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(54) **Title**: ENGINEERED YEAST WITH IMPROVED GROWTH UNDER LOW AERATION

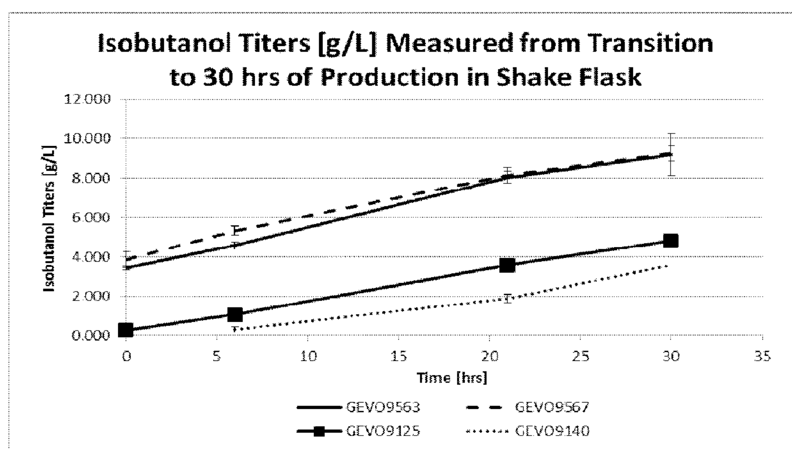

FIGURE 5

(57) **Abstract**: The present application relates to recombinant yeast microorganisms comprising biosynthetic pathways and methods of using said recombinant yeast microorganisms to produce various beneficial pyruvate-derived metabolites, e.g., isobutanol. In some embodiments, the recombinant yeast microorganisms may comprise a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced pyruvate decarboxylase (PDC) activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from NDE1 and NDE2.

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ENGINEERED YEAST WITH IMPROVED GROWTH UNDER LOW AERATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 61/664,267, filed June 26, 2012, which is herein incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

[0002] Recombinant microorganisms and methods of producing such microorganisms are provided. Also provided are methods of producing beneficial metabolites derived from pyruvate by contacting a suitable substrate with the recombinant microorganisms and enzymatic preparations therefrom.

DESCRIPTION OF TEXT FILE SUBMITTED ELECTRONICALLY

[0003] The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: GEVO_083_01WO_SeqList_ST25.txt, date recorded: June 24, 2013, file size: 120 kilobytes).

BACKGROUND

[0004] Wild-type yeasts such as *Saccharomyces cerevisiae* produce ethanol from pyruvate via the activity of an endogenous enzymatic conversion mediated by pyruvate decarboxylase (PDC). Metabolic engineers aiming to develop recombinant yeast microorganisms for the production of the advanced biofuel candidate, isobutanol, face a dilemma – while elimination of PDC activity is necessary to eliminate the production of ethanol and increase isobutanol yields (*See, e.g.*, commonly-owned US Patent No. 8,017,375), this modification comes at a cost. These modified yeasts are generally unable to grow anaerobically.

[0005] To overcome this growth defect, the present inventors identified modifications which allow PDC-deficient mutants to grow anaerobically. By virtue of one or more of these modifications, the resulting yeast strains exhibit improved growth under low aeration conditions, allowing for more economical production of

desired pyruvate-derived metabolites (*e.g.*, isobutanol).

SUMMARY OF THE INVENTION

[0006] The present inventors have discovered that one or more modifications can be introduced into PDC-deficient yeast strains to improve growth under low aeration conditions.

[0007] In a first aspect, the present application relates to a recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2*.

[0008] In one embodiment, the recombinant yeast microorganism includes a mutation in at least one gene selected from *NDE1* and *NDE2*. In a further embodiment, the mutation is a point mutation. In some embodiments, the point mutation results in a truncation of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In certain embodiments, the truncation occurs within 100 amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In further embodiments, the truncation occurs within 50, within 20, within 10, within 7, or within 5 amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In a specific embodiment, the truncation removes 1, 2, 3, 4, 5, or 6 amino acids from the N-terminus of the Nde1p polypeptide, *e.g.*, resulting in the removal of M, M-I, M-I-R, M-I-R-Q, M-I-R-Q-S, or M-I-R-Q-S-L. In a specific embodiment, the truncation occurs at the Q4 position of a polypeptide encoded by *NDE1*. In other embodiments, the recombinant yeast microorganism includes a partial deletion in at least one gene selected from *NDE1* and *NDE2*. In another embodiment, the recombinant yeast microorganism comprises a complete deletion of at least one gene selected from *NDE1* and *NDE2*. In yet another embodiment, the recombinant yeast microorganism includes a modification of the regulatory region associated with at least one gene selected from *NDE1* and

NDE2. In yet another embodiment, the recombinant yeast microorganism comprises a modification of the transcriptional regulator of at least one gene selected from *NDE1* and *NDE2.*

[0009] In another aspect, the present application relates to a recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YNL295W.* In some embodiments, the modification of *YNL295W* is a mutation, disruption, or deletion of *YNL295W.* In one embodiment, the modification of *YNL295W* is a mutation of *YNL295W.* In certain embodiments, the modification of *YNL295W* is a point mutation of *YNL295W.* In a specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an amino acid substitution at an amino acid position which is within 5 Angstroms of T441 of the protein encoded by *YNL295W.* In a specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an amino acid substitution at the T441 of the protein encoded by *YNL295W*, wherein said T441 residue is replaced with a residue selected from the group consisting of arginine, histidine, lysine, aspartic acid, glutamic acid, serine, asparagine, glutamine, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine. In a further specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an amino acid substitution at the T441 residue of the protein encoded by *YNL295W*, wherein said T441 residue is replaced with an alanine residue.

[0010] In another aspect, the present application relates to a recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism

is engineered to comprise at least one modification of *YJL055W*. In some embodiments, the modification of *YJL055W* is a mutation, disruption, or deletion of *YJL055W*. In one embodiment, the modification of *YJL055W* is a mutation of *YJL055W*. In certain embodiments, the modification of *YJL055W* is a point mutation of *YJL055W*. In a specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which results in an amino acid substitution at an amino acid position which is within 5 Angstroms of D99 of the protein encoded by *YJL055W*. In a specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which results in an amino acid substitution at the D99 of the protein encoded by *YJL055W*, wherein said D99 residue is replaced with a residue selected from the group consisting of arginine, histidine, lysine, glutamic acid, serine, threonine, asparagine, glutamine, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine. In a further specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which results in an amino acid substitution at the D99 residue of the protein encoded by *YJL055W*, wherein said D99 residue is replaced with a glutamic acid residue.

[0011] In various embodiments disclosed herein, the yeast microorganism is engineered to reduce PDC activity by deleting, disrupting, or mutating one or more genes encoding for pyruvate decarboxylase and/or a positive transcriptional regulator thereof. In one embodiment, said pyruvate decarboxylase gene targeted for disruption, deletion, or mutation is selected from the group consisting of *PDC1*, *PDC5*, and *PDC6*, or homologs or variants thereof. In an exemplary embodiment, all three of *PDC1*, *PDC5*, and *PDC6* are targeted for disruption, deletion, or mutation. In yet another embodiment, a positive transcriptional regulator of the *PDC1*, *PDC5*, and/or *PDC6* genes is targeted for disruption, deletion or mutation. In one embodiment, said positive transcriptional regulator is *PDC2*, or homologs or variants thereof.

[0012] As described herein, the modifications identified herein are relevant to improving growth characteristics in PDC-deficient yeast engineered for the production of variety of pyruvate-derived metabolites. In some embodiments, the pyruvate-derived metabolite is selected from the group consisting of isobutanol, 2-butanol, 1-butanol, 2-butanone, 2,3-butanediol, acetoin, diacetyl, valine, leucine, pantothenic acid, isobutylene, 3-methyl-1-butanol, 4-methyl-1-pentanol, coenzyme A, lactic acid, and malic acid.

[0013] In a preferred embodiment, the pyruvate-derived metabolite is isobutanol. Accordingly, in another aspect, the present application relates to a recombinant yeast microorganism comprising an isobutanol producing metabolic pathway, wherein said isobutanol producing metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the conversion of pyruvate to isobutanol, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2.*

[0014] In another aspect, the present application relates to a recombinant yeast microorganism comprising an isobutanol producing metabolic pathway, wherein said isobutanol producing metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the conversion of pyruvate to isobutanol, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YNL295W* as set forth herein.

[0015] In another aspect, the present application relates to a recombinant yeast microorganism comprising an isobutanol producing metabolic pathway, wherein said isobutanol producing metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the conversion of pyruvate to isobutanol, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YJL055W* as set forth herein.

[0016] In various embodiments described herein, the recombinant yeast microorganisms of the application may further be engineered to reduce or eliminate the expression and/or activity of one or more enzymes selected from a glycerol-3-phosphate dehydrogenase (GPD), a 3-keto acid reductase (3-KAR), or an aldehyde dehydrogenase (ALDH).

[0017] In one embodiment, the isobutanol producing metabolic pathway comprises at least one exogenous gene encoding a polypeptide that catalyzes a step in the conversion of pyruvate to isobutanol. In another embodiment, the

isobutanol producing metabolic pathway comprises at least two exogenous genes encoding polypeptides that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least three exogenous genes encoding polypeptides that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least four exogenous genes encoding polypeptides that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least five exogenous genes encoding polypeptides that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, all of the isobutanol producing metabolic pathway steps in the conversion of pyruvate to isobutanol are converted by exogenously encoded enzymes.

[0018] In one embodiment, one or more of the isobutanol pathway genes encodes an enzyme that is localized to the cytosol. In one embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least one isobutanol pathway enzyme localized in the cytosol. In another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least two isobutanol pathway enzymes localized in the cytosol. In yet another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least three isobutanol pathway enzymes localized in the cytosol. In yet another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least four isobutanol pathway enzymes localized in the cytosol. In an exemplary embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with five isobutanol pathway enzymes localized in the cytosol. In yet another exemplary embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with all isobutanol pathway enzymes localized in the cytosol.

[0019] In various embodiments described herein, the isobutanol pathway genes may encode enzyme(s) selected from the group consisting of acetolactate synthase (ALS), ketol-acid reductoisomerase (KARI), dihydroxyacid dehydratase (DHAD), 2-keto-acid decarboxylase, *e.g.*, keto-isovalerate decarboxylase (KIVD), and alcohol dehydrogenase (ADH). In one embodiment, the KARI is an NADH-dependent KARI (NKR). In another embodiment, the ADH is an NADH-dependent ADH. In yet

another embodiment, the KARI is an NADH-dependent KARI (NKR) and the ADH is an NADH-dependent ADH.

[0020] As described herein, in preferred embodiments, the recombinant microorganisms of the application are recombinant yeast microorganisms.

[0021] In some embodiments, the recombinant yeast microorganisms may be members of the Saccharomyces clade, *Saccharomyces sensu stricto* microorganisms, Crabtree-negative yeast microorganisms, Crabtree-positive yeast microorganisms, post-WGD (whole genome duplication) yeast microorganisms, pre-WGD (whole genome duplication) yeast microorganisms, and non-fermenting yeast microorganisms.

[0022] In some embodiments, the recombinant microorganisms may be yeast recombinant microorganisms of the Saccharomyces clade.

[0023] In some embodiments, the recombinant microorganisms may be *Saccharomyces sensu stricto* microorganisms. In one embodiment, the *Saccharomyces sensu stricto* is selected from the group consisting of *S. cerevisiae, S. kudriavzevii, S. mikatae, S. bayanus, S. uvarum, S. carocanis* and hybrids thereof.

[0024] In some embodiments, the recombinant microorganisms may be Crabtree-negative recombinant yeast microorganisms. In one embodiment, the Crabtree-negative yeast microorganism is classified into a genera selected from the group consisting of *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Issatchenkia*, *Hansenula*, or *Candida*. In additional embodiments, the Crabtree-negative yeast microorganism is selected from *Saccharomyces kluyveri, Kluyveromyces lactis, Kluyveromyces marxianus, Pichia anomala, Pichia stipitis, Pichia kudriavzevii, Hansenula anomala, Candida utilis* and *Kluyveromyces waltii*.

[0025] In some embodiments, the recombinant microorganisms may be Crabtree-positive recombinant yeast microorganisms. In one embodiment, the Crabtree-positive yeast microorganism is classified into a genera selected from the group consisting of *Saccharomyces, Kluyveromyces, Zygosaccharomyces, Debaryomyces, Candida, Pichia* and *Schizosaccharomyces*. In additional embodiments, the Crabtree-positive yeast microorganism is selected from the group consisting of *Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces bayanus, Saccharomyces paradoxus, Saccharomyces castelli, Kluyveromyces thermotolerans, Candida glabrata, Z. bailli, Z. rouxii, Debaryomyces hansenii, Pichia pastorius, Schizosaccharomyces pombe*, and *Saccharomyces uvarum*.

[0026] In some embodiments, the recombinant microorganisms may be post-WGD (whole genome duplication) yeast recombinant microorganisms. In one embodiment, the post-WGD yeast recombinant microorganism is classified into a genera selected from the group consisting of *Saccharomyces* or *Candida*. In additional embodiments, the post-WGD yeast is selected from the group consisting of *Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces bayanus, Saccharomyces paradoxus, Saccharomyces castelli*, and *Candida glabrata*.

[0027] In some embodiments, the recombinant microorganisms may be pre-WGD (whole genome duplication) yeast recombinant microorganisms. In one embodiment, the pre-WGD yeast recombinant microorganism is classified into a genera selected from the group consisting of *Saccharomyces, Kluyveromyces, Candida, Pichia, Issatchenkia, Debaryomyces, Hansenula, Pachysolen, Yarrowia* and *Schizosaccharomyces*. In additional embodiments, the pre-WGD yeast is selected from the group consisting of *Saccharomyces kluyveri, Kluyveromyces thermotolerans, Kluyveromyces marxianus, Kluyveromyces waltii, Kluyveromyces lactis, Candida tropicalis, Pichia pastoris, Pichia anomala, Pichia stipitis, Issatchenkia orientalis, Issatchenkia occidentalis, Debaryomyces hansenii, Hansenula anomala, Pachysolen tannophilis, Yarrowia lipolytica*, and *Schizosaccharomyces pombe*.

[0028] In some embodiments, the recombinant microorganisms may be microorganisms that are non-fermenting yeast microorganisms, including, but not limited to those, classified into a genera selected from the group consisting of *Tricosporon*, *Rhodotorula*, *Myxozyma*, or *Candida*. In a specific embodiment, the non-fermenting yeast is *C. xestobii*.

[0029] In another aspect, the present invention provides methods of producing a pyruvate-derived metabolite using a recombinant microorganism as described herein. In one embodiment, the method includes cultivating the recombinant microorganism in a culture medium containing a feedstock providing a carbon source until the pyruvate-derived metabolite is produced and optionally, recovering the pyruvate-derived metabolite. In one embodiment, the microorganism produces the pyruvate-derived metabolite from a carbon source at a yield of at least about 5 percent theoretical. In another embodiment, the microorganism produces the pyruvate-derived metabolite at a yield of at least about 10 percent, at least about 15 percent, about least about 20 percent, at least about 25 percent, at least about 30

percent, at least about 35 percent, at least about 40 percent, at least about 45 percent, at least about 50 percent, at least about 55 percent, at least about 60 percent, at least about 65 percent, at least about 70 percent, at least about 75 percent, at least about 80 percent, at least about 85 percent, at least about 90 percent, at least about 95 percent, or at least about 97.5 percent theoretical. In an exemplary embodiment, the pyruvate-derived metabolite is isobutanol.

[0030] In one embodiment, the recombinant microorganism converts the carbon source to the pyruvate-derived metabolite under aerobic conditions. In another embodiment, the recombinant microorganism converts the carbon source to the pyruvate-derived metabolite under microaerobic conditions. In yet another embodiment, the recombinant microorganism converts the carbon source to the pyruvate-derived metabolite under anaerobic conditions.

BRIEF DESCRIPTION OF DRAWINGS

[0031] Illustrative embodiments of the invention are illustrated in the drawings, in which:

[0032] **Figure 1** illustrates an exemplary embodiment of an isobutanol pathway.

[0033] **Figure 2** illustrates an exemplary embodiment of an NADH-dependent isobutanol pathway.

[0034] **Figure 3** illustrates various biosynthetic pathways which use pyruvate as an intermediate (adapted from Liu *et al.*, 2010, *Annu. Rev. Genet*. 44: 53-69).

[0035] **Figure 4** illustrates the OD_{600} measured during aerobic fermentation of the anaerobically-growing isolates GEVO9563 and GEVO9567 as compared to the parent strain GEVO9140 and the comparator control strain, GEVO9125.

[0036] **Figure 5** illustrates the isobutanol titers of the anaerobic strains GEVO9563 and GEVO9567 over the course of the 30 hour production phase under aerobic conditions as compared to GEVO9125 and GEVO9140.

[0037] **Figure 6** illustrates the specific isobutanol titers [g/L/OD] of the anaerobic strains GEVO9563 and GEVO9567 throughout the course of the 30 hour production phase under aerobic conditions as compared to GEVO9125 and GEVO9140.

[0038] **Figure 7** illustrates the estimated specific isobutanol productivity (g/g-h) of the anaerobic strains GEVO9563 and GEVO9567, the parent, GEVO9140, and the comparator control strain GEVO9125 calculated from 6 hours to 21 hours of

production in a single shake flask fermentation.

DETAILED DESCRIPTION

[0039] As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polynucleotide" includes a plurality of such polynucleotides and reference to "the microorganism" includes reference to one or more microorganisms, and so forth.

[0040] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods, devices and materials are described herein.

[0041] Any publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[0042] The term "microorganism" includes prokaryotic and eukaryotic microbial species from the Domains Archaea, Bacteria and Eucarya, the latter including yeast and filamentous fungi, protozoa, algae, or higher Protista. The terms "microbial cells" and "microbes" are used interchangeably with the term microorganism.

[0043] The term "prokaryotes" is art recognized and refers to cells which contain no nucleus or other cell organelles. The prokaryotes are generally classified in one of two domains, the Bacteria and the Archaea. The definitive difference between organisms of the Archaea and Bacteria domains is based on fundamental differences in the nucleotide base sequence in the 16S ribosomal RNA.

[0044] The term "Archaea" refers to a categorization of organisms of the division Mendosicutes, typically found in unusual environments and distinguished from the rest of the prokaryotes by several criteria, including the number of ribosomal proteins and the lack of muramic acid in cell walls. On the basis of ssrRNA analysis, the Archaea consist of two phylogenetically-distinct groups: Crenarchaeota and Euryarchaeota. On the basis of their physiology, the Archaea can be organized into three types: methanogens (prokaryotes that produce methane); extreme halophiles

(prokaryotes that live at very high concentrations of salt (NaCl); and extreme (hyper) thermophiles (prokaryotes that live at very high temperatures). Besides the unifying archaeal features that distinguish them from Bacteria (*i.e.*, no murein in cell wall, ester-linked membrane lipids, etc.), these prokaryotes exhibit unique structural or biochemical attributes which adapt them to their particular habitats. The Crenarchaeota consist mainly of hyperthermophilic sulfur-dependent prokaryotes and the Euryarchaeota contain the methanogens and extreme halophiles.

[0045] "Bacteria", or "eubacteria", refers to a domain of prokaryotic organisms. Bacteria include at least eleven distinct groups as follows: (1) Gram-positive (gram+) bacteria, of which there are two major subdivisions: (1) high G+C group (*Actinomycetes, Mycobacteria, Micrococcus*, others) (2) low G+C group (*Bacillus, Clostridia, Lactobacillus, Staphylococci, Streptococci, Mycoplasmas*); (2) Proteobacteria, *e.g.*, Purple photosynthetic +non-photosynthetic Gram-negative bacteria (includes most "common" Gram-negative bacteria); (3) Cyanobacteria, *e.g.*, oxygenic phototrophs; (4) Spirochetes and related species; (5) Planctomyces; (6) Bacteroides, Flavobacteria; (7) Chlamydia; (8) Green sulfur bacteria; (9) Green non-sulfur bacteria (also anaerobic phototrophs); (10) Radioresistant micrococci and relatives; (11) Thermotoga and Thermosipho thermophiles.

[0046] "Gram-negative bacteria" include cocci, nonenteric rods, and enteric rods. The genera of Gram-negative bacteria include, for example, *Neisseria, Spirillum, Pasteurella, Brucella, Yersinia, Francisella, Haemophilus, Bordetella, Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Vibrio, Pseudomonas, Bacteroides, Acetobacter, Aerobacter, Agrobacterium, Azotobacter, Spirilla, Serratia, Vibrio, Rhizobium, Chlamydia, Rickettsia, Treponema*, and *Fusobacterium*.

[0047] "Gram positive bacteria" include cocci, nonsporulating rods, and sporulating rods. The genera of gram positive bacteria include, for example, *Actinomyces, Bacillus, Clostridium, Corynebacterium, Erysipelothrix, Lactobacillus, Listeria, Mycobacterium, Myxococcus, Nocardia, Staphylococcus, Streptococcus*, and *Streptomyces*.

[0048] The term "genus" is defined as a taxonomic group of related species according to the Taxonomic Outline of Bacteria and Archaea (Garrity, G.M., Lilburn, T.G., Cole, J.R., Harrison, S.H., Euzeby, J., and Tindall, B.J. (2007) The Taxonomic Outline of Bacteria and Archaea. TOBA Release 7.7, March 2007. Michigan State University Board of Trustees.

[0049] The term “species” is defined as a collection of closely related organisms with greater than 97% 16S ribosomal RNA sequence homology and greater than 70% genomic hybridization and sufficiently different from all other organisms so as to be recognized as a distinct unit.

[0050] The terms "recombinant microorganism," “modified microorganism,” and "recombinant host cell" are used interchangeably herein and refer to microorganisms that have been genetically modified to express or to overexpress endogenous polynucleotides, to express heterologous polynucleotides, such as those included in a vector, in an integration construct, or which have an alteration in expression of an endogenous gene. By "alteration" it is meant that the expression of the gene, or level of a RNA molecule or equivalent RNA molecules encoding one or more polypeptides or polypeptide subunits, or activity of one or more polypeptides or polypeptide subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the alteration. For example, the term "alter" can mean "inhibit," but the use of the word "alter" is not limited to this definition. It is understood that the terms "recombinant microorganism" and "recombinant host cell" refer not only to the particular recombinant microorganism but to the progeny or potential progeny of such a microorganism. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0051] The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein results from transcription and translation of the open reading frame sequence. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired product encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantitated by qRT-PCR or by Northern hybridization (see Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). Protein encoded by a selected sequence can be quantitated by various methods, *e.g.*, by ELISA, by assaying for the biological activity of the protein, or by employing assays that are

independent of such activity, such as western blotting or radioimmunoassay, using antibodies that recognize and bind the protein. See Sambrook *et al.*, 1989, *supra.*

[0052] The term "overexpression" refers to an elevated level (*e.g.*, aberrant level) of mRNAs encoding for a protein(s), and/or to elevated levels of protein(s) in cells as compared to similar corresponding unmodified cells expressing basal levels of mRNAs or having basal levels of proteins. In particular embodiments mRNA(s) or protein(s) may be overexpressed by at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 8-fold, 10-fold, 12-fold, 15-fold or more in microorganisms engineered to exhibit increased gene mRNA, protein, and/or activity.

[0053] As used herein and as would be understood by one of ordinary skill in the art, "reduced activity and/or expression" of a protein such as an enzyme can mean either a reduced specific catalytic activity of the protein (*e.g.* reduced activity) and/or decreased concentrations of the protein in the cell (*e.g.* reduced expression). As would be understood by one or ordinary skill in the art, the reduced activity of a protein in a cell may result from decreased concentrations of the protein in the cell.

[0054] The term "wild-type microorganism" describes a cell that occurs in nature, *i.e.*, a cell that has not been genetically modified. A wild-type microorganism can be genetically modified to express or overexpress a first target enzyme. This microorganism can act as a parental microorganism in the generation of a microorganism modified to express or overexpress a second target enzyme. In turn, the microorganism modified to express or overexpress a first and a second target enzyme can be modified to express or overexpress a third target enzyme.

[0055] Accordingly, a "parental microorganism" functions as a reference cell for successive genetic modification events. Each modification event can be accomplished by introducing a nucleic acid molecule in to the reference cell. The introduction facilitates the expression or overexpression of a target enzyme. It is understood that the term "facilitates" encompasses the activation of endogenous polynucleotides encoding a target enzyme through genetic modification of *e.g.*, a promoter sequence in a parental microorganism. It is further understood that the term "facilitates" encompasses the introduction of heterologous polynucleotides encoding a target enzyme in to a parental microorganism

[0056] The term "engineer" refers to any manipulation of a microorganism that results in a detectable change in the microorganism, wherein the manipulation includes but is not limited to inserting a polynucleotide and/or polypeptide

heterologous to the microorganism and mutating a polynucleotide and/or polypeptide native to the microorganism.

[0057] The term "mutation" as used herein indicates any modification of a nucleic acid and/or polypeptide which results in an altered nucleic acid or polypeptide. Mutations include, for example, point mutations, deletions, or insertions of single or multiple residues in a polynucleotide, which includes alterations arising within a protein-encoding region of a gene as well as alterations in regions outside of a protein-encoding sequence, such as, but not limited to, regulatory or promoter sequences. A genetic alteration may be a mutation of any type. For instance, the mutation may constitute a point mutation, a frame-shift mutation, a nonsense mutation, an insertion, or a deletion of part or all of a gene. In addition, in some embodiments of the modified microorganism, a portion of the microorganism genome has been replaced with a heterologous polynucleotide. In some embodiments, the mutations are naturally-occurring. In other embodiments, the mutations are identified and/or enriched through artificial selection pressure. In still other embodiments, the mutations in the microorganism genome are the result of genetic engineering.

[0058] The term "biosynthetic pathway", also referred to as "metabolic pathway", refers to a set of anabolic or catabolic biochemical reactions for converting one chemical species into another. Gene products belong to the same "metabolic pathway" if they, in parallel or in series, act on the same substrate, produce the same product, or act on or produce a metabolic intermediate (*i.e.*, metabolite) between the same substrate and metabolite end product.

[0059] As used herein, the term “isobutanol producing metabolic pathway” refers to an enzyme pathway which produces isobutanol from pyruvate.

[0060] The term “NADH-dependent” as used herein with reference to an enzyme, *e.g.*, KARI and/or ADH, refers to an enzyme that catalyzes the reduction of a substrate coupled to the oxidation of NADH with a catalytic efficiency that is greater than the reduction of the same substrate coupled to the oxidation of NADPH at equal substrate and cofactor concentrations.

[0061] The term “exogenous” as used herein with reference to various molecules, *e.g.*, polynucleotides, polypeptides, enzymes, etc., refers to molecules that are not normally or naturally found in and/or produced by a given yeast, bacterium, organism, microorganism, or cell in nature.

[0062] On the other hand, the term "endogenous" or "native" as used herein with reference to various molecules, *e.g.*, polynucleotides, polypeptides, enzymes, etc., refers to molecules that are normally or naturally found in and/or produced by a given yeast, bacterium, organism, microorganism, or cell in nature.

[0063] The term "heterologous" as used herein in the context of a modified host cell refers to various molecules, *e.g.*, polynucleotides, polypeptides, enzymes, etc., wherein at least one of the following is true: (a) the molecule(s) is/are foreign ("exogenous") to (*i.e.*, not naturally found in) the host cell; (b) the molecule(s) is/are naturally found in (*e.g.*, is "endogenous to") a given host microorganism or host cell but is either produced in an unnatural location or in an unnatural amount in the cell; and/or (c) the molecule(s) differ(s) in nucleotide or amino acid sequence from the endogenous nucleotide or amino acid sequence(s) such that the molecule differing in nucleotide or amino acid sequence from the endogenous nucleotide or amino acid as found endogenously is produced in an unnatural (*e.g.*, greater than naturally found) amount in the cell.

[0064] The term "feedstock" is defined as a raw material or mixture of raw materials supplied to a microorganism or fermentation process from which other products can be made. For example, a carbon source, such as biomass or the carbon compounds derived from biomass are a feedstock for a microorganism that produces a biofuel in a fermentation process. However, a feedstock may contain nutrients other than a carbon source.

[0065] The term "substrate" or "suitable substrate" refers to any substance or compound that is converted or meant to be converted into another compound by the action of an enzyme. The term includes not only a single compound, but also combinations of compounds, such as solutions, mixtures and other materials which contain at least one substrate, or derivatives thereof. Further, the term "substrate" encompasses not only compounds that provide a carbon source suitable for use as a starting material, such as any biomass derived sugar, but also intermediate and end product metabolites used in a pathway associated with a recombinant microorganism as described herein.

[0066] The term "fermentation" or "fermentation process" is defined as a process in which a microorganism is cultivated in a culture medium containing raw materials, such as feedstock and nutrients, wherein the microorganism converts raw materials, such as a feedstock, into products.

[0067] The term "volumetric productivity" or "production rate" is defined as the amount of product formed per volume of medium per unit of time. Volumetric productivity is reported in gram per liter per hour (g/L/h).

[0068] The term "specific productivity" or "specific production rate" is defined as the amount of product formed per volume of medium per unit of time per amount of cells. Specific productivity is reported in gram (or milligram) per gram cell dry weight per hour (g/g h).

[0069] The term "yield" is defined as the amount of product obtained per unit weight of raw material and may be expressed as g product per g substrate (g/g). Yield may be expressed as a percentage of the theoretical yield. "Theoretical yield" is defined as the maximum amount of product that can be generated per a given amount of substrate as dictated by the stoichiometry of the metabolic pathway used to make the product. For example, the theoretical yield for one typical conversion of glucose to isobutanol is 0.41 g/g. As such, a yield of isobutanol from glucose of 0.39 g/g would be expressed as 95% of theoretical or 95% theoretical yield.

[0070] The term "titer" is defined as the strength of a solution or the concentration of a substance in solution. For example, the titer of a biofuel in a fermentation broth is described as g of biofuel in solution per liter of fermentation broth (g/L).

[0071] "Aerobic conditions" are defined as conditions under which the oxygen concentration in the fermentation medium is sufficiently high for an aerobic or facultative anaerobic microorganism to use as a terminal electron acceptor.

[0072] In contrast, "anaerobic conditions" are defined as conditions under which the oxygen concentration in the fermentation medium is too low for the microorganism to use as a terminal electron acceptor. Anaerobic conditions may be achieved by sparging a fermentation medium with an inert gas such as nitrogen until oxygen is no longer available to the microorganism as a terminal electron acceptor. Alternatively, anaerobic conditions may be achieved by the microorganism consuming the available oxygen of the fermentation until oxygen is unavailable to the microorganism as a terminal electron acceptor. Methods for the production of isobutanol under anaerobic conditions are described in commonly owned and co-pending publication, US 2010/0143997, the disclosures of which are herein incorporated by reference in its entirety for all purposes.

[0073] "Aerobic metabolism" refers to a biochemical process in which oxygen is used as a terminal electron acceptor to make energy, typically in the form of ATP,

from carbohydrates. Aerobic metabolism occurs e.g. via glycolysis and the TCA cycle, wherein a single glucose molecule is metabolized completely into carbon dioxide in the presence of oxygen.

[0074] In contrast, "anaerobic metabolism" refers to a biochemical process in which oxygen is not the final acceptor of electrons contained in NADH. Anaerobic metabolism can be divided into anaerobic respiration, in which compounds other than oxygen serve as the terminal electron acceptor, and substrate level phosphorylation, in which the electrons from NADH are utilized to generate a reduced product via a "fermentative pathway."

[0075] In "fermentative pathways", NAD(P)H donates its electrons to a molecule produced by the same metabolic pathway that produced the electrons carried in NAD(P)H. For example, in one of the fermentative pathways of certain yeast strains, NAD(P)H generated through glycolysis transfers its electrons to pyruvate, yielding ethanol. Fermentative pathways are usually active under anaerobic conditions but may also occur under aerobic conditions, under conditions where NADH is not fully oxidized via the respiratory chain. For example, above certain glucose concentrations, Crabtree positive yeasts produce large amounts of ethanol under aerobic conditions.

[0076] The term "byproduct" or "by-product" means an undesired product related to the production of an amino acid, amino acid precursor, chemical, chemical precursor, biofuel, biofuel precursor, higher alcohol, or higher alcohol precursor.

[0077] The term "substantially free" when used in reference to the presence or absence of a protein activity (3-KAR enzymatic activity, ALDH enzymatic activity, PDC enzymatic activity, GPD enzymatic activity, etc.) means the level of the protein activity is substantially less than that of the same protein activity in the wild-type host, wherein less than about 50% of the wild-type level is preferred and less than about 30% is more preferred. The activity may be less than about 20%, less than about 10%, less than about 5%, or less than about 1% of wild-type activity. Microorganisms which are "substantially free" of a particular protein activity (3-KAR enzymatic activity, ALDH enzymatic activity, PDC enzymatic activity, GPD enzymatic activity, etc.) may be created through recombinant means or identified in nature.

[0078] The term "non-fermenting yeast" is a yeast species that fails to demonstrate an anaerobic metabolism in which the electrons from NADH are utilized to generate a reduced product via a fermentative pathway such as the production of

ethanol and CO_2 from glucose. Non-fermentative yeast can be identified by the "Durham Tube Test" (J.A. Barnett, R.W. Payne, and D. Yarrow. 2000. Yeasts Characteristics and Identification. 3rd edition. p. 28-29. Cambridge University Press, Cambridge, UK) or by monitoring the production of fermentation productions such as ethanol and CO_2.

[0079] The term "polynucleotide" is used herein interchangeably with the term "nucleic acid" and refers to an organic polymer composed of two or more monomers including nucleotides, nucleosides or analogs thereof, including but not limited to single stranded or double stranded, sense or antisense deoxyribonucleic acid (DNA) of any length and, where appropriate, single stranded or double stranded, sense or antisense ribonucleic acid (RNA) of any length, including siRNA. The term "nucleotide" refers to any of several compounds that consist of a ribose or deoxyribose sugar joined to a purine or a pyrimidine base and to a phosphate group, and that are the basic structural units of nucleic acids. The term "nucleoside" refers to a compound (as guanosine or adenosine) that consists of a purine or pyrimidine base combined with deoxyribose or ribose and is found especially in nucleic acids. The term "nucleotide analog" or "nucleoside analog" refers, respectively, to a nucleotide or nucleoside in which one or more individual atoms have been replaced with a different atom or with a different functional group. Accordingly, the term polynucleotide includes nucleic acids of any length, DNA, RNA, analogs and fragments thereof. A polynucleotide of three or more nucleotides is also called nucleotidic oligomer or oligonucleotide.

[0080] It is understood that the polynucleotides described herein include "genes" and that the nucleic acid molecules described herein include "vectors" or "plasmids." Accordingly, the term "gene", also called a "structural gene" refers to a polynucleotide that codes for a particular sequence of amino acids, which comprise all or part of one or more proteins or enzymes, and may include regulatory (non-transcribed) DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. The transcribed region of the gene may include untranslated regions, including introns, 5'-untranslated region (UTR), and 3'-UTR, as well as the coding sequence.

[0081] The term "operon" refers to two or more genes which are transcribed as a single transcriptional unit from a common promoter. In some embodiments, the genes comprising the operon are contiguous genes. It is understood that

transcription of an entire operon can be modified (*i.e.*, increased, decreased, or eliminated) by modifying the common promoter. Alternatively, any gene or combination of genes in an operon can be modified to alter the function or activity of the encoded polypeptide. The modification can result in an increase in the activity of the encoded polypeptide. Further, the modification can impart new activities on the encoded polypeptide. Exemplary new activities include the use of alternative substrates and/or the ability to function in alternative environmental conditions.

[0082] A "vector" is any means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include viruses, bacteriophage, pro-viruses, plasmids, phagemids, transposons, and artificial chromosomes such as YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), and PLACs (plant artificial chromosomes), and the like, that are "episomes," that is, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine -conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can be an organism which comprises one or more of the above polynucleotide constructs such as an agrobacterium or a bacterium.

[0083] "Transformation" refers to the process by which a vector is introduced into a host cell. Transformation (or transduction, or transfection), can be achieved by any one of a number of means including chemical transformation (e.g. lithium acetate transformation), electroporation, microinjection, biolistics (or particle bombardment-mediated delivery), or agrobacterium mediated transformation.

[0084] The term "enzyme" as used herein refers to any substance that catalyzes or promotes one or more chemical or biochemical reactions, which usually includes enzymes totally or partially composed of a polypeptide, but can include enzymes composed of a different molecule including polynucleotides.

[0085] The term “protein,” “peptide,” or “polypeptide” as used herein indicates an organic polymer composed of two or more amino acidic monomers and/or analogs thereof. As used herein, the term “amino acid” or “amino acidic monomer” refers to any natural and/or synthetic amino acids including glycine and both D or L optical isomers. The term “amino acid analog” refers to an amino acid in which one or more individual atoms have been replaced, either with a different atom, or with a different

functional group. Accordingly, the term polypeptide includes amino acidic polymer of any length including full length proteins, and peptides as well as analogs and fragments thereof. A polypeptide of three or more amino acids is also called a protein oligomer or oligopeptide

[0086] The term "homolog," used with respect to an original polynucleotide or polypeptide of a first family or species, refers to distinct polynucleotides or polypeptides of a second family or species which are determined by functional, structural or genomic analyses to be a polynucleotide or polypeptide of the second family or species which corresponds to the original polynucleotide or polypeptide of the first family or species. Most often, homologs will have functional, structural or genomic similarities. Techniques are known by which homologs of a polynucleotide or polypeptide can readily be cloned using genetic probes and PCR. Identity of cloned sequences as homolog can be confirmed using functional assays and/or by genomic mapping of the genes.

[0087] A polypeptide has "homology" or is "homologous" to a second polypeptide if the amino acid sequence encoded by a gene has a similar amino acid sequence to that of the second gene. Alternatively, a polypeptide has homology to a second polypeptide if the two polypeptides have "similar" amino acid sequences. (Thus, the terms "homologous polypeptides" or “homologous proteins” are defined to mean that the two polypeptides have similar amino acid sequences).

[0088] The term “analog” or “analogous” refers to polynucleotide or polypeptide sequences that are related to one another in function only and are not from common descent or do not share a common ancestral sequence. Analogs may differ in sequence but may share a similar structure, due to convergent evolution. For example, two enzymes are analogs or analogous if the enzymes catalyze the same reaction of conversion of a substrate to a product, are unrelated in sequence, and irrespective of whether the two enzymes are related in structure.

Recombinant Yeast Microorganisms With Improved Growth Under Anaerobic Conditions

[0089] To engineer a yeast biocatalyst that produces isobutanol at high yield, it is necessary to reduce the yeast’s ability to produce ethanol, as unwanted ethanol production diverts carbon flow away from the desired pyruvate-derived compound, isobutanol. One disclosed method for reducing ethanol production is the disruption,

mutation, or deletion of one or more endogenous pyruvate decarboxylase (PDC) encoding genes. *See* commonly owned US Pat. No. 8,017,375, which is herein incorporated by reference in its entirety.

[0090] When the production of ethanol is reduced by the disruption, mutation, or deletion of endogenous PDC genes in Crabtree-positive yeast such as *S. cerevisiae*, a number of problems arise which prohibit the use of such strains in industrial processes. One significant problem in particular is that the modified yeast are generally unable to grow anaerobically.

[0091] To overcome this growth defect, the present inventors identified modifications which allow PDC-deficient mutants to grow anaerobically. By virtue of one or more of these modifications, the resulting yeast strains exhibit improved growth under low aeration conditions, allowing for more economical production of desired pyruvate-derived metabolites (*e.g.*, isobutanol).

[0092] As used herein, a "pyruvate-derived metabolite" can include any biosynthetic pathway which uses pyruvate as the starting material and/or as an intermediate. As is understood in the art, yeast cells convert sugars to produce pyruvate, which is then utilized in a number of pathways of cellular metabolism. In recent years, yeast cells have been engineered to produce a number of desirable products via pyruvate-driven biosynthetic pathways. Amongst the biosynthetic pathways which derive from pyruvate include pathways for the production of isobutanol, 2-butanol, 1-butanol, 2-butanone, 2,3-butanediol, acetoin, diacetyl, valine, leucine, pantothenic acid, isobutylene, 3-methyl-1-butanol, 4-methyl-1-pentanol, coenzyme A, lactic acid, and malic acid. Engineered biosynthetic pathways for the synthesis of these beneficial pyruvate-derived metabolites are found in Table 1 and **Figure 3**.

Table 1. Pyruvate-Derived Biosynthetic Pathways.

Biosynthetic Pathway	***Reference***[a]
Isobutanol	US 2009/0226991 (Feldman *et al.*), US 2011/0020889 (Feldman *et al.*), and US 2010/0143997 (Buelter *et al.*)
1-Butanol	US 2010/0159546 (Aristidou *et al.*), WO/2010/017230 (Lynch), WO/2010/031772 (Wu *et al.*), and KR2011002130 (Lee *et al.*)
2-Butanol	WO/2007/130518 (Donaldson *et al.*), WO/2007/130521 (Donaldson *et al.*), and WO/2009/134276 (Donaldson *et al.*)
2-Butanone	WO/2007/130518 (Donaldson *et al.*), WO/2007/130521 (Donaldson *et al.*), and WO/2009/134276 (Donaldson *et al.*)
2-3-Butanediol	WO/2007/130518 (Donaldson *et al.*), WO/2007/130521 (Donaldson

	et al.), and WO/2009/134276 (Donaldson *et al.*)
Acetoin	WO/2007/130518 (Donaldson *et al.*), WO/2007/130521 (Donaldson *et al.*), and WO/2009/134276 (Donaldson *et al.*)
Diacetyl	Gonzalez *et al.*, 2000, *J. Biol. Chem* 275: 35876-85 and Ehsani *et al.*, 2009, *App. Environ. Micro.* 75: 3196-205
Valine	WO/2001/021772 (Yocum *et al.*) and McCourt *et al.*, 2006, *Amino Acids* 31: 173-210
Leucine	WO/2001/021772 (Yocum *et al.*) and McCourt *et al.*, 2006, *Amino Acids* 31: 173-210
Pantothenic Acid	WO/2001/021772 (Yocum *et al.*)
3-Methyl-1-Butanol	WO/2008/098227 (Liao *et al.*), Atsumi *et al.*, 2008, *Nature* 451: 86-89, and Connor *et al.*, 2008, *Appl. Environ. Microbiol.* 74: 5769-5775
4-Methyl-1-Pentanol	WO/2010/045629 (Liao *et al.*), Zhang *et al.*, 2008, *Proc Natl Acad Sci USA* 105:20653-20658
Coenzyme A	WO/2001/021772 (Yocum *et al.*)
Lactic Acid	WO/2007/032792 (Suominen *et al.*), US 2010/0137551 (Rajgarhia *et al.*)
Malic Acid	US 2008/0090273 (Winkler *et al.*)

[a] – The contents of each of the references in this table are herein incorporated by reference in their entireties for all purposes.

[0093] Each of the products listed in Table 1 are derived from pyruvate. As would be understood in the art, another compound which is derived from pyruvate is ethanol, the production of which can divert carbon flow away from the desired pyruvate-derived compound, thereby reducing optimal yield of the desired pyruvate-derived compound. To support the commercial production of pyruvate-derived metabolites, recombinant yeast strains with reduced PDC activity have been constructed. *See, e.g.*, commonly-owned US Patent No. 8,017,375. The commercial production of desired pyruvate-derived metabolites further depends in part, on the ability of the yeast to grow under conditions which are economically favored, *i.e.*, growth under anaerobic conditions. PDC-deficient yeast, however, generally do not tolerate growth under anaerobic conditions.

[0094] As described herein, the present inventors have discovered that one or more modifications can be introduced into PDC-deficient yeast strains to improve growth under low aeration conditions. Specifically, as shown in Example 3, the present inventors have found that modifications to *NDE1* and/or *NDE2* result in improved growth under low aeration conditions. Specifically, the present inventors have determined that deletion, disruption, or mutation of a gene selected from *NDE1* and *NDE2* improves the growth of PDC-deficient yeast under low aeration conditions.

[0095] Accordingly, in a first aspect, the present application relates to a

recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2*.

[0096] Thus, the application relates to recombinant yeast cells engineered to provide reduced endogenous expression and/or activity of a protein encoded by *NDE1* and/or *NDE2* (*i.e.*, herein referred to as Nde1 or Nde1p, or in the case of *NDE2*, as Nde2 or Nde2p). In general, cells that have a reduced expression and/or activity of Nde1 and/or Nde2 or homologs thereof will exhibit an enhanced ability to grow under low aeration conditions.

[0097] Nde1p and Nde2p are mitochondrial external NADH dehydrogenases (*i.e.*, NADH dehydrogenase, external). *See* Luttik *et al.*, 1998, *J. Biol. Chem.* 273(38): 24529-34. *See also* Small *et al.*, 1998, *J. Bacteriol.* 180(16): 4051-55. In yeast, Nde1p and Nde2p contribute to the reoxidation of cytosolic NADH by the mitochondria. *See* Overkamp *et al.*, 2000, *J. Bacteriol.* 182(10): 2823-30. Nde1p and Nde2p are located in the inner mitochondrial membrane with catalytic sites facing the intermembranal space. *See* Pahlman *et al.*, 2002, *J. Biol. Chem.* 277(31): 27991-95. Accordingly, disruption or mutation of Nde1p and/or Nde2p at these catalytic sites would generally be expected to reduce their enzymatic activity. In Nde1p/Nde2p double mutants, oxidation of external NADH is completely absent, confirming their external location. *See* Melo *et al.*, 2004, *Microbiol. Mol. Biol. Rev.* 68(4): 603-16.

[0098] As described herein, deletion, disruption, or mutation of one or more endogenous genes encoding for Nde1p and/or Nde2p and/or a positive transcriptional regulator thereof can be made to improve the growth of yeast cells under low aeration conditions. In one embodiment, the recombinant yeast microorganism includes a mutation in at least one gene selected from *NDE1* and *NDE2*. In a further embodiment, the mutation is a point mutation. In some embodiments, the point mutation results in a truncation of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In certain embodiments, the truncation

occurs within 100 amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In further embodiments, the truncation occurs within 50, within 20, within 10, within 7, or within 5 amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In a specific embodiment, the truncation removes 1, 2, 3, 4, 5, or 6 amino acids from the N-terminus of the Nde1p polypeptide, *e.g.*, resulting in the removal of M, M-I, M-I-R, M-I-R-Q, M-I-R-Q-S, or M-I-R-Q-S-L. In a specific embodiment, the truncation occurs at the Q4 position of a polypeptide encoded by *NDE1*. In another embodiment, the recombinant yeast microorganism includes a partial deletion in at least one gene selected from *NDE1* and *NDE2*. In another embodiment, the recombinant yeast microorganism comprises a complete deletion of at least one gene selected from *NDE1* and *NDE2*. In yet another embodiment, the recombinant yeast microorganism includes a modification of the regulatory region associated with at least one gene selected from *NDE1* and *NDE2*. In yet another embodiment, the recombinant yeast microorganism comprises a modification of the transcriptional regulator of at least one gene selected from *NDE1* and *NDE2*.

[0099] As is understood by those skilled in the art, however, there are several additional mechanisms for reducing or disruption the expression and/or activity of a protein encoded by *NDE1* and/or *NDE2*, including, but not limited to, the use of a regulated promoter, use of a weak constitutive promoter, disruption of one of the two copies of the gene in a diploid yeast, disruption of both copies of the gene in a diploid yeast, expression of an anti-sense nucleic acid, expression of an siRNA, overexpression of a negative regulator of the endogenous promoter, alteration of the activity of an endogenous or heterologous gene, use of a heterologous gene with lower specific activity, the like or combinations thereof.

[00100] One aspect of the application is therefore directed to a recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In one

embodiment, the Nde1 polypeptide comprises SEQ ID NO: 2. In another embodiment, the Nde2 polypeptide comprises SEQ ID NO: 4. Homologs of Nde1 and Nde2 are known to occur in yeast other than *S. cerevisiae*. Accordingly, in additional embodiments, a Nde1 and/or Nde2 polypeptide derived from a yeast selected from *Ajellomyces, Arthroderma, Ashbya, Aspergillus, Botryotinia, Candida, Chaetomium, Clavispora, Coccidioides, Debaryomyces, Gibberella, Glomerella, Grosmannia, Issatchenkia, Kluyveromyces, Leptosphaeria, Lodderomyces, Magnaporthe, Metarhizium, Meyerozyma, Mycosphaerella, Nectria, Neosartorya, Neurospora, Paracoccidioides, Penicillium, Phaeosphaeria, Pichia, Podospora, Pyrenophora, Saccharomyces, Scheffersomyces, Schizosaccharomyces, Sclerotinia, Sordaria, Talaromyces, Trichoderma, Trichophyton, Tuber, Uncinocarpus, Verticillium, Yarrowia* or *Zygosaccharomyces* may be disrupted, deleted, or mutated. Sequences for a variety of Nde1 homologs from yeast other than *S. cerevisiae* are available in the art, *e.g.*, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, and SEQ ID NO: 16. Likewise, sequences for a variety of Nde2 homologs from yeast other than *S. cerevisiae* are available in the art, *e.g.*, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, and SEQ ID NO: 28.

[00101] Additionally shown in Example 3 are observations made by the present inventors regarding the presence of *YNL295W* (SEQ ID NO: 29) and *YJL055W* (SEQ ID NO: 31) mutations in yeast strains exhibiting improved growth under low aeration conditions. Specifically, a T441 to A alteration was observed in *YNL295W*, while a D99 to E substitution was seen in *YJL055W*.

[00102] Accordingly, in another aspect, the present application relates to a recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YNL295W*. In some embodiments, the modification of *YNL295W* is a mutation, disruption, or deletion of *YNL295W*. In one embodiment, the modification of *YNL295W* is a mutation of *YNL295W*. In certain embodiments, the modification of *YNL295W* is a point

mutation of *YNL295W*. In a specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an amino acid substitution at an amino acid position which is within 5 Angstroms of T441 of the protein encoded by *YNL295W*. In a specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an amino acid substitution at the T441 of the protein encoded by *YNL295W*, wherein said T441 residue is replaced with a residue selected from the group consisting of arginine, histidine, lysine, aspartic acid, glutamic acid, serine, asparagine, glutamine, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine. In a further specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an amino acid substitution at the T441 residue of the protein encoded by *YNL295W*, wherein said T441 residue is replaced with an alanine residue.

[00103] In an additional aspect, the present application relates to a recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YJL055W*. In some embodiments, the modification of *YJL055W* is a mutation, disruption, or deletion of *YJL055W*. In one embodiment, the modification of *YJL055W* is a mutation of *YJL055W*. In certain embodiments, the modification of *YJL055W* is a point mutation of *YJL055W*. In a specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which results in an amino acid substitution at an amino acid position which is within 5 Angstroms of D99 of the protein encoded by *YJL055W*. In a specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which results in an amino acid substitution at the D99 of the protein encoded by *YJL055W*, wherein said D99 residue is replaced with a residue selected from the group consisting of arginine, histidine, lysine, glutamic acid, serine, threonine, asparagine, glutamine, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine. In a further specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which

results in an amino acid substitution at the D99 residue of the protein encoded by *YJL055W*, wherein said D99 residue is replaced with a glutamic acid residue.

[00104] In various aspects, the recombinant yeast microorganism is engineered to reduce PDC activity by deleting, disrupting, or mutating one or more genes encoding for pyruvate decarboxylase and/or a positive transcriptional regulator thereof. In one embodiment, said pyruvate decarboxylase gene targeted for disruption, deletion, or mutation is selected from the group consisting of *PDC1*, *PDC5*, and *PDC6*, or homologs or variants thereof. In an exemplary embodiment, all three of *PDC1*, *PDC5*, and *PDC6* are targeted for disruption, deletion, or mutation. In yet another embodiment, a positive transcriptional regulator of the *PDC1*, *PDC5*, and/or *PDC6* is targeted for disruption, deletion or mutation. In one embodiment, said positive transcriptional regulator is *PDC2*, or homologs or variants thereof.

[00105] As is understood by those skilled in the art, there are several additional mechanisms available for reducing or disrupting the activity of a protein encoded by *PDC1*, *PDC5*, *PDC6*, and/or *PDC2*, including, but not limited to, the use of a regulated promoter, use of a weak constitutive promoter, disruption of one of the two copies of the gene in a diploid yeast, disruption of both copies of the gene in a diploid yeast, expression of an anti-sense nucleic acid, expression of an siRNA, over expression of a negative regulator of the endogenous promoter, alteration of the activity of an endogenous or heterologous gene, use of a heterologous gene with lower specific activity, the like or combinations thereof.

Isobutanol Producing Recombinant Microorganisms

[00106] In certain exemplary embodiments, the present application relates to a recombinant yeast microorganism comprising an engineered isobutanol producing metabolic pathway. In recent years, yeast cells have been engineered to produce increased quantities of isobutanol, an important commodity chemical and biofuel candidate. Engineered yeast for the production of isobutanol are described in a variety of commonly-owned patents, *e.g.*, U.S. Pat. Nos. 8,017,375, 8,017,376, 8,071,358, 8,097,440, 8,133,715, 8,153,415, and 8,158,404, and co-pending publications, US 2009/0226990, US 2010/0143997, US 2011/0020889, US 2011/0076733, US 2011/0201090, US 2012/0045809, and US 2012/0034666.

[00107] As described herein, the present invention relates to recombinant microorganisms for producing isobutanol, wherein said recombinant microorganisms

comprise an isobutanol producing metabolic pathway. In one embodiment, the isobutanol producing metabolic pathway to convert pyruvate to isobutanol can be comprised of the following reactions:

1. 2 pyruvate → acetolactate + CO_2
2. acetolactate + NAD(P)H → 2,3-dihydroxyisovalerate + $NAD(P)^+$
3. 2,3-dihydroxyisovalerate → alpha-ketoisovalerate
4. alpha-ketoisovalerate → isobutyraldehyde + CO_2
5. isobutyraldehyde +NAD(P)H → isobutanol + NADP

[00108] In one embodiment, these reactions are carried out by the enzymes 1) Acetolactate synthase (ALS), 2) Ketol-acid reductoisomerase (KARI), 3) Dihydroxy-acid dehydratase (DHAD), 4) 2-keto-acid decarboxylase, *e.g.*, Keto-isovalerate decarboxylase (KIVD), and 5) an Alcohol dehydrogenase (ADH) (**Figure 1**). In some embodiments, the recombinant microorganism may be engineered to overexpress one or more of these enzymes. In an exemplary embodiment, the recombinant microorganism is engineered to overexpress all of these enzymes.

[00109] Alternative pathways for the production of isobutanol in yeast have been described in WO/2007/050671 and in Dickinson *et al.*, 1998, *J Biol Chem* 273:25751-6. These and other isobutanol producing metabolic pathways are within the scope of the present application. In one embodiment, the isobutanol producing metabolic pathway comprises five substrate to product reactions. In another embodiment, the isobutanol producing metabolic pathway comprises six substrate to product reactions. In yet another embodiment, the isobutanol producing metabolic pathway comprises seven substrate to product reactions.

[00110] As described above, the present inventors have discovered that one or more modifications can be introduced into PDC-deficient yeast strains to improve growth under low aeration conditions. Specifically, as shown in Example 3, the present inventors have found that modifications to *NDE1* and/or *NDE2* can be made to improve growth under low aeration conditions. Specifically, the present inventors have determined that deletion, disruption, or mutation of a gene selected from *NDE1* and *NDE2* improves the growth of PDC-deficient yeast under low aeration conditions, thereby resulting in PDC-deficient yeast strains with improved growth characteristics, allowing for more economical production of desired pyruvate-derived metabolites. In an exemplary embodiment, the pyruvate-derived metabolite is isobutanol.

[00111] Accordingly, in another aspect, the present application relates to a recombinant yeast microorganism comprising an isobutanol producing metabolic pathway, wherein said isobutanol producing metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the conversion of pyruvate to isobutanol, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2.*

[00112] In one embodiment, the recombinant yeast microorganism includes a mutation in at least one gene selected from *NDE1* and *NDE2*. In a further embodiment, the mutation is a point mutation. In some embodiments, the point mutation results in a truncation of a polypeptide encoded by a gene selected from *NDE1* and *NDE2.* In certain embodiments, the truncation occurs within 100 amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In further embodiments, the truncation occurs within 50, within 20, within 10, within 7, or within 5 amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*. In a specific embodiment, the truncation removes 1, 2, 3, 4, 5, or 6 amino acids from the N-terminus of the Nde1p polypeptide, *e.g.*, resulting in the removal of M, M-I, M-I-R, M-I-R-Q, M-I-R-Q-S, or M-I-R-Q-S-L. In a specific embodiment, the truncation occurs at the Q4 position of a polypeptide encoded by *NDE1*. In another embodiment, the recombinant yeast microorganism includes a partial deletion in at least one gene selected from *NDE1* and *NDE2*. In another embodiment, the recombinant yeast microorganism comprises a complete deletion of at least one gene selected from *NDE1* and *NDE2*. In yet another embodiment, the recombinant yeast microorganism includes a modification of the regulatory region associated with at least one gene selected from *NDE1* and *NDE2.* In yet another embodiment, the recombinant yeast microorganism comprises a modification of the transcriptional regulator of at least one gene selected from *NDE1* and *NDE2.*

[00113] In one embodiment, the Nde1 polypeptide comprises SEQ ID NO: 2. In another embodiment, the Nde2 polypeptide comprises SEQ ID NO: 4. Homologs of Nde1 and Nde2 are known to occur in yeast other than *S. cerevisiae*. Accordingly, in additional embodiments, a Nde1 and/or Nde2 polypeptide derived from a yeast

selected from *Ajellomyces, Arthroderma, Ashbya, Aspergillus, Botryotinia, Candida, Chaetomium, Clavispora, Coccidioides, Debaryomyces, Gibberella, Glomerella, Grosmannia, Issatchenkia, Kluyveromyces, Leptosphaeria, Lodderomyces, Magnaporthe, Metarhizium, Meyerozyma, Mycosphaerella, Nectria, Neosartorya, Neurospora, Paracoccidioides, Penicillium, Phaeosphaeria, Pichia, Podospora, Pyrenophora, Saccharomyces, Scheffersomyces, Schizosaccharomyces, Sclerotinia, Sordaria, Talaromyces, Trichoderma, Trichophyton, Tuber, Uncinocarpus, Verticillium, Yarrowia* or *Zygosaccharomyces* may be disrupted, deleted, or mutated. Sequences for a variety of Nde1 homologs from yeast other than *S. cerevisiae* are available in the art, *e.g.*, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, and SEQ ID NO: 16. Likewise, sequences for a variety of Nde2 homologs from yeast other than *S. cerevisiae* are available in the art, *e.g.*, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, and SEQ ID NO: 28.

[00114] Additionally shown in Example 3 are observations made by the present inventors regarding the presence of *YNL295W* (SEQ ID NO: 29) and *YJL055W* (SEQ ID NO: 31) mutations in yeast strains exhibiting improved growth under low aeration conditions. Specifically, a T441 to A alteration was observed in *YNL295W*, while a D99 to E substitution was seen in *YJL055W*.

[00115] Accordingly, in another aspect, the present application relates to a recombinant yeast microorganism comprising an isobutanol producing metabolic pathway, wherein said isobutanol producing metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the conversion of pyruvate to isobutanol, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YNL295W*. In some embodiments, the modification of *YNL295W* is a mutation, disruption, or deletion of *YNL295W*. In one embodiment, the modification of *YNL295W* is a mutation of *YNL295W*. In certain embodiments, the modification of *YNL295W* is a point mutation of *YNL295W*. In a specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an amino acid substitution at an amino acid position which is within 5 Angstroms of T441 of the protein encoded by *YNL295W*. In a specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an

amino acid substitution at the T441 of the protein encoded by *YNL295W*, wherein said T441 residue is replaced with a residue selected from the group consisting of arginine, histidine, lysine, aspartic acid, glutamic acid, serine, asparagine, glutamine, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine. In a further specific embodiment, the modification of *YNL295W* is a point mutation of *YNL295W* which results in an amino acid substitution at the T441 residue of the protein encoded by *YNL295W*, wherein said T441 residue is replaced with an alanine residue.

[00116] In an additional aspect, the present application relates to a recombinant yeast microorganism comprising an isobutanol producing metabolic pathway, wherein said isobutanol producing metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the conversion of pyruvate to isobutanol, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YJL055W*. In some embodiments, the modification of *YJL055W* is a mutation, disruption, or deletion of *YJL055W*. In one embodiment, the modification of *YJL055W* is a mutation of *YJL055W*. In certain embodiments, the modification of *YJL055W* is a point mutation of *YJL055W*. In a specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which results in an amino acid substitution at an amino acid position which is within 5 Angstroms of D99 of the protein encoded by *YJL055W*. In a specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which results in an amino acid substitution at the D99 of the protein encoded by *YJL055W*, wherein said D99 residue is replaced with a residue selected from the group consisting of arginine, histidine, lysine, glutamic acid, serine, threonine, asparagine, glutamine, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine. In a further specific embodiment, the modification of *YJL055W* is a point mutation of *YJL055W* which results in an amino acid substitution at the D99 residue of the protein encoded by *YJL055W*, wherein said D99 residue is replaced with a glutamic acid residue

[00117] In one embodiment, the yeast microorganism comprising an isobutanol producing metabolic pathway has reduced or no pyruvate decarboxylase (PDC) activity. PDC catalyzes the decarboxylation of pyruvate to acetaldehyde, which is

then reduced to ethanol by ADH via an oxidation of NADH to NAD+. Ethanol production is the main pathway to oxidize the NADH from glycolysis. Deletion, disruption, or mutation of this pathway increases the pyruvate and the reducing equivalents (NADH) available for an isobutanol biosynthetic pathway. Accordingly, deletion, disruption, or mutation of one or more genes encoding for pyruvate decarboxylase and/or a positive transcriptional regulator thereof can further increase the yield of isobutanol. In one embodiment, said pyruvate decarboxylase gene targeted for disruption, deletion, or mutation is selected from the group consisting of *PDC1*, *PDC5*, and *PDC6*, or homologs or variants thereof. In another embodiment, all three of *PDC1*, *PDC5*, and *PDC6* are targeted for disruption, deletion, or mutation. In yet another embodiment, a positive transcriptional regulator of the *PDC1*, *PDC5*, and/or *PDC6* is targeted for disruption, deletion or mutation. In one embodiment, said positive transcriptional regulator is *PDC2*, or homologs or variants thereof.

[00118] As is understood by those skilled in the art, there are several additional mechanisms available for reducing or disrupting the activity of a protein encoded by *PDC1*, *PDC5*, *PDC6*, and/or *PDC2*, including, but not limited to, the use of a regulated promoter, use of a weak constitutive promoter, disruption of one of the two copies of the gene in a diploid yeast, disruption of both copies of the gene in a diploid yeast, expression of an anti-sense nucleic acid, expression of an siRNA, over expression of a negative regulator of the endogenous promoter, alteration of the activity of an endogenous or heterologous gene, use of a heterologous gene with lower specific activity, the like or combinations thereof

[00119] As described herein, strains that naturally produce low levels of pyruvate decarboxylase can also have applicability for producing increased levels of isobutanol. As would be understood by one skilled in the art equipped with the instant disclosure, strains that naturally produce low levels of pyruvate decarboxylase may inherently exhibit low or undetectable levels of pyruvate decarboxylase activity, a trait which may be favorable for the production of isobutanol.

[00120] In various embodiments described herein, the recombinant microorganism comprises an engineered isobutanol producing metabolic pathway. In one embodiment, the isobutanol producing metabolic pathway comprises at least one exogenous gene encoding a polypeptide that catalyzes a step in the conversion of

pyruvate to isobutanol. In another embodiment, the isobutanol producing metabolic pathway comprises at least two exogenous genes encoding polypeptides that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least three exogenous genes encoding polypeptides that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least four exogenous genes encoding polypeptides that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least five exogenous genes encoding polypeptides that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, all of the isobutanol producing metabolic pathway steps in the conversion of pyruvate to isobutanol are converted by exogenously encoded enzymes.

[00121] In one embodiment, one or more of the isobutanol pathway genes encodes an enzyme that is localized to the cytosol. In one embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least one isobutanol pathway enzyme localized in the cytosol. In another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least two isobutanol pathway enzymes localized in the cytosol. In yet another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least three isobutanol pathway enzymes localized in the cytosol. In yet another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least four isobutanol pathway enzymes localized in the cytosol. In an exemplary embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with five isobutanol pathway enzymes localized in the cytosol. Isobutanol producing metabolic pathways in which one or more genes are localized to the cytosol are described in commonly owned and co-pending publication, US 2011/0076733, which is herein incorporated by reference in its entirety for all purposes.

[00122] As is understood in the art, a variety of organisms can serve as sources for the isobutanol pathway enzymes, including, but not limited to, *Saccharomyces* spp., including *S. cerevisiae* and *S. uvarum*, *Kluyveromyces* spp., including *K. thermotolerans*, *K. lactis*, and *K. marxianus*, *Pichia* spp., *Hansenula* spp., including

H. polymorpha, *Candida* spp., *Trichosporon* spp., *Yamadazyma* spp., including *Y.* spp. *stipitis*, *Torulaspora pretoriensis*, *Issatchenkia orientalis*, *Schizosaccharomyces* spp., including *S. pombe*, *Cryptococcus* spp., *Aspergillus* spp., *Neurospora* spp., or *Ustilago* spp. Sources of genes from anaerobic fungi include, but not limited to, *Piromyces* spp., *Orpinomyces* spp., or *Neocallimastix* spp. Sources of prokaryotic enzymes that are useful include, but not limited to, *Escherichia* spp., *Zymomonas* spp., *Staphylococcus* spp., *Bacillus* spp., *Clostridium* spp., *Corynebacterium* spp., *Pseudomonas* spp., *Slackia* spp., *Lactococcus* spp., *Enterobacter* spp., *Streptococcus* spp., *Salmonella* spp., *Bacteroides* spp., *Methanococcus* spp., *Erythrobacter* spp., *Sphingomonas* spp., *Sphingobium* spp., and *Novosphingobium* spp.

[00123] In some embodiments, one or more of these enzymes can be encoded by native genes. Alternatively, one or more of these enzymes can be encoded by heterologous genes.

[00124] For example, acetolactate synthases capable of converting pyruvate to acetolactate may be derived from a variety of sources (*e.g.*, bacterial, yeast, Archaea, etc.), including *B. subtilis* (GenBank Accession No. Q04789.3), *L. lactis* (GenBank Accession No. NP_267340.1), *S. mutans* (GenBank Accession No. NP_721805.1), *K. pneumoniae* (GenBank Accession No. ZP_06014957.1), *C. glutamicum* (GenBank Accession No. P42463.1), *E. cloacae* (GenBank Accession No. YP_003613611.1), *M. maripaludis* (GenBank Accession No. ABX01060.1), *M. grisea* (GenBank Accession No. AAB81248.1), *T. stipitatus* (GenBank Accession No. XP_002485976.1), or *S. cerevisiae ILV2* (GenBank Accession No. NP_013826.1). Additional acetolactate synthases capable of converting pyruvate to acetolactate are described in commonly owned US Publication No. 2011/0076733 (now US Patent No. 8,232,089), which is herein incorporated by reference in its entirety. A review article characterizing the biosynthesis of acetolactate from pyruvate via the activity of acetolactate synthases is provided by Chipman *et al.*, 1998, *Biochimica et Biophysica Acta* 1385: 401-19, which is herein incorporated by reference in its entirety. Chipman *et al.* provide an alignment and consensus for the sequences of a representative number of acetolactate synthases. Motifs shared in common between the majority of acetolactate synthases include:

SGPG(A/C/V)(T/S)N (SEQ ID NO: 33),

GX(P/A)GX(V/A/T) (SEQ ID NO: 34),

GX(Q/G)(T/A)(L/M)G(Y/F/W)(A/G)X(P/G)(W/A)AX(G/T)(A/V) (SEQ ID NO: 35), and

GD(G/A)(G/S/C)F (SEQ ID NO: 36)

motifs at amino acid positions corresponding to the 163-169, 240-245, 521-535, and 549-553 residues, respectively, of the *S. cerevisiae ILV2*. Thus, a protein harboring one or more of these amino acid motifs can generally be expected to exhibit acetolactate synthase activity.

[00125] Ketol-acid reductoisomerases capable of converting acetolactate to 2,3-dihydroxyisovalerate may be derived from a variety of sources (*e.g.*, bacterial, yeast, Archaea, etc.), including *E. coli* (GenBank Accession No. EGB30597.1), *L. lactis* (GenBank Accession No. YP_003353710.1), *S. exigua* (GenBank Accession No. ZP_06160130.1), *C. curtam* (GenBank Accession No. YP_003151266.1), *Shewanella sp.* (GenBank Accession No. YP_732498.1), *V. fischeri* (GenBank Accession No. YP_205911.1), *M. maripaludis* (GenBank Accession No. YP_001097443.1), *B. subtilis* (GenBank Accession No. CAB14789), *S. pombe* (GenBank Accession No. NP_001018845), *B. thetaiotamicron* (GenBank Accession No. NP_810987), or *S. cerevisiae ILV5* (GenBank Accession No. NP_013459.1). Additional ketol-acid reductoisomerases capable of converting acetolactate to 2,3-dihydroxyisovalerate are described in commonly owned US Publication No. 2011/0076733 (now US Patent No. 8,232,089), which is herein incorporated by reference in its entirety. An alignment and consensus for the sequences of a representative number of ketol-acid reductoisomerases is provided in commonly owned and co-pending US Publication No. 2010/0143997, which is herein incorporated by reference in its entirety. Motifs shared in common between the majority of ketol-acid reductoisomerases include:

G(Y/C/W)GXQ(G/A) (SEQ ID NO: 37),

(F/Y/L)(S/A)HG(F/L) (SEQ ID NO: 38),

V(V/I/F)(M/L/A)(A/C)PK (SEQ ID NO: 39),

D(L/I)XGE(Q/R)XXLXG (SEQ ID NO: 40), and

S(D/N/T)TA(E/Q/R)XG (SEQ ID NO: 41)

motifs at amino acid positions corresponding to the 89-94, 175-179, 194-200, 262-272, and 459-465 residues, respectively, of the *E. coli* ketol-acid reductoisomerase encoded by *ilvC*. Thus, a protein harboring one or more of these amino acid motifs can generally be expected to exhibit ketol-acid reductoisomerase activity.

[00126] To date, all known, naturally existing ketol-acid reductoisomerases are known to use NADPH as a cofactor. In certain embodiments, a ketol-acid reductoisomerase which has been engineered to used NADH as a cofactor may be utilized to mediate the conversion of acetolactate to 2,3-dihydroxyisovalerate. Engineered NADH-dependent KARI enzymes ("NKRs") and methods of generating such NKRs are disclosed in commonly owned and co-pending US Publication No. 2010/0143997.

[00127] In accordance with the invention, any number of mutations can be made to a KARI enzyme, and in a preferred aspect, multiple mutations can be made to a KARI enzyme to result in an increased ability to utilize NADH for the conversion of acetolactate to 2,3-dihydroxyisovalerate. Such mutations include point mutations, frame shift mutations, deletions, and insertions, with one or more (*e.g.*, one, two, three, four, five or more, etc.) point mutations preferred.

[00128] Mutations may be introduced into naturally existing KARI enzymes to create NKRs using any methodology known to those skilled in the art. Mutations may be introduced randomly by, for example, conducting a PCR reaction in the presence of manganese as a divalent metal ion cofactor. Alternatively, oligonucleotide directed mutagenesis may be used to create the NKRs which allows for all possible classes of base pair changes at any determined site along the encoding DNA molecule. In general, this technique involves annealing an oligonucleotide complementary (except for one or more mismatches) to a single stranded nucleotide sequence coding for the KARI enzyme of interest. The mismatched oligonucleotide is then extended by DNA polymerase, generating a double-stranded DNA molecule which contains the desired change in sequence in one strand. The changes in sequence can, for example, result in the deletion, substitution, or insertion of an amino acid. The double-stranded polynucleotide can then be inserted into an appropriate expression vector, and a mutant or modified polypeptide can thus be produced. The above-described oligonucleotide directed mutagenesis can, for example, be carried out via PCR.

[00129] Dihydroxy acid dehydratases capable of converting 2,3-dihydroxyisovalerate to α-ketoisovalerate may be derived from a variety of sources (*e.g.*, bacterial, yeast, Archaea, etc.), including *E. coli* (GenBank Accession No. YP_026248.1), *L. lactis* (GenBank Accession No. NP_267379.1), *S. mutans* (GenBank Accession No. NP_722414.1), *M. stadtmanae* (GenBank Accession No. YP_448586.1), *M. tractuosa* (GenBank Accession No. YP_004053736.1),

Eubacterium SCB49 (GenBank Accession No. ZP_01890126.1), *G. forsetti* (GenBank Accession No. YP_862145.1), *Y. lipolytica* (GenBank Accession No. XP_502180.2), *N. crassa* (GenBank Accession No. XP_963045.1), or *S. cerevisiae ILV3* (GenBank Accession No. NP_012550.1). Additional dihydroxy acid dehydratases capable of 2,3-dihydroxyisovalerate to α-ketoisovalerate are described in commonly owned US Publication No. 2011/0076733 (now US Patent No. 8,232,089). Motifs shared in common between the majority of dihydroxy acid dehydratases include:

SLXSRXXIA (SEQ ID NO: 42),
CDKXXPG (SEQ ID NO: 43),
GXCXGXXTAN (SEQ ID NO: 44),
GGSTN (SEQ ID NO: 45),
GPXGXPGMRXE (SEQ ID NO: 46),
ALXTDGRXSG (SEQ ID NO: 47), and
GHXXPEA (SEQ ID NO: 48)

motifs at amino acid positions corresponding to the 93-101, 122-128, 193-202, 276-280, 482-491, 509-518, and 526-532 residues, respectively, of the *E. coli* dihydroxy acid dehydratase encoded by *ilv*D. Thus, a protein harboring one or more of these amino acid motifs can generally be expected to exhibit dihydroxy acid dehydratase activity.

[00130] 2-keto-acid decarboxylases capable of converting α-ketoisovalerate to isobutyraldehyde may be derived from a variety of sources (*e.g.*, bacterial, yeast, Archaea, etc.), including *L. lactis kiv*D (GenBank Accession No. YP_003353820.1), *E. cloacae* (GenBank Accession No. P23234.1), *M. smegmatis* (GenBank Accession No. A0R480.1), *M. tuberculosis* (GenBank Accession No. O53865.1), *M. avium* (GenBank Accession No. Q742Q2.1, *A. brasilense* (GenBank Accession No. P51852.1), *L. lactis kdc*A (GenBank Accession No. AAS49166.1), *S. epidermidis* (GenBank Accession No. NP_765765.1), *M. caseolyticus* (GenBank Accession No. YP_002560734.1), *B. megaterium* (GenBank Accession No. YP_003561644.1), *S. cerevisiae ARO10* (GenBank Accession No. NP_010668.1), or *S. cerevisiae THI3* (GenBank Accession No. CAA98646.1). Additional 2-keto-acid decarboxylases capable of converting α-ketoisovalerate to isobutyraldehyde are described in commonly owned US Publication No. 2011/0076733 (now US Patent No. 8,232,089).

Motifs shared in common between the majority of 2-keto-acid decarboxylases include:

FG(V/I)(P/S)G(D/E)(Y/F) (SEQ ID NO: 49),

(T/V)T(F/Y)G(V/A)G(E/A)(L/F)(S/N) (SEQ ID NO: 50),

N(G/A)(L/I/V)AG(S/A)(Y/F)AE (SEQ ID NO: 51),

(V/I)(L/I/V)XI(V/T/S)G (SEQ ID NO: 52), and

GDG(S/A)(L/F/A)Q(L/M)T (SEQ ID NO: 53)

motifs at amino acid positions corresponding to the 21-27, 70-78, 81-89, 93-98, and 428-435 residues, respectively, of the *L. lactis* 2-keto-acid decarboxylase encoded by *kiv*D. Thus, a protein harboring one or more of these amino acid motifs can generally be expected to exhibit 2-keto-acid decarboxylase activity.

[00131] Alcohol dehydrogenases capable of converting isobutyraldehyde to isobutanol may be derived from a variety of sources (*e.g.*, bacterial, yeast, Archaea, etc.), including *L. lactis* (GenBank Accession No. YP_003354381), *B. cereus* (GenBank Accession No. YP_001374103.1), *N. meningitidis* (GenBank Accession No. CBA03965.1), *S. sanguinis* (GenBank Accession No. YP_001035842.1), *L. brevis* (GenBank Accession No. YP_794451.1), *B. thuringiensis* (GenBank Accession No. ZP_04101989.1), *P. acidilactici* (GenBank Accession No. ZP_06197454.1), *B. subtilis* (GenBank Accession No. EHA31115.1), *N.* crassa (GenBank Accession No. CAB91241.1) or *S. cerevisiae ADH6* (GenBank Accession No. NP_014051.1). Additional alcohol dehydrogenases capable of converting isobutyraldehyde to isobutanol are described in commonly owned US Publication Nos. 2011/0076733 (now US Patent No. 8,232,089) and 2011/0201072. Motifs shared in common between the majority of alcohol dehydrogenases include:

C(H/G)(T/S)D(L/I)H (SEQ ID NO: 54),

GHEXXGXV (SEQ ID NO: 55),

(L/V)(Q/K/E)(V/I/K)G(D/Q)(R/H)(V/A) (SEQ ID NO: 56),

CXXCXXC (SEQ ID NO: 57),

(C/A)(A/G/D)(G/A)XT(T/V) (SEQ ID NO: 58), and

G(L/A/C)G(G/P)(L/I/V)G (SEQ ID NO: 59)

motifs at amino acid positions corresponding to the 39-44, 59-66, 76-82, 91-97, 147-152, and 171-176 residues, respectively, of the *L. lactis* alcohol dehydrogenase encoded by *adh*A. Thus, a protein harboring one or more of these amino acid motifs can generally be expected to exhibit alcohol dehydrogenase activity.

[00132] In an exemplary embodiment, pathway steps 2 and 5 of the isobutanol pathway may be carried out by KARI and ADH enzymes that utilize NADH (rather than NADPH) as a cofactor. It has been found previously that utilization of NADH-dependent KARI (NKR) and ADH enzymes to catalyze pathway steps 2 and 5, respectively, surprisingly enables production of isobutanol at theoretical yield and/or under anaerobic conditions. *See, e.g.*, commonly owned and co-pending patent publication US 2010/0143997. An example of an NADH-dependent isobutanol pathway is illustrated in **Figure 2**. Thus, in one embodiment, the recombinant microorganisms of the present invention may use an NKR to catalyze the conversion of acetolactate to produce 2,3-dihydroxyisovalerate. In another embodiment, the recombinant microorganisms of the present invention may use an NADH-dependent ADH to catalyze the conversion of isobutyraldehyde to produce isobutanol. In yet another embodiment, the recombinant microorganisms of the present invention may use both an NKR to catalyze the conversion of acetolactate to produce 2,3-dihydroxyisovalerate, and an NADH-dependent ADH to catalyze the conversion of isobutyraldehyde to produce isobutanol.

[00133] In another embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to isobutanol. In one embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to isobutyraldehyde. In another embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to keto-isovalerate. In another embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to 2,3-dihydroxyisovalerate. In another embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to acetolactate.

[00134] Furthermore, any of the genes encoding the foregoing enzymes (or any others mentioned herein (or any of the regulatory elements that control or modulate expression thereof)) may be optimized by genetic/protein engineering techniques, such as directed evolution or rational mutagenesis, which are known to those of ordinary skill in the art. Such action allows those of ordinary skill in the art to optimize the enzymes for expression and activity in yeast.

The Microorganism in General

[00135] As described herein, the recombinant microorganisms of the present invention can express a plurality of heterologous and/or native enzymes involved in pathways for the production of a pyruvate-derived metabolite (*e.g.*, isobutanol).

[00136] As described herein, "engineered" or "modified" microorganisms are produced via the introduction of genetic material into a host or parental microorganism of choice and/or by modification of the expression of native genes, thereby modifying or altering the cellular physiology and biochemistry of the microorganism. Through the introduction of genetic material and/or the modification of the expression of native genes the parental microorganism acquires new properties, *e.g.*, the ability to produce a new, or greater quantities of, an intracellular and/or extracellular metabolite. As described herein, the introduction of genetic material into and/or the modification of the expression of native genes in a parental microorganism results in a new or modified ability to produce a desired pyruvate-derived metabolite (*e.g.*, isobutanol) from a suitable carbon source. The genetic material introduced into and/or the genes modified for expression in the parental microorganism contains gene(s), or parts of genes, coding for one or more of the enzymes involved in a biosynthetic pathway for the production of a pyruvate-derived metabolite (*e.g.*, isobutanol) and may also include additional elements for the expression and/or regulation of expression of these genes, *e.g.*, promoter sequences.

[00137] In addition to the introduction of a genetic material into a host or parental microorganism, an engineered or modified microorganism can also include the alteration, disruption, deletion or knocking-out of a gene or polynucleotide to alter the cellular physiology and biochemistry of the microorganism. Through the alteration, disruption, deletion or knocking-out of a gene or polynucleotide, the microorganism acquires new or improved properties (*e.g.*, the ability to produce a new metabolite or greater quantities of an intracellular metabolite, to improve the flux of a metabolite down a desired pathway, and/or to reduce the production of by-products).

[00138] Recombinant microorganisms provided herein may also produce metabolites in quantities not available in the parental microorganism. A "metabolite" refers to any substance produced by metabolism or a substance necessary for or taking part in a particular metabolic process. A metabolite can be an organic compound that is a starting material (*e.g.*, glucose or pyruvate), an intermediate

(*e.g.*, 2-ketoisovalerate), or an end product (*e.g.*, isobutanol) of metabolism. Metabolites can be used to construct more complex molecules, or they can be broken down into simpler ones. Intermediate metabolites may be synthesized from other metabolites, perhaps used to make more complex substances, or broken down into simpler compounds, often with the release of chemical energy.

[00139] The disclosure identifies specific genes useful in the methods, compositions and organisms of the disclosure; however it will be recognized that absolute identity to such genes is not necessary. For example, changes in a particular gene or polynucleotide comprising a sequence encoding a polypeptide or enzyme can be performed and screened for activity. Typically such changes comprise conservative mutations and silent mutations. Such modified or mutated polynucleotides and polypeptides can be screened for expression of a functional enzyme using methods known in the art.

[00140] Due to the inherent degeneracy of the genetic code, other polynucleotides which encode substantially the same or functionally equivalent polypeptides can also be used to clone and express the polynucleotides encoding such enzymes.

[00141] As will be understood by those of skill in the art, it can be advantageous to modify a coding sequence to enhance its expression in a particular host. The genetic code is redundant with 64 possible codons, but most organisms typically use a subset of these codons. The codons that are utilized most often in a species are called optimal codons, and those not utilized very often are classified as rare or low-usage codons. Codons can be substituted to reflect the preferred codon usage of the host, in a process sometimes called "codon optimization" or "controlling for species codon bias."

[00142] Optimized coding sequences containing codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al.*, 1989, *Nucl Acids Res.* 17: 477-508) can be prepared, for example, to increase the rate of translation or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, as compared with transcripts produced from a non-optimized sequence. Translation stop codons can also be modified to reflect host preference. For example, typical stop codons for *S. cerevisiae* and mammals are UAA and UGA, respectively. The typical stop codon for monocotyledonous plants is UGA, whereas insects and *E. coli* commonly use UAA as the stop codon (Dalphin *et al.*, 1996, *Nucl Acids Res.* 24:

216-8). Methodology for optimizing a nucleotide sequence for expression in a plant is provided, for example, in U.S. Pat. No. 6,015,891, and the references cited therein.

[00143] Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given enzyme of the disclosure. The native DNA sequence encoding the biosynthetic enzymes described above are referenced herein merely to illustrate an embodiment of the disclosure, and the disclosure includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the disclosure. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The disclosure includes such polypeptides with different amino acid sequences than the specific proteins described herein so long as the modified or variant polypeptides have the enzymatic anabolic or catabolic activity of the reference polypeptide. Furthermore, the amino acid sequences encoded by the DNA sequences shown herein merely illustrate embodiments of the disclosure.

[00144] In addition, homologs of enzymes useful for generating a pyruvate-derived metabolite (*e.g.*, isobutanol) are encompassed by the microorganisms and methods provided herein.

[00145] As used herein, two proteins (or a region of the proteins) are substantially homologous when the amino acid sequences have at least about 30%, 40%, 50% 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity. To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In one embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, typically at least 40%, more typically at least 50%, even more typically at least 60%, and even more typically at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the

corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[00146] When "homologous" is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (*e.g.*, charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of homology may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art (See, *e.g.*, Pearson W.R., 1994, *Methods in Mol Biol* 25: 365-89).

[00147] The following six groups each contain amino acids that are conservative substitutions for one another: 1) Serine (S), Threonine (T); 2) Aspartic Acid (D), Glutamic Acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Alanine (A), Valine (V), and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[00148] Sequence homology for polypeptides, which is also referred to as percent sequence identity, is typically measured using sequence analysis software. See commonly owned and co-pending application US 2009/0226991. A typical algorithm used comparing a molecule sequence to a database containing a large number of sequences from different organisms is the computer program BLAST. When searching a database containing sequences from a large number of different organisms, it is typical to compare amino acid sequences. Database searching using amino acid sequences can be measured by algorithms described in commonly owned U.S. Pat. No. 8,017,375.

[00149] It is understood that a range of microorganisms can be modified to include a recombinant metabolic pathway suitable for the production of a desired pyruvate-derived metabolite (*e.g.*, isobutanol). In various embodiments, microorganisms may

be selected from yeast microorganisms. Yeast microorganisms for the production of a desired pyruvate-derived metabolite (*e.g.*, isobutanol) may be selected based on certain characteristics:

[00150] One characteristic may include the property that the microorganism is selected to convert various carbon sources into a desired pyruvate-derived metabolite (*e.g.*, isobutanol). The term "carbon source" generally refers to a substance suitable to be used as a source of carbon for prokaryotic or eukaryotic cell growth. Examples of suitable carbon sources are described in commonly owned U.S. Pat. No. 8,017,375. Accordingly, in one embodiment, the recombinant microorganism herein disclosed can convert a variety of carbon sources, including but not limited to glucose, galactose, mannose, xylose, arabinose, lactose, sucrose, and mixtures thereof, to one or more pyruvate-derived metabolites (*e.g.*, isobutanol).

[00151] The recombinant microorganism may thus further include a pathway for the production of a desired pyruvate-derived metabolite (*e.g.*, isobutanol) from five-carbon (pentose) sugars including xylose. Most yeast species metabolize xylose via a complex route, in which xylose is first reduced to xylitol via a xylose reductase (XR) enzyme. The xylitol is then oxidized to xylulose via a xylitol dehydrogenase (XDH) enzyme. The xylulose is then phosphorylated via a xylulokinase (XK) enzyme. This pathway operates inefficiently in yeast species because it introduces a redox imbalance in the cell. The xylose-to-xylitol step uses primarily NADPH as a cofactor (generating NADP+), whereas the xylitol-to-xylulose step uses NAD+ as a cofactor (generating NADH). Other processes must operate to restore the redox imbalance within the cell. This often means that the organism cannot grow anaerobically on xylose or other pentose sugars. Accordingly, a yeast species that can efficiently ferment xylose and other pentose sugars into a desired fermentation product is therefore very desirable.

[00152] Thus, in one aspect, the recombinant microorganism is engineered to express a functional exogenous xylose isomerase. Exogenous xylose isomerases (XI) functional in yeast are known in the art. *See*, *e.g.*, Rajgarhia *et al.*, U.S. Pat. No. 7,943,366, which is herein incorporated by reference in its entirety. In an embodiment according to this aspect, the exogenous XI gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell. In a preferred embodiment, the recombinant microorganism further has a deletion or disruption of a native gene that encodes for an enzyme (*e.g.*, XR and/or XDH) that

catalyzes the conversion of xylose to xylitol. In a further preferred embodiment, the recombinant microorganism also contains a functional, exogenous xylulokinase (XK) gene operatively linked to promoter and terminator sequences that are functional in the yeast cell. In one embodiment, the xylulokinase (XK) gene is overexpressed.

[00153] In one embodiment, the microorganism has reduced glycerol-3-phosphate dehydrogenase (GPD) activity. GPD catalyzes the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) via the oxidation of NADH to NAD+. Glycerol is then produced from G3P by Glycerol-3-phosphatase (GPP). Glycerol production is a secondary pathway to oxidize excess NADH from glycolysis. Reduction or elimination of this pathway would increase the pyruvate and reducing equivalents (NADH) available for the production of a pyruvate-derived metabolite (*e.g.*, isobutanol). Thus, disruption, deletion, or mutation of the genes encoding for glycerol-3-phosphate dehydrogenases can further increase the yield of the desired metabolite (*e.g.*, isobutanol). Yeast strains with reduced GPD activity are described in commonly owned and co-pending US Patent Publication Nos. 2011/0020889 and 2011/0183392.

[00154] In yet another embodiment, the microorganism has reduced 3-keto acid reductase (3-KAR) activity. 3-KARs catalyze the conversion of 3-keto acids (*e.g.*, acetolactate) to 3-hydroxyacids (*e.g.*, DH2MB). Yeast strains with reduced 3-KAR activity are described in commonly owned U.S. Pat. Nos. 8,133,715, 8,153,415, and 8,158,404, which are herein incorporated by reference in their entireties.

[00155] In yet another embodiment, the microorganism has reduced aldehyde dehydrogenase (ALDH) activity. Aldehyde dehydrogenases catalyze the conversion of aldehydes (*e.g.*, isobutyraldehyde) to acid by-products (*e.g.*, isobutyrate). Yeast strains with reduced ALDH activity are described in commonly owned U.S. Pat. Nos. 8,133,715, 8,153,415, and 8,158,404, which are herein incorporated by reference in their entireties.

[00156] In one embodiment, the yeast microorganisms may be selected from the "*Saccharomyces* Yeast Clade", as described in commonly owned U.S. Pat. No. 8,017,375.

[00157] The term "Saccharomyces *sensu stricto*" taxonomy group is a cluster of yeast species that are highly related to *S. cerevisiae* (Rainieri *et al.*, 2003, *J. Biosci Bioengin* 96: 1-9). Saccharomyces *sensu stricto* yeast species include but are not limited to *S. cerevisiae, S. kudriavzevii, S. mikatae, S. bayanus, S. uvarum, S.*

carocanis and hybrids derived from these species (Masneuf *et al.*, 1998, *Yeast* 7: 61-72).

[00158] An ancient whole genome duplication (WGD) event occurred during the evolution of the hemiascomycete yeast and was discovered using comparative genomic tools (Kellis *et al.*, 2004, *Nature* 428: 617-24; Dujon *et al.*, 2004, *Nature* 430:35-44; Langkjaer *et al.*, 2003, *Nature* 428: 848-52; Wolfe *et al.*, 1997, *Nature* 387: 708-13). Using this major evolutionary event, yeast can be divided into species that diverged from a common ancestor following the WGD event (termed "post-WGD yeast" herein) and species that diverged from the yeast lineage prior to the WGD event (termed "pre-WGD yeast" herein).

[00159] Accordingly, in one embodiment, the yeast microorganism may be selected from a post-WGD yeast genus, including but not limited to Saccharomyces and Candida. The favored post-WGD yeast species include: *S. cerevisiae, S. uvarum, S. bayanus, S. paradoxus, S. castelli*, and *C. glabrata*.

[00160] In another embodiment, the yeast microorganism may be selected from a pre-whole genome duplication (pre-WGD) yeast genus including but not limited to Saccharomyces, Kluyveromyces, Candida, Pichia, Issatchenkia, Debaryomyces, Hansenula, Yarrowia and, Schizosaccharomyces. Representative pre-WGD yeast species include: *S. kluyveri, K. thermotolerans, K. marxianus, K. waltii, K. lactis, C. tropicalis, P. pastoris, P. anomala, P. stipitis, I. orientalis, I. occidentalis, I. scutulata, D. hansenii, H. anomala, Y. lipolytica*, and *S. pombe*.

[00161] A yeast microorganism may be either Crabtree-negative or Crabtree-positive as described in described in commonly owned U.S. Pat. No. 8,017,375. In one embodiment the yeast microorganism may be selected from yeast with a Crabtree-negative phenotype including but not limited to the following genera: *Saccharomyces, Kluyveromyces, Pichia, Issatchenkia, Hansenula*, and *Candida*. Crabtree-negative species include but are not limited to: *S. kluyveri, K. lactis, K. marxianus, P. anomala, P. stipitis, I. orientalis, I. occidentalis, I. scutulata, H. anomala*, and *C. utilis*. In another embodiment, the yeast microorganism may be selected from yeast with a Crabtree-positive phenotype, including but not limited to Saccharomyces, Kluyveromyces, Zygosaccharomyces, Debaryomyces, Pichia and Schizosaccharomyces. Crabtree-positive yeast species include but are not limited to: *S. cerevisiae, S. uvarum, S. bayanus, S. paradoxus, S. castelli, K.*

thermotolerans, C. glabrata, Z. bailli, Z. rouxii, D. hansenii, P. pastorius, and *S. pombe.*

[00162] Another characteristic may include the property that the microorganism is that it is non-fermenting. In other words, it cannot metabolize a carbon source anaerobically while the yeast is able to metabolize a carbon source in the presence of oxygen. Nonfermenting yeast refers to both naturally occurring yeasts as well as genetically modified yeast. During anaerobic fermentation with fermentative yeast, the main pathway to oxidize the NADH from glycolysis is through the production of ethanol. Ethanol is produced by alcohol dehydrogenase (ADH) via the reduction of acetaldehyde, which is generated from pyruvate by pyruvate decarboxylase (PDC). In one embodiment, a fermentative yeast can be engineered to be non-fermentative by the reduction or elimination of the native PDC activity. Thus, most of the pyruvate produced by glycolysis is not consumed by PDC and is available for the isobutanol pathway. Deletion of this pathway increases the pyruvate and the reducing equivalents available for the biosynthetic pathway. Fermentative pathways contribute to low yield and low productivity of pyruvate-derived metabolites such as isobutanol. Accordingly, deletion of one or more *PDC* genes may increase yield and productivity of isobutanol.

[00163] In some embodiments, the recombinant microorganisms may be microorganisms that are non-fermenting yeast microorganisms, including, but not limited to those, classified into a genera selected from the group consisting of *Tricosporon*, *Rhodotorula*, *Myxozyma*, or *Candida*. In a specific embodiment, the non-fermenting yeast is *C. xestobii*.

Methods in General

Identification of Enzyme Homologs

[00164] Any method can be used to identify genes that encode for enzymes that are homologous to the genes described herein (*e.g.*, Nde1 and/or Nde2 homologs). Generally, genes that are homologous or similar to the enzymes described herein may be identified by functional, structural, and/or genetic analysis. In most cases, homologous or similar genes and/or homologous or similar enzymes will have functional, structural, or genetic similarities.

[00165] Techniques known to those skilled in the art may be suitable to identify additional homologous genes and homologous enzymes. Generally, analogous genes and/or analogous enzymes can be identified by functional analysis and will have functional similarities. Techniques known to those skilled in the art may be suitable to identify analogous genes and analogous enzymes. For example, to identify homologous or analogous genes, proteins, or enzymes, techniques may include, but not limited to, cloning a gene by PCR using primers based on a published sequence of a gene/enzyme or by degenerate PCR using degenerate primers designed to amplify a conserved region among related protein-encoding genes. Further, one skilled in the art can use techniques to identify homologous or analogous genes, proteins, or enzymes with functional homology or similarity. Techniques include examining a cell or cell culture for the catalytic activity of an enzyme through *in vitro* enzyme assays for said activity (*e.g.*, as described herein or in Kiritani, K. *Branched-Chain Amino Acid*s Methods Enzymology, 1970), then isolating the enzyme with said activity through purification, determining the protein sequence of the enzyme through techniques such as Edman degradation, design of PCR primers to the likely nucleic acid sequence, amplification of said DNA sequence through PCR, and cloning of said nucleic acid sequence. To identify homologous or similar genes and/or homologous or similar enzymes, analogous genes and/or analogous enzymes or proteins, techniques also include comparison of data concerning a candidate gene or enzyme with databases such as BRENDA, KEGG, or MetaCYC. The candidate gene or enzyme may be identified within the above mentioned databases in accordance with the teachings herein.

Identification of Endogenous Polypeptides

[00166] In various embodiments, the endogenous nucleic acid or polypeptide identified herein is the *S. cerevisiae* version of the nucleic acid or polypeptide (*e.g.*, *NDE1, NDE2, PDC1, PDC2, PDC5, PDC6, GPD1, GPD2, YMR226c, ALD6, etc.*). Any method can be used to identify genes that encode for the endogenous polypeptide of interest in a variety of yeast strains. Generally, genes that are homologous or similar to the endogenous polypeptide of interest can be identified by functional, structural, and/or genetic analysis. Homologous or similar polypeptides will generally have functional, structural, or genetic similarities.

[00167] The chromosomal location of the genes encoding endogenous *S. cerevisiae* polypeptides (*e.g.*, Nde1, Nde2, Pdc1, Pdc2, Pdc5, Pdc6, Gpd1, Gpd2, Ymr226c, Ald6, etc.) may be syntenic to chromosomes in many related yeast [Byrne, K.P. and K. H. Wolfe (2005) "The Yeast Gene Order Browser: combining curated homology and syntenic context reveals gene fate in polyploid species." Genome Res. **15**(10):1456-61. Scannell, D. R., K. P. Byrne, J. L. Gordon, S. Wong, and K. H. Wolfe (2006) "Multiple rounds of speciation associated with reciprocal gene loss in polyploidy yeasts." *Nature* 440: 341-5. Scannell, D. R., A. C. Frank, G. C. Conant, K. P. Byrne, M. Woolfit, and K. H. Wolfe (2007)" Independent sorting-out of thousands of duplicated gene pairs in two yeast species descended from a whole-genome duplication." *Proc Natl Acad Sci U S A* 104: 8397-402]. Using this syntenic relationship, species-specific versions of these genes are readily identified in a variety of yeast, including but not limited to, *Ashbya gossypii*, *Candida glabrata*, *Kluyveromyces lactis*, *Kluyveromyces polyspora*, *Kluyveromyces thermotolerans*, *Kluyveromyces waltii*, *Saccharomyces kluyveri*, *Saccharomyces castelli*, *Saccharomyces bayanus*, and *Zygosaccharomyces rouxii*.

[00168] As will be understood by one skilled in the art, this technique is therefore additionally suitable for the identification homologous Nde1, Nde2, Pdc1, Pdc2, Pdc5, Pdc6, Gpd1, Gpd2, Ymr226c, and Ald6 polypeptides in yeast other than *S. cerevisiae*.

Genetic Insertions and Deletions

[00169] Any method can be used to introduce a nucleic acid molecule into yeast and many such methods are well known. For example, transformation and electroporation are common methods for introducing nucleic acid into yeast cells. *See, e.g.*, Gietz *et al.*, 1992, *Nuc Acids Res.* 27: 69-74; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163-8; and Becker *et al.*, 1991, *Methods in Enzymology* 194: 182-7.

[00170] In an embodiment, the integration of a gene of interest into a DNA fragment or target gene of a yeast microorganism occurs according to the principle of homologous recombination. According to this embodiment, an integration cassette containing a module comprising at least one yeast marker gene and/or the gene to be integrated (internal module) is flanked on either side by DNA fragments homologous to those of the ends of the targeted integration site (recombinogenic sequences). After transforming the yeast with the cassette by appropriate methods,

a homologous recombination between the recombinogenic sequences may result in the internal module replacing the chromosomal region in between the two sites of the genome corresponding to the recombinogenic sequences of the integration cassette. (Orr-Weaver *et al.*, 1981, *PNAS USA* 78: 6354-58).

[00171] In an embodiment, the integration cassette for integration of a gene of interest into a yeast microorganism includes the heterologous gene under the control of an appropriate promoter and terminator together with the selectable marker flanked by recombinogenic sequences for integration of a heterologous gene into the yeast chromosome. In an embodiment, the heterologous gene includes an appropriate native gene desired to increase the copy number of a native gene(s). The selectable marker gene can be any marker gene used in yeast, including but not limited to, *HIS3*, *TRP1*, *LEU2*, *URA3*, *bar*, *ble*, *hph*, and *kan*. The recombinogenic sequences can be chosen at will, depending on the desired integration site suitable for the desired application.

[00172] In another embodiment, integration of a gene into the chromosome of the yeast microorganism may occur via random integration (Kooistra *et al.*, 2004, *Yeast* 21: 781-792).

[00173] Additionally, in an embodiment, certain introduced marker genes are removed from the genome using techniques well known to those skilled in the art. For example, URA3 marker loss can be obtained by plating *URA3* containing cells in FOA (5-fluoro-orotic acid) containing medium and selecting for FOA resistant colonies (Boeke *et al.*, 1984, *Mol. Gen. Genet* 197: 345-47).

[00174] The exogenous nucleic acid molecule contained within a yeast cell of the disclosure can be maintained within that cell in any form. For example, exogenous nucleic acid molecules can be integrated into the genome of the cell or maintained in an episomal state that can stably be passed on ("inherited") to daughter cells. Such extra-chromosomal genetic elements (such as plasmids, mitochondrial genome, *etc.*) can additionally contain selection markers that ensure the presence of such genetic elements in daughter cells. Moreover, the yeast cells can be stably or transiently transformed. In addition, the yeast cells described herein can contain a single copy, or multiple copies of a particular exogenous nucleic acid molecule as described above.

Reduction of Enzymatic Activity

[00175] Yeast microorganisms within the scope of the invention may have reduced enzymatic activity such as reduced NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR activity. The term "reduced" as used herein with respect to a particular polypeptide activity refers to a lower level of polypeptide activity than that measured in a comparable yeast cell of the same species. The term reduced also refers to the elimination of polypeptide activity as compared to a comparable yeast cell of the same species. Thus, yeast cells lacking activity for an endogenous NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR are considered to have reduced activity for NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR since most, if not all, comparable yeast strains have at least some activity for NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR. Such reduced NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR activities can be the result of lower NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR concentration (*e.g.*, via reduced expression), lower specific activity of the NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR, or a combination thereof. Many different methods can be used to make yeast having reduced NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR activity. For example, a yeast cell can be engineered to have a disrupted NDE1-, NDE2-, PDC-, GPD-, ALDH-, or 3-KAR-encoding locus using common mutagenesis or knock-out technology. *See*, *e.g.*, Methods in Yeast Genetics (1997 edition), Adams, Gottschling, Kaiser, and Stems, Cold Spring Harbor Press (1998). In addition, a yeast cell can be engineered to partially or completely remove the coding sequence for a particular NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR gene. Furthermore, the promoter sequence and/or associated regulatory elements can be mutated, disrupted, or deleted to reduce the expression of a NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR gene. Moreover, certain point-mutation(s) can be introduced which results in a NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR protein with reduced activity. Also included within the scope of this invention are yeast strains which when found in nature, are substantially free of one or more NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR activities.

[00176] Alternatively, antisense technology can be used to reduce NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR activity. For example, yeasts can be engineered to contain a cDNA that encodes an antisense molecule that prevents a NDE1, NDE2, PDC, GPD, ALDH, or 3-KAR from being made. The term "antisense molecule" as used herein encompasses any nucleic acid molecule that contains sequences that correspond to the coding strand of an endogenous polypeptide. An antisense

molecule also can have flanking sequences (*e.g.*, regulatory sequences). Thus antisense molecules can be ribozymes or antisense oligonucleotides. A ribozyme can have any general structure including, without limitation, hairpin, hammerhead, or axhead structures, provided the molecule cleaves RNA.

Overexpression of Heterologous Genes

[00177] Methods for overexpressing a polypeptide from a native or heterologous nucleic acid molecule are well known. Such methods include, without limitation, constructing a nucleic acid sequence such that a regulatory element promotes the expression of a nucleic acid sequence that encodes the desired polypeptide. Typically, regulatory elements are DNA sequences that regulate the expression of other DNA sequences at the level of transcription. Thus, regulatory elements include, without limitation, promoters, enhancers, and the like. For example, the exogenous genes can be under the control of an inducible promoter or a constitutive promoter. Moreover, methods for expressing a polypeptide from an exogenous nucleic acid molecule in yeast are well known. For example, nucleic acid constructs that are used for the expression of exogenous polypeptides within Kluyveromyces and Saccharomyces are well known (*see*, *e.g.*, U.S. Pat. Nos. 4,859,596 and 4,943,529, for *Kluyveromyces* and, *e.g.*, Gellissen *et al.*, Gene 190(1):87-97 (1997) for *Saccharomyces*). Yeast plasmids have a selectable marker and an origin of replication. In addition certain plasmids may also contain a centromeric sequence. These centromeric plasmids are generally a single or low copy plasmid. Plasmids without a centromeric sequence and utilizing either a 2 micron (*S. cerevisiae*) or 1.6 micron (*K. lactis*) replication origin are high copy plasmids. The selectable marker can be either prototrophic, such as *HIS3*, *TRP1*, *LEU2*, *URA3* or *ADE2*, or antibiotic resistance, such as, *bar*, *ble*, *hph*, or *kan*.

[00178] In another embodiment, heterologous control elements can be used to activate or repress expression of endogenous genes. Additionally, when expression is to be repressed or eliminated, the gene for the relevant enzyme, protein or RNA can be eliminated by known deletion techniques.

[00179] As described herein, any yeast within the scope of the disclosure can be identified by selection techniques specific to the particular polypeptide (*e.g.* an isobutanol pathway enzyme) being expressed, over-expressed or repressed. Methods of identifying the strains with the desired phenotype are well known to those

skilled in the art. Such methods include, without limitation, PCR, RT-PCR, and nucleic acid hybridization techniques such as Northern and Southern analysis, altered growth capabilities on a particular substrate or in the presence of a particular substrate, a chemical compound, a selection agent and the like. In some cases, immunohistochemistry and biochemical techniques can be used to determine if a cell contains a particular nucleic acid by detecting the expression of the encoded polypeptide. For example, an antibody having specificity for an encoded enzyme can be used to determine whether or not a particular yeast cell contains that encoded enzyme. Further, biochemical techniques can be used to determine if a cell contains a particular nucleic acid molecule encoding an enzymatic polypeptide by detecting a product produced as a result of the expression of the enzymatic polypeptide. For example, transforming a cell with a vector encoding acetolactate synthase and detecting increased acetolactate concentrations compared to a cell without the vector indicates that the vector is both present and that the gene product is active. Methods for detecting specific enzymatic activities or the presence of particular products are well known to those skilled in the art. For example, the presence of acetolactate can be determined as described by Hugenholtz and Starrenburg, 1992, *Appl. Micro. Biot.* 38:17-22.

Increase of Enzymatic Activity

[00180] Yeast microorganisms of the invention may be further engineered to have increased activity of enzymes (*e.g.*, increased activity of enzymes involved in an isobutanol producing metabolic pathway). The term “increased” as used herein with respect to a particular enzymatic activity refers to a higher level of enzymatic activity than that measured in a comparable yeast cell of the same species. For example, overexpression of a specific enzyme can lead to an increased level of activity in the cells for that enzyme. Increased activities for enzymes involved in glycolysis or the isobutanol pathway would result in increased productivity and yield of isobutanol.

[00181] Methods to increase enzymatic activity are known to those skilled in the art. Such techniques may include increasing the expression of the enzyme by increased copy number and/or use of a strong promoter, introduction of mutations to relieve negative regulation of the enzyme, introduction of specific mutations to increase specific activity and/or decrease the K_M for the substrate, or by directed

evolution. See, *e.g.*, Methods in Molecular Biology (vol. 231), ed. Arnold and Georgiou, Humana Press (2003).

Methods of Using Recombinant Yeast Microorganisms for Production of Pyruvate-Derived Metabolites

[00182] For a biocatalyst to produce a beneficial metabolite most economically, it is desirable to produce said metabolite at a high yield. Preferably, the only product produced is the desired metabolite, as extra products (*i.e.* by-products) lead to a reduction in the yield of the desired metabolite and an increase in capital and operating costs, particularly if the extra products have little or no value. These extra products also require additional capital and operating costs to separate these products from the desired metabolite.

[00183] In one aspect, the present invention provides a method of producing a pyruvate-derived metabolite from a recombinant microorganism described herein. In one embodiment, the recombinant microorganism comprises a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2*.

[00184] In a further exemplary embodiment, the pyruvate-derived metabolite is isobutanol. Thus, in a related aspect, the present invention provides a method of producing isobutanol from a recombinant yeast microorganism described herein. In one embodiment, the recombinant yeast microorganism comprises an isobutanol producing metabolic pathway, wherein said isobutanol producing metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the conversion of pyruvate to isobutanol, wherein said recombinant yeast microorganism is engineered to comprise reduced PDC activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2*.

[00185] In a method to produce a pyruvate-derived metabolite (*e.g.*, isobutanol) from a carbon source, the recombinant yeast microorganism is cultured in an appropriate culture medium containing a carbon source. In certain embodiments, the method further includes isolating the pyruvate-derived metabolite (*e.g.*, isobutanol) from the culture medium. For example, a pyruvate-derived metabolite (*e.g.*, isobutanol) may be isolated from the culture medium by any method known to those skilled in the art, such as distillation, pervaporation, or liquid-liquid extraction.

[00186] In one embodiment, the recombinant microorganism may produce the pyruvate-derived metabolite (*e.g.*, isobutanol) from a carbon source at a yield of at least 5 percent theoretical. In another embodiment, the microorganism may produce the pyruvate-derived metabolite (*e.g.*, isobutanol) from a carbon source at a yield of at least about 10 percent, at least about 15 percent, about least about 20 percent, at least about 25 percent, at least about 30 percent, at least about 35 percent, at least about 40 percent, at least about 45 percent, at least about 50 percent, at least about 55 percent, at least about 60 percent, at least about 65 percent, at least about 70 percent, at least about 75 percent, at least about 80 percent, at least about 85 percent, at least about 90 percent, at least about 95 percent, or at least about 97.5% theoretical. In a specific embodiment, the pyruvate-derived metabolite is isobutanol.

Distillers Dried Grains Comprising Spent Yeast Biocatalysts

[00187] In an economic fermentation process, as many of the products of the fermentation as possible, including the co-products that contain biocatalyst cell material, should have value. Insoluble material produced during fermentations using grain feedstocks, like corn, is frequently sold as protein and vitamin rich animal feed called distillers dried grains (DDG). *See, e.g.*, commonly owned and co-pending U.S. Publication No. 2009/0215137, which is herein incorporated by reference in its entirety for all purposes. As used herein, the term “DDG” generally refers to the solids remaining after a fermentation, usually consisting of unconsumed feedstock solids, remaining nutrients, protein, fiber, and oil, as well as spent yeast biocatalysts or cell debris therefrom that are recovered by further processing from the fermentation, usually by a solids separation step such as centrifugation.

[00188] Distillers dried grains may also include soluble residual material from the fermentation, or syrup, and are then referred to as "distillers dried grains and solubles" (DDGS). Use of DDG or DDGS as animal feed is an economical use of the

spent biocatalyst following an industrial scale fermentation process.

[00189] Accordingly, in one aspect, the present invention provides an animal feed product comprised of DDG derived from a fermentation process for the production of a beneficial pyruvate-derived metabolite (*e.g.*, isobutanol), wherein said DDG comprise a spent yeast biocatalyst of the present invention. In an exemplary embodiment, said spent yeast biocatalyst has been engineered to comprise reduced PDC activity. In another embodiment, said spent yeast biocatalyst has additionally been engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2*.

[00190] In certain additional embodiments, the DDG comprising a spent yeast biocatalyst of the present invention comprise at least one additional product selected from the group consisting of unconsumed feedstock solids, nutrients, proteins, fibers, and oils.

[00191] In another aspect, the present invention provides a method for producing DDG derived from a fermentation process using a yeast biocatalyst (*e.g.*, a recombinant yeast microorganism of the present invention), said method comprising: (a) cultivating said yeast biocatalyst in a fermentation medium comprising at least one carbon source; (b) harvesting insoluble material derived from the fermentation process, said insoluble material comprising said yeast biocatalyst; and (c) drying said insoluble material comprising said yeast biocatalyst to produce the DDG.

[00192] In certain additional embodiments, the method further comprises step (d) of adding soluble residual material from the fermentation process to said DDG to produce DDGS. In some embodiments, said DDGS comprise at least one additional product selected from the group consisting of unconsumed feedstock solids, nutrients, proteins, fibers, and oils.

[00193] This invention is further illustrated by the following examples that should not be construed as limiting. The contents of all references, patents, and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference for all purposes.

EXAMPLES

Example 1: Isolation of Mutants with Improved Growth Under Anaerobic Conditions

[00194] The following example illustrates how the isolation of spontaneous mutants of Pdc-deficient yeast capable of growth under anaerobic conditions were

obtained.

[00195] Strains described in the examples are listed in Table 2.

Table 2. Genotype of Strains Disclosed in Examples.

Strain	Genotype
GEVO9125	MATa ura3Δ **adh6Δ**::P_{PGK1}:Bs_alsS1_coSc:T_{CYC1}:loxP:P_{TEF1}:hph:T_{Kl_URA3}:loxP **ald6Δ**::P_{PGK1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Kl_URA3:T_{CYC1}:P_{CCW12}:Ec_ilvC_coSc$^{P2D1\text{-}A1\text{-}his6}$ **gpd2Δ**:: $P_{PDC1(-628)}$: Ll_ilvD_coSc4: P_{TDH3}:Sc_AFT1: T_{CYC1}:loxP: P_{CCW12}:Ec_ilvC_coSc$^{P2D1\text{-}A1\text{-}his6}$ **tma29Δ**::loxP **gpd1Δ**::P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:$P_{PDC1(-750)}$:Ll_kivD_coSc5:T_{GPD1} **pdc1Δ**::P_{CUP1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:T_{Kl_URA3} **pdc6Δ**::P_{TEF1}:Ll_ilvD:P_{TDH3}:Ec_ilvC_coSC$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA **pdc5Δ**::P_{TDH3}:FRED_AFT1-2:T_{PGK1}:loxP:P_{TEF1}:ble:T_{URA3}:loxP **2μ**:[P_{Sc_TEF1}Ll_ilvD_coSc:$T_{Sc_ADH1\ (inverted)}$, $P_{Sc_PDC1\text{-}750}$Ec_NKR$^{P2D1\text{-}A1\text{-}his6}$: P_{Sc_TPI1}**G418R**:P_{Sc_ENO2}Ll_adhARE1:T_{Sc_CYC1}] {evolved for C2i, glucose derepression and ~0.1h-1 growth rate in YNB50D medium} (GEVO7931 + pGV3602/pGV3634 #8)
GEVO9140	MAT a ura3Δ **pdc1Δ**::P_{CUP1}-Bs_alsS_coSc-T_{CYC1}-P_{PGK1}-Ll_kivd2_coEc-T_{Kl_URA3} **pdc6Δ**::T_{Kl_URA3} **pdc5Δ**::P_{PGK1}:Bs_alsS_coSc:T_{CYC1}:loxP **tma29Δ**::T_{CYC1}:loxP:$P_{PDC1(-750)}$:Ll_kivD_coSc5:(T_{TMA29}) **adh6Δ**::P_{ADH1}:FRED_AFT1-2:T_{CYC1} **2μ**:[P_{Sc_TEF1}Ll_ilvD_coSc:T_{Sc_ADH1} (inverted), $P_{Sc_PDC1\text{-}750}$:Ec_NKR$^{P2D1\text{-}A1\text{-}his6}$: P_{Sc_TPI1}**G418R**:P_{Sc_ENO2}:Ll_adhA^{RE1}:T_{Sc_CYC1}]
GEVO9563 & GEVO9567	MAT a ura3Δ **pdc1Δ**::P_{CUP1}-Bs_alsS_coSc-T_{CYC1}-P_{PGK1}-Ll_kivd2_coEc-T_{Kl_URA3} **pdc6Δ**::T_{Kl_URA3} **pdc5Δ**::P_{PGK1}:Bs_alsS_coSc:T_{CYC1}:loxP **tma29Δ**::T_{CYC1}:loxP:$P_{PDC1(-750)}$:Ll_kivD_coSc5:(T_{TMA29}) **adh6Δ**::P_{ADH1}:FRED_AFT1-2:T_{CYC1} **2μ**:[P_{Sc_TEF1}Ll_ilvD_coSc:T_{Sc_ADH1} (inverted), $P_{Sc_PDC1\text{-}750}$:Ec_NKR$^{P2D1\text{-}A1\text{-}his6}$: P_{Sc_TPI1}**G418R**:P_{Sc_ENO2}:Ll_adhA^{RE1}:T_{Sc_CYC1}] Selected by growth on YPD in anaerobic chamber

[00196] In this example, spontaneous mutants were selected from GEVO9140 (Table 2), a strain incapable of growth under anaerobic conditions – GEVO9140 harbors disruptions in *PDC1*, *PDC5*, and *PDC6*, and expresses the *B. subtilis als*S, an engineered NKR from *E. coli* (*Ec_NKR*$^{P2D1\text{-}A1}$, *See, e.g.*, US Patent No. 8,097,440), the *L. lactis ilv*D, the *L. lactis kiv*D, and an engineered ADH from *L. lactis* (*Ll_adhA*^{RE1}, *See, e.g.*, US Patent No. 8,133,715). The isolation of spontaneous mutants of GEVO9140 capable of growing under anaerobic conditions were obtained as follows:

[00197] An overnight culture of GEVO9140 was grown in YPE media (10 g/L Difco Yeast Extract, 20 g/L Difco Peptone, and 2% EtOH), from which a concentrated cell

suspension of GEVO9140 was obtained. 50 µL (4.6 x 10^7 cfu) of the cell suspension was plated onto YPD plates [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose]. The YPD plates were cultivated at 30°C in the anaerobic chamber for 10 days. From these plates, several colonies representing Pdc-deficient yeast capable of growth under anaerobic conditions were chosen for further analysis as described in Example 2.

Example 2: Fermentations With Selected Mutants of GEVO9140

[00198] The following example illustrates the growth of selected mutants of GEVO9140.

[00199] In this example, the growth of two mutants derived from GEVO9140 – GEVO9563 and GEVO9567 – was compared to the parental strain and a comparator strain, GEVO9125. GEVO9125, like GEVO9140, is a strain which harbors disruptions in *PDC1, PDC5,* and *PDC6*, and expresses similar isobutanol pathway genes. GEVO9125 is incapable of growth under anaerobic conditions.

[00200] In this experiment, cultures of GEVO9125, GEVO9140, GEVO9563, and GEVO9567 were incubated in liquid medium comprising 80 g/L glucose at 33°C and 250 rpm for 42-48 hrs. At this point, they were sampled for GC analysis and transitioned to 33°C and 75 rpm.

[00201] **Figure 4** illustrates the growth (as measured at OD_{600}) of the anaerobic strains GEVO9563 and GEVO9567 over the course of 30 hours under aerobic conditions as compared to GEVO9125 and GEVO9140. The anaerobic strains GEVO9563 and GEVO9567 grew faster on glucose than the parent GEVO9140 and as well or better than GEVO9125 in this experiment.

[00202] **Figure 5** illustrates the isobutanol titers [g/L] of the anaerobic strains GEVO9563 and GEVO9567 over the course of the 30 hour production phase under aerobic conditions as compared to GEVO9125 and GEVO9140. GEVO9563 and GEVO9567 produced approximately 4 g/L isobutanol during growth phase, while the parent, GEVO9140, and the comparator control strain GEVO9125 produced approximately 0.3 g/L isobutanol at transition. Importantly, GEVO9563 and GEVO9567 produced approximately 9 g/L isobutanol during the 30 hour production phase, while the parent, GEVO9140, and the comparator control strain GEVO9125 produced 3.5 g/L and 4.8 g/L isobutanol, respectively.

[00203] **Figure 6** illustrates the specific isobutanol titers [g/L/OD] of the anaerobic

strains GEVO9563 and GEVO9567 throughout the course of the 30 hour production phase under aerobic conditions as compared to GEVO9125 and GEVO9140. GEVO9563 and GEVO9567 exhibited specific isobutanol titers at transition of 1.1 g/L/OD and 0.75 g/L/OD, respectively, while the parent, GEVO9140, and the comparator control strain GEVO9125 exhibited specific isobutanol titers at transition of approximately 0.1 g/L/OD. Further, GEVO9563 and GEVO9567 had specific isobutanol titers at 30 hours of production of 2.0 g/L/OD and 1.5 g/L/OD, respectively, while the parent, GEVO9140, and the comparator control strain GEVO9125 had specific isobutanol titers at transition of 0.7 g/L/OD and 0.8 g/L/OD, respectively.

[00204] **Figure 7** illustrates the estimated specific isobutanol productivity (g/g-h) of the anaerobic strains GEVO9563 and GEVO9567, the parent, GEVO9140, and the comparator control strain GEVO9125 calculated from 6 hours to 21 hours of production in a single shake flask fermentation. The anaerobic isolates GEVO9563 and GEVO9567 had estimated specific isobutanol productivity of 0.075 g/g-h and 0.045 g/g-h, respectively, while GEVO9140 and GEVO9125 had estimated specific isobutanol productivities of 0.047 g/g-h and 0.043 g/g-h, respectively.

Example 3: Genome Sequencing of GEVO9567

[00205] The following example demonstrates the identification of a *NDE1* mutation in GEVO9567.

[00206] To identify relevant mutations that occurred during the evolutionary engineering of GEVO9567, sequencing of GEVO9567 as well as parental strain GEVO9140 was performed. These data were used to identify mutations linked to the improved growth under low aeration in GEVO9567.

[00207] Sequencing revealed that GEVO9567 harbors a C → T mutation at the tenth nucleotide of the Nde1p coding sequence, *i.e.*, at the fourth codon. This mutation results in a change from glutamine to a premature stop codon (*i.e.*, Q4 → STOP), resulting from a CAA to TAA codon alteration.

[00208] While the disruption of the *NDE1* locus is at least responsible, in part, for the anaerobic-tolerant phenotype of GEVO9567, the evolution system also selected for two additional modifications that can contribute to the beneficial properties observed in GEVO9567. Specifically, a T441 to A alteration was observed in *YNL295W*, while a D99 to E substitution was seen in *YJL055W*, a protein involved in

the metabolism of purine and pyrimidine base analogs. *See* Stepchenkova *et al.*, 2005, *BMC Genetics* 6: 31. *See also* Ko *et al.*, 2008, *Yeast* 25(2): 155-60.

[00209] The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood there from as modifications will be obvious to those skilled in the art.

[00210] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

[00211] The disclosures, including the claims, figures and/or drawings, of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entireties.

WHAT IS CLAIMED IS:

1. A recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced pyruvate decarboxylase (PDC) activity, and wherein said recombinant yeast microorganism is engineered to reduce or eliminate the expression or activity of an endogenous polypeptide encoded by a gene selected from *NDE1* and *NDE2.*

2. The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is engineered to comprise a mutation in at least one gene selected from *NDE1* and *NDE2*.

3. The recombinant yeast microorganism of claim 2, wherein said mutation is a point mutation.

4. The recombinant yeast microorganism of claim 3, wherein said point mutation results in a truncation of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*.

5. The recombinant yeast microorganism of claim 4, wherein said truncation occurs within twenty amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2.*

6. The recombinant yeast microorganism of claim 4, wherein said truncation occurs within ten amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*.

7. The recombinant yeast microorganism of claim 4, wherein said truncation occurs within five amino acids of the N-terminus of a polypeptide encoded by a gene selected from *NDE1* and *NDE2*.

8. The recombinant yeast microorganism of claim 4, wherein said truncation occurs at the Q4 position of a polypeptide encoded by *NDE1.*

9. The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is engineered to comprise a partial deletion of at least one gene selected from *NDE1* and *NDE2*.

10. The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is engineered to comprise a complete deletion of at least one gene selected from *NDE1* and *NDE2*.

11. The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is engineered to comprise a disruption in at least one gene selected from *NDE1* and *NDE2*.

12. The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is engineered to comprise a deletion, disruption, or mutation of a regulatory region associated with at least one gene selected from *NDE1* and *NDE2*.

13. The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is engineered to comprise at least one modification of a transcriptional regulator of at least one gene selected from *NDE1* and *NDE2*.

14. A recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced pyruvate decarboxylase (PDC) activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YNL295W*.

15. The recombinant yeast microorganism of claim 14, wherein said modification of *YNL295W* is a mutation, disruption, or deletion of *YNL295W*.

16. The recombinant yeast microorganism of claim 15, wherein said mutation of *YNL295W* is a point mutation of *YNL295W*.

17. The recombinant yeast microorganism of claim 16, wherein said point mutation of *YNL295W* results in an amino acid substitution at an amino acid position which is within 5 Angstroms of T441 of the protein encoded by *YNL295W*.

18. The recombinant yeast microorganism of claim 17, wherein said T441 residue is replaced with an alanine residue.

19. A recombinant yeast microorganism comprising a metabolic pathway for the production of a pyruvate-derived metabolite, wherein said metabolic pathway comprises at least one exogenous gene and/or at least one overexpressed endogenous gene encoding an enzyme that catalyzes a pathway step in the production of the pyruvate-derived metabolite, wherein said recombinant yeast microorganism is engineered to comprise reduced pyruvate decarboxylase (PDC) activity, and wherein said recombinant yeast microorganism is engineered to comprise at least one modification of *YJL055W*.

20. The recombinant yeast microorganism of claim 19, wherein said modification of *YJL055W* is a mutation, disruption, or deletion of *YJL055W*.

21. The recombinant yeast microorganism of claim 20, wherein said mutation of *YJL055W* is a point mutation of *YJL055W*.

22. The recombinant yeast microorganism of claim 21, wherein said point mutation of *YJL055W* results in an amino acid substitution at an amino acid position which is within 5 Angstroms of D99 of the protein encoded by *YJL055W*.

23. The recombinant yeast microorganism of claim 22, wherein said D99 residue is replaced with a glutamic acid residue.

24. The recombinant yeast microorganism according to any of the preceding claims, wherein the reduction in PDC activity results from the deletion, disruption, or

mutation of one or more genes encoding for pyruvate decarboxylase and/or a positive transcriptional regulator thereof.

25. The recombinant yeast microorganism of claim 24, wherein said pyruvate decarboxylase gene targeted for disruption, deletion, or mutation is selected from the group consisting of *PDC1*, *PDC5*, and *PDC6*, or homologs or variants thereof.

26. The recombinant yeast microorganism of claim 25, wherein all three of *PDC1*, *PDC5*, and *PDC6* are targeted for disruption, deletion, or mutation.

27. The recombinant yeast microorganism of claim 24, wherein said positive transcriptional regulator is *PDC2*, or homologs or variants thereof.

28. The recombinant yeast microorganism according to any of the preceding claims, wherein said recombinant yeast microorganism is engineered to reduce glycerol-3-phosphate dehydrogenase (GPD) activity.

29. The recombinant yeast microorganism according to any of the preceding claims, wherein said recombinant yeast microorganism is engineered to reduce 3-keto acid reductase (3-KAR) activity.

30. The recombinant yeast microorganism according to any of the preceding claims, wherein said recombinant yeast microorganism is engineered to reduce aldehyde dehydrogenase (ALDH) activity.

31. The recombinant microorganism of any one of the preceding claims, wherein said pyruvate-derived metabolite is selected from the group consisting of isobutanol, 2-butanol, 1-butanol, 2-butanone, 2,3-butanediol, acetoin, diacetyl, valine, leucine, pantothenic acid, isobutylene, 3-methyl-1-butanol, 4-methyl-1-pentanol, coenzyme A, lactic acid, and malic acid.

32. A method of producing a pyruvate-derived metabolite, comprising:

(a) providing a recombinant yeast microorganism according to any of the preceding claims; and

(b) cultivating the recombinant yeast microorganism in a culture medium containing a feedstock providing a carbon source, until the pyruvate-derived metabolite is produced.

33. The method of claim 32, wherein said recombinant yeast microorganism converts the carbon source to the pyruvate-derived metabolite under anaerobic conditions.

34. The method of claim 32, wherein said recombinant yeast microorganism converts the carbon source to the pyruvate-derived metabolite under microaerobic conditions.

35. The method of any of claims 32-34, wherein said pyruvate-derived metabolite is isobutanol.

FIGURES

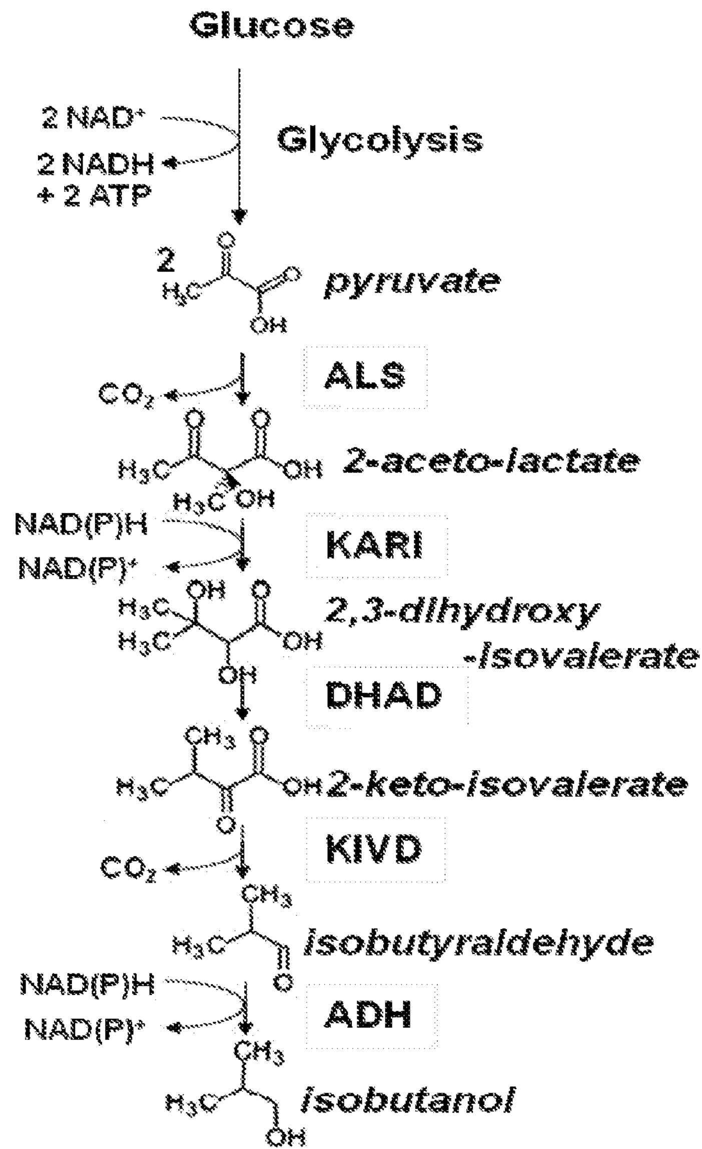

FIGURE 1

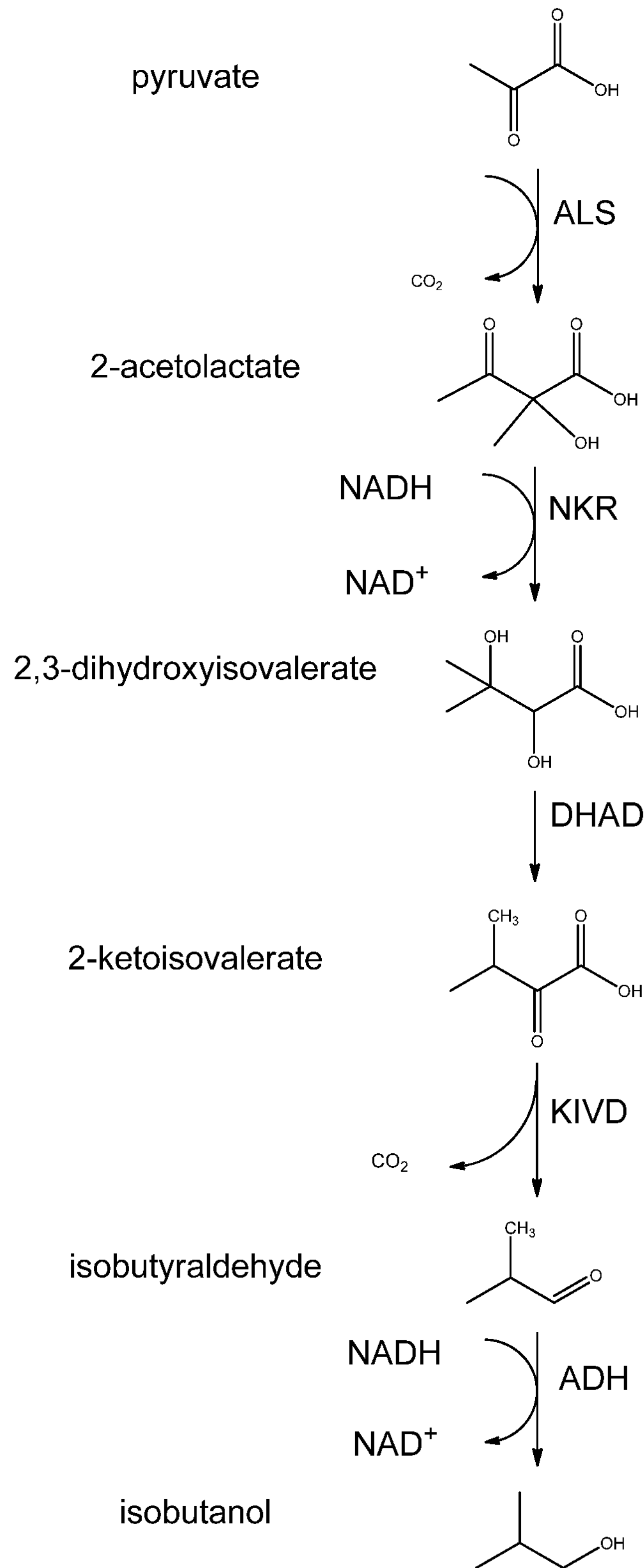

FIGURE 2

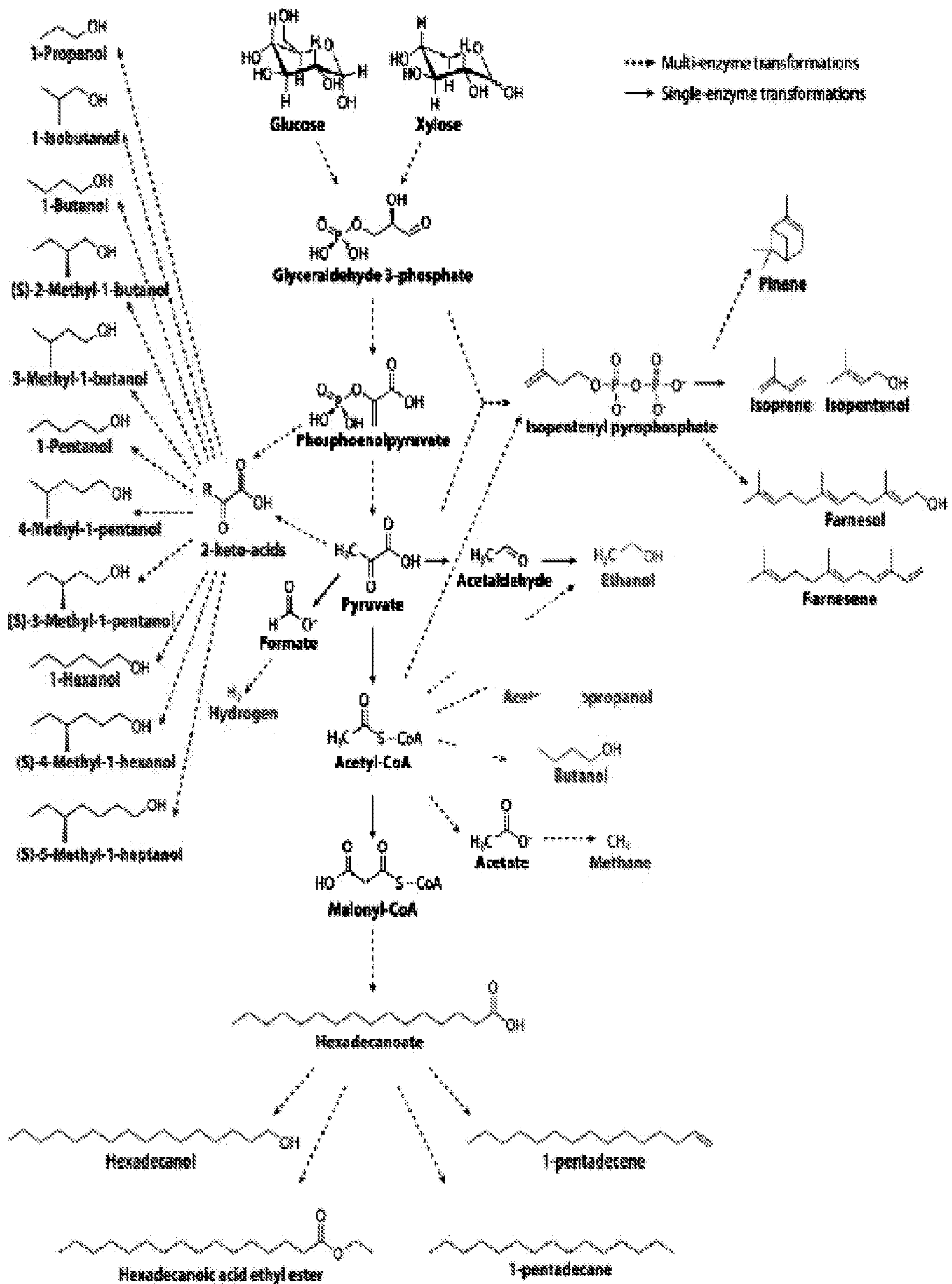

FIGURE 3

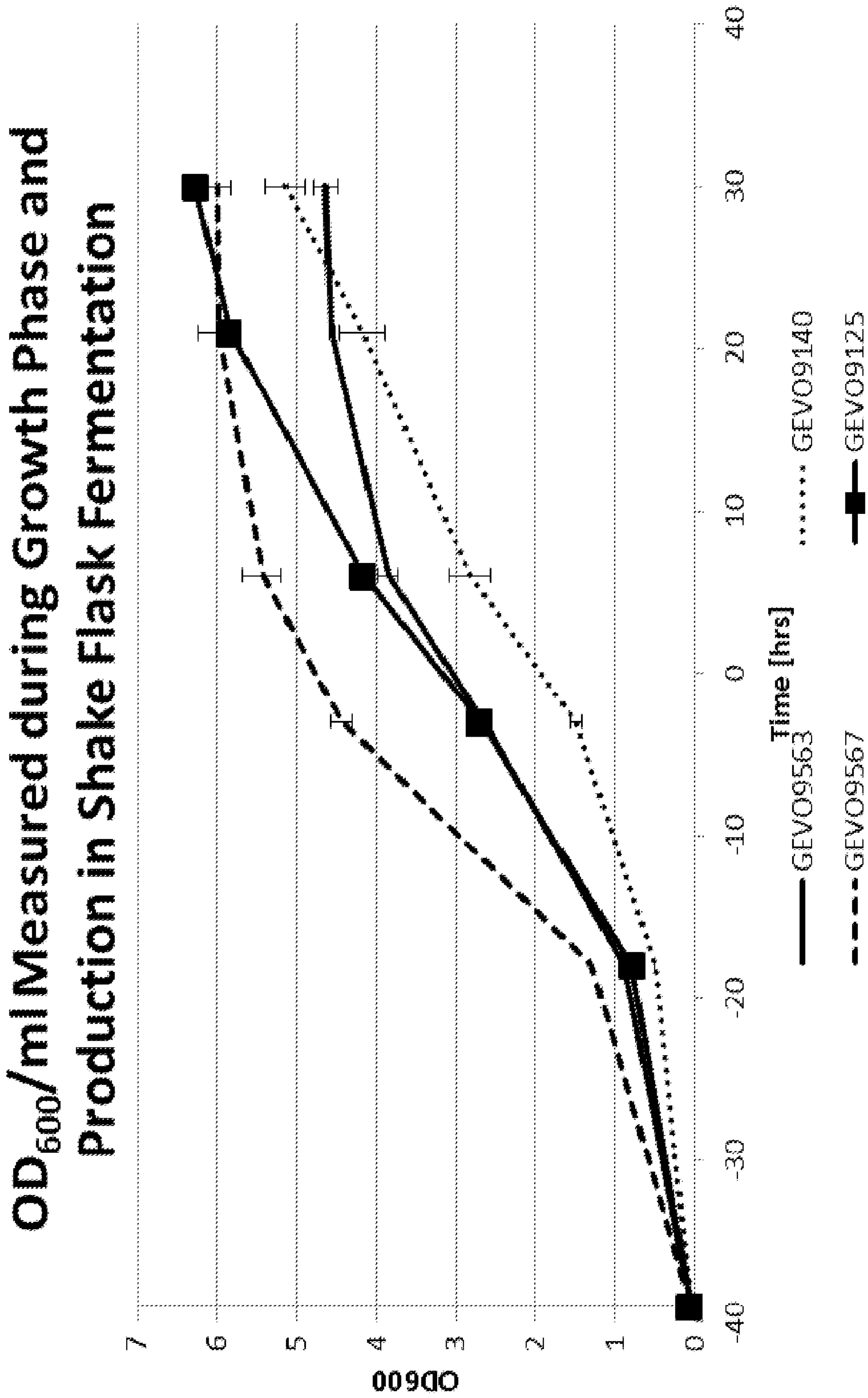

FIGURE 4

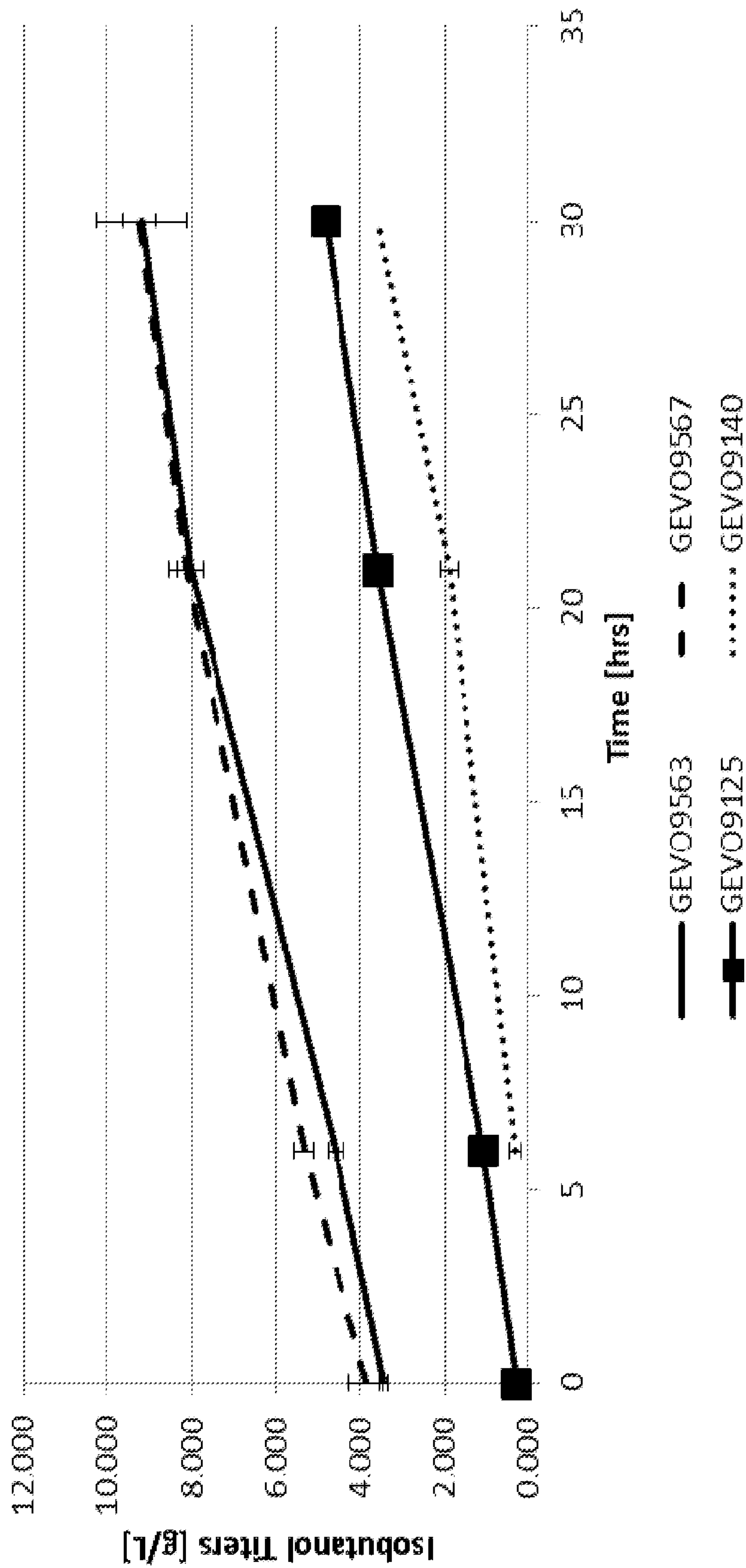

FIGURE 5

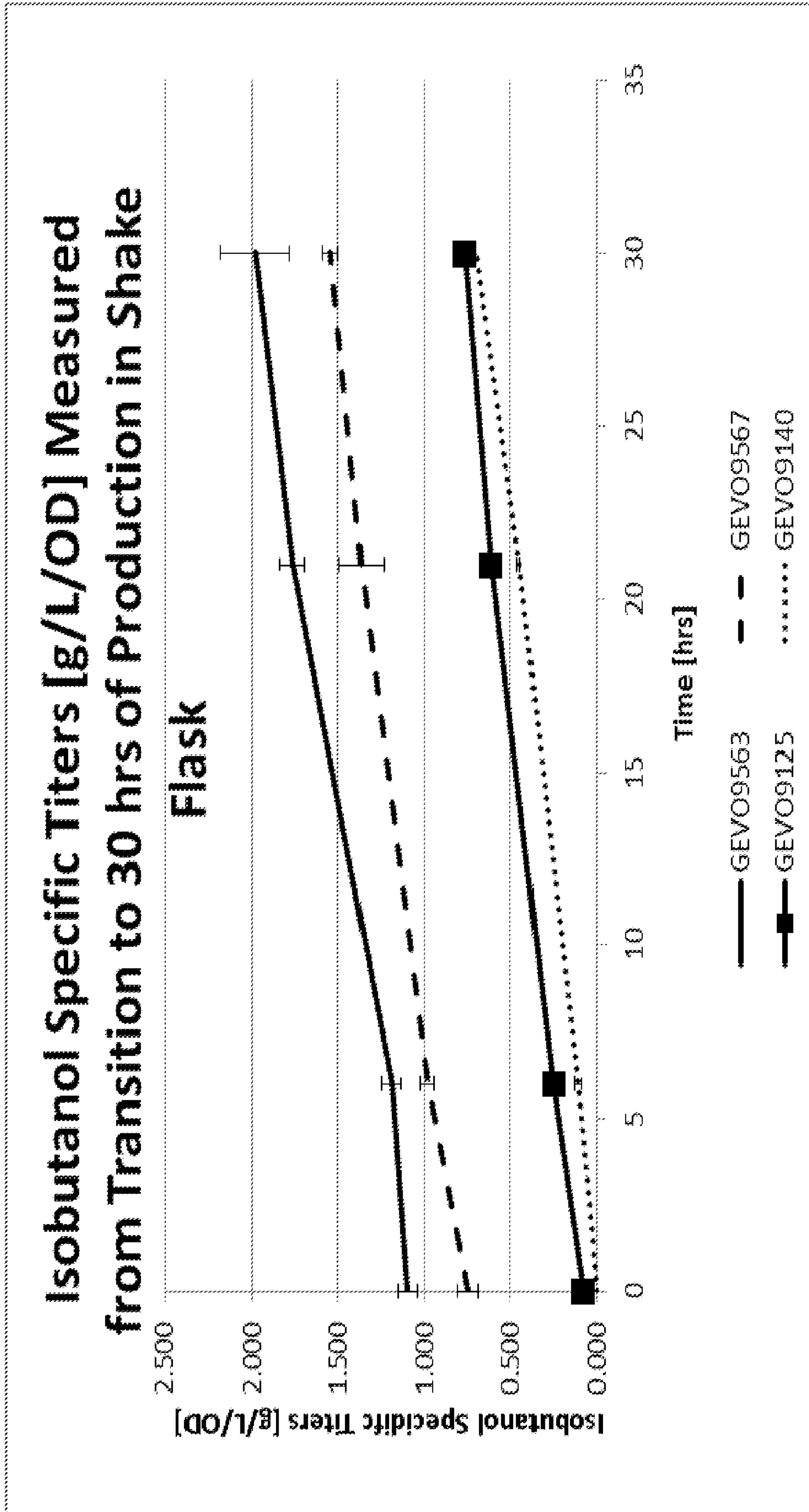

FIGURE 6

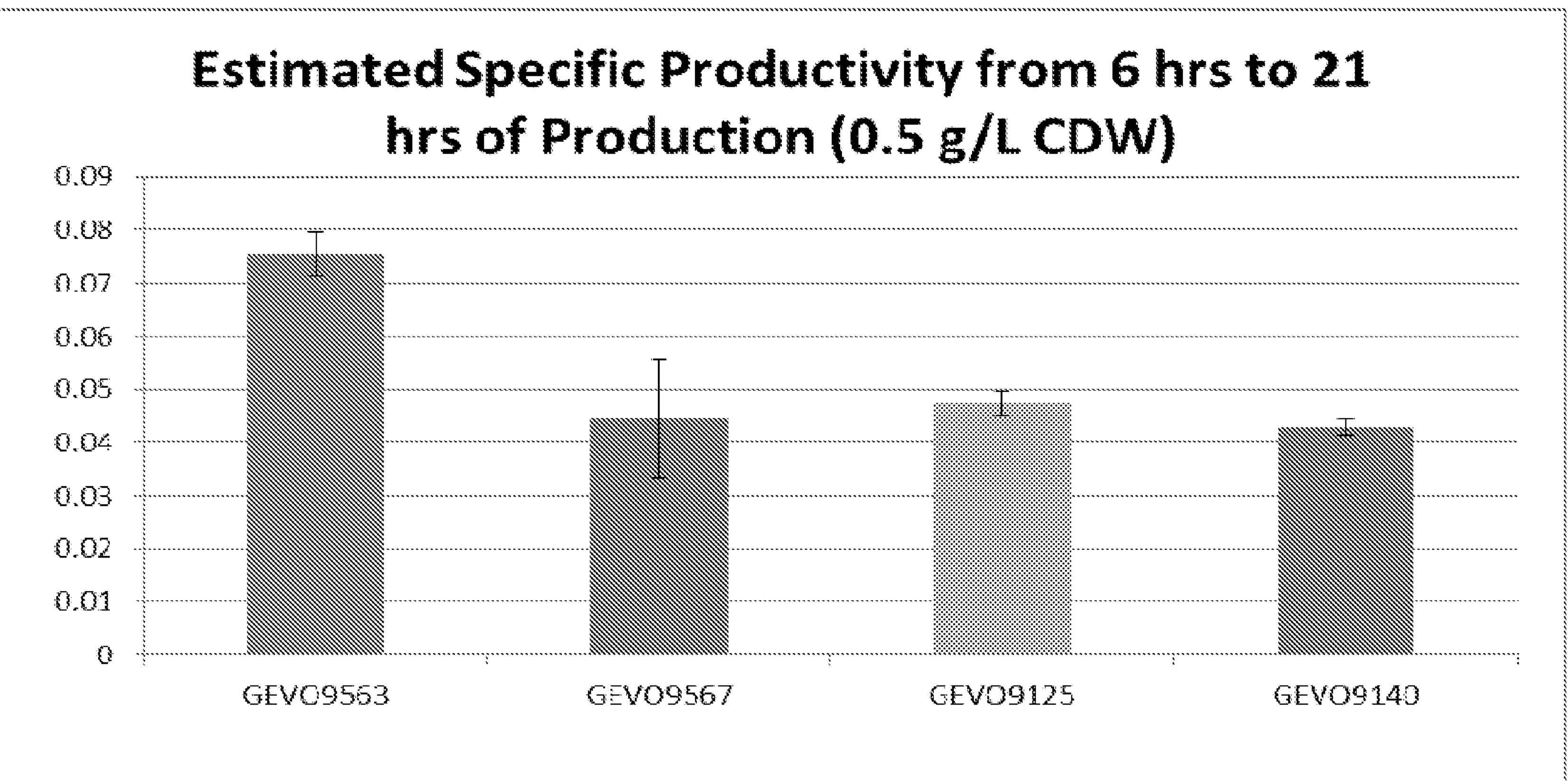

FIGURE 7