologous thiolase gene, when cultivated under the same conditions.

Exemplary thiolase genes that can be used with the host cells and methods of use described herein include, but are not limited to, the *Clostridium acetobutylicum* thiolase gene comprising SEQ ID NO: 1 (which encodes the thiolase of SEQ ID NO: 3), the *Lactobacillus reuteri* thiolase gene encoding the thiolase of SEQ ID NO: 26, the *Lactobacillus brevis* thiolase gene comprising SEQ ID NO: 41 (which encodes the thiolase of SEQ ID NO: 42), the *Propionibacterium freudenreichii* thiolase gene comprising SEQ ID NO: 39 (which encodes the thiolase of SEQ ID NO: 40), an *E. coli* thiolase (NP_416728, Martin et al., *Nat. Biotechnology* 21:796-802 (2003)), a *S. cerevisiae* thiolase (NP_015297, Hiser et al., *J. Biol. Chem.* 269:31383-31389 (1994)), a *C. pasteurianum* thiolase (e.g., protein ID ABAI8857.l), a *C. beijerinckii* thiolase (e.g., protein ID EAP59904.1 or EAP59931.1), a *Clostridium perfringens* thiolase (e.g., protein ID ABG86544.l,
ABG83108.I), a *Clostridium difficile* thiolase (e.g., protein ID CAJ67900.1 or ZP_01231975.1), a *Thermoanaerobacterium thermosaccharolyticum* thiolase (e.g., protein ID CAB07500.1), a *Thermoanaerobacter tengcongensis* thiolase (e.g., A.L.M23825.1), a *Carboxydothermus hydrogenoformans* thiolase (e.g., protein ID ABB13995.I), a *Desulfotomaculum reducens* MI-I thiolase (e.g., protein ID EAR45123.1), or a *Candida tropicalis* thiolase (e.g., protein ID BAA02716.1 or BAA02715.1).

In one aspect, the thiolase has at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the thiolase encoded by any thiolase gene described herein (e.g., any thiolase of SEQ ID NO: 3, 26, 40 or 42). In one aspect, the thiolase sequence differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two amino acids, or by one amino acid from the thiolase encoded by any thiolase gene described herein (e.g., any thiolase of SEQ ID NO: 3, 26, 40 or 42). In one aspect, the thiolase comprises or consists of the amino acid sequence of the thiolase encoded by any thiolase gene described herein (e.g., any thiolase of SEQ ID NO: 3, 26, 40 or 42), allelic variant, or a fragment thereof having thiolase activity. In one aspect, the thiolase has an amino acid substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids. In some aspects, the total number of amino acid substitutions, deletions and/or insertions is not more than 10, e.g., not more than 9, 8, 7, 6, 5, 4, 3, 2, or 1.

In one aspect, the coding sequence of the heterologous thiolase gene hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the full-length complementary strand of the coding sequence of any thiolase gene described herein (e.g., any thiolase gene of SEQ ID NO: 1, 2, 25, 39, or 41).

In one aspect, the coding sequence of the heterologous thiolase gene has at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity with the coding sequence of any thiolase gene described herein (e.g., any thiolase gene of SEQ ID NO: 1, 2, 25, 39, or 41).

In one aspect, the coding sequence of the heterologous thiolase gene comprises the coding sequence of any thiolase gene described herein (e.g., any thiolase gene of SEQ ID NO: 1, 2, 25, 39, or 41). In one aspect, the coding sequence of the heterologous thiolase gene comprises a subsequence of any thiolase gene described herein (e.g., any thiolase
gene of SEQ ID NO: 1, 2, 25, 39, or 41), wherein the subsequence encodes a polypeptide having thiolase activity. In one aspect, the number of nucleotides residues in the subsequence is at least 75%, e.g., at least 80%, 85%, 90%, or 95% of the number of the referenced sequence.

Polynucleotides encoding the thiolase may be obtained from microorganisms of any genus, as described supra.

The thiolases can also include fused polypeptides or cleavable fusion polypeptides, as described supra.

The thiolase genes can also be used to design nucleic acid probes to identify and clone DNA encoding thiolases from strains of different genera or species, as described supra.

Techniques used to isolate or clone a polynucleotide encoding a thiolase are described supra.

The thiolase genes may also be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, etc.) as described supra.

**Acetoacetate decarboxylases**

In some aspects, the host cells comprise a heterologous gene that encodes an acetoacetate decarboxylase. The acetoacetate decarboxylase can be any acetoacetate decarboxylase that is suitable for the host cells and their methods of use described herein, such as a naturally occurring acetoacetate decarboxylase or a variant thereof that retains acetoacetate decarboxylase activity. In one aspect, the acetoacetate decarboxylase is present in the cytosol of the host cells.

In some aspects, the host cells comprising a heterologous acetoacetate decarboxylase gene have an increased level of acetoacetate decarboxylase activity compared to the host cells without the heterologous acetoacetate decarboxylase gene, when cultivated under the same conditions. In some aspects, the host cells have an increased level of acetoacetate decarboxylase activity of at least 5%, e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at 500% compared to the host cells without the heterologous acetoacetate decarboxylase gene, when cultivated under the same conditions.

Exemplary acetoacetate decarboxylase genes that can be used with the host cells and methods of use described herein include, but are not limited to, a *Clostridium beijerinckii* acetoacetate decarboxylase of SEQ ID NO: 16 (which encodes the
acetoacetate decarboxylase of SEQ ID NO: 18), a *Lactobacillus salivarius* acetoacetate decarboxylase gene comprising SEQ ID NO: 43 (which encodes the acetoacetate decarboxylase of SEQ ID NO: 44), a *Lactobacillus plantarum* acetoacetate decarboxylase gene comprising SEQ ID NO: 45 (which encodes the acetoacetate decarboxylase of SEQ ID NO: 46), a *C. acetobutylicum* acetoacetate decarboxylase gene (NP_149328.1, which encodes the acetoacetate decarboxylase of SEQ ID NO: 36; see Petersen and Bennett, *Appl. Environ. Microbiol* 56:3491-3498 (1990)) and a *Clostridium saccharoperbutylacetonicum* acetoacetate decarboxylase (AAP42566.1, Kosaka, et al., *Biosci. Biotechnol Biochem*. 71:58-68 (2007)).

In one aspect, the acetoacetate decarboxylase has at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the acetoacetate decarboxylase encoded by any acetoacetate decarboxylase gene described herein (e.g., any acetoacetate decarboxylase of SEQ ID NO: 18, 36, 44, or 46). In one aspect, the acetoacetate decarboxylase sequence differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two amino acids, or by one amino acid from the acetoacetate decarboxylase encoded by any acetoacetate decarboxylase gene described herein (e.g., any acetoacetate decarboxylase of SEQ ID NO: 18, 36, 44, or 46). In one aspect, the acetoacetate decarboxylase comprises or consists of the amino acid sequence of the acetoacetate decarboxylase encoded by any acetoacetate decarboxylase gene described herein (e.g., any acetoacetate decarboxylase of SEQ ID NO: 18, 36, 44, or 46), allelic variant, or a fragment thereof having acetoacetate decarboxylase activity. In one aspect, the acetoacetate decarboxylase has an amino acid substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids. In some aspects, the total number of amino acid substitutions, deletions and/or insertions is not more than 10, e.g., not more than 9, 8, 7, 6, 5, 4, 3, 2, or 1.

In one aspect, the coding sequence of the heterologous acetoacetate decarboxylase gene hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the full-length complementary strand of the coding sequence of any acetoacetate decarboxylase gene described herein (e.g., any acetoacetate decarboxylase gene of SEQ ID NO: 16, 17, 35, 43, or 45).

In one aspect, the coding sequence of the heterologous acetoacetate decarboxylase gene has at least 65%, e.g., at least 70%, at least 75%, at least 80%, at
least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity with the coding sequence of any acetoacetate decarboxylase gene described herein (e.g., any acetoacetate decarboxylase gene of SEQ ID NO: 16, 17, 35, 43, or 45). In one aspect, the coding sequence of the heterologous acetoacetate decarboxylase gene comprises the coding sequence of any acetoacetate decarboxylase gene described herein (e.g., any acetoacetate decarboxylase gene of SEQ ID NO: 16, 17, 35, 43, or 45). In one aspect, the coding sequence of the heterologous acetoacetate decarboxylase gene comprises a subsequence of any acetoacetate decarboxylase gene described herein. In one aspect, the number of nucleotides residues in the subsequence is at least 75%, e.g., at least 80%, 85%, 90%, or 95% of the number of the referenced sequence.

Polynucleotides encoding the acetoacetate decarboxylase may be obtained from microorganisms of any genus, as described supra.

The acetoacetate decarboxylases can also include fused polypeptides or cleavable fusion polypeptides, as described supra.

The acetoacetate decarboxylase genes can also be used to design nucleic acid probes to identify and clone DNA encoding acetoacetate decarboxylases from strains of different genera or species, as described supra.

Techniques used to isolate or clone a polynucleotide encoding a acetoacetate decarboxylase are described supra.

The acetoacetate decarboxylase genes may also be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, etc.) as described supra.

**Isopropanol dehydrogenases**

In some aspects, the host cells comprise a heterologous gene that encodes an isopropanol dehydrogenase. The isopropanol dehydrogenase can be any isopropanol dehydrogenase that is suitable for the host cells and their methods of use described herein, such as a naturally occurring isopropanol dehydrogenase or a variant thereof that retains isopropanol dehydrogenase activity. In one aspect, the isopropanol dehydrogenase is present in the cytosol of the host cells.

In some aspects, the host cells comprising a heterologous isopropanol dehydrogenase gene have an increased level of isopropanol dehydrogenase activity compared to the host cells without the heterologous isopropanol dehydrogenase gene,
when cultivated under the same conditions. In some aspects, the host cells have an increased level of isopropanol dehydrogenase activity of at least 5%, e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at 500% compared to the host cells without the heterologous isopropanol dehydrogenase gene, when cultivated under the same conditions.

Exemplary isopropanol dehydrogenase genes that can be used with the host cells and methods of use described herein include, but are not limited to, a *Clostridium beijerinckii* isopropanol dehydrogenase of SEQ ID NO: 19 (which encodes the isopropanol dehydrogenase of SEQ ID NO: 21), a *Thermoanaerobacter ethanolicus* isopropanol dehydrogenase gene comprising SEQ ID NO: 22 (which encodes the isopropanol dehydrogenase of SEQ ID NO: 24), a *Lactobacillus fermentum* isopropanol dehydrogenase gene comprising SEQ ID NO: 47 (which encodes the isopropanol dehydrogenase of SEQ ID NO: 48), a *Lactobacillus antri* isopropanol dehydrogenase gene comprising SEQ ID NO: 37 (which encodes the isopropanol dehydrogenase of SEQ ID NO: 38), a *Thermoanaerobacter brockii* isopropanol dehydrogenase (P14941 :1, Hanai et al., *Appl. Environ. Microbiol.* 73:7814-7818 (2007); Peretz et al., *Anaerobe* 3:259-270 (1997)), a *Ralstonia eutropha* n-propanol dehydrogenase (formerly *Alcaligenes eutrophus*) (YP_299391 :1, Steinbuchel and Schlegel et al., *Eur. J. Biochem.* 141 :555-564 (1984)), a *Burkholderia* sp. AIU 652 isopropanol dehydrogenase, and a *Phytoponas* species isopropanol dehydrogenase (AAP39869.1, Uttaro and Opperdoes et al., *Mol. Biochem. Parasitol.* 85:213-219 (1997)).

In one aspect, the isopropanol dehydrogenase has at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the isopropanol dehydrogenase encoded by any isopropanol dehydrogenase gene described herein (e.g., any isopropanol dehydrogenase of SEQ ID NO: 21, 24, 38, or 48). In one aspect, the isopropanol dehydrogenase sequence differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two amino acids, or by one amino acid from the isopropanol dehydrogenase encoded by any isopropanol dehydrogenase gene described herein (e.g., any isopropanol dehydrogenase of SEQ ID NO: 21, 24, 38, or 48). In one aspect, the isopropanol dehydrogenase comprises or consists of the amino acid sequence of the isopropanol dehydrogenase encoded by any isopropanol dehydrogenase gene described herein (e.g., any isopropanol dehydrogenase of SEQ ID NO: 21, 24, 38, or 48), allelic variant, or a fragment thereof having isopropanol
dehydrogenase activity. In one aspect, the isopropanol dehydrogenase has an amino acid substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids. In some aspects, the total number of amino acid substitutions, deletions and/or insertions is not more than 10, e.g., not more than 9, 8, 7, 6, 5, 4, 3, 2, or 1.

In one aspect, the coding sequence of the heterologous isopropanol dehydrogenase gene hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with with the full-length complementary strand of the coding sequence of any isopropanol dehydrogenase gene described herein (e.g., any isopropanol dehydrogenase gene of SEQ ID NO: 19, 20, 22, 23, 37, or 47).

In one aspect, the coding sequence of the heterologous isopropanol dehydrogenase gene has at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity with the coding sequence of any isopropanol dehydrogenase gene described herein (e.g., any isopropanol dehydrogenase gene of SEQ ID NO: 19, 20, 22, 23, 37, or 47).

In one aspect, the coding sequence of the heterologous isopropanol dehydrogenase gene comprises the coding sequence of any isopropanol dehydrogenase gene described herein (e.g., any isopropanol dehydrogenase gene of SEQ ID NO: 19, 20, 22, 23, 37, or 47). In one aspect, the coding sequence of the heterologous isopropanol dehydrogenase gene comprises a subsequence of any isopropanol dehydrogenase gene described herein. In one aspect, the number of nucleotides residues in the subsequence is at least 75%, e.g., at least 80%, 85%, 90%, or 95% of the number of the referenced sequence.

Polynucleotides encoding the isopropanol dehydrogenase may be obtained from microorganisms of any genus, as described supra.

The isopropanol dehydrogenases can also include fused polypeptides or cleavable fusion polypeptides, as described supra.

The isopropanol dehydrogenase genes can also be used to design nucleic acid probes to identify and clone DNA encoding isopropanol dehydrogenases from strains of different genera or species, as described supra.

Techniques used to isolate or clone a polynucleotide encoding an isopropanol dehydrogenase are described supra.

The isopropanol dehydrogenase genes may also be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water,
etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, etc.) as described supra.

Expression Vectors and Nucleic Acid Constructs

The *Lactobacillus* host cells described herein may utilize expression vectors comprising the coding sequence of one or more (e.g., two, several) heterologous isopropanol pathway genes (e.g., the coding sequence of a thiolase, butyrate-acetoacetate CoA transferase, acetoacetate decarboxylase, and/or isopropanol dehydrogenase described herein) linked to one or more control sequences that direct expression in a suitable host cell under conditions compatible with the control sequence(s). Such expression vectors may be used in any of the host cells and methods described herein. The polynucleotides described herein may be manipulated in a variety of ways to provide for expression of a desired polypeptide. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more (e.g., two, several) convenient restriction sites to allow for insertion or substitution of the polynucleotide at such sites. Alternatively, the polynucleotide(s) may be expressed by inserting the polynucleotide(s) 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 virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

In one aspect, each gene coding sequence is contained on an independent vector. In one aspect, at least two of the gene coding sequences are contained on a single vector. In one aspect, at least three of the gene coding sequences are contained on a single vector. In one aspect, at least four of the gene coding sequences are contained on a single vector. In one aspect, all the gene coding sequences are contained on a single vector. Polynucleotides encoding heteromeric subunits of a protein complex (e.g., a CoA-transferase) may be contained in a single heterologous polynucleotide on a single vector or alternatively contained in separate heterologous polynucleotides on separate vectors.

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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 expression vector may contain any suitable promoter sequence that is recognized by a host cell for expression of any isopropanol pathway gene described herein. The promoter sequence contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any polynucleotide 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 the host cell.

Each gene coding sequence described herein may be operably linked to a promoter that is foreign to the gene. For example, in one aspect, the gene coding sequence encoding a thiolase, butyrate-acetoacetate CoA transferase, acetoacetate decarboxylase, and/or isopropanol dehydrogenase is operably linked to promoter foreign to the polynucleotide.

As described supra, polynucleotides encoding heteromeric subunits of a protein complex may be contained in a single heterologous polynucleotide (e.g., a single plasmid), or alternatively contained in separate heterologous polynucleotides (e.g., on separate plasmids). For example, in one aspect, the first heterologous polynucleotide encoding a first subunit, and the second heterologous polynucleotide encoding a second subunit are contained in a single heterologous polynucleotide operably linked to a promoter that is foreign to both the heterologous polynucleotide encoding the first subunit and the heterologous polynucleotide encoding the second subunit. In one aspect, the first heterologous polynucleotide encoding a first subunit, and the second heterologous polynucleotide encoding a second subunit are each contained in separate unlinked heterologous polynucleotides, wherein the heterologous polynucleotide encoding the first subunit is operably linked to a foreign promoter, and the heterologous polynucleotide encoding the second subunit is operably linked to a foreign promoter. The promoters in the foregoing may be the same or different.
Examples of suitable promoters for directing the transcription of the nucleic acid constructs in a bacterial host cell are the promoters obtained from the Bacillus amyloliquetaciens alpha-amyrase gene (amyQ), Bacillus licheniformis alpha-amyrase gene (amyL), Bacillus licheniformis penicillinase gene (penP), Bacillus stearothermophilus maltogenic amylase gene (amyM), Bacillus subtilis levansucrase gene (sacB), Bacillus subtilis xylA and xylB genes, E. coli lac operon, E. coli trc promoter (Egon et al., 1988, Gene 69: 301-315), Streptomyces coelicolor agarase gene (dagA), and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. USA 75: 3727-3731), as well as the tac promoter (DeBoer et al., 1983, Proc. Natl. Acad. Sci. USA 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert et al., 1980, Scientific American, 242: 74-94; and in Sambrook et al., 1989, supra. Additional exemplary synthetic promoters useful for gene expression in Lactobacillus can be found in Rud et al., 2006, Microbiology 152, 101 1-1019.

The control sequence may also be a suitable transcription terminator sequence, which is recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3'-terminus of the polynucleotide encoding the polypeptide. Any terminator that is functional in the host cell of choice may be used.

The control sequence may also be a suitable leader sequence, when transcribed is a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5'-terminus of the polynucleotide encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used.

The control sequence may also be a polyadenylation sequence; a sequence operably linked to the 3'-terminus of the polynucleotide and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell of choice may be used.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the polypeptide. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. The foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, the foreign signal peptide coding sequence may simply replace the
natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used.

Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for Bacillus NCIB 11837 maltogenic amylase, Bacillus licheniformis subtilisin, Bacillus licheniformis beta-lactamase, Bacillus stearothermophilus alpha-amylase, Bacillus stearothermophilus neutral proteases (nprT, nprS, nprM), and Bacillus subtilis prsA. Further signal peptides are described by Simonen and Palva, 1993, Microbiological Reviews 57: 109-137.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for Bacillus subtilis alkaline protease (aprE), Bacillus subtilis neutral protease (nprT), Myceliophthora thermophila laccase (WO 95/33836), Rhizomucor miehei aspartic proteinase, and Saccharomyces cerevisiae alpha-factor.

Where both signal peptide and propeptide sequences are present at the N-terminus of a polypeptide, the propeptide sequence is positioned next to the N-terminus of a polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that allow the 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. Other examples of regulatory sequences are those that allow for gene amplification.

The vectors may contain one or more (e.g., two, 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 the dal genes from Bacillus subtilis or Bacillus licheniformis, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, erythromycin, kanamycin, spectinomycin, or tetracycline resistance.
The vectors may contain one or more (e.g., two, several) elements that permit integration of the vector into the host cell's genome or autonomous replication of the vector in the cell 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 non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing 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 contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to 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 polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination. Integration also my use techniques known in the art, such as the use of temperature sensitive plasmids.

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" means a polynucleotide 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, and pUB1 10, pE194, pTA1060, and pAMβI permitting replication in Bacillus.

More than one copy of a polynucleotide described herein may be inserted into a host cell to increase production of a polypeptide. 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 polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

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

A construct or vector (or multiple constructs or vectors) comprising the one or more (e.g., two, several) heterologous isopropanol pathway genes may be introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

The introduction of a construct or vector containing one or more heterologous isopropanol pathway genes into a Lactobacillus cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, Mol. Gen. Genet. 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, J. Bacteriol. 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, J. Mol. Biol. 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, Biotechniques 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, J. Bacteriol. 169: 5271-5278).

Gene Disruptions

The Lactobacillus host cell may also comprise one or more (e.g., two, several) gene disruptions, e.g., to divert sugar metabolism from undesired products to isopropanol. In one aspect, the Lactobacillus host cells comprise a disrupted endogenous acetate kinase gene. In some of these aspects, the Lactobacillus host cells produce a greater amount of isopropanol compared to the cell without the disruption when cultivated under identical conditions. In some aspects, the endogenous gene is inactivated.

The disrupted gene may be any suitable endogenous gene encoding an acetate kinase. Examples of endogenous genes encoding the acetate kinase include the Lactobacillus reuteri gene having the coding sequence shown in SEQ ID NO: 59, which encode subunits having the amino acid sequences of SEQ ID NO: 60. Additional endogenous acetate kinase genes from Lactobacillus are described in U.S. Provisional Application No. 61/653,908, the content of which is hereby incorporated by reference.

In some aspects, the endogenous gene encodes an acetate kinase having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 60. In some aspects, the endogenous gene encodes an acetate kinase having a sequence that differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two
amino acids, or by one amino acid from SEQ ID NO: 60. In some aspects, the endogenous
gene encodes an acetate kinase comprising or consisting of SEQ ID NO: 60.

In other aspects of the host cells and related methods, the coding sequence of the
endogenous gene encoding an acetate kinase has at least 60%, e.g., at least 70%, at least
75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at
least 99%, or 100% sequence identity to SEQ ID NO: 59. In some aspects, the coding
sequence of the endogenous gene comprises or consists of SEQ ID NO: 59.

In other aspects of the host cells and related methods, the coding sequence of the
gene encoding the acetate kinase hybridizes under at least low stringency conditions,
medium stringency conditions, medium-high stringency conditions, high stringency
conditions, or very high stringency conditions with the full-length complementary strand of
SEQ ID NO: 59.

The *Lactobacillus* host cells comprising a disrupted endogenous acetate kinase
gene may be constructed using methods well known in the art, including those methods
described herein. A portion of the gene can be disrupted such as the coding region or a
control sequence required for expression of the coding region. Such a control sequence of
the gene may be a promoter sequence or a functional part thereof, *i.e.*, a part that is
sufficient for affecting expression of the gene. For example, a promoter sequence may be
inactivated resulting in no expression or a weaker promoter may be substituted for the
native promoter sequence to reduce expression of the coding sequence. Other control
sequences for possible modification include, but are not limited to, a leader, propeptide
sequence, signal sequence, transcription terminator, and transcriptional activator.

The *Lactobacillus* host cells comprising a disrupted endogenous acetate kinase
gene may be constructed by gene deletion techniques to eliminate or reduce expression of
the gene. Gene deletion techniques enable the partial or complete removal of the gene
thereby eliminating their expression. In such methods, deletion of the gene is accomplished
by homologous recombination using a plasmid that has been constructed to contiguously
contain the 5' and 3' regions flanking the gene.

The *Lactobacillus* host cells comprising a disrupted endogenous acetate kinase
gene may also be constructed by introducing, substituting, and/or removing one or more
(several) nucleotides in the gene or a control sequence thereof required for the
transcription or translation thereof. For example, nucleotides may be inserted or removed
for the introduction of a stop codon, the removal of the start codon, or a frame-shift of the
open reading frame. Such a modification may be accomplished by site-directed
mutagenesis or PCR generated mutagenesis in accordance with methods known in the art.

The *Lactobacillus* host cells comprising a disrupted endogenous acetate kinase gene may also be constructed by gene disruption techniques by inserting into the gene a disruptive nucleic acid construct comprising a nucleic acid fragment homologous to the gene that will create a duplication of the region of homology and incorporate construct DNA between the duplicated regions. Such a gene disruption can eliminate gene expression if the inserted construct separates the promoter of the gene from the coding region or interrupts the coding sequence such that a non-functional gene product results. A disrupting construct may be simply a selectable marker gene accompanied by 5' and 3' regions homologous to the gene. The selectable marker enables identification of transformants containing the disrupted gene.

The *Lactobacillus* host cells comprising a disrupted endogenous acetate kinase gene may also be constructed by the process of gene conversion (see, for example, Iglesias and Trautner, *Molecular General Genetics* 1983, 189, 73-76). For example, in the gene conversion method, a nucleotide sequence corresponding to the gene is mutagenized *in vitro* to produce a defective nucleotide sequence, which is then transformed into the *Lactobacillus* strain to produce a defective gene. By homologous recombination, the defective nucleotide sequence replaces the endogenous gene. It may be desirable that the defective nucleotide sequence also comprises a marker for selection of transformants containing the defective gene.

The *Lactobacillus* host cells comprising a disrupted endogenous acetate kinase gene may also be constructed by established anti-sense techniques using a nucleotide sequence complementary to the nucleotide sequence of the gene (Parish and Stoker, *FEMS Microbiol. Lett.* 1997, 154, 151-157). More specifically, expression of the gene by a *Lactobacillus* strain may be reduced or inactivated by introducing a nucleotide sequence complementary to the nucleotide sequence of the gene, which may be transcribed in the strain and is capable of hybridizing to the mRNA produced in the strain. Under conditions allowing the complementary anti-sense nucleotide sequence to hybridize to the mRNA, the amount of protein translated is thus reduced or eliminated.

The *Lactobacillus* host cells comprising a disrupted endogenous acetate kinase gene may also be constructed by established RNA interference (RNAi) techniques (see, for example, WO 2005/056772 and WO 2008/080017).
The *Lactobacillus* host cells comprising a disrupted endogenous acetate kinase gene may be further constructed by random or specific mutagenesis using methods well known in the art, including, but not limited to, chemical mutagenesis (see, for example, Hopwood, *The Isolation of Mutants* in *Methods in Microbiology* (J.R. Norris and D.W. Ribbons, eds.) pp. 363-433, Academic Press, New York, 1970). Modification of the gene may be performed by subjecting the parent strain to mutagenesis and screening for mutant strains in which expression of the gene has been reduced or inactivated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, use of a suitable oligonucleotide, or subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing methods.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N'-nitroso-N-nitrosoguanidine (NTG) O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the parent strain to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and selecting for mutants exhibiting reduced or no expression of the gene.

A nucleotide sequence homologous or complementary to a gene described herein may be used from other microbial sources to disrupt the corresponding gene in a *Lactobacillus* strain of choice.

In one aspect, the modification of a gene in the *Lactobacillus* host cell is unmarked with a selectable marker. Removal of the selectable marker gene may be accomplished by culturing the mutants on a counter-selection medium. Where the selectable marker gene contains repeats flanking its 5' and 3' ends, the repeats will facilitate the looping out of the selectable marker gene by homologous recombination when the mutant strain is submitted to counter-selection. The selectable marker gene may also be removed by homologous recombination by introducing into the mutant strain a nucleic acid fragment comprising 5' and 3' regions of the defective gene, but lacking the selectable marker gene, followed by selecting on the counter-selection medium. By homologous recombination, the defective gene containing the selectable marker gene is replaced with the nucleic acid fragment lacking the selectable marker gene. Other methods known in the art may also be used.
Methods of producing isopropanol

The Lactobacillus host cells described herein may be used for the production of isopropanol. In one aspect is a method of producing isopropanol, comprising: (a) cultivating any one of the Lactobacillus host cells described herein in a medium under suitable conditions to produce the isopropanol; and (b) recovering the isopropanol.

The Lactobacillus host cells comprising an active isopropanol pathway may be cultivated in a nutrient medium suitable for isopropanol production using methods well known in the art. For example, the cell may be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable fermentation medium and under conditions allowing isopropanol production.

The Lactobacillus host cells may produce isopropanol in a fermentable medium comprising any one or more (e.g., two, several) sugars, such as glucose, fructose, sucrose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides. In some instances, the fermentable medium is derived from a natural source, such as sugar cane, starch, or cellulose, and may be the result of pretreating the source by enzymatic hydrolysis (saccharification). In some aspects, the fermentable medium comprises sugar cane juice. Suitable media are available from commercial suppliers, may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection), or may be prepared from commercially available ingredients.

In one aspect, the Lactobacillus host cells are cultivated in the presence of fructose, such as at least 0.1%, 0.25%, 0.5%, 0.75%, 1%, 2%, 3%, 4%, 5% or 10% fructose. In one aspect, the Lactobacillus host cells are cultivated in the presence of 1,2-propanediol, such as at least 0.1%, 0.25%, 0.5%, 0.75%, 1%, 2%, 3%, 4%, 5% or 10% 1,2-propanediol.

In addition to the appropriate carbon sources from one or more (e.g., two, several) sugar(s), the fermentable medium may contain other nutrients or stimulators known to those skilled in the art, such as macronutrients (e.g., nitrogen sources) and micronutrients (e.g., vitamins, mineral salts, and metallic cofactors). In some aspects, the carbon source can be preferentially supplied with at least one nitrogen source, such as yeast extract, N₂, peptone (e.g., Bacto™ Peptone), or soytone (e.g., Bacto™ Soytone). Nonlimiting examples of vitamins include multivitamins, biotin, pantothenate, nicotinic acid, meso-inositol, thiamine, pyridoxine, para-aminobenzoic acid, folic acid, riboflavin, and Vitamins A, B, C, D, and E. Examples of mineral salts and metallic cofactors include, but are not limited to Na, P, K, Mg, S, Ca, Fe, Zn, Mn, Co and Cu.
Suitable conditions used for the methods of propanol production may be determined by one skilled in the art in light of the teachings herein. In some aspects of the methods, the host cells are cultivated for about 12 hours to about 216 hours, such as about 24 hours to about 144 hours, or about 36 hours to about 96 hours. The temperature is typically between about 26°C to about 60°C, e.g., about 34°C to about 50°C, and at a pH of about 3.0 to about 8.0, such as about 3.0 to about 7.0, about 3.0 to about 6.0, about 3.0 to about 5.0, about 3.5 to about 4.5, about 4.0 to about 8.0, about 4.0 to about 7.0, about 4.0 to about 6.0, about 4.0 to about 5.0, about 5.0 to about 8.0, about 5.0 to about 7.0, or about 5.0 to about 6.0 or less than about 6.0, less than about 5.0, less than about 4.5, less than about 4.0, less than about 3.5, less than about 3.0, or less than about 2.5. In some aspects of the methods, the resulting intracellular pH of the host cell is about 2.0 to about 8.0, such as about 2.0 to about 7.0, about 2.0 to about 6.0, about 2.0 to about 5.0, about 1.5 to about 4.5, about 3.0 to about 8.0, such as about 3.0 to about 7.0, about 3.0 to about 6.0, about 3.0 to about 5.0, about 3.5 to about 4.5, about 4.0 to about 8.0, about 4.0 to about 7.0, about 4.0 to about 6.0, about 4.0 to about 5.0, about 5.0 to about 8.0, about 5.0 to about 7.0, or about 5.0 to about 6.0, or less than about 6.0, less than about 5.0, less than about 4.5, less than about 4.0, less than about 3.5, less than about 3.0, or less than about 2.5. Cultivation may be performed under anaerobic, microaerobic, or aerobic conditions, as appropriate. In some aspects, the cultivation is performed under anaerobic conditions.

Cultivation may be performed under anaerobic, substantially anaerobic (microaerobic), or aerobic conditions, as appropriate. Briefly, anaerobic refers to an environment devoid of oxygen, substantially anaerobic (microaerobic) refers to an environment in which the concentration of oxygen is less than air, and aerobic refers to an environment wherein the oxygen concentration is approximately equal to or greater than that of the air. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains less than 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/C₀₂ mixture or other suitable non-oxygen gas or gases. In some embodiments, the cultivation is performed under anaerobic conditions or substantially anaerobic conditions.

The methods of described herein can employ any suitable fermentation operation mode. For example, batch mode fermentation may be used with a close system where
culture media and host microorganism, set at the beginning of fermentation, have no additional input except for the reagents certain reagents, e.g., for pH control, foam control or others required for process sustenance. The process described herein can also be employed in Fed-batch or continuous mode.

The methods described herein may be practiced in several bioreactor configurations, such as stirred tank, bubble column, airlift reactor and others known to those skilled in the art. The methods may be performed in free cell culture or in immobilized cell culture as appropriate. Any material support for immobilized cell culture may be used, such as alginates, fibrous bed, or argyle materials such as chrysotile, montmorillonite KSF and montmorillonite K-10.

In one aspect of the methods, the isopropanol is produced at a titer greater than about 5 g/L, e.g., greater than about 10 g/L, 25 g/L, 50 g/L, 75 g/L, 100 g/L, 125 g/L, 150 g/L, 160 g/L, 170 g/L, 180 g/L, 190 g/L, 200 g/L, 210 g/L, 225 g/L, 250 g/L, 275 g/L, 300 g/L, 325 g/L, 350 g/L, 400 g/L, or 500/g/L; or between about 10 g/L and about 500 g/L, e.g., between about 50 g/L and about 350 g/L, about 100 g/L and about 300 g/L, about 150 g/L and about 250 g/L, about 175 g/L and about 225 g/L, or about 190 g/L and about 210 g/L.

In one aspect of the methods, the isopropanol is produced at a titer greater than about 0.01 gram per gram of carbohydrate, e.g., greater than about 0.02, 0.05, 0.75, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 gram per gram of carbohydrate.

In one aspect of the methods, the amount of produced isopropanol is at least 5%, e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, or at least 100% greater compared to cultivating the host cell without the heterologous polynucleotides encoding the butyrate-acetoacetate CoA transferase when cultivated under the same conditions.

The isopropanol can be optionally recovered from the fermentation medium using any procedure known in the art including, but not limited to, chromatography (e.g., size exclusion chromatography, adsorption chromatography, ion exchange chromatography), electrophoretic procedures, differential solubility, osmosis, distillation, extraction (e.g., liquid-liquid extraction), pervaporation, extractive filtration, membrane filtration, membrane separation, reverse, or ultrafiltration. In one aspect, the isopropanol is separated from other fermented material and purified by conventional methods of distillation. Accordingly, in one aspect, the method further comprises purifying the recovered isopropanol by distillation.

The recombinant isopropanol may also be purified by the chemical conversion of impurities (contaminants) to products more easily removed from isopropanol by the procedures described above (e.g., chromatography, electrophoretic procedures, differential
solubility, distillation, or extraction) and/or by direct chemical conversion of impurities to isopropanol. For example, in one aspect, the method further comprises purifying the recovered isopropanol by converting acetone contaminant to isopropanol. Conversion of acetone to isopropanol may be accomplished using any suitable reducing agent known in the art (e.g., lithium aluminium hydride (LiAlH₄), a sodium species (such as sodium amalgam or sodium borohydride (NaBH₄)), tin species (such as Sn(ll) chloride), hydrazine, zinc-mercury amalgam (Zn(Hg)), diisobutylaluminum hydride (DIBAH), oxalic acid (C₂H₂O₄), formic acid (HCOOH), Ascorbic acid, iron species (such as iron(lll) sulfate), and the like).

In some aspects of the methods, the recombinant isopropanol preparation before and/or after being optionally purified is substantially pure. With respect to the methods of producing isopropanol, "substantially pure" intends a recovered preparation that contains no more than 15% impurity, wherein impurity intends compounds other than propanol but does not include the n-propanol isomer. This, a substantially pure preparation of isopropanol may contain n-propanol in excess of 15%. In one variation, a substantially pure preparation is provided wherein the preparation contains no more than 25% impurity, or no more than 20% impurity, or no more than 10% impurity, or no more than 5% impurity, or no more than 3% impurity, or no more than 1% impurity, or no more than 0.5% impurity.

The Lactobacillus host cells may also be engineered to coproduce isopropanol and n-propanol, as described in PCT/US2011/58405, the content of which is hereby incorporated in its entirety (particularly the discussions on coproduction of isopropanol and n-propanol).

The isopropanol produced by any of the methods described herein may be converted to propylene. Propylene can be produced by the chemical dehydration of isopropanol using acidic catalysts known in the art, such as acidic alumina, zeolites, and other metallic oxides; acidic organic-sulfonic acid resins; mineral acids such as phosphoric and sulfuric acids; and Lewis acids such as boron trifluoride and aluminum compounds (March, Jerry. Advanced Organic Chemistry. New York: John Wiley and Sons, 1992). Suitable temperatures for dehydration of isopropanol to propylene typically range from about 180°C to about 600°C, e.g., 300°C to about 500°C, or 350°C to about 450°C.

The dehydration reaction of isopropanol is typically conducted in an adiabatic or isothermal reactor, which can also be a fixed or a fluidized bed reactor; and can be optimized using residence time ranging from about 0.1 to about 60 seconds, e.g., from about 1 to about 30 seconds. Non-converted alcohol can be recycled to the dehydration reactor.

In one aspect is a method of producing propylene, comprising: (a) cultivating a
recombinant *Lactobacillus* host cell described herein (e.g., a recombinant *Lactobacillus* host cell comprising heterologous polynucleotides encoding a butyrate-acetoacetate CoA transferase) in a medium under suitable conditions to produce isopropanol; (b) recovering the isopropanol; (c) dehydrating the isopropanol under suitable conditions to produce propylene; and (d) recovering the propylene.

Contaminants that may be generated during dehydration may be removed through purification using techniques known in the art. For example, propylene can be washed with water or a caustic solution to remove acidic compounds like carbon dioxide and/or fed into beds to absorb polar compounds like water or for the removal of, e.g., carbon monoxide. Alternatively, a distillation column can be used to separate higher hydrocarbons such as propane, butane, butylene and higher compounds. The separation of propylene from contaminants like ethylene may be carried out by methods known in the art, such as cryogenic distillation.

Suitable assays to test for the production of isopropanol and propylene for the methods of production and host cells described herein can be performed using methods known in the art. For example, final isopropanol product and intermediates (e.g., acetone), as well as 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 isopropanol in the fermentation broth can also be tested with the culture supernatant. Byproducts and residual sugar in the fermentation medium (e.g., glucose) can be quantified by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., *Biotechnol. Bioeng.* 90:775 -779 (2005)), or using other suitable assay and detection methods well known in the art.

The propylene produced from isopropanol may be further converted to polypropylene or polypropylene copolymers by polymerization processes known in the art. Suitable temperatures typically range from about 105°C to about 300°C for bulk polymerization, or from about 50°C to about 100°C for polymerization in suspension. Alternatively, polypropylene can be produced in a gas phase reactor in the presence of a polymerization catalyst such as Ziegler-Natta or metalocene catalysts with temperatures ranging from about 60°C to about 80°C.

The following examples are provided by way of illustration and are not intended to be limiting of the invention.
Examples

Chemicals used as buffers and substrates were commercial products of at least reagent grade.

Base Host Strains

*Lactobacillus reuteri SJ10655 (Q4ZXV)*

Strain *Lactobacillus reuteri* DSM20016 was obtained from a public strain collection. This strain was subcultured in MRS medium, and an aliquot frozen as SJ10468. SJ10468 was inoculated into MRS medium, propagated without shaking for one day at 37°C, and spread on MRS agar plates to obtain single colonies. After two days of growth at 37°C, a single colony was reisolated on a MRS agar plate, the plate incubated at 37°C for three days, and the cell growth on the plate was scraped off and stored in the strain collection as SJ10655 (alternative name: 04ZXV).

The same cell growth was used to inoculate a 10 ml MRS culture, which was incubated without shaking at 37°C for 3 days, whereafter cells were harvested by centrifugation and genomic DNA was prepared using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) and sent for genome sequencing.

The genome sequence revealed that the isolate SJ10655 (04ZXV) has a genome essentially identical to that of JCM1112, rather than to that of the closely related strain DSM20016. JCM1112 and DSM20016 are derived from the same original isolate, *L. reuteri* F275 (Morita et al. DNA research, 2008, 15, 151-161.)

*Lactobacillus reuteri SJ11044*

Strain *Lactobacillus reuteri* SJ1 1044 was derived from strain SJ10655 (04ZXV) as described in U.S. Provisional Application No. 61/653,908.

*Lactobacillus reuteri SJ11294 (SJ11400)*

*L. reuteri* strain SJ1 1294 (SJ1 1400) is a modified version of 04ZXV (supra) which has improved transformation efficiency from a disrupted gene encoding a specificity subunit (LAR_0818) of a type I restriction modification system, as described in U.S. Provisional Application No. 61/648,958.

*Lactobacillus reuteri TRGU975 and SJ11538*

*L. reuteri* strains TRGU975 and SJ1 1538 are modified versions of *L. reuteri* strains 04ZXV and SJ1 1400, respectively, having a disrupted acetate kinase gene (SEQ ID NO: 59), as described in U.S. Provisional Application No. 61/653,908.


Escherichia coli TG1

TG1 is a commonly used cloning strain and was obtained from a commercial supplier; it has the following genotype: F'[traD36 lacIq Δ(lacZ) M15 proA+B+] glnV (supE) thi-1 Δ(mcrB-hsdSM)5 (rK- mK- McrB-) thi Δ(lac-proAB).
Media

LB plates were composed of 37 g LB agar (Sigma cat no. L3027) and double distilled water to 1L.

LBP4GS plates were composed of 37 g LB agar (Sigma cat no. L3027), 0.5% starch (Merck cat. no. 101252), 0.01 M K2PO4, 0.4% glucose, and double distilled water to 1L.

TY bouillon medium was composed of 20 g tryptone (Difco cat no. 211699), 5 g yeast extract (Difco cat no. 212750), 7*10^{-3} g ferrocyanide, 1*10^{-3} g manganese(II)-chloride, 1.5*10^{-3} g magnesium sulfate, and double distilled water to 1L.

Minimal medium (MM) was composed of 20 g glucose, 1.1 g KH2PO4, 8.9 g K2HP04; 1.0 g (NH4)2SO4; 0.5 g Na-citrate; 5.0 g MgSO4-7H2O; 4.8 mg MnSO4-7H2O; 2 mg thiamine; 0.4 mg/L biotin; 0.135 g FeCl3-6H2O; 10 mg ZnCl2-6H2O; 10 mg CaCl2-6H2O; 10 mg Na2MoO4-2H2O; 9.5 mg CuSO4-5H2O; 2.5 mg H3BO3; and double distilled water to 1L, pH adjusted to 7 with HCl.

MRS medium was obtained from Difco™, as either Difco™ Lactobacilli MRS Agar or Difco™ Lactobacilli MRS Broth, having the following compositions—Difco™ Lactobacilli MRS Agar: Proteose Peptone No. 3 (10.0 g), Beef Extract (10.0 g), Yeast Extract (5.0 g), Dextrose (20.0 g), Polysorbate 80 (1.0 g), Ammonium Citrate (2.0 g), Sodium Acetate (5.0 g), Magnesium Sulfate (0.1 g), Manganese Sulfate (0.05 g), Dipotassium Phosphate (2.0 g), Agar (15.0 g) and water to 1L. Difco™ Lactobacilli MRS Broth: Consists of the same ingredients without the agar.

LC (Lactobacillus Carrying) medium (LCM) was composed of Trypticase (10 g), Tryptose (3 g), Yeast extract (5 g), KH2PO4 (3 g), Tween 80 (1 ml), sodium-acetate (1 g), ammonium citrate (1.5 g), Cystein-HCl (0.2 g), MgSO4-7H2O (12 mg), FeSO4-7H2O (0.68 mg), MnSO4-7H2O (25 mg), and double distilled water to 1L, pH adjusted to 7.0. Sterile glucose is added after autoclaving, to 1% (5 ml of a 20% glucose stock solution/100 ml medium).

Example 1: Transformation protocol for Lactobacillus strains.

Lactobacillus strains

Unless noted otherwise, plasmid DNA constructed in E. coli was purified from 2 ml of an overnight culture grown in TY medium, and supplemented with appropriate antibiotics using a QiAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) as described by the manufacturer. The plasmid DNA was recovered in a volume of 50 microliters, and one microliter of this plasmid preparation was used for electroporation of Lactobacillus.

Plasmid DNA was transformed into Lactobacillus strains by electroporation. The L.
reuteri strains were prepared for electroporation as follows: The strain was inoculated from a frozen stock culture into LCM medium, and incubated without shaking at 37°C overnight. A 5 ml aliquot was transferred into 500 ml LCM medium and incubated at 37°C without shaking until OD$_{600}$ reached approximately 0.8. The cells were harvested by centrifugation as above, resuspended and washed 2 times in 50 ml of ion-exchanged sterile water at room temperature, and harvested by centrifugation. The cells were finally gently resuspended in 2.5 ml of 30% PEG1500, and 50 microliter aliquots were quickly frozen in an alcohol/dry ice bath, and stored at -80°C until use. Variations to the electroporation procedures below are described in the respective examples.

Electroporation procedure A: The frozen cells were thawed on ice, and 2 microliter of a DNA suspension in TE buffer was added. 40 microliters of the mixture was transferred to an ice-cold 2 mm electroporation cuvette, kept on ice for 1-3 minutes, and electroporation carried out in a BioRad Gene Pulser™ with a setting of 1.5 kV; 25 microFarad; 400 Ohms. 500 microliter of LCM was added, and the mixture incubated without shaking for 2 hours at 37°C before plating. Cells were plated on either LCM agar plates (LCM medium solidified with % agar) or MRS agar plates, supplemented with the required antibiotics, and incubated in an anaerobic chamber (Oxoid; equipped with Anaerogen sachet).

Electroporation procedure B: The frozen cells were thawed on ice, and 1 microliter of a DNA suspension in TE buffer was added. 40 microliters of the mixture was transferred to an ice-cold 1 mm electroporation cuvette, kept on ice for 1-3 minutes, and electroporation carried out in a BioRad Gene Pulser™ with a setting of 1.2 kV; 25 microFarad; 400 Ohms. 500 microliter of LCM was added, and the mixture incubated without shaking for 4 hours at 37 °C before plating on MRS agar plates, supplemented with the required antibiotics, and incubation in an anaerobic chamber.

E. coli strains

Transformation of E. coli was conducted by electroporation using either a BioRad Gene Pulser™ (BioRad, Hercules, CA, USA) as described by the manufacturer, or by using chemically competent cells prepared following ordinary textbook procedures commonly known in the art.

Example 2: Peptide-inducible pSIP expression vectors.

The peptide-inducible expression vectors pSIP409, pSIP410, and pSIP411 (Sørvig, et al. Microbiology 2005, 151, 2439-2449.) were received from Lars Axelsson, Nofima Mat AS, Norway. pSIP409 and pSIP410 were transformed into E. coli SJ2 by electroporation,
selecting erythromycin resistance (150 microgram/ml) on LB agar plates at 37°C. Two transformants containing pSIP409 were kept as SJ10517 and SJ10518, and two transformants containing pSIP410 were kept as SJ10519 and SJ10520.

pSIP411 was transformed into naturally competent *Bacillus subtilis* DN1885 cells, essentially as described (Yasbin et al. *J Bacteriol* 1975, 121, 296-304), selecting for erythromycin resistance (5 microgram/ml) on LBPGS plates at 37°C. Two such transformants were kept as SJ10513 and SJ10514.

pSIP411 was in addition transformed into *E. coli* MG1655 by electroporation, selecting erythromycin resistance (200 microgram/ml) on LB agar plates at 37°C, and two transformants kept as SJ10542 and SJ10543.

For use in induction of gene expression from these vectors in *Lactobacillus*, the inducing peptide, here named M-19-R and having the following amino acid sequence: "Met-Ala-Gly-Asn-Ser-Ser-Asn-Phe-Ile-His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg", was obtained from "Polypeptide Laboratories France, 7 rue de Boulogne, 67100 Strasbourg, France".

**Example 3: Construction of pVS2-based vectors pSJ10600 and pSJ10603 for constitutive expression.**

A set of constitutive expression vectors were constructed based on the plasmid pVS2 (von Wright et al., *Appl. Environ. Microbiol.* 1987, 53, 1584-1588) and promoters described by Rud et al. (Rud et al. *Microbiology* 2006, 152, 101 1-1019). A DNA fragment containing the *P11* promoter with a selection of flanking restriction sites, and another fragment containing P27 with a selection of flanking restriction sites, was chemically synthesized by Geneart AG (Regensburg, Germany).

The DNA fragment containing *P11* with flanking restriction sites, and the DNA fragment containing P27 with flanking restriction sites are shown in SEQ ID NOs: 49 and 50, respectively. Both DNA fragments were obtained in the form of DNA preparations, where the fragments had been inserted into the standard Geneart vector, pMA. The vector containing *P11* was transformed into *E. coli* SJ2 cells, and a transformant kept as SJ10560, containing plasmid pSJ10560. The vector containing P27 was transformed into *E. coli* SJ2 cells, and a transformant kept as SJ10561, containing plasmid pSJ10561.

The promoter-containing fragments, in the form of 176 bp HindIII fragments, were excised from the Geneart vectors and ligated to HindIII-digested pUC19. The *P11*-containing fragment was excised from the vector prepared from SJ10560, ligated to pUC19, and correct transformants of *E. coli* SJ2 were kept as SJ10585 and SJ10586, containing pSJ10585 and pSJ10586, respectively. The P27 containing fragment was
excised from the vector prepared from SJ10561, ligated to pUC19, and correct transformants of \textit{E. coli} SJ2 were kept as SJ10587 and SJ10588, containing pSJ10587 and pSJ10588, respectively.

Plasmid pVS2 was obtained in \textit{Lactobacillus plantarum} NC8, a strain kept as SJ10491, extracted from this strain by standard plasmid preparation procedures known in the art, and transformed into \textit{E. coli} MG1655 selecting erythromycin resistance (200 microgram/ml) on LB agar plates at 37°C. Two such transformants were kept as SJ10583 and SJ10584.

To insert \textit{p11} into pVS2, the \textit{p11}-containing 176 bp HindII fragment was excised and purified by agarose gel electrophoresis from pSJ10585, and ligated to HindII I-digested pVS2, which had been prepared from SJ10583. The ligation mixture was transformed by electroporation into \textit{E. coli} MG1655, selecting erythromycin resistance (200 microgram/ml) on LB agar plates, and two transformants, which both harbor plasmids with the promoter insert in one particular of the two possible orientations, were kept as SJ10600 and SJ10601, containing pSJ10600 (Figure 2) and pSJ10601.

Another transformant, having the promoter insert in the other of the two possible orientations, was kept as SJ10602, containing pSJ10602. The plasmid preparation from SJ10602 appeared to contain less DNA than the comparable preparations from SJ10600 and SJ10601, and, upon further work, pSJ10602 appeared to be rather unstable, with deletion derivatives dominating in the plasmid population.

To insert \textit{p27} into pVS2, the \textit{p27}-containing 176 bp HindII fragment was excised and purified by agarose gel electrophoresis from pSJ10588, and ligated to HindII II-digested pVS2, which had been prepared from SJ10583. The ligation mixture was transformed by electroporation into \textit{E. coli} MG1655, selecting erythromycin resistance (200 microgram/ml) on LB agar plates, and two transformants, which both harbor plasmids with the promoter insert in one particular of the two possible orientations, were kept as SJ10603 and SJ10604, containing pSJ10603 (Figure 3) and pSJ10604.

Another transformant, having the promoter insert in the other of the two possible orientations, was kept as SJ10605, containing pSJ10605. The promoter orientation in this plasmid is the same as in pSJ10602, described above. The plasmid preparation from SJ10605 appeared to contain less DNA than the comparable preparations from SJ10603 and SJ10604, and, upon further work, pSJ10605 appeared to be rather unstable, with deletion derivatives dominating in the plasmid population.
Example 4: Cloning of isopropanol pathway genes.

Cloning of a *Clostridium acetobutylicum* thiolase gene and construction of vector pSJ10705

The 1176 bp coding sequence (without stop codon) of a thiolase gene identified in *Clostridium acetobutylicum* was designed for optimized expression in the three organisms *Escherichia coli*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* and synthetically constructed into pSJ10705. The DNA fragment containing the codon optimized coding sequence was designed with the sequence 5'-AAGCTTTC-3' immediately prior to the start codon (to add a HindIII site and convert the start region to a Ncol-compatible BspHI site), and the sequence 5' - TAGTCTAGACTCGAGGAATTCGGTACC-3' immediately downstream (to add a stop codon, and restriction sites XbaI-XhoI-EcoRI-Kpnl).

The resulting sequence was then submitted to and synthesized by Geneart AG (Regenburg, Germany) and delivered in the pMA backbone vector containing the β-lactamase encoding gene blaTEM-1. The DNA preparation delivered from Geneart was transformed into *E. coli* SJ2 by electroporation, selecting ampicillin resistance (200 microgram/ml) and two transformants kept, as Sj10705 (SJ2/pSJ10705) and Sj10706 (SJ2/pSJ10706).

The wild-type nucleotide sequence (WT), codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the C. *acetobutylicum* thiolase gene are SEQ ID NOs: 1, 2, and 3, respectively. The coding sequence is 1179 bp including the stop codon and the encoded predicted protein is 392 amino acids. Using the SignalP program (Nielsen et al., 1997, *Protein Engineering* 10: 1-6), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 392 amino acids with a predicted molecular mass of 41.4 kDa and an isoelectric pH of 7.08.

Cloning of a *Lactobacillus reuteri* thiolase gene and construction of vector pSJ10694

The 1176 bp thiolase coding sequence (without stop codon) from *Lactobacillus reuteri* was amplified from chromosomal DNA of SJ10468 (supra) using primers 671826 and 671827 shown below.

Primer 671826:
5'-AGTCAAGCTTCCATGGAGAAGGTCTTACATTGTGC-3'  (SEQ ID NO: 51)

Primer 671827:
5'-ATGCGGTACCGAATTCGAGTGCTAGACTAAATTTTCTTAAGCAGAACC-3'  (SEQ ID NO: 52)

The PCR reaction was programmed for 94°C for 2 minutes; and then 19 cycles each at 95°C for 30 seconds, 59°C for 1 minute, and 72°C for 2 minute; then one cycle at 72°C
for 5 minutes. A PCR amplified fragment of approximately 1.2 kb was digested with Ncol +
EcoRI, purified by agarose gel electrophoresis, and then ligated to the agarose gel
electrophoresis purified EcoRI-Ncol vector fragment of plasmid pSIP409. The ligation
mixture was transformed into *E. coli* SJ2, selecting ampicillin resistance (200
microgram/ml), and a transformant, deemed correct by restriction digest and DNA
sequencing, was kept as SJ10694 (SJ2/pSJ10694).

The codon-optimized nucleotide sequence (CO), and deduced amino acid
sequence of the *L. reuteri* thiolase gene are SEQ ID NOs: 25 and 26, respectively. The
coding sequence is 1179 bp including the stop codon and the encoded predicted protein is
392 amino acids. Using the SignalP program (Nielsen et al., 1997, *Protein Engineering* 10:
1-6), no signal peptide in the sequence was predicted. Based on this program, the
predicted mature protein contains 392 amino acids with a predicted molecular mass of 41.0
kDa and an isoelectric pH of 5.4.

Cloning of a *Propionibacterium freudenreichii* thiolase gene and construction of vector
PSJ10676

The 1152 bp coding sequence (without stop codon) of a thiolase gene identified in
*Propionibacterium freudenreichii* was optimized for expression in the three organisms
*Escherichia coli*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* and synthetically
constructed into pSJ10676. The DNA fragment containing the codon optimized CDS was
designed with the sequence 5'-AAGCTTTC-3' immediately prior to the start codon (to add a
HindIII site and convert the start region to a Ncol-compatible BspHI site), and the sequence
5'-TAGTCTAGACTCGAGAATTCGTACC-3' (SEQ ID NO: 53) immediately downstream
(to add a stop codon, and restriction sites Xbal-Xhol-EcoRI-KpnI).

The resulting sequence was then submitted to and synthesized by Geneart AG
(Regenburg, Germany) and delivered in the pMA backbone vector containing the β-
lactamase encoding gene blaTEM-1. The DNA preparation delivered from Geneart was
transformed into *E. coli* SJ2 by electroporation, selecting ampicillin resistance (200
microgram/ml) and two transformants kept, as SJ10676 (SJ2/pSJ10676) and SJ10677
(SJ2/pSJ10677).

The codon-optimized nucleotide sequence (CO), and deduced amino acid
sequence of the *P. freudenreichii* thiolase gene are SEQ ID NOs: 39 and 40, respectively.
The coding sequence is 1155 bp including the stop codon and the encoded predicted protein is 384 amino acids. Using the SignalP program (Nielsen et al., 1997, *Protein
Engineering* 10: 1-6), no signal peptide in the sequence was predicted. Based on this
program, the predicted mature protein contains 384 amino acids with a predicted molecular
mass of 39.8 kDa and an isoelectric pH of 6.1.

Cloning of a Lactobacillus brevis thiolase gene and construction of vector pSJ10699

The 1167 bp coding sequence (without stop codon) of a thiolase gene identified in Lactobacillus brevis was optimized for expression in the three organisms Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri and synthetically constructed into pSJ10699. The DNA fragment containing the codon optimized CDS was designed with the sequence 5'-AAGCTTCC-3' immediately prior to the start codon (to add a HindIII site and convert the start region to a Ncol site), and the sequence 5'-TAGTCTAGACTCGAGGAATTCGGTACC-3' (SEQ ID NO: 53) immediately downstream (to add a stop codon, and restriction sites Xbal-Xhol-EcoRI-Kpnl).

The resulting sequence was then submitted to and synthesized by Geneart AG (Regenburg, Germany) and delivered in the pMA backbone vector containing the β-lactamase encoding gene blaTEM-1. The DNA preparation delivered from Geneart was transformed into E. coli SJ2 by electroporation, selecting ampicillin resistance (200 microgram/ml) and two transformants kept, as SJ10699 (SJ2/pSJ10699) and SJ10700 (SJ2/pSJ10700).

The codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the L. brevis thiolase gene are SEQ ID NOs: 41 and 42, respectively. The coding sequence is 1170 bp including the stop codon and the encoded predicted protein is 389 amino acids. Using the SignalP program (Nielsen et al., 1997, Protein Engineering 10: 1-6), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 389 amino acids with a predicted molecular mass of 40.4 kDa and an isoelectric pH of 6.5.

Cloning of B. subtilis succinyl-CoA:acetoacetate transferase genes and construction of vectors pSJ10695 and pSJ10697

The 699 bp coding sequence (without stop codon) of the scoA subunit of the B. subtilis succinyl-CoA:acetoacetate transferase and the 648 bp coding sequence of the scoB subunit of the B. subtilis succinyl-CoA:acetoacetate transferase were designed for optimized expression in the three organisms Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri and synthetically constructed into pSJ10695 and pSJ10697, respectively.

The DNA fragment containing the codon-optimized scoA coding sequence was designed with the sequence 5'-AAGCTTCTCGAGACTACAGGAATT TAGCC-3' (SEQ ID NO: 54) immediately prior to the start codon (to add a HindIII site, a Lactobacillus
RBS, and to have the start codon within a Ncol site), and an EcoRI restriction site immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10695 (SJ2/pSJ10695) and SJ10696 (SJ2/pSJ10696).

The wild-type nucleotide sequence (WT), codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the *B. subtilis* scoA subunit of the succinyl-CoA:acetoacetate transferase are SEQ ID NOs: 4, 5, and 6, respectively. The coding sequence is 702 bp including the stop codon and the encoded predicted protein is 233 amino acids. Using the SignalP program (Nielsen et al., *supra*), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 233 amino acids with a predicted molecular mass of 25.1 kDa and an isoelectric pH of 6.50.

The DNA fragment containing the codon optimized scoB coding sequence was designed with the sequence 5'-GAATT CACTA TTACA AGGAG ATTTT AGTC-3' (SEQ ID NO: 55) immediately prior to the start codon (to add a EcoRI site, a *Lactobacillus* RBS, and to have the start codon within a Ncol-compatible BspHI site), and Eagl and KpnI restriction sites immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10697 (SJ2/pSJ10697) and SJ10698 (SJ2/pSJ10698).

The wild-type nucleotide sequence (WT), codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the *B. subtilis* scoB subunit of the succinyl-CoA:acetoacetate transferase are SEQ ID NOs: 7, 8, and 9, respectively. The coding sequence is 651 bp including the stop codon and the encoded predicted protein is 216 amino acids. Using the SignalP program (Nielsen et al., *supra*), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 216 amino acids with a predicted molecular mass of 23.4 kDa and an isoelectric pH of 5.07.

**Cloning of *B. mojavensis* succinyl-CoA:acetoacetate transferase genes and construction of vectors**

The 711 bp coding sequence (without stop codon) of the scoA subunit of the *B. mojavensis* succinyl-CoA:acetoacetate transferase and the 654 bp coding sequence (without stop codon) of the scoB subunit of the *B. mojavensis* succinyl-CoA:acetoacetate transferase were designed for optimized expression in the three organisms *Escherichia coli*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* and synthetically constructed into pSJ10721 and pSJ10723, respectively.

The DNA fragment containing the codon-optimized scoA coding sequence was designed with the sequence 5'-AAGCT TCTCG AGACT ATTAC AAGGA GATTT TAGCC-3' - 52 -
The wild-type nucleotide sequence (WT), codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the \textit{B. mojavensis} scoA subunit of the succinyl-CoA:acetoacetate transferase are SEQ ID NOs: 10, 11, and 12, respectively. The coding sequence is 714 bp including the stop codon and the encoded predicted protein is 237 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 237 amino acids with a predicted molecular mass of 25.5 kDa and an isoelectric pH of 5.82.

The DNA fragment containing the codon optimized scoB nucleotide coding sequence was designed with the sequence 5'-GAATT CACTA TTACA AGGAG ATTTT AGTC-3' (SEQ ID NO: 55) immediately prior to the start codon (to add a EcoRI site, a \textit{Lactobacillus} RBS, and to have the start codon within a Ncol site), and an EcoRI restriction site immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10723 (SJ2/pSJ10723) and SJ10724 (SJ2/pSJ10724).

The wild-type nucleotide sequence (WT), codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the \textit{B. mojavensis} scoB subunit of the succinyl-CoA:acetoacetate transferase are SEQ ID NOs: 13, 14, and 15, respectively. The coding sequence is 657 bp including the stop codon and the encoded predicted protein is 218 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 218 amino acids with a predicted molecular mass of 23.7 kDa and an isoelectric pH of 5.40.

Cloning of \textit{E. coli} acetoacetyl-CoA transferase genes and construction of vectors pSJ10715 and PSJ10717

The 648 bp coding sequence (without stop codon) of the \textit{atoA} subunit (uniprot:P76459) of the \textit{E. coli} acetyl-CoA transferase and the 660 bp coding sequence (without stop codon) of the \textit{atoD} subunit (uniprot:P76458) of the \textit{E. coli} acetyl-CoA transferase were optimized for expression in the three organisms \textit{Escherichia coli}, \textit{Lactobacillus plantarum}, and \textit{Lactobacillus reuteri} and synthetically constructed into pSJ10715 and pSJ10717, respectively.

The DNA fragment containing the codon-optimized \textit{atoA} subunit nucleotide coding
sequence was designed with the sequence 5’-AAGCT TCTCG AGACT ATTAC AAGGA GATTT TAGCC-3’ (SEQ ID NO: 54) immediately prior to the start codon (to add HindI1 and XhoI sites, a Lactobacillus RBS, and to have the start codon within a NcoI site), and an EcoRI restriction site immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10715 (SJ2/pSJ10715) and SJ10716 (SJ2/pSJ10716).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the E. coli atoA subunit of the acetoacetyl-CoA transferase are SEQ ID NOs: 27 and 28, respectively. The coding sequence is 651 bp including the stop codon and the encoded predicted protein is 216 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 216 amino acids with a predicted molecular mass of 23.0 kDa and an isoelectric pH of 5.9.

The DNA fragment containing the codon optimized atoD nucleotide coding sequence was designed with the sequence 5’-GAATT CACTA TTACA AGGAG ATTTT AGTC-3’ (SEQ ID NO: 55) immediately prior to the start codon (to add a EcoRI site, a Lactobacillus RBS, and to have the start codon within a NcoI-compatible BspHI site), and EagI and KpnI restriction sites immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10717 (SJ2/pSJ10717) and SJ10718 (SJ2/pSJ10718).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the E. coli atoD subunit of the acetoacetyl-CoA transferase are SEQ ID NOs: 29 and 30, respectively. The coding sequence is 663 bp including the stop codon and the encoded predicted protein is 220 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 220 amino acids with a predicted molecular mass of 23.5 kDa and an isoelectric pH of 4.9.

Cloning of Clostridium acetobutylicum acetoacetyl-CoA transferase genes and construction of vectors pSJ10727 and pSJ10731

The 654 bp coding sequence (without stop codon) of the ctfA subunit (uniprot:P33752) of the C. acetobutylicum acetyl-CoA transferase and the 663 bp coding sequence (without stop codon) of the ctfB subunit (uniprot:P23673) of the C. acetobutylicum acetyl-CoA transferase were optimized for expression in the three organisms Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri and synthetically constructed into pSJ10727 and pSJ10731, respectively.
The DNA fragment containing the codon optimized ctfA subunit coding sequence was designed with the sequence 5’-AAGCT TCTCG AGACT ATTAC AAGGA GATTTC TAGTC-3’ (SEQ ID NO: 56) immediately prior to the start codon (to add HindIII and XhoI sites, a Lactobacillus RBS, and to have the start codon within a NcoI-compatible BspHI site), and an EcoRI restriction site immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10727 (SJ2/pSJ10727) and SJ10728 (SJ2/pSJ10728).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the C. acetobutylicum ctfA subunit of the acetoacetyl-CoA transferase are SEQ ID NOs: 31 and 32, respectively. The coding sequence is 657 bp including the stop codon and the encoded predicted protein is 218 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 218 amino acids with a predicted molecular mass of 23.6 kDa and an isoelectric pH of 9.3.

The DNA fragment containing the codon optimized ctfB subunit coding sequence was designed with the sequence 5’-GAATT CACTA TTACA AGGAG ATTTTT AGTC-3’ (SEQ ID NO: 55) immediately prior to the start codon (to add an EcoRI site, a Lactobacillus RBS, and to have the start codon within a NcoI-compatible BspHI site), and EagI and KpnI restriction sites immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10731 (SJ2/pSJ10731) and SJ10732 (SJ2/pSJ10732).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the C. acetobutylicum ctfB subunit of the acetoacetyl-CoA transferase are SEQ ID NOs: 33 and 34, respectively. The coding sequence is 666 bp including the stop codon and the encoded predicted protein is 221 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 221 amino acids with a predicted molecular mass of 23.6 kDa and an isoelectric pH of 8.5.

Cloning of Eremococcus coleocola butyrate-acetoacetate CoA transferase genes and construction of vectors pSJ11474 and pSJ11476

The 651 bp coding sequence (without stop codon) of the subunit A (UniProt:E4KQS7) of the Eremococcus coleocola putative butyrate-acetoacetate CoA transferase and the 639 bp coding sequence (without stop codon) of the subunit (UniProt:E4KQS6) of the Eremococcus coleocola putative butyrate-acetoacetate CoA transferase were optimized for expression in the three organisms Escherichia coli,
Lactobacillus plantarum, and Lactobacillus reuteri.

Two different codon-optimized nucleotide sequences (CO) designated D1396V and D1396W (SEQ ID NOs: 61 and 62, respectively) encode the subunit A (SEQ ID NO: 63). The coding sequence is 654 bp including the stop codon and the encoded predicted protein is 217 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 217 amino acids with a predicted molecular mass of 24 kDa and an isoelectric pH of 4.8.

Two different codon-optimized nucleotide sequences (CO) designated D1396X and D1396Y (SEQ ID NOs: 64 and 65, respectively) encode the subunit B (SEQ ID NO: 66). The coding sequence is 642 bp including the stop codon and the encoded predicted protein is 213 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 213 amino acids with a predicted molecular mass of 23 kDa and an isoelectric pH of 4.2.

Two different combinations of subunit A and subunit B encoding genes were designed. In the first combination, the DNA sequence 5'-GGATC CCTCG AGACT ATTAC AAGGA GATTTC TAGTC-3' (SEQ ID NO: 77) was inserted in front of the first subunit gene (D1396V), followed by the sequence 5'-TAGAA TTCAC TATTA CAAGG AGATT TAGC C-3' (SEQ ID NO: 78), the second subunit gene (D1396X), and the sequence 5'-TAGCG GCCGG GTACC-3' (SEQ ID NO: 79).

In the second combination, the DNA sequence 5'-GGATC CCTCG AGACT ATTAC AAGGA GATTTC TAGTC-3' (SEQ ID NO: 77) was inserted in front of the first subunit gene (D1396W), followed by the sequence 5'-TAGAA TTCAC TATTA CAAGG AGATT TAGC C-3' (SEQ ID NO: 78), the second subunit gene (D1396Y), and the sequence 5'-TAGCG GCCGG GTACC-3' (SEQ ID NO: 79).

The designed constructs were obtained from Geneart AG and transformed as described above, resulting in SJ1 1474 (SJ2/pSJ1 1474) and SJ1 1475 (SJ2/pSJ1 1475), both harbouring the second combination (D1396W + D1396Y) of genes above, and SJ1 1476 (SJ2/pSJ1 1476) and SJ1 1477 (SJ2/pSJ1 1477), both harboring the first combination (D1396V + D1396X) of genes above.

Cloning of Alicyclobacillus sp-18711 putative succinyl-CoA:acetoacetate transferase genes and construction of vector pSJ1 1472

The 696 bp coding sequence (without stop codon) of the ScoA subunit of the Alicyclobacillus succinyl-CoA:acetoacetate transferase and the 657 bp coding sequence
(without stop codon) of the ScoB subunit of the *Alicyclobacillus succinyl-CoA:acetoacetate transferase* were optimized for expression in the three organisms *Escherichia coli*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*.

A codon-optimized nucleotide sequence (CO) designated D1396R (SEQ ID NO: 67) encodes the ScoA subunit (SEQ ID NO: 68). The coding sequence is 699 bp including the stop codon and the encoded protein is 232 amino acids. Using the SignalP program (Nielsen et al., *supra*), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 232 amino acids with a predicted molecular mass of 25 kDa and an isoelectric pH of 5.2.

A codon-optimized nucleotide sequence (CO) designated D1396T (SEQ ID NO: 69) encodes the ScoB subunit (SEQ ID NO: 70). The coding sequence is 660 bp including the stop codon and the encoded protein is 219 amino acids. Using the SignalP program (Nielsen et al., *supra*), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 219 amino acids with a predicted molecular mass of 23 kDa and an isoelectric pH of 4.7.

To combine the ScoA and ScoB encoding genes, the DNA sequence 5'-'GGATC CCTCG AGACT ATTAC AAGGA GATT TAGTC-3' (SEQ ID NO: 77) was inserted in front of the first subunit gene (D1396R), followed by the sequence 5'-TAGAA TTCAC TATTA CAAGG AGATT TTAGC C-3' (SEQ ID NO: 78), the second subunit gene (D1396T), and the sequence 5'-TACCG GCCGG GTACC-3' (SEQ ID NO: 79).

The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ11472 (SJ2/pSJ1 1472) and SJ11473 (SJ2/pSJ1 1473).

**Cloning of *Helicobacter pylori* succinyl-CoA:acetoacetate transferase genes and construction of vectors pSJ11466 and pSJ11468**

The 696 bp coding sequence (without stop codon) of the ScoA subunit (UniProt:032639) of the *H. pylori* succinyl-CoA:acetoacetate transferase and the 621 bp coding sequence (without stop codon) of the ScoB subunit (UniProt P56007) of the *H. pylori* succinyl-CoA:acetoacetate transferase were optimized for expression in the three organisms *Escherichia coli*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*.

Two different codon-optimized nucleotide sequences (CO) designated D1396J and D1396K (SEQ ID NOs: 71 and 72, respectively) encode the ScoA subunit (SEQ ID NO: 73). The coding sequence is 699 bp including the stop codon and the encoded predicted protein is 232 amino acids. Using the SignalP program (Nielsen et al., *supra*), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 232 amino acids with a predicted molecular mass of 25 kDa and an
isoelectric pH of 5.9.

Two different codon-optimized nucleotide sequences (CO) designated D1396M and D1396N (SEQ ID NOs: 74 and 75, respectively) encode the ScoB subunit (SEQ ID NO: 76). The coding sequence is 624 bp including the stop codon and the encoded predicted protein is 207 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 207 amino acids with a predicted molecular mass of 22 kDa and an isoelectric pH of 5.3.

Two different combinations of ScoA and ScoB encoding genes were designed. In the first combination, the DNA sequence 5'-GGATC CCTCG AGACT ATTAC AAGGA GATTTC TAGTC-3' (SEQ ID NO: 77) was inserted in front of the first subunit gene (D1396J), followed by the sequence 5'-TAGAA TTCAC TATTA CAAGG AGATT TTAGC C-3' (SEQ ID NO: 78), the second subunit gene (D1396M), and the sequence 5'-TAGCG GCCGG GTACC-3' (SEQ ID NO: 79).

In the second combination, the DNA sequence 5'-GGATC CCTCG AGACT ATTAC AAGGA GATTTC TAGTC-3' (SEQ ID NO: 77) was inserted in front of the first subunit gene (D1396K), followed by the sequence 5'-TAGAA TTCAC TATTA CAAGG AGATT TTAGC C-3' (SEQ ID NO: 78), the second subunit gene (D1396N), and the sequence 5'-TAGCG GCCGG GTACC-3' (SEQ ID NO: 79).

The designed constructs were obtained from Geneart AG and transformed as described above, resulting in SJ1 1466 (SJ2/pSJ1 1466) and SJ1 1467 (SJ2/pSJ1 1467), both harbouring the first combination (D1396J + D1396M) of genes above, and SJ1 1468 (SJ2/pSJ1 1468) and SJ1 1469 (SJ2/pSJ1 1469), both harboring the second combination (D1396K + D1396N) of genes above.

Cloning of a *Clostridium acetobutylicum* acetoacetate decarboxylase gene and construction of vector pSJ1071 1

The 777 bp coding sequence (without stop codon) of the acetoacetate decarboxylase (uniprot:P23670) from *C. acetobutylicum* was optimized for expression in the three organisms *Escherichia coli*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* and synthetically constructed into pSJ1071 1.

The DNA fragment containing the codon-optimized acetoacetate decarboxylase coding sequence (adc) was designed with the sequence 5'-AAGCT TCGGC CGACT ATTAC AAGGA GATTTC TAGCC-3' (SEQ ID NO: 57) immediately prior to the start codon (to add HindIII and Eagl sites and a *Lactobacillus* RBS), and a Kpnl restriction site immediately downstream. The designed construct was obtained from Geneart AG and
transformed as described above, resulting in SJ1071 1 (SJ2/pSJ1071 1) and SJ10712 (SJ2/pSJ10712).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the C. acetobutylicum acetoacetate decarboxylase gene are SEQ ID NOs: 35 and 36, respectively. The coding sequence is 780 bp including the stop codon and the encoded predicted protein is 259 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 259 amino acids with a predicted molecular mass of 27.5 kDa and an isoelectric pH of 6.2.

Cloning of a Clostridium beijerinckii acetoacetate decarboxylase gene and construction of vector PSJ10713

The 738 bp coding sequence (without stop codon) of the acetoacetate decarboxylase (uniprot:Q716S5) from C. beijerinckii was optimized for expression in the three organisms Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri and synthetically constructed into pSJ10713.

The DNA fragment containing the codon optimized acetoacetate decarboxylase coding sequence (adc Cb) was designed with the sequence 5'-AAGCT TCGGC CGACT ATTAC AAGGA GATT TAGCC-3' (SEQ ID NO: 57) immediately prior to the start codon (to add HindII and EagI sites and a Lactobacillus RBS), and a KpnI restriction site immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10713 (SJ2/pSJ10713) and SJ10714 (SJ2/pSJ10714).

The wild-type nucleotide sequence (WT), codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the C. beijerinckii acetoacetate decarboxylase gene is SEQ ID NO: 16, 17, and 18, respectively. The coding sequence is 741 bp including the stop codon and the encoded predicted protein is 246 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 246 amino acids with a predicted molecular mass of 27.5 kDa and an isoelectric pH of 6.18.

Cloning of a Lactobacillus salivarius acetoacetate decarboxylase gene and construction of vector pSJ10707

The 831 bp CDS (without stop codon) of the acetoacetate decarboxylase (SWISSPROT:Q1 WVG5) from L. salivarius was optimized for expression in the three organisms Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri and
synthetically constructed into pSJ10707.

The DNA fragment containing the codon optimized acetoacetate decarboxylase CDS (adc Ls) was designed with the sequence 5'-AAGCT TCGGC CGACT ATTAC AAGGA GATT'T TAGAC-3' (SEQ ID NO: 57) immediately prior to the start codon (to add Hindi 11 and Eagl sites and a Lactobacillus RBS), and a Kpnl restriction site immediately downstream. The constructs were obtained from Geneart AG and transformed as previously described, resulting in SJ10707 (SJ2/pSJ 10707) and SJ10708 (SJ2/pSJ 10708).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the L. salivarius acetoacetate decarboxylase gene is SEQ ID NO: 43 and 44, respectively. The coding sequence is 834 bp including the stop codon and the encoded predicted protein is 277 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 277 amino acids with a predicted molecular mass of 30.9 kDa and an isoelectric pH of 4.6.

Cloning of a Lactobacillus plantarum acetoacetate decarboxylase gene and construction of vector pSJ10701

The 843 bp CDS (without stop codon) of the acetoacetate decarboxylase (SWISSPROT:Q890G0) from L. plantarum was optimized for expression in the three organisms Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri and synthetically constructed into pSJ10701.

The DNA fragment containing the codon optimized acetoacetate decarboxylase CDS (adc Lp) was designed with the sequence 5'-AAGCT TCGGC CGACT ATTAC AAGGA GATT'T TAGCC-3' (SEQ ID NO: 57) immediately prior to the start codon (to add Hindi 11 and Eagl sites and a Lactobacillus RBS), and a Kpnl restriction site immediately downstream. The constructs were obtained from Geneart AG and transformed as previously described, resulting in SJ10701 (SJ2/pSJ 10701) and SJ10702 (SJ2/pSJ 10702).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the L. plantarum acetoacetate decarboxylase gene is SEQ ID NO: 45 and 46, respectively. The coding sequence is 846 bp including the stop codon and the encoded predicted protein is 281 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 281 amino acids with a predicted molecular mass of 30.8 kDa and an isoelectric pH of 4.7.

Cloning of a Thermoanaerobacter ethanolicus isopropanol dehydrogenase gene and
construction of vector pSJ10719

The 1056 bp coding sequence (without stop codon) of the isopropanol dehydrogenase (uniprot:Q2MJT8) from T. ethanolicus was optimized for expression in the three organisms Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri and synthetically constructed into pSJ10719.

The DNA fragment containing the codon optimized isopropanol dehydrogenase coding sequence (adh Te) was designed with the sequence 5'-GGTAC CACTA TTACA AGGAG ATTTT AGTC-3' (SEQ ID NO: 58) immediately prior to the start codon (to add a KpnI site, a Lactobacillus RBS, and to have the start codon within a Ncol-compatible BspHI site), and Xmal and HindIII restriction sites immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10719 (SJ2/pSJ10719) and SJ10720 (SJ2/pSJ 10720).

The wild-type nucleotide sequence (WT), codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the T. ethanolicus isopropanol dehydrogenase gene is SEQ ID NO: 22, 23, and 24, respectively. The coding sequence is 1059 bp including the stop codon and the encoded predicted protein is 352 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 352 amino acids with a predicted molecular mass of 37.7 kDa and an isoelectric pH of 6.23.

Cloning of a Clostridium beijerinckii isopropanol dehydrogenase gene and construction of vector pSJ 10725

The 1053 bp coding sequence (without stop codon) of the isopropanol dehydrogenase (uniprot:P25984) from C. beijerinckii was optimized for expression in the three organisms Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri and synthetically constructed into pSJ10725.

The DNA fragment containing the codon optimized isopropanol dehydrogenase coding sequence (adh Cb) was designed with the sequence 5'-GGTAC CACTA TTACA AGGAG ATTTT AGTC-3' (SEQ ID NO: 58) immediately prior to the start codon (to add a KpnI site, a Lactobacillus RBS, and to have the start codon within a Ncol-compatible BspHI site), and Xmal and HindIII restriction sites immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10725 (SJ2/pSJ 10725) and SJ10726 (SJ2/pSJ 10726).

The wild-type nucleotide sequence (WT), codon-optimized nucleotide sequence (CO), and deduced amino acid sequence of the C. beijerinckii isopropanol dehydrogenase gene is SEQ ID NO: 19, 20, and 21, respectively. The coding sequence is 1056 bp
including the stop codon and the encoded predicted protein is 351 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 351 amino acids with a predicted molecular mass of 37.8 kDa and an isoelectric pH of 6.64.

Cloning of a *Lactobacillus antri* isopropanol dehydrogenase gene and construction of vector pSJ10709

The 1068 bp coding sequence (without stop codon) of the isopropanol dehydrogenase (SWISSPROT:C8P9V7) from *L. antri* was optimized for expression in the three organisms *Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri* and synthetically constructed into pSJ10709.

The DNA fragment containing the codon-optimized isopropanol dehydrogenase coding sequence (sadh La) was designed with the sequence 5'-GGTAC CACTA TTACA AGGAG ATTTT AGTC-3' (SEQ ID NO: 58) immediately prior to the start codon (to add a KpnI site and a *Lactobacillus* RBS), and Xmal and Hind III restriction sites immediately downstream. The designed construct was obtained from Geneart AG and transformed as described above, resulting in SJ10709 (SJ2/pSJ 10709) and SJ10710 (SJ2/pSJ10710).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the *L. antri* isopropanol dehydrogenase gene is SEQ ID NO: 37 and 38, respectively. The coding sequence is 1071 bp including the stop codon and the encoded predicted protein is 356 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 356 amino acids with a predicted molecular mass of 38.0 kDa and an isoelectric pH of 4.9.

Cloning of a *Lactobacillus fermentum* isopropanol dehydrogenase gene and construction of vector pSJ10703

The 1068 bp CDS (without stop codon) of the isopropanol dehydrogenase (SWISSPROT:B2GDH6) from *L. fermentum* was optimized for expression in the three organisms *Escherichia coli, Lactobacillus plantarum, and Lactobacillus reuteri* and synthetically constructed into pSJ10703.

The DNA fragment containing the codon optimized isopropanol dehydrogenase CDS (sadh Lf) was designed with the sequence 5'-GGTAC CACTA TTACA AGGAG ATTTT AGTC-3' (SEQ ID NO: 58) immediately prior to the start codon (to add a KpnI site and a *Lactobacillus* RBS), and Xmal and Hind III restriction sites immediately downstream. The constructs were obtained from Geneart AG and transformed as previously described,
resulting in SJ10703 (SJ2/pSJ 10703) and SJ10704 (SJ2/pSJ 10704).

The codon-optimized nucleotide sequence (CO) and deduced amino acid sequence of the \textit{L. fermentum} isopropanol dehydrogenase gene is SEQ ID NO: 47 and 48, respectively. The coding sequence is 1071 bp including the stop codon and the encoded predicted protein is 356 amino acids. Using the SignalP program (Nielsen et al., supra), no signal peptide in the sequence was predicted. Based on this program, the predicted mature protein contains 356 amino acids with a predicted molecular mass of 37.9 kDa and an isoelectric pH of 5.2.

\textbf{Example 5: Construction and transformation of pathway constructs for isopropanol production in \textit{E. coli}.}

\textbf{Construction of pSJ10843 containing a \textit{C. beijerinckii} acetoacetate decarboxylase gene and a \textit{C. beijerinckii} alcohol dehydrogenase gene}

Plasmids pSJ10725 and pSJ10713 were digested individually with KpnI+AlwNI. Plasmid pSJ10725 was further digested with PvuII to reduce the size of unwanted fragments. The resulting 1689 bp fragment of pSJ10725 and the 2557 bp fragment of pSJ10713 were each purified using gel electrophoresis and subsequently ligated as outlined herein. An aliquot of the ligation mixture was used for transformation of \textit{E. coli} SJ2 chemically competent cells, and transformants selected on LB plates with 200 microgram/ml ampicillin. Four colonies, picked among more than 100 transformants, were all deemed to contain the desired recombinant plasmid by restriction analysis using HindIII, and two of these were kept, resulting in SJ10843 (SJ2/pSJ 10843) and SJ10844 (SJ2/pSJ 10844).

\textbf{Construction of pSJ10841 containing a \textit{C. acetobutylicum} acetoacetate decarboxylase gene and a \textit{C. beijerinckii} alcohol dehydrogenase gene}

Plasmids pSJ10725 and pSJ10711 were digested individually with KpnI+AlwNI; in addition, pSJ10725 was digested with PvuII to reduce the size of unwanted fragments. The resulting 1689 bp fragment of pSJ10725 and the 2596 bp fragment of pSJ10711 were each purified using gel electrophoresis and subsequently ligated as outlined herein. An aliquot of the ligation mixture was used for transformation of \textit{E. coli} SJ2 chemically competent cells, and transformants selected on LB plates with 200 microgram/ml ampicillin. 4 colonies, picked among more than 100 transformants, were all deemed to contain the desired recombinant plasmid by restriction analysis using BsgI, and two of these were kept, resulting in SJ10841 (SJ2/pSJ 10841) and SJ10842 (SJ2/pSJ 10842).
Construction of pSJ10748 containing a *B. subtilis* succinyl-CoA:acetoacetate transferase genes

Plasmids pSJ10697 and pSJ10695 were each digested with EcoRI and Kpnl. The resulting 690 bp fragment of pSJ10697 and the 3106 bp fragment of pSJ10695 were each purified using gel electrophoresis and subsequently ligated as outlined herein.

An aliquot of the ligation mixture was used for transformation of *E. coli* SJ2 by electroporation, and transformants selected on LB plates with 200 microgram/ml ampicillin. 3 colonies, picked among more than 50 transformants, were all deemed to contain the desired recombinant plasmid by restriction analysis using *Pvu*I, and two of these were kept, resulting in SJ10748 (SJ2/pSJ10748) and SJ10749 (SJ2/pSJ10749).

Construction of pSJ10777 containing a *B. mojavensis* succinyl-CoA:acetoacetate transferase genes

Plasmids pSJ10723 and pSJ10721 were each digested with EcoRI + Kpnl. The resulting 696 bp fragment of pSJ10723 and the 3118 bp fragment of pSJ10721 were each purified using gel electrophoresis and subsequently ligated as outlined herein.

An aliquot of the ligation mixture was used for transformation of *E. coli* SJ2 chemically competent cells, and transformants selected on LB plates with 200 microgram/ml ampicillin. 4 colonies, picked among more than 500 transformants, were analyzed and one, deemed to contain the desired recombinant plasmid by restriction analysis using *Pvu*I, was kept, resulting in SJ10777 (SJ2/pSJ10777).

Construction of pSJ10750 containing a *E. coli* acetoacetyl-CoA transferase genes

Plasmids pSJ10717 and pSJ10715 were each digested with EcoRI + Kpnl. The resulting 702 bp fragment of pSJ10717 and the 3051 bp fragment of pSJ10715 were each purified using gel electrophoresis and subsequently ligated as outlined herein.

An aliquot of the ligation mixture was used for transformation of *E. coli* SJ2 by electroporation, and transformants selected on LB plates with 200 microgram/ml ampicillin. 3 colonies, picked among more than 50 transformants, were all deemed to contain the desired recombinant plasmid by restriction analysis using *Apa*LI, and two of these were kept, resulting in SJ10750 (SJ2/pSJ10750) and SJ10751 (SJ2/pSJ10751).

Construction of pSJ10752 containing a *Clostridium acetobutylicum* acetoacetyl-CoA transferase genes

Plasmids pSJ10731 and pSJ10727 were each digested with EcoRI + Kpnl. The resulting 705 bp fragment of pSJ10731 and the 3061 bp fragment of pSJ10727 were each purified using gel electrophoresis and subsequently ligated as outlined herein.
An aliquot of the ligation mixture was used for transformation of *E. coli* SJ2 by electroporation, and transformants selected on LB plates with 200 microgram/ml ampicillin. 3 colonies, picked among more than 50 transformants, were all deemed to contain the desired recombinant plasmid by restriction analysis using PvuI, and two of these were kept, resulting in SJ10752 (SJ2/pSJ 10752) and SJ10753 (SJ2/pSJ 10753).

Construction of expression vector pSJ10798 containing a *Clostridium acetobutylicum* thiolase gene

Plasmid pSJ10705 was digested with BspHI and EcoRI, whereas pSJ10600 was digested with Ncol and EcoRI. The resulting 1193 bp fragment of pSJ10705 and the 5147 bp fragment of pSJ10600 were each purified using gel electrophoresis and subsequently ligated as outlined herein.

An aliquot of the ligation mixture was used for transformation of *E. coli* TG1 by electroporation, and transformants selected on LB plates with 200 microgram/ml erythromycin. 3 of 4 colonies analyzed were deemed to contain the desired recombinant plasmid by restriction analysis using Nsil as well as DNA sequencing, and two of these were kept, resulting in SJ10798 (TG1/pSJ10798) and SJ10799 (TG1/pSJ10799).

Construction of expression vector pSJ10796 containing a *L. reuteri* thiolase gene

Plasmid pSJ10694 was digested with Ncol and EcoRI, and the resulting 1.19 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and EcoRI, and the 5.2 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Nsil, and two of these, further verified by DNA sequencing, were kept, resulting in SJ10796 (TG1/pSJ10796) and SJ10797 (TG1/pSJ10797).

Construction of expression vector pSJ10802 containing a *Lactobacillus reuteri* thiolase gene

Plasmid pSJ10694 was digested with Ncol and EcoRI, and the resulting 1.19 kb fragment purified using gel electrophoresis. Plasmid pSJ10603 was digested with Ncol and EcoRI, and the 5.2 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Nsil, and two of these, further verified by DNA sequencing,
were kept, resulting in SJ10802 (TG1/pSJ 10802) and SJ10803 (TG1/pSJ10803).

Construction of expression vector pSJ10795 containing a *Propionibacterium freudenreichii* thiolase gene

Plasmid pSJ10676 was digested with BspHI and EcoRI, and the resulting 1.17 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and EcoRI, and the 5.2 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using NsI, and one of these, further verified by DNA sequencing, was kept, resulting in SJ10795 (TG1/pSJ10795).

Construction of expression vector pSJ10743 containing a *Lactobacillus brevis* thiolase gene

Plasmid pSJ10699 was digested with Ncol and EcoRI, and the resulting 1.18 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and EcoRI, and the 5.2 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into MG1655 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. 16 of the resulting colonies were analyzed and two, deemed to contain the desired recombinant plasmid by restriction analysis using Clal and further verified by DNA sequencing, were kept, resulting in SJ10743 (TG1/pSJ10743) and SJ10757 (TG1/pSJ10757).

Construction of expression vector pSJ10886 containing a *Bacillus subtilis* succinyl-CoA:acetoacetate transferase genes

Plasmid pSJ10748 was digested with Ncol and Kpnl, and the resulting 1.4 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and Kpnl, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using HindIII, and two of these, further verified by DNA sequencing, were kept, resulting in SJ10886 (TG1/pSJ10886) and SJ10887 (TG1/pSJ10887).

Construction of expression vector pSJ10888 containing *E. coli* acetoacyl-CoA transferase genes
Plasmid pSJ10750 was digested with Ncol and Kpnl, and the resulting 1.35 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and Kpnl, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using HindIII, and two of these, further verified by DNA sequencing, were kept, resulting in SJ10888 (TG1/pSJ10888) and SJ10889 (TG1/pSJ10889).

Construction of expression vector pSJ10756 containing a *C. beijerinckii* acetoacetate decarboxylase gene

Plasmid pSJ10713 was digested with Eagl and Kpnl, and the resulting 0.77 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Eagl and Kpnl, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and one, deemed to contain the desired recombinant plasmid by restriction analysis using Clal and verified by DNA sequencing, was kept as SJ10756 (TG1/pSJ10756).

Construction of expression vector pSJ10754 containing a *C. acetobutylicum* acetoacetate decarboxylase gene

Plasmid pSJ10711 was digested with Eagl and Kpnl, and the resulting 0.81 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Eagl and Kpnl, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into MG1655 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed, three deemed to contain the desired recombinant plasmid by restriction analysis using Clal and two, verified by DNA sequencing, were kept as SJ10754 (MG1655/pSJ 10754) and SJ10755 (MG1655/pSJ 10755).

Construction of expression vector pSJ10780 containing a *L. salivarius* acetoacetate decarboxylase gene

Plasmid pSJ10707 was digested with Pcil and Kpnl, and the resulting 0.84 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and
Kpnl, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into MG1655 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed, all deemed to contain the desired recombinant plasmid by restriction analysis using Clal and two, verified by DNA sequencing, were kept as SJ10780 (MG1655/pSJ10780) and SJ10781 (MG1655/pSJ10781).

Construction of expression vector pSJ10778 containing a L. plantarum acetoacetate decarboxylase gene

Plasmid pSJ10701 was digested with Ncol and Kpnl, and the resulting 0.85 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and Kpnl, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into MG1655 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed, all deemed to contain the desired recombinant plasmid by restriction analysis using Clal and two, verified by DNA sequencing, were kept as SJ10778 (MG1655/pSJ10778) and SJ10779 (MG1655/pSJ10779).

Construction of expression vector pSJ10768 containing a Lactobacillus antri isopropanol dehydrogenase gene

Plasmid pSJ10709 was digested with Kpnl and Xmal, and the resulting 1.1 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Xmal and Kpnl, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and two deemed to contain the desired recombinant plasmid by restriction analysis using Clal and verified by DNA sequencing, were kept as SJ10768 (TG1/pSJ10768) and SJ10769 (TG1/pSJ10769).

Construction of expression vectors pSJ10745, pSJ10763, pSJ10764, and pSJ10767, containing a Thermoanaerobacter ethanolicus isopropanol dehydrogenase gene

Plasmid pSJ10719 was digested with BspHI and Xmal, and the resulting 1.06 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and Xmal, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed and ligated. The ligation mixture was transformed into MG1655.
electrocompetent cells, and one of the resulting colonies, deemed to contain the desired recombinant plasmid by restriction analysis using Clal and verified by DNA sequencing, was kept as SJ10745 (MG1655/pSJ 10745). The ligation mixture was also transformed into electrocompetent E. coli JM103, where two of four colonies were deemed to contain the desired plasmid by restriction analysis using Clal, and these kept as SJ10763 (JM103/pSJ10763) and SJ10764 (JM103/pSJ10764).

Finally, the ligation mixture was transformed into electrocompetent TG1, where three of four colonies were deemed to contain the desired plasmid by restriction analysis using Clal, and one, SJ10767 (JM103/pSJ10767), was verified by DNA sequencing.

**Construction of expression vector pSJ10782 containing a Clostridium beijerinckii isopropanol dehydrogenase gene**

Plasmid pSJ10725 was digested with BspHI and Xmal, and the resulting 1.06 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Ncol and Xmal, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into MG1655 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and two, deemed to contain the desired recombinant plasmid by restriction analysis using Clal and verified by DNA sequencing, were kept as SJ10782 (TG1/pSJ10782) and SJ10783 (TG1/pSJ10783).

**Construction of expression vector pSJ10762 containing a Lactobacillus fermentum isopropanol dehydrogenase gene**

Plasmid pSJ10703 was digested with BspHI and Xmal, and the resulting 1.1 kb fragment purified using gel electrophoresis. Plasmid pSJ10600 was digested with Xmal and Ncol, and the resulting 5.1 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into JM103 as well as TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Transformants were analyzed and two (one from each host strain), deemed to contain the desired recombinant plasmid by restriction analysis using Clal and verified by DNA sequencing, were kept as SJ10762 (JM103/pSJ10762) and SJ10765 (TG1/pSJ10765). Transformant SJ10766 (JM103/pSJ10766) was also verified to contain the Lactobacillus fermentum isopropanol dehydrogenase gene.

**Construction of expression vector pSJ10954 containing a C. acetobutylicum thiolase gene, B. mojavensis succinyl-CoA:acetoacetate transferase genes (both subunits), a C. beijerinckii acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase**
Plasmid pSJ10798 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10777 was digested with Xhol and Eagl, and the resulting 1.43 kb fragment purified using gel electrophoresis. Plasmid pSJ10843 was digested with Eagl and Xmal, and the resulting 1.85 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ10954 (TG1/pSJ10954) and SJ10955 (TG1/pSJ10955).

Construction of expression vector pSJ10956 containing a C. acetobutylicum thiolase gene, B. mojavensis succinyl-CoA:acetoacetate transferase genes (both subunits), a C. acetobutylicum acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase gene

Plasmid pSJ10798 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10777 was digested with Xhol and Eagl, and the resulting 1.43 kb fragment purified using gel electrophoresis. Plasmid pSJ10841 was digested with Eagl and Xmal, and the resulting 1.89 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ10956 (TG1/pSJ10956) and SJ10957 (TG1/pSJ10957).

From an independent construction process (digestion, fragment purification, ligation, transformation by electroporation) one transformant, deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, was kept as SJ10926 (TG1 pSJ10926).

Construction of expression vector pSJ10942 containing a C. acetobutylicum thiolase gene, B. subtilis succinyl-CoA:acetoacetate transferase genes (both subunits), a C. beijerinckii acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase gene

Plasmid pSJ10798 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10748 was digested with Xhol and Eagl, and the resulting 1.43 kb fragment purified using gel electrophoresis. Plasmid
pSJ10843 was digested with Eagl and Xmal, and the resulting 1.85 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ10942 (TG1/pSJ10942) and SJ10943 (TG1/pSJ10943).

Construction of expression vector pSJ10944 containing a *C. acetobutylicum* thiolase gene, *B. subtilis* succinyl-CoA:acetoacetate transferase genes (both subunits), a *C. acetobutylicum* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene

Plasmid pSJ10798 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10748 was digested with Xhol and Eagl, and the resulting 1.43 kb fragment purified using gel electrophoresis. Plasmid pSJ10841 was digested with Eagl and Xmal, and the resulting 1.89 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ10944 (TG1/pSJ10944) and SJ10945 (TG1/pSJ10945).

Construction of expression vector pSJ10946 containing a *C. acetobutylicum* thiolase gene, an *E. coli* acetoacetyl-CoA transferase genes (both subunits), a *C. beijerinckii* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene

Plasmid pSJ10798 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10750 was digested with Xhol and Eagl, and the resulting 1.37 kb fragment purified using gel electrophoresis. Plasmid pSJ10843 was digested with Eagl and Xmal, and the resulting 1.85 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ10946 (TG1/pSJ10946) and SJ10947 (TG1/pSJ10947).
Construction of expression vector pSJ10948 containing a *C. acetobutylicum* thiolase gene, *E. coli* acetoacetyl-CoA transferase genes (both subunits), a *C. acetobutylicum* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene.

Plasmid pSJ10798 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10750 was digested with Xhol and Eagl, and the resulting 1.37 kb fragment purified using gel electrophoresis. Plasmid pSJ10841 was digested with Eagl and Xmal, and the resulting 1.89 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ10948 (TG1/pSJ10948) and SJ10949 (TG1/pSJ10949).

Construction of expression vector pSJ10950 containing a *C. acetobutylicum* thiolase gene, *C. acetobutylicum* acetoacetyl-CoA transferase genes (both subunits), a *C. beijerinckii* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene.

Plasmid pSJ10798 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10752 was digested with Xhol and Eagl, and the resulting 1.38 kb fragment purified using gel electrophoresis. Plasmid pSJ10843 was digested with Eagl and Xmal, and the resulting 1.85 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ10950 (TG1/pSJ10950) and SJ10951 (TG1/pSJ10951).

Construction of expression vector pSJ10952 containing a *C. acetobutylicum* thiolase gene, *C. acetobutylicum* acetoacetyl-CoA transferase genes (both subunits), a *C. acetobutylicum* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene.

Plasmid pSJ10798 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10752 was digested with Xhol and Eagl, and the resulting 1.38 kb fragment purified using gel electrophoresis. Plasmid pSJ10841 was digested with Eagl and Xmal, and the resulting 1.89 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation
mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ10952 (TG1/pSJ10952) and SJ10953 (TG1/pSJ10953).

Construction of expression vector pSJ10790 containing a C. acetobutylicum thiolase gene, B. mojavensis succinyl-CoA:acetoacetate transferase genes (both subunits), a C. beijerinckii acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase gene under control of the P 11 promoter

Plasmid pTRGU00178 (see US Provisional Patent Application No. 61/408,138, filed October 29, 2010) was digested with Ncol and BamHi, and the resulting 1.2 kb fragment purified using gel electrophoresis. pTRGU00178 was also digested with BamHi and Sail, and the resulting 2.1 kb fragment purified using gel electrophoresis. pSIP409 was digested with Ncol and Xhol, and the resulting 5.7 kb fragment purified using gel electrophoresis. The three purified fragments were mixed, ligated, and the ligation mixture transformed into SJ2 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Two transformants, deemed to contain the desired recombinant plasmid by restriction analysis using EcoRI, BgIII, and HindIII, were kept as SJ10562 (SJ2/pSJ 10562) and SJ10563 (SJ2/pSJ 10563).

Plasmid pSJ10562 was digested with XbaI and NotI, and the resulting 7.57 kb fragment purified using gel electrophoresis. Plasmid pTRGU00200 (supra) was digested with XbaI and NotI, and the resulting 2.52 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into MG1655 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Two transformants, deemed to contain the desired recombinant plasmid by restriction analysis using NotI + XbaI, were kept as SJ10593 (MG1655/pSJ 10593) and SJ10594 (MG1655/pSJ 10594).

Plasmid pTRGU00200 was digested with EcoRI and BamHI, and the resulting 1.2 kb fragment purified using gel electrophoresis. pSJ10600 was digested with EcoRI and BamHI, and the resulting 5.2 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into MG1655 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Two transformants, deemed to contain the desired recombinant plasmid by restriction analysis using EcoRI + BamHI, were kept as SJ10690 (MG1655/pSJ 10690) and SJ10691 (MG1655/pSJ 10691).
Plasmid pSJ10593 was digested with BamHI and XbaI, and the resulting 3.25 kb fragment purified using gel electrophoresis. pSJ10690 was digested with BamHI and XbaI, and the resulting 6.3 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Two transformants, deemed to contain the desired recombinant plasmid by restriction analysis using NsiI, were kept as SJ10790 (TG1/pSJ10790) and SJ10791 (TG1/pSJ10791).

Construction of pSJ10792 containing a C. acetobutylicum thiola gene, B. mojavensis succinyl-CoA:acetoacetate transferase genes (both subunits), a C. beijerinckii acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase gene under control of the P27 promoter.

Plasmid pTRGU00200 was digested with EcoRI and BamHI, and the resulting 1.2 kb fragment purified using gel electrophoresis. pSJ10603 was digested with EcoRI and BamHI, and the resulting 5.2 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into MG1655 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Two transformants, deemed to contain the desired recombinant plasmid by restriction analysis using EcoRI + BamHI, were kept as SJ10692 (MG1655/pSJ 10692) and SJ10693 (MG1655/pSJ 10693).

Plasmid pSJ10593 was digested with BamHI and XbaI, and the resulting 3.25 kb fragment purified using gel electrophoresis. pSJ10692 was digested with BamHI and XbaI, and the resulting 6.3 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Two transformants, deemed to contain the desired recombinant plasmid by restriction analysis using NsiI, were kept as SJ10792 (TG1/pSJ10792) and SJ10793 (TG1/pSJ10793).

Construction of expression vector pSJ11208 containing a L. reuteri thiola gene, B. mojavensis succinyl-CoA:acetoacetate transferase genes (both subunits), a C. beijerinckii acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase gene.

Plasmid pSJ10796 was digested with XhoI and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10954 was digested with XhoI and Xmal, and the resulting 3.28 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Three of the resulting colonies were analyzed and deemed to contain the desired
recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ1 1208 (TG1/pSJ1 1208) and SJ1 1209 (TG1/pSJ1 1209).

Construction of expression vector pSJ1 1204 containing a L. reuteri thiolase gene, B. subtilis succinyl-CoA:acetoacetate transferase genes (both subunits), a C. beijerinckii acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase gene

Plasmid pSJ10796 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10942 was digested with Xhol and Xmal, and the resulting 3.26 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ1 1204 (TG1/pSJ1 1204) and SJ1 1205 (TG1/pSJ1 1205).

Construction of expression vector pSJ1 1339 containing a L. reuteri thiolase gene, B. subtilis succinyl-CoA:acetoacetate transferase genes (both subunits), a C. beijerinckii acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase gene

Plasmid pSJ10802 was digested with Nhel and Xhol, and the resulting 1.5 kb fragment purified using gel electrophoresis. Plasmid pSJ10802 was separately digested with Xmal and Nhel, and the resulting 4.8 kb fragment purified using gel electrophoresis. Plasmid pSJ10942 was digested with Xhol and Xmal, and the resulting 3.26 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed, two were deemed to contain the desired recombinant plasmid by restriction analysis using EcoRI + Nhel, and these were kept as SJ1 1339 (TG1/pSJ1 1339) and SJ1 1340 (TG1/pSJ1 1340).

Construction of expression vector pSJ1 1230 containing a L. reuteri thiolase gene, E. coli acetoacetyl-CoA transferase genes (both subunits), a C. beijerinckii acetoacetate decarboxylase gene, and a C. beijerinckii alcohol dehydrogenase gene

Plasmid pSJ10796 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10946 was digested with Xhol and Xmal, and the resulting 3.23 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 electrocompetent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed, two were deemed to contain the desired recombinant plasmid by restriction analysis using EcoRI + Nhel, and these were kept as SJ1 1230 (TG1/pSJ1 1230) and SJ1 1231 (TG1/pSJ1 1231).
37°C. Seven of the resulting colonies were analyzed and 5 deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, and two of these were kept, resulting in SJ1 1230 (TG1/pSJ1 1230) and SJ1 1231 (TG1/pSJ 11231).

Construction of expression vector pSJ1 1206 containing a *L. reuteri* thiolase gene, *C. acetobutyllicum* acetoacetyl-CoA transferase genes (both subunits), a *C. beijerinckii* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene.

Plasmid pSJ10796 was digested with Xhol and Xmal, and the resulting 6.3 kb fragment purified using gel electrophoresis. Plasmid pSJ10951 was digested with Xhol and Xmal, and the resulting 3.23 kb fragment purified using gel electrophoresis. The purified fragments were mixed, ligated, and the ligation mixture transformed into TG1 chemically competent cells, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed and two, deemed to contain the desired recombinant plasmid by restriction analysis using Xbal, were kept as SJ1 1206 (TG1/pSJ1 1206) and SJ1 1207 (TG1/pSJ1 1207).

Construction of expression vectors pSJ1 1492 and pSJ1 1533 containing a *L. reuteri* thiolase gene, *Eremococcus coleocola* butyrate-acetoacetate CoA transferase genes (both subunits), a *C. beijerinckii* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene.

Each of the two different combinations of codon-optimized *Eremococcus coleocola* butyrate-acetoacetate CoA transferase genes were excised from plasmids pSJ1 1474 and pSJ1 1476, respectively, by digestion with Xhol and Eagl, and the resulting 1.35 kb fragments purified using gel electrophoresis. Plasmid pSJ1 1231 was digested with Xhol and Eagl, and the resulting 8.15 kb fragment purified using gel electrophoresis. Each of the *Eremococcus coleocola* butyrate-acetoacetate CoA transferase gene fragments was individually ligated to the pSJ1 1231 fragment, and the ligation mixture transformed into *E. coli* TG1 by electroporation, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the colonies resulting from ligation with the pSJ1 1474 fragment were analyzed, three were deemed to contain the desired recombinant plasmid by restriction analysis using Ncol, and two were kept as SJ1 1492 (TG1/pSJ1 1492) and SJ1 1493 (TG1/pSJ1 1493). 16 of the colonies resulting from ligation with the pSJ1 1476 fragment were analyzed, four deemed to contain the desired recombinant plasmid by restriction analysis using Ncol, and two were kept as SJ1 1533 (TG1/pSJ1 1533) and SJ1 1539 (TG1/pSJ1 1539).

Construction of expression vector pSJ1 1490 containing a *L. reuteri* thiolase gene.
*Alicyclobacillus* succinyl-CoA:acetoacetate transferase genes (both subunits), a *C. beijerinckii* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene.

The combination of codon-optimized *Alicyclobacillus* succinyl-CoA:acetoacetate transferase genes was excised from plasmid pSJ1 1472, by digestion with Xhol and Eagl, and the resulting 1.35 kb fragment purified using gel electrophoresis. Plasmid pSJ1 1231 was digested with Xhol and Eagl, and the resulting 8.15 kb fragment purified using gel electrophoresis. The *Alicyclobacillus* succinyl-CoA:acetoacetate transferase gene fragment was ligated to the pSJ1 1231 fragment, and the ligation mixture transformed into *E. coli* TG1 by electroporation, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Four of the resulting colonies were analyzed, two were deemed to contain the desired recombinant plasmid by restriction analysis using BglIII, and these were kept as SJ1 1490 (TG1/pSJ1 1490) and SJ1 1491 (TG1/pSJ1 1491).

Construction of expression vectors pSJ1 1540 and pSJ1 1513 containing a *L. reuteri* thiolase gene, *Helicobacter pylori* succinyl-CoA:acetoacetate transferase genes (both subunits), a *C. beijerinckii* acetoacetate decarboxylase gene, and a *C. beijerinckii* alcohol dehydrogenase gene.

Each of the two different combinations of codon-optimized *Helicobacter pylori* succinyl-CoA:acetoacetate transferase genes were excised from plasmids pSJ 11466 and pSJ 1468, respectively, by digestion with Xhol and Eagl, and the resulting 1.35 kb fragments purified using gel electrophoresis. Plasmid pSJ1 1231 was digested with Xhol and Eagl, and the resulting 8.15 kb fragment purified using gel electrophoresis. Each of the *Helicobacter pylori* succinyl-CoA:acetoacetate transferase gene fragments was individually ligated to the pSJ1 1231 fragment, and the ligation mixture transformed into *E. coli* TG1 by electroporation, selecting erythromycin resistance (200 microgram/ml) on LB plates at 37°C. Seven of the colonies resulting from ligation with the pSJ1 1466 fragment were analyzed, five were deemed to contain the desired recombinant plasmid by restriction analysis using BsaHI, and two were kept as SJ1 1540 (TG1/pSJ1 1540) and SJ1 1541 (TG1/pSJ1 11541). 4 of the colonies resulting from ligation with the pSJ1 1468 fragment were analyzed, three deemed to contain the desired recombinant plasmid by restriction analysis using Ndel and BglIII, and two were kept as SJ1 1513 (TG1/pSJ1 1513) and SJ1 1514 (TG1/pSJ1 1514).

**Example 6:** Comparison of isopropanol and acetone production in *E. coli* hosts having an active isopropanol pathway with different CoA-transferase genes

*E. coli* strains comprising an active isopropanol pathway with selected CoA-
transferase constructs described above were inoculated in 10 ml LB + 1 % glucose + 100 microgram/ml erythromycin, and incubated with shaking at 37°C overnight. Supernatants were then analyzed for 1-propanol, isopropanol, acetone, and ethanol by GC-FID. Samples were diluted 1+1 with 0.05% tetrahydrofuran in methanol and analyzed using the GC parameters are listed in Table 1.

Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Approx. Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC column</td>
<td>DB-WAX 30m – 0.25mm i.d – 0.50 µm film part-no 122-7033 from J&amp;W Scientific</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>Temp. gradient</td>
<td>0 – 4.5 min: 50°C 4.5 – 9.93 min: 50 – 240°C linear gradient</td>
</tr>
<tr>
<td>Detection</td>
<td>FID</td>
</tr>
<tr>
<td>Internal standard</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>External standards</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetone (Analytical grade)</td>
</tr>
<tr>
<td></td>
<td>1-propanol (Analytical grade)</td>
</tr>
<tr>
<td></td>
<td>2-propanol (HPLC grade)</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
</tr>
</tbody>
</table>

In total, four cultures were inoculated with each strain and the average titers (the sum of isopropanol and acetone, as both these components are downstream metabolites relative to the acetoacetyl CoA transferase portion of the isopropanol pathway) for the different strains are presented in Table 2 below.

This experiment shows that using an E. coli host, all genes tested, except for the Helicobacter pylori derived genes, give rise to substantial isopropanol + acetone production. In this host, constructs encoding the E. coli acetoacetyl-CoA transferase, the C. acetobutylicum acetoacetyl-CoA transferase, and the Eremococcus coleocola butyrate-acetoacetate CoA transferase produce similar levels of isopropanol + acetone.

Table 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Heterologous active isopropanol pathway</th>
<th>Heterologous CoA-transferase gene</th>
<th>Encoded CoA-transferase sequence (SEQ ID NOs)</th>
<th>Isopropanol + acetone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ11600</td>
<td>–</td>
<td>None</td>
<td>–</td>
<td>0.002</td>
</tr>
<tr>
<td>SJ10796</td>
<td>–</td>
<td>None</td>
<td>–</td>
<td>0.002</td>
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<tr>
<td>SJ11204</td>
<td>Yes</td>
<td>β. subtilis succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.126</td>
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<tr>
<td>SJ11205</td>
<td>Yes</td>
<td>β. subtilis succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.123</td>
</tr>
<tr>
<td>SJ11339</td>
<td>Yes</td>
<td>β. subtilis succinyl-</td>
<td>6 + 9</td>
<td>0.072</td>
</tr>
</tbody>
</table>
Table 3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Transformation DNA</th>
<th>Transformation Method</th>
<th>isopropanol pathway</th>
<th>Disrupted ack gene</th>
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<tbody>
<tr>
<td>SJ11011</td>
<td>SJ 10655</td>
<td>pSJ 10600</td>
<td>WO20 12/058603</td>
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<td>No</td>
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<tr>
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<td>SJ 10655</td>
<td>pSJ 10600</td>
<td>WO20 12/058603</td>
<td>–</td>
<td>No</td>
</tr>
<tr>
<td>SJ11360</td>
<td>SJ 11294</td>
<td>pSJ 10600</td>
<td>Protocol B</td>
<td>–</td>
<td>No</td>
</tr>
</tbody>
</table>

Example 7: Construction of *Lactobacillus reuteri* strains containing isopropanol pathway constructs.

*L. reuteri* strains containing an active isopropanol pathway with selected CoA-transferase constructs described above, were generated by transformation as depicted in Table 3 below.
<table>
<thead>
<tr>
<th>SJ 11361</th>
<th>SJ 11294</th>
<th>pSJ 10600</th>
<th>Protocol B</th>
<th>–</th>
<th>No</th>
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</thead>
<tbody>
<tr>
<td>SJ 11322</td>
<td>SJ 11044</td>
<td>pSJ 11231</td>
<td>Protocol A (w/ E. coli acetoacetyl-CoA transferase)</td>
<td>Yes</td>
<td>No</td>
</tr>
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<td>SJ 11276</td>
<td>SJ 11044</td>
<td>pSJ 11230</td>
<td>Protocol A (w/ E. coli acetoacetyl-CoA transferase)</td>
<td>Yes</td>
<td>No</td>
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<td>SJ 11388</td>
<td>SJ 11294</td>
<td>pSJ 11231</td>
<td>Protocol B (w/ E. coli acetoacetyl-CoA transferase)</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>SJ 11389</td>
<td>SJ 11294</td>
<td>pSJ 11231</td>
<td>Protocol B (w/ E. coli acetoacetyl-CoA transferase)</td>
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<td>No</td>
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<td>SJ 11721</td>
<td>SJ 11400</td>
<td>pSJ 11230</td>
<td>Protocol B (w/ E. coli acetoacetyl-CoA transferase)</td>
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<td>No</td>
</tr>
<tr>
<td>SJ 11722</td>
<td>SJ 11400</td>
<td>pSJ 11231</td>
<td>Protocol B (w/ E. coli acetoacetyl-CoA transferase)</td>
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<td>No</td>
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<td>SJ 11569</td>
<td>SJ 11400</td>
<td>PSJ 11533</td>
<td>Protocol B (w/ Eremococcus coleocola butyrate-acetoacetate CoA transferase)</td>
<td>Yes</td>
<td>No</td>
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<td>SJ 11400</td>
<td>PSJ 11539</td>
<td>Protocol B (w/ Eremococcus coleocola butyrate-acetoacetate CoA transferase)</td>
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<td>No</td>
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<td>SJ 11400</td>
<td>pSJ 11492</td>
<td>Protocol B (w/ Eremococcus coleocola butyrate-acetoacetate CoA transferase)</td>
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<td>No</td>
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<td>pSJ 11492</td>
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<td>SJ 11525</td>
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<td>Yes</td>
<td>No</td>
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<td>SJ 11400</td>
<td>pSJ 11490</td>
<td>Protocol B (w/ Alicyclobacillus succinyl-CoA: acetoacetate transferase)</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>SJ 11529</td>
<td>SJ 11400</td>
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<td>Yes</td>
<td>No</td>
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<td>SJ 11400</td>
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<tr>
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<td>SJ 11294</td>
<td>PSJ 11339</td>
<td>Protocol B</td>
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<td>No</td>
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<td>SJ 11373</td>
<td>SJ 11294</td>
<td>PSJ 11339</td>
<td>Protocol B</td>
<td>Yes ( (w/ \beta . \text{subtilis}) ) succinyl-CoA:acetoacetate transferase</td>
<td>No</td>
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<td>SJ 11374</td>
<td>SJ 11294</td>
<td>PSJ 11340</td>
<td>Protocol B</td>
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<td>No</td>
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<td>SJ 11294</td>
<td>PSJ 11340</td>
<td>Protocol B</td>
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<td>No</td>
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<td>SJ 11274</td>
<td>SJ 11400</td>
<td>pSJ 11208</td>
<td>WO20 12/058603</td>
<td>Yes ( (w/ \beta . \text{mojavensis}) ) succinyl-CoA:acetoacetate transferase</td>
<td>No</td>
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<td>SJ 11275</td>
<td>SJ 11400</td>
<td>pSJ 11209</td>
<td>WO20 12/058603</td>
<td>Yes ( (w/ \beta . \text{mojavensis}) ) succinyl-CoA:acetoacetate transferase</td>
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<td>pSJ 11206</td>
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<td>SJ 11044</td>
<td>pSJ 11207</td>
<td>WO20 12/058603</td>
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<td>SJ 11252</td>
<td>SJ 10655</td>
<td>pSJ 11207</td>
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<td>No</td>
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<td>SJ 11426</td>
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<td>SJ 11422</td>
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<td>Protocol B</td>
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<td>Yes</td>
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<td>SJ 11423</td>
<td>TRGU975</td>
<td>pSJ 11231</td>
<td>Protocol B</td>
<td>Yes ( (w/ \text{E. coli}) ) acetoacetyl-CoA transferase</td>
<td>Yes</td>
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<td>SJ 11784</td>
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<td>pSJ 11231</td>
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<td>SJ 11785</td>
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<td>Yes</td>
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<td>PSJ 11539</td>
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<td>pSJ 11493</td>
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<tr>
<td>SJ 11618</td>
<td>TRGU975</td>
<td>pSJ 11490</td>
<td>Protocol B</td>
<td>(w/ *Alicyclobacillus succinyl-CoA: acetoacetate transferase)</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ 11619</td>
<td>TRGU975</td>
<td>pSJ 11491</td>
<td>Protocol B</td>
<td>(w/ *Alicyclobacillus succinyl-CoA: acetoacetate transferase)</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ 11577</td>
<td>TRGU975</td>
<td>PSJ 11540</td>
<td>Protocol B</td>
<td>(w/ *Helicobacter pylori succinyl-CoA:acetoacetate transferase)</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ 11578</td>
<td>TRGU975</td>
<td>pSJ 11541</td>
<td>Protocol B</td>
<td>(w/ *Helicobacter pylori succinyl-CoA:acetoacetate transferase)</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ 11622</td>
<td>TRGU975</td>
<td>PSJ 11513</td>
<td>Protocol B</td>
<td>(w/ *Helicobacter pylori succinyl-CoA:acetoacetate transferase)</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ 11623</td>
<td>TRGU975</td>
<td>pSJ 11514</td>
<td>Protocol B</td>
<td>(w/ *Helicobacter pylori succinyl-CoA:acetoacetate transferase)</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ 11624</td>
<td>TRGU975</td>
<td>pSJ 11204</td>
<td>Protocol B</td>
<td>(w/ *B. subtilis succinyl-CoA:acetoacetate transferase)</td>
<td>Yes</td>
</tr>
</tbody>
</table>
**Example 8: Comparison of isopropanol production in L. reuteri hosts having an active isopropanol pathway with different CoA-transferase genes**

*L. reuteri* strains comprising an active isopropanol pathway with selected CoA-transferase constructs described above were inoculated (from stationary 30°C overnight cultures in MRS medium with 10 microgram/ml erythromycin) into 5 separate 2 ml cultures (MRS + 10 microgram/ml erythromycin). The 2 ml cultures were incubated in closed tubes without shaking, at 30 °C for 3 days, whereafter supernatants were analyzed for 1-propanol, isopropanol, acetone, and ethanol as described *supra*. The average resulting isopropanol titers are shown in Table 4 below.

Unlike the *E. coli* isopropanol production results above, *L. reuteri* showed significant variability in isopropanol production depending on selection of the CoA-transferase gene. It is evident that both pairs of *Eremococcus coleocola* butyrate-acetoacetate CoA transferase genes tested confer a surprisingly higher isopropanol production capability on *L. reuteri* strains compared to the other CoA-transferase sequences tested.

Table 4.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Heterologous active isopropanol pathway</th>
<th>Heterologous CoA-transferase gene</th>
<th>Encoded CoA-transferase sequence (SEQ ID NOs)</th>
<th>Isopropanol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ11625</td>
<td>Yes (w/ <em>B. subtilis</em> succinyl-CoA:acetoacetate transferase)</td>
<td>Yes</td>
<td>Protocol B</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ11628</td>
<td>Yes (w/ <em>B. mojavensis</em> succinyl-CoA:acetoacetate transferase)</td>
<td>Yes</td>
<td>Protocol B</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ11629</td>
<td>Yes (w/ <em>B. mojavensis</em> succinyl-CoA:acetoacetate transferase)</td>
<td>Yes</td>
<td>Protocol B</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ11626</td>
<td>Yes (w/ <em>C. acetobutylicum</em> acetoacetyl-CoA transferase)</td>
<td>Yes</td>
<td>Protocol B</td>
<td>Yes</td>
</tr>
<tr>
<td>SJ11627</td>
<td>Yes (w/ <em>C. acetobutylicum</em> acetoacetyl-CoA transferase)</td>
<td>Yes</td>
<td>Protocol B</td>
<td>Yes</td>
</tr>
<tr>
<td>Strain</td>
<td>Result</td>
<td>Organism/Enzyme Description</td>
<td>IC50</td>
<td>EC50</td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
<td>-------------------------------------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>SJ11569</td>
<td>Yes</td>
<td><em>Eremococcus Coleocola</em> butyrate-acetoacetate CoA transferase</td>
<td>63 + 66</td>
<td>0.018</td>
</tr>
<tr>
<td>SJ11570</td>
<td>Yes</td>
<td><em>Eremococcus Coleocola</em> butyrate-acetoacetate CoA transferase</td>
<td>63 + 66</td>
<td>0.018</td>
</tr>
<tr>
<td>SJ11527</td>
<td>Yes</td>
<td><em>Eremococcus Coleocola</em> butyrate-acetoacetate CoA transferase</td>
<td>63 + 66</td>
<td>0.018</td>
</tr>
<tr>
<td>SJ11528</td>
<td>Yes</td>
<td><em>Eremococcus Coleocola</em> butyrate-acetoacetate CoA transferase</td>
<td>63 + 66</td>
<td>0.015</td>
</tr>
<tr>
<td>SJ11525</td>
<td>Yes</td>
<td><em>Alicyclobacillus</em> succinyl-CoA:acetoacetate transferase</td>
<td>68 + 70</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11526</td>
<td>Yes</td>
<td><em>Alicyclobacillus</em> succinyl-CoA:acetoacetate transferase</td>
<td>68 + 70</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11571</td>
<td>Yes</td>
<td><em>Helicobacter pylori</em> succinyl-CoA:acetoacetate transferase</td>
<td>73 + 76</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11572</td>
<td>Yes</td>
<td><em>Helicobacter pylori</em> succinyl-CoA:acetoacetate transferase</td>
<td>73 + 76</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11529</td>
<td>Yes</td>
<td><em>Helicobacter pylori</em> succinyl-CoA:acetoacetate transferase</td>
<td>73 + 76</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11530</td>
<td>Yes</td>
<td><em>Helicobacter pylori</em> succinyl-CoA:acetoacetate transferase</td>
<td>73 + 76</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11322</td>
<td>Yes</td>
<td><em>E. coli</em> acetoacetyl-CoA transferase</td>
<td>28 + 30</td>
<td>0.013</td>
</tr>
<tr>
<td>SJ11276</td>
<td>Yes</td>
<td><em>E. coli</em> acetoacetyl-CoA transferase</td>
<td>28 + 30</td>
<td>0.014</td>
</tr>
<tr>
<td>SJ11277</td>
<td>Yes</td>
<td><em>E. coli</em> acetoacetyl-CoA transferase</td>
<td>28 + 30</td>
<td>0.014</td>
</tr>
<tr>
<td>SJ11388</td>
<td>Yes</td>
<td><em>E. coli</em> acetoacetyl-CoA transferase</td>
<td>28 + 30</td>
<td>0.010</td>
</tr>
<tr>
<td>SJ11389</td>
<td>Yes</td>
<td><em>E. coli</em> acetoacetyl-CoA transferase</td>
<td>28 + 30</td>
<td>0.006</td>
</tr>
<tr>
<td>SJ11721</td>
<td>Yes</td>
<td><em>E. coli</em> acetoacetyl-CoA transferase</td>
<td>28 + 30</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11722</td>
<td>Yes</td>
<td><em>E. coli</em> acetoacetyl-CoA transferase</td>
<td>28 + 30</td>
<td>0.010</td>
</tr>
<tr>
<td>SJ11270</td>
<td>Yes</td>
<td>β. <em>subtilis</em> succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.003</td>
</tr>
<tr>
<td>SJ11271</td>
<td>Yes</td>
<td>β. <em>subtilis</em> succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.003</td>
</tr>
<tr>
<td>SJ11372</td>
<td>Yes</td>
<td>β. <em>subtilis</em> succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.003</td>
</tr>
<tr>
<td>SJ11373</td>
<td>Yes</td>
<td>β. <em>subtilis</em> succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.003</td>
</tr>
<tr>
<td>SJ11374</td>
<td>Yes</td>
<td>β. <em>subtilis</em> succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.003</td>
</tr>
<tr>
<td>SJ11375</td>
<td>Yes</td>
<td>β. <em>subtilis</em> succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.003</td>
</tr>
<tr>
<td>SJ11274</td>
<td>Yes</td>
<td>β. <em>mojavensis</em> succinyl-CoA:acetoacetate transferase</td>
<td>12 + 15</td>
<td>0.005</td>
</tr>
<tr>
<td>SJ11275</td>
<td>Yes</td>
<td>β. <em>mojavensis</em> succinyl-CoA:acetoacetate transferase</td>
<td>12 + 15</td>
<td>0.008</td>
</tr>
<tr>
<td>SJ11252</td>
<td>Yes</td>
<td>C. acetobutylicum acetate-CoA transferase</td>
<td>32 + 34</td>
<td>0.008</td>
</tr>
<tr>
<td>SJ11272</td>
<td>Yes</td>
<td>C. acetobutylicum acetate-CoA transferase</td>
<td>32 + 34</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Example 9: Comparison of isopropanol production in *L. reuteri* hosts having an active isopropanol pathway with different CoA-transferase genes and a disrupted endogenous acetate kinase gene.

*L. reuteri* strains with a disrupted acetate kinase gene and comprising an active isopropanol pathway with selected CoA-transferase constructs described above were inoculated (from stationary 30°C overnight cultures in MRS medium with 10 microgram/ml erythromycin) into ten separate 2 ml cultures: five in MRS + 10 microgram/ml erythromycin + 0.5 % fructose, and five in MRS + 10 microgram/ml erythromycin, with no fructose added. The 2 ml cultures were incubated in closed tubes without shaking, at 30°C for 3 days, whereafter supernatants were analyzed for 1-propanol, isopropanol, acetone, and ethanol as described supra. The average resulting isopropanol titers are shown in Table 5 below.

As in Example 8 above, the *L. reuteri* showed significant variability in isopropanol production depending on selection of the CoA-transferase gene. It is evident that both pairs of *Eremococcus coleocola* butyrate-acetoacetate CoA transferase genes tested confer a surprisingly higher isopropanol production capability on *L. reuteri* strains compared to the other CoA-transferase sequences tested. This experiment demonstrates that using an acetate kinase disrupted *L. reuteri* host strain comprising heterologous sequences encoding the *Eremococcus coleocola* butyrate-acetoacetate CoA transferase are capable of even higher isopropanol titers (up to 0.041%).

Table 5.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>butyrate-acetoacetate CoA transferase</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ11778</td>
<td>Yes</td>
<td><em>Eremococcus coleocola</em> butyrate-acetoacetate CoA transferase</td>
<td>63 + 66</td>
<td>0.024</td>
</tr>
<tr>
<td>SJ11779</td>
<td>Yes</td>
<td><em>Eremococcus coleocola</em> butyrate-acetoacetate CoA transferase</td>
<td>63 + 66</td>
<td>0.024</td>
</tr>
<tr>
<td>SJ11620</td>
<td>Yes</td>
<td><em>Eremococcus coleocola</em> butyrate-acetoacetate CoA transferase</td>
<td>63 + 66</td>
<td>0.024</td>
</tr>
<tr>
<td>SJ11621</td>
<td>Yes</td>
<td><em>Eremococcus coleocola</em> butyrate-acetoacetate CoA transferase</td>
<td>63 + 66</td>
<td>0.032</td>
</tr>
<tr>
<td>SJ11618</td>
<td>Yes</td>
<td><em>Alicyclobacillus</em> succinyl-CoA:acetoacetate transferase</td>
<td>68 + 70</td>
<td>0.002</td>
</tr>
<tr>
<td>SJ11619</td>
<td>Yes</td>
<td><em>Alicyclobacillus</em> succinyl-CoA:acetoacetate transferase</td>
<td>68 + 70</td>
<td>0.002</td>
</tr>
<tr>
<td>SJ11577</td>
<td>Yes</td>
<td><em>Helicobacter pylori</em> succinyl-CoA:acetoacetate transferase</td>
<td>73 + 76</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11578</td>
<td>Yes</td>
<td><em>Helicobacter pylori</em> succinyl-CoA:acetoacetate transferase</td>
<td>73 + 76</td>
<td>0.001</td>
</tr>
<tr>
<td>SJ11622</td>
<td>Yes</td>
<td><em>Helicobacter pylori</em> succinyl-CoA:acetoacetate transferase</td>
<td>73 + 76</td>
<td>Nd</td>
</tr>
<tr>
<td>SJ11623</td>
<td>Yes</td>
<td><em>Helicobacter pylori</em> succinyl-CoA:acetoacetate transferase</td>
<td>73 + 76</td>
<td>Nd</td>
</tr>
<tr>
<td>SJ11624</td>
<td>Yes</td>
<td><em>β. subtilis</em> succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.005</td>
</tr>
<tr>
<td>SJ11625</td>
<td>Yes</td>
<td><em>β. subtilis</em> succinyl-CoA:acetoacetate transferase</td>
<td>6 + 9</td>
<td>0.006</td>
</tr>
<tr>
<td>SJ11628</td>
<td>Yes</td>
<td><em>β. mojavensis</em> succinyl-CoA:acetoacetate transferase</td>
<td>12 + 15</td>
<td>0.006</td>
</tr>
<tr>
<td>SJ11629</td>
<td>Yes</td>
<td><em>β. mojavensis</em> succinyl-CoA:acetoacetate transferase</td>
<td>12 + 15</td>
<td>0.007</td>
</tr>
<tr>
<td>SJ11626</td>
<td>Yes</td>
<td><em>C. acetobutylicum</em> acetocetyl-CoA transferase</td>
<td>32 + 34</td>
<td>0.002</td>
</tr>
<tr>
<td>SJ11627</td>
<td>Yes</td>
<td><em>C. acetobutylicum</em> acetocetyl-CoA transferase</td>
<td>32 + 34</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Although the foregoing has been described in some detail by way of illustration and example for the purposes of clarity of understanding, it is apparent to those skilled in the art that any equivalent aspect or modification may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention.

In some aspects, the invention may be described by the following numbered paragraphs:

[1] A recombinant *Lactobacillus* host cell comprising an active isopropanol pathway, wherein the cell comprises a first heterologous polynucleotide encoding a polypeptide
having at least 75% sequence identity to SEQ ID NO: 63, and a second heterologous polynucleotide encoding a polypeptide having at least 75% sequence identity to SEQ ID NO: 66; and wherein the cell is capable of producing isopropanol.

[2] The recombinant host cell of paragraph [1], wherein the first heterologous polynucleotide encodes a polypeptide having at least 80%, e.g., at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 63; and the second heterologous polynucleotide encodes a polypeptide having at least 80%, e.g., at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 66.

[3] The recombinant host cell of paragraph [1], wherein the first heterologous polynucleotide encodes a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 63; and the second heterologous polynucleotide encodes a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 66.

[4] The recombinant host cell of any one of paragraphs [1]-[3], wherein the first heterologous polynucleotide comprises a coding sequence that hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 61 or 62; and the second heterologous polynucleotide comprises a coding sequence that hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 64 or 65.

[5] The recombinant host cell of any one of paragraphs [1]-[4], wherein the first heterologous polynucleotide comprises a coding sequence having at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 61 or 62; and the second heterologous polynucleotide comprises a coding sequence having at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at
least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 64 or 65.

[6] The recombinant host cell of any one of paragraphs [1]-[4], wherein the first heterologous polynucleotide comprises the nucleotide sequence of SEQ ID NO: 61 or 62; and the second heterologous polynucleotide comprises the nucleotide sequence of SEQ ID NO: 64 or 65.

[7] The recombinant host cell of any one of paragraphs [1]-[6], wherein the first heterologous polynucleotide is operably linked to a foreign promoter and the second heterologous polynucleotide is operably linked to a foreign promoter.

[8] The recombinant host cell of any one of paragraphs [1]-[7], wherein the first heterologous polynucleotide and the second heterologous polynucleotide encode for polypeptides that form a protein complex having butyrate-acetoacetate CoA transferase activity.

[9] The recombinant host cell of any one of paragraphs [1]-[7], wherein the cell produces or is capable of producing a greater amount of isopropanol compared to the cell without the first and second heterologous polynucleotides, when cultivated under identical conditions.

[10] The recombinant host cell of paragraph [9], wherein the cell produces or is capable of producing at least 10% more (e.g., at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 75%, at least 100%, or at least 200% more) isopropanol compared to the cell without the first and second heterologous polynucleotides, when cultivated under identical conditions.

[11] The recombinant host cell of any one of paragraphs [1]-[10], wherein the cell comprises one or more heterologous isopropanol pathway genes selected from:
   a heterologous thiolase gene;
   a heterologous acetoacetate decarboxylase gene; and
   a heterologous isopropanol dehydrogenase gene.

[12] The recombinant host cell of any one of paragraphs [1]-[10], wherein the cell comprises a heterologous thiolase gene; a heterologous acetoacetate decarboxylase gene; and a heterologous isopropanol dehydrogenase gene.
[13] The recombinant host cell of any one of paragraphs [1]-[12], wherein the cell comprises a disruption to an endogenous gene encoding an acetate kinase.

[14] The recombinant host cell of paragraph [13], wherein the cell comprises a disruption to an endogenous gene encoding an acetate kinase having at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 60.

[15] The recombinant host cell of paragraph [13] or [14], wherein the cell comprises a disruption to an endogenous gene encoding an acetate kinase having an amino acid sequence comprising or consisting of SEQ ID NO: 60.

[16] The recombinant host cell of any one of paragraphs [13]-[15], wherein the coding sequence of the gene encoding the acetate kinase has at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 59.

[17] The recombinant host cell of any one of paragraphs [13]-[16], wherein the coding sequence of the gene encoding the acetate kinase comprises or consists of SEQ ID NO: 59.

[18] The recombinant host cell of any one of paragraphs [13]-[16], wherein the disruption occurs in the coding sequence of the gene encoding the acetate kinase.

[19] The recombinant host cell of any one of paragraphs [13]-[16], wherein the disruption occurs in a promoter sequence of the gene encoding the acetate kinase.

[20] The recombinant host cell of any one of paragraphs [13]-[19], wherein the cell produces at least 25% less (e.g., at least 50% less, at least 60% less, at least 70% less, at least 80% less, or at least 90% less) of the acetate kinase compared to the cell without the disruption when cultivated under identical conditions.

[21] The recombinant host cell of any one of paragraphs [13]-[19], wherein the endogenous gene encoding an acetate kinase is inactivated.
[22] The recombinant host cell of any one of paragraphs [13]-[21], wherein the cell produces a decreased amount of acetate (e.g., at least 25% less, at least 50% less, at least 60% less, at least 70% less, at least 80% less, or at least 90% less) compared to the cell without the disruption when cultivated under identical conditions.

[23] The recombinant host cell of any one of paragraphs [13]-[22], wherein the cell produces a greater amount of isopropanol compared to the cell without the disruption when cultivated under identical conditions.

[24] The recombinant host cell of any one of paragraphs [13]-[22], wherein the cell is capable of producing at least 10% more (e.g., at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 75%, at least 100%, or at least 200% more) isopropanol compared to the cell without the disruption, when cultivated under identical conditions.

[25] The recombinant host cell of any one of paragraphs [1]-[24], wherein the cell is selected from Lactobacillus plantarum, Lactobacillus fructivorans, and Lactobacillus reuteri.

[26] The recombinant host cell of paragraph [25], wherein the cell is a Lactobacillus reuteri cell.

[27] A composition comprising the recombinant host cell of any one of paragraphs [1]-[26].

[28] The composition of paragraph [27], wherein the composition comprises a fermentable medium.

[29] The composition of paragraph [28], wherein the fermentable medium comprises sucrose, glucose and/or fructose.

[30] The composition of paragraph [28], wherein the fermentable medium comprises sugarcane juice (e.g., non-sterilized sugarcane juice).

[31] The composition of any of paragraphs [27]-[30], further comprising isopropanol.
[32] The composition of paragraph [31], wherein the isopropanol is at a titer greater than about 0.01 g/L, e.g., greater than about 0.02 g/L, 0.05 g/L, 0.075 g/L, 0.1 g/L, 0.5 g/L, 1 g/L, 2 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 55 g/L, 60 g/L, 65 g/L, 70 g/L, 75 g/L, 80 g/L, 85 g/L, 90 g/L, 95 g/L, 100 g/L, 125 g/L, 150 g/L, 200 g/L, or 250 g/L.

[33] A method of producing isopropanol, comprising:
   (a) cultivating the recombinant *Lactobacillus* host cell of any one of paragraphs [1]-[26] in a medium under suitable conditions to produce isopropanol; and
   (b) recovering the isopropanol.

[34] The method of paragraph [33], wherein the medium is a fermentable medium.

[35] The method of paragraph [34], wherein the fermentable medium comprises sucrose, glucose and/or fructose.

[36] The method of paragraph [34], wherein the fermentable medium comprises sugarcane juice (e.g., non-sterilized sugarcane juice).

[37] The method of any of paragraphs [33]-[36], wherein the produced isopropanol after step (a) and before step (b) is at a titer greater than about 0.01 g/L, e.g., greater than about 0.02 g/L, 0.05 g/L, 0.075 g/L, 0.1 g/L, 0.5 g/L, 1 g/L, 2 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 55 g/L, 60 g/L, 65 g/L, 70 g/L, 75 g/L, 80 g/L, 85 g/L, 90 g/L, 95 g/L, 100 g/L, 125 g/L, 150 g/L, 200 g/L, or 250 g/L.

[38] The method of any of paragraphs [33]-[37], further comprising purifying the recovered isopropanol by distillation.

[39] The method of any of paragraphs [33]-[38], further comprising purifying the recovered isopropanol by converting acetone contaminant to isopropanol in the presence of a reducing agent.

[40] The method of any of paragraphs [33]-[39], wherein the resulting isopropanol is substantially pure.
[41] A method of producing propylene, comprising:
   (a) cultivating the recombinant host cell of any of paragraphs [1]-[26] in a medium under suitable conditions to produce isopropanol;
   (b) recovering the isopropanol;
   (c) dehydrating the isopropanol under suitable conditions to produce propylene; and
   (d) recovering the propylene.

[42] The method of paragraph [41], wherein the medium is a fermentable medium.

[43] The method of paragraph [42], wherein the fermentable medium comprises sucrose, glucose and/or fructose.

[44] The method of paragraph [42], wherein the fermentable medium comprises sugarcane juice (e.g., non-sterilized sugarcane juice).

[45] The method of any of paragraphs [41]-[44], wherein the produced isopropanol after step (a) and before step (b) is at a titer greater than about 0.01 g/L, e.g., greater than about 0.02 g/L, 0.05 g/L, 0.075 g/L, 0.1 g/L, 0.5 g/L, 1 g/L, 2 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 55 g/L, 60 g/L, 65 g/L, 70 g/L, 75 g/L, 80 g/L, 85 g/L, 90 g/L, 95 g/L, 100 g/L, 125 g/L, 150 g/L, 200 g/L, or 250 g/L.

[46] The method of any one of paragraphs [41]-[45], wherein dehydrating the isopropanol comprises treating the isopropanol with an acid catalyst.
Claims

What is claimed is:

1. A recombinant *Lactobacillus* host cell comprising an active isopropanol pathway, wherein the cell comprises a first heterologous polynucleotide encoding a polypeptide having at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 63; and a second heterologous polynucleotide encoding a polypeptide having at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 66; and wherein the cell is capable of producing isopropanol.

2. The recombinant host cell of claim 1, wherein the first heterologous polynucleotide encodes a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 63; and the second heterologous polynucleotide encodes a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 66.

3. The recombinant host cell of claim 1 or 2, wherein the first heterologous polynucleotide comprises the nucleotide sequence of SEQ ID NO: 61 or 62; and the second heterologous polynucleotide comprises the nucleotide sequence of SEQ ID NO: 64 or 65.

4. The recombinant host cell of any one of claims 1-3, wherein the first heterologous polynucleotide is operably linked to a foreign promoter and the second heterologous polynucleotide is operably linked to a foreign promoter.

5. The recombinant host cell of any one of claims 1-4, wherein the first heterologous polynucleotide and the second heterologous polynucleotide encode for polypeptides that form a protein complex having butyrate-acetoacetate CoA transferase activity.

6. The recombinant host cell of any one of claims 1-5, wherein the cell produces or is capable of producing a greater amount of isopropanol (e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) isopropanol.
50%, at least 75%, at least 100%, or at least 200% more) compared to the cell without the first and second heterologous polynucleotides, when cultivated under identical conditions.

7. The recombinant host cell of any one of claims 1-6, wherein the cell comprises one or more heterologous isopropanol pathway genes selected from:
   a heterologous thiolase gene;
   a heterologous acetoacetate decarboxylase gene; and
   a heterologous isopropanol dehydrogenase gene.

8. The recombinant host cell of any one of claims 1-7, wherein the cell comprises a disruption to an endogenous gene encoding an acetate kinase.

9. The recombinant host cell of claim 8, wherein the cell comprises a disruption to an endogenous gene encoding an acetate kinase having at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 60.

10. The recombinant host cell of claim 8 or 9, wherein the cell comprises a disruption to an endogenous gene encoding an acetate kinase having an amino acid sequence comprising or consisting of SEQ ID NO: 60.

11. The recombinant host cell of any one of claims 1-10, wherein the cell is selected from *Lactobacillus plantarum*, *Lactobacillus fructivorans*, and *Lactobacillus reuteri*.

12. The recombinant host cell of claim 11, wherein the cell is a *Lactobacillus reuteri* cell.

13. A composition comprising the recombinant host cell of any one of claims 1-12 and a fermentable medium.

14. A method of producing isopropanol, comprising:
   (a) cultivating the recombinant *Lactobacillus* host cell of any one of claims 1-12 in a medium under suitable conditions to produce isopropanol; and
   (b) recovering the isopropanol.

15. The method of claim 14, wherein the medium is a fermentable medium.
16. The method of claim 15, wherein the fermentable medium comprises sucrose, glucose and/or fructose.

17. The method of claim 15, wherein the fermentable medium comprises sugarcane juice (e.g., non-sterilized sugarcane juice).

18. The method of any of claims 14-17, wherein the produced isopropanol after step (a) and before step (b) is at a titer greater than about 0.01 g/L, e.g., greater than about 0.02 g/L, 0.05 g/L, 0.075 g/L, 0.1 g/L, 0.5 g/L, 1 g/L, 2 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 55 g/L, 60 g/L, 65 g/L, 70 g/L, 75 g/L, 80 g/L, 85 g/L, 90 g/L, 95 g/L, 100 g/L, 125 g/L, 150 g/L, 200 g/L, or 250 g/L.

19. A method of producing propylene, comprising:
   (a) cultivating the recombinant host cell of any of claims 1-12 in a medium under suitable conditions to produce isopropanol;
   (b) recovering the isopropanol;
   (c) dehydrating the isopropanol under suitable conditions to produce propylene;
   and
   (d) recovering the propylene.
Glucose
→ Glycolysis
→ Pyruvate
→ Acetyl-CoA
   ↓ Thiolase
   ↓ Acetoacetyl-CoA
      ↓ CoA-transferase
      ↓ Acetoacetate
         ↓ Acetoacetate decarboxylase
         ↓ Acetone
            ↓ Isopropanol dehydrogenase
               ↓ Isopropanol

Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. C12N15/74 C12P7/02

**ADD.**

According to International Patent Classification (IPC) into both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

EPO-Internal, Sequence Search, BIOSIS, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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Further documents are listed in the continuation of Box C.

See patent family annex.

**Date of the actual completion of the international search**

10 January 2014

**Date of mailing of the international search report**

23/01/2014

**Name and mailing address of the ISA**

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Fax. (+31-70) 340-3016

**Authorized officer**

Offermann, Stefanie
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