(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 September 2013 (26.09.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/142338 A1

(51) International Patent Classification:
C12P 7/16 (2006.01) *C12N 9/02* (2006.01)
C12N 1/18 (2006.01) *C12N 9/88* (2006.01)
C12N 9/04 (2006.01)

(21) International Application Number:
PCT/US2013/032159

(22) International Filing Date:
15 March 2013 (15.03.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/615,174 23 March 2012 (23.03.2012) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: ACETATE SUPPLEMENTION OF MEDIUM FOR BUTANOLOGENS

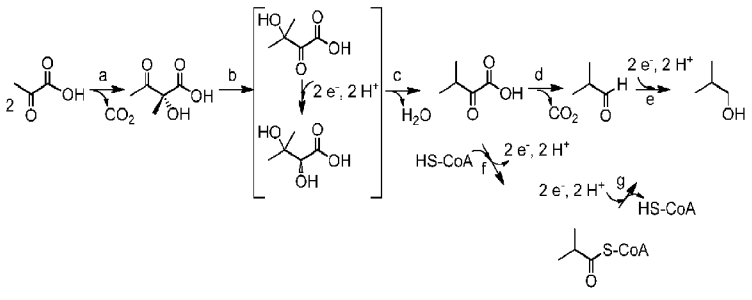

FIG. 1

(57) **Abstract**: The invention relates to the fields of industrial microbiology and alcohol production. More specifically, the invention relates to improved production of butanol isomers by recombinant microorganisms containing an engineered butanol pathway and disrupted activity of the genes in pathways for the production of by-products during the fermentation when the microorganisms are grown in a fermentation medium containing acetate. In embodiments, recombinant microorganisms have an increased growth rate in a fermentation medium containing acetate as a C2 supplement.

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ACETATE SUPPLEMENTION OF MEDIUM FOR BUTANOLOGENS

FIELD OF THE INVENTION

[0001] The invention relates to the fields of industrial microbiology and alcohol production. More specifically, the invention relates to improvements in fermentative production of butanol isomers by recombinant microorganisms containing an engineered butanol pathway when the microorganisms are grown in a fermentation medium containing acetate.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application Serial No. 61/615,174, filed on March 23, 2012, and incorporated herein by reference in its entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTTED ELECTRONICALLY VIA EFS-WEB

[0003] The content of the electronically submitted Sequence Listing (20120315_CL5681USNP_SEQLIST_ST25; SIZE: 294,486 ; DATE OF CREATION: March 14, 2013), filed herewith, is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0004] Butanol is an important industrial chemical with a variety of applications, where its potential as a fuel or fuel additive is particularly significant. Butanol is favored as a fuel or fuel additive because it yields only CO_2 and little or no SO_x or NO_x when burned in the standard internal combustion engine. Although butanol is a four-carbon alcohol, it has an energy content similar to that of gasoline and can be blended with any fossil fuel. Additionally, butanol is less corrosive than ethanol, the most preferred fuel additive to date.

[0005] Butanol also has the potential of impacting hydrogen distribution problems in the fuel cell industry. Fuel cells today are plagued by safety concerns associated with hydrogen transport and distribution. Butanol, however, can be easily reformed for its

hydrogen content and can be distributed through existing gas stations in the purity required for either fuel cells or vehicles. Butanol is also useful as a feedstock chemical in the plastics industry and as a food grade extractant in the food and flavor industry. Each year 10 to 12 billion pounds of butanol are produced by petrochemical means and the need for this commodity chemical will likely increase in the future.

[0006] Methods for the chemical synthesis of isobutanol are known, such as oxo synthesis, catalytic hydrogenation of carbon monoxide (*Ullmann's Encyclopedia of Industrial Chemistry*, 6th edition, Wiley-VCH Verlag GmbH and Co., Weinheim, Germany, Vol. 5, pp. 716-719 (2003)) and Guerbet condensation of methanol with n-propanol (Carlini *et al.*, *J. Molec. Catal. A. Chem.*, *220*:215-220 (2004)). These processes use starting materials derived from petrochemicals, are generally expensive and not environmentally friendly. The production of isobutanol from plant-derived raw materials could minimize the use of fossil fuels.

[0007] Isobutanol is produced biologically in minute quantities as a by-product of yeast fermentation. It is a minor component of "fusel oil" that forms as a result of the incomplete metabolism of amino acids by yeast. Isobutanol is specifically produced from catabolism of L-valine. After the amine group of L-valine is harvested as a nitrogen source, the resulting α-keto acid is decarboxylated and reduced to isobutanol by enzymes of the so-called Ehrlich pathway (Dickinson *et al., J. Biol. Chem. 273*:25752-25756, 1998) ("Dickinson"). Addition of exogenous L-valine to the fermentation medium increases the yield of isobutanol, as described by Dickinson, wherein it is reported that a yield of isobutanol of 3 g/L is obtained by providing L-valine at a concentration of 20 g/L in the fermentation broth. However, the use of valine as a feedstock would be cost prohibitive for industrial scale isobutanol production.

[0008] Microorganisms expressing engineered biosynthetic pathways for producing butanol, including isobutanol, directly from sugars have been described previously in, *e.g.*, U.S. Patent Nos. 7,851,188 and 7,993,889. Such butanologens may further include disruption of certain genes involved in the formation of by-products during fermentation in order to maximize the yield of butanol isomers. The genes involved in the by-product formation include the genes necessary for ethanol formation (*see* U.S. Patent Appl. Pub. No. 20090305363) and isobutyric acid formation. (*see* PCT Patent Appl. Pub. No. WO2012/129555). Microorganisms in which genes necessary for ethanol formation (*e.g.*, PDC gene) are discrupted require an exogeneous C2 supplement for proper growth. This

requirement for a C2 supplement is usually met by adding small amounts of ethanol to the culture medium. For example, U.S. Patent Application Publication No. 20090305363, incorporated herein by reference, describes PDC knockout yeast strains that were unable to grow in a medium containing 2% glucose as carbon source, but were found to grow very well in a medium containing glucose supplemented with a small amount of ethanol.

[0009] Under some circumstances, butanologens with disruptions in genes necessary for isobutyric acid production (e.g., ALD6) in addition to PDC gene disruptions may have altered ability to grow and produce butanol, even when ethanol is used as a C2 supplement. Although approaches to such challenges have been described in the art, for example by engineering the strain for reduced C2 dependence (see, for example, US App. Pub. No. 20120156735), alternative or supplemental methods to replace or supplement such strategies would represent an advance in the art.

SUMMARY OF THE INVENTION

[0010] Provided herein are methods for producing butanol comprising:

a. providing a recombinant host cell comprising:
    i. an engineered butanol biosynthetic pathway; and
b. contacting the host cell of a) with a fermentation medium comprising:
    i. a fermentable carbon substrate; and
    ii. acetate;

wherein said recombinant host cell has been engineered to reduce or eliminate pyruvate decarboxylase (PDC) activity and, optionally, aldehyde dehydrogenase activity; and whereby butanol is produced directly from the fermentable carbon substrate via the engineered butanol biosynthetic pathway. In embodiments, the acetate is added to the fermentation medium. In embodiments, the acetate is present in an amount sufficient for growth of the host cell. In embodiments, the acetate is present in an amount sufficient for improved butanol production. In embodiments, the acetate is added to the fermentation medium. In embodiments, the acetate is from a renewable feedstock source.

[0011] One embodiment of the invention is directed to a method for producing butanol comprising:

a. providing a recombinant host cell comprising:
    i. an engineered butanol biosynthetic pathway; and

b. contacting the host cell of a) with a fermentation medium comprising:

i. a fermentable carbon substrate; and

ii. acetate as C2 supplement in an amount sufficient for growth of the host cell of a) and for butanol production, wherein the acetate is added to the fermentation medium;

wherein said recombinant host cell has been engineered to reduce or eliminate pyruvate decarboxylase (PDC) activity and aldehyde dehydrogenase activity; and whereby butanol is produced directly from the fermentable carbon substrate via the engineered butanol biosynthetic pathway.

[0012] One embodiment of the invention is directed to a method for producing isobutanol comprising the following substrate to product conversions:

a) pyruvate to acetolactate (pathway step a);

b) the acetolactate from a) to 2,3-dihydroxyisovalerate (pathway step b);

c) the 2,3-dihydroxyisovalerate from b) to α-ketoisovalerate (pathway step c);

d) the α-ketoisovalerate from c) to isobutyraldehyde (pathway step d); and

e) the isobutyraldehyde from d) to isobutanol (pathway step e);

and wherein

i) the substrate to product conversion of step a) is performed by an acetolactate synthase enzyme;

ii) the substrate to product conversion of step b) is performed by an acetohydroxy acid isomeroreductase enzyme;

iii) the substrate to product conversion of step c) is performed by a dihydroxyacid dehydratase dehydratase enzyme;

iv) the substrate to product conversion of step d) is performed by an α-ketoacid decarboxylase enzyme; and

v) the substrate to product conversion of step e) is performed by an alcohol dehydrogenase enzyme;

whereby isobutanol is produced directly from pyruvate via the engineered biosynthetic pathway.

[0013] One embodiment of the invention is directed to a method for producing isobutanol comprising the following substrate to product conversions:

a) pyruvate to acetolactate (pathway step a);

b) the acetolactate from a) to 2,3-dihydroxyisovalerate (pathway step b);

c) the 2,3-dihydroxyisovalerate from b) to α-ketoisovalerate (pathway step c);
d) the α-ketoisovalerate from c) to isobutyryl-CoA (pathway step f); and
e) the isobutyryl-CoA from d) to isobutyraldehyde (pathway step g);
f) the isobutyraldehyde from e) to isobutanol (pathway step e);
and wherein
i) the substrate to product conversion of step a) is performed by an acetolactate synthase enzyme;
ii) the substrate to product conversion of step b) is performed by an acetohydroxy acid isomeroreductase enzyme;
iii) the substrate to product conversion of step c) is performed by a dihydroxyacid dehydratase dehydratase enzyme;
iv) the substrate to product conversion of step d) is performed by a branched-chain keto acid dehydrogenase enzyme;
v) the substrate to product conversion of step e) is performed by an acelylating aldehyde dehydrogenase enzyme; and
vi) the substrate to product conversion of step f) is performed by an alcohol dehydrogenase enzyme;

whereby isobutanol is produced directly from pyruvate via the engineered biosynthetic pathway.

[0014] One embodiment of the invention is directed to a composition comprising:

a) a recombinant host cell comprising:
i) an engineered butanol biosynthetic pathway; and
b) a fermentation medium comprising:
i) a fermentable carbon substrate; and
ii) acetate in an amount sufficient for growth of the host cell of a) and for butanol production;

wherein said recombinant host cell has been engineered to reduce or eliminate pyruvate decarboxylase (PDC) activity and aldehyde dehydrogenase activity.

In embodiments, the acetate is added to the fermentation medium. In embodiments, the acetate is from a renewable feedstock source. In embodiments, both the acetate and carbon substrate are from a renewable feedstock source.

[0015] One embodiment of the invention is directed to a composition, wherein the fermentation medium further comprises butanol.

[0016] One embodiment of the invention is directed to a method for producing butanol comprising maintaining the composition above under conditions whereby butanol is produced directly from the fermentable carbon substrate via the engineered butanol biosynthetic pathway.

[0017] One embodiment of the invention is directed to butanol produced from the method disclosed herein.

[0018] In some embodiments, the polypeptide having pyruvate decarboxylase activity is PDC1, PDC5, PDC6 or combinations thereof. In some embodiments, the polypeptide having aldehyde dehydrogenase activity is ALD2, ALD3, ALD4, ALD5, ALD6 or combinations thereof.

[0019] In some embodiments, the host cell has been engineered or evolved to comprise a reduced or eliminated requirement for exogenous two-carbon substrate supplementation for their growth.

[0020] In some embodiments, the host cell comprises a heterologous polynucleotide encoding a polypeptide with phosphoketolase activity, a heterologous polynucleotide encoding a polypeptide with phosphotransacetylase activity, or both.

[0021] In some embodiments, the recombinant host cell is a bacteria or yeast.

[0022] In some embodiments, the recombinant host cell is a whole cell catalyst subjected to conditions for isobutanol production.

[0023] In some embodiments, the butanol produced is isobutanol. In some embodiments, the butanol produced is 1-butanol.

[0024] In some embodiments, the method further comprises recovering the butanol. In some embodiments, the recovery is by distillation, liquid-liquid extraction, adsorption, decantation, pervaporation or combinations thereof. In some embodiments, the method also comprises removing solids from the fermentation medium. In some embodiments, the removing step occurs before the recovery step. In some embodiments, the removing is by centrifugation, filtration or decantation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Figure 1 depicts different isobutanol biosynthetic pathways. The steps labeled "a", "b", "c", "d", "e", "f" and "g" represent substrate to product conversions described below. Step "a" may be catalyzed, for example, by acetolactate synthase. Step "b" may

be catalyzed, for example, by acetohydroxyacid reductoisomerase. Step "c" may be catalyzed, for example, by dihydroxyacid dehydratase. Step "d" may be catalyzed, for example, by branched-chain keto acid decarboxylase. Step "e" may be catalyzed, for example, by branched chain alcohol dehydrogenase. Step "f" may be catalyzed, for example, by branched chain keto acid dehydrogenase. Step "g" may be catalyzed, for example, by acetylating aldehyde dehydrogenase.

[0026] Figure 2 depicts the 1-butanol biosynthetic pathway. The steps labeled "a", "b", "c", "d", "e", and "f" represent substrate to product conversions described below. Step "a" may be catalyzed, for example, by acetyl-CoA acetyl transferase. Step "b" may be catalyzed, for example, by 3-hydroxybutyryl-CoA dehydrogenase. Step "c" may be catalyzed, for example, by crotonase. Step "d" may be catalyzed, for example, by butyryl-CoA dehydrogenase. Step "e" may be catalyzed, for example, by butyraldehyde dehydrogenase. Step "f" may be catalyzed, for example, by butanol dehydrogenase.

[0027] .

DETAILED DESCRIPTION

[0028] All documents cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued or foreign patents, or any other documents, are each entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited documents.

[0029] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the claims.

[0030] Provided herein are methods for fermentative production of butanol isomers using recombinant microorganisms expressing engineered butanol biosynthetic pathways and having one or more disruptions of genes involved in by-product formation during the fermentation process, wherein acetate is added to the fermentation medium.

[0031] As disclosed herein, applicants have discovered that butanologens having PDC gene deletions (and optionally ALD6 gene deletions) have an improved growth rate or butanol isomer production when they are grown in a fermentation medium with acetate as

an exogeneous C2 supplement. Since acetate is less expensive than ethanol, using acetate as a C2 supplement reduces the production cost of the butanol isomers.

[0100] In embodiments, host cells with a PDC-KO phenotype may comprise a reduced or eliminated requirement for exogenous two-carbon substrate supplementation for their growth. For example, host cells with a PDC-KO phenotype may be engineered (including, but not limited to, for example, to comprise heterologous polynucleotides encoding a polypeptide with phosphoketolase activity and/or a heterologous polynucleotide encoding a polypeptide with phosphotransacetylase activity) or evolved to reduce or eliminate the requirement for C2 supplements for growth. While in such embodiments, at least in theory, acetate may not be absolutely required to satisfy a C2 auxotrophy, as demonstrated in the Examples, methods provided herein can provide improvements in butanol production employing such host cells.

[0032] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present application including the definitions will control. Also, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. All publications, patents and other references mentioned herein are incorporated by reference in their entireties for all purposes.

[0033] Although methods and materials similar or equivalent to those disclosed herein can be used in practice or testing of the present invention, suitable methods and materials are disclosed below. The materials, methods and examples are illustrative only and are not intended to be limiting. Other features and advantages of the invention will be apparent from the detailed description and from the claims. In order to further define this invention, the following terms and definitions are herein provided.

[0034] As used herein, the terms "comprises," "comprising," "includes," "including," "has," "having," "contains," "containing" or any other variation thereof, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. For example, a composition, a mixture, a process, a method, an article, or an apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent to such composition, mixture, process, method, article, or apparatus. Further, unless expressly stated to the contrary, "or" refers to an inclusive "or"

and not to an exclusive "or." For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

[0035] As used herein, the term "consists of" or variations such as "consist of" or "consisting of," as used throughout the specification and claims, indicate the inclusion of any recited integer or group of integers, but that no additional integer or group of integers may be added to the specified method, structure, or composition.

[0036] As used herein, the term "consists essentially of" or variations such as "consist essentially of" or "consisting essentially of," as used throughout the specification and claims, indicate the inclusion of any recited integer or group of integers, and the optional inclusion of any recited integer or group of integers that do not materially change the basic or novel properties of the specified method, structure or composition. *See* M.P.E.P. § 2111.03.

[0037] Also, the indefinite articles "a" and "an" preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances, *i.e.*, occurrences of the element or component. Therefore "a" or "an" should be read to include one or at least one, and the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

[0038] The term "invention" or "present invention" as used herein is a non-limiting term and is not intended to refer to any single embodiment of the particular invention but encompasses all possible embodiments as described in the application.

[0039] As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention or the reaction conditions described herein refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source or purity of the ingredients employed to make the compositions or to carry out the methods; and the like. The term "about" also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities. In one embodiment, the term "about" means within 10% of the reported numerical value, and sometimes within 5% of the reported numerical value.

[0040] In some instances, "biomass" as used herein refers to the cell biomass of the fermentation product-producing microorganism, typically provided in units g/L dry cell weight (dcw).

[0041] The term "fermentation product" includes any desired product of interest, including, but not limited to 1-butanol, isobutanol, etc.

[0042] The term "butanol isomer" or "butanol" refers to 1-butanol, isobutanol or mixtures thereof. Isobutanol is also known as 2-methyl-1-propanol.

[0043] The term "butanol biosynthetic pathway" as used herein refers to an enzyme pathway to produce 1-butanol or isobutanol. For example, butanol biosynthetic pathways are disclosed in U.S. Pat. No. 7,993,889, which is incorporated herein by reference.

[0044] The term "isobutanol biosynthetic pathway" refers to the enzymatic pathway to produce isobutanol. From time to time "isobutanol biosynthetic pathway" is used synonymously with "isobutanol production pathway."

[0045] The term "1-butanol biosynthetic pathway" as used herein refers to an enzyme pathway to produce 1-butanol.

[0046] A "recombinant host cell" is defined as a host cell that has been genetically manipulated to express a biosynthetic production pathway, wherein the host cell either produces a biosynthetic product in greater quantities relative to an unmodified host cell or produces a biosynthetic product that is not ordinarily produced by an unmodified host cell.

[0047] The term "engineered" as applied to a butanol biosynthetic pathway refers to the butanol biosynthetic pathway that is manipulated, such that the carbon flux from pyruvate through the engineered butanol biosynthetic pathway is maximized, thereby producing an increased amount of butanol directly from the fermentable carbon substrate. Such engineering includes expression of heterologous polynucleotides or polypeptides, overexpression of endogenous polynucleotides or polypeptides, cytosolic localization of proteins that do not naturally localize to cytosol, increased cofactor availability, decreased activity of competitive pathways, etc.

[0048] The term "fermentable carbon substrate" refers to a carbon source capable of being metabolized by the microorganisms such as those disclosed herein. Suitable fermentable carbon substrates include, but are not limited to, sugars, including monosaccharides, such as glucose, fructose, arabinose or xylose; oligosaccharides such as

lactose or sucrose; polysaccharides, such as starch, cellulose, lignocellulose or hemicellulose; one-carbon substrates, fatty acids; and a combination of these.

[0049] "Fermentation medium" as used herein means a mixture of water, fermentable carbon substrates, dissolved solids, fermentation product and all other constituents of the material held in the fermentation vessel in which the fermentation product is being made by the reaction of fermentable carbon substrates to fermentation products, water and carbon dioxide (CO_2) by the microorganisms present. From time to time, as used herein the term "fermentation broth" and "fermentation mixture" can be used synonymously with "fermentation medium."

[0050] The term "aerobic conditions" as used herein means growth conditions in the presence of oxygen.

[0051] The term "microaerobic conditions" as used herein means growth conditions with low levels of dissolved oxygen. For example, the oxygen level may be less than about 1% of air-saturation.

[0052] The term "anaerobic conditions" as used herein means growth conditions in the absence of oxygen.

[0053] The term "carbon substrate" refers to a carbon source capable of being metabolized by the recombinant host cells disclosed herein. Non-limiting examples of carbon substrates are provided herein and include, but are not limited to, monosaccharides, oligosaccharides, polysaccharides, ethanol, lactate, succinate, glycerol, carbon dioxide, acetate, methanol, glucose, fructose, sucrose, xylose, arabinose, dextrose, and mixtures thereof.

[0054] The term "C2 supplement" refers to a carbon source having two carbon atoms, when added to the fermentation medium, the C2 supplement increases the growth and/or butanol production of butanologens having disruption of the activity of the proteins involved in the formation of the by-products during the fermentation process. Non-limiting examples of C2 supplements include acetate and ethanol.

[0055] The term "butanologen" as used herein refers to a microorganism capable of producing butanol isomers. Such microorganisms are typically recombinant microorganisms comprising an engineered butanol biosynthetic pathway. The term "isobutanologen" as used herein refers to a microorganism capable of producing isobutanol isomers. Such microorganisms are typically recombinant microorganisms comprising an engineered isobutanol biosynthetic pathway.

[0056] The term "PDC knock-out" as used herein refers to a host cell comprising disruptions, deletions, mutations, and/or substitutions in the polynucleotide or gene encoding a polypeptide having PDC activity, or in the endogenous polypeptides having PDC1, PDC5 or PDC6 activity or any combinations thereof, such that PDC activity is eliminated or reduced.

[0057] The term "ALD knock-out" as used herein refers to a host cell comprising disruptions, deletions, mutations, and/or substitutions in the polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity, or in the endogenous polypeptides having ALD2, ALD3, ALD4, ALD5 or ALD6 activity or any combinations thereof, such that ALD activity is eliminated or reduced.

[0058] As used herein, the term "improved butanol production" refers to an improvement in a butanol production parameter including, but not limited to, an increase in at least one of yield, effective rate, effective titer or specific productivity or a decrease in yield of at least one byproduct such as, for example, isobutyric acid. Increases or decreases associated with acetate are determined relative to the appropriate control method in the absence of acetate.

[0059] As used herein, the term "yield" refers to the amount of product per amount of carbon source in g/g. The yield may be exemplified for glucose as the carbon source. It is understood unless otherwise noted that yield is expressed as a percentage of the theoretical yield. In reference to a microorganism or metabolic pathway, "theoretical yield" is defined as the maximum amount of product that can be generated per total 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 isopropanol is 0.33 g/g. As such, a yield of isopropanol from glucose of 0.297 g/g would be expressed as 90% of theoretical or 90% theoretical yield. It is understood that while in the present disclosure the yield is exemplified for glucose as a carbon source, the invention can be applied to other carbon sources and the yield may vary depending on the carbon source used. One skilled in the art can calculate yields on various carbon sources.

[0060] The term "effective titer" as used herein, refers to the total amount of butanol isomer produced by fermentation per liter of fermentation medium. The total amount of butanol isomer includes: (i) the amount of butanol in the fermentation medium; (ii) the

amount of butanol isomer recovered from the organic extractant; and (iii) the amount of butanol isomer recovered from the gas phase, if gas stripping is used.

[0061] The term "effective rate" as used herein, refers to the total amount of butanol isomer produced by fermentation per liter of fermentation medium per hour of fermentation.

[0062] The term "specific productivity" as used herein, refers to the g of butanol isomer produced per g of dry cell weight of cells per unit time.

[0063] The term "growth rate" as used herein, refers to the rate at which the microorganisms grow in the culture medium. The growth rate of the recombinant microorganisms can be monitored, for example, by measuring the optical density at 600 nanometers. The doubling time may be calculated from the logarithmic part of the growth curve and used as a measure of the growth rate.

Polypeptides and Polynucleotides for Use in the Invention

[0064] As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein, " "amino acid chain" or any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" may be used instead of, or interchangeably with any of these terms. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis. The polypeptides used in this invention comprise full-length polypeptides and fragments thereof.

[0065] By an "isolated" polypeptide or a fragment, variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purposes of the invention, as are native or

recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.

[0066] Polypeptides and other enzymes suitable for use in the present invention and fragments thereof are encoded by polynucleotides. The term "polynucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, *e.g.*, messenger RNA (mRNA), virally-derived RNA, or plasmid DNA (pDNA). A polynucleotide may comprise a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond, such as found in peptide nucleic acids (PNA)). The term "nucleic acid" refers to any one or more nucleic acid segments, *e.g.*, DNA or RNA fragments, present in a polynucleotide. Polynucleotides according to the present invention further include such molecules produced synthetically. Polynucleotides of the invention may be native to the host cell or heterologous. In addition, a polynucleotide or a nucleic acid may be or may include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

[0067] In certain embodiments, the polynucleotide or nucleic acid is DNA. In the case of DNA, a polynucleotide comprising a nucleic acid, which encodes a polypeptide normally may include a promoter and/or other transcription or translation control elements operably associated with one or more coding regions. An operable association is when a coding region for a gene product, *e.g.*, a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide. Suitable promoters and other transcription control regions are disclosed herein.

[0068] A polynucleotide sequence can be referred to as "isolated," in which it has been removed from its native environment. For example, a heterologous polynucleotide encoding a polypeptide or polypeptide fragment having enzymatic activity (*e.g.*, the ability to convert a substrate to xylulose) contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. An isolated polynucleotide fragment in the form of a polymer of DNA can be comprised of one or more segments of cDNA, genomic DNA, or synthetic DNA.

[0069] The term "gene" refers to a nucleic acid fragment that is capable of being expressed as a specific protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence.

[0070] As used herein, a "coding region" or "ORF" is a portion of nucleic acid which consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a coding region, if present, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, 5' and 3' non-translated regions, and the like, are not part of a coding region. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence that influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences can include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

[0071] A variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from viral systems (particularly an internal ribosome entry site, or IRES). In other embodiments, a polynucleotide of the present invention is RNA, for example, in the form of messenger RNA (mRNA). RNA of the present invention may be single stranded or double stranded.

[0072] As used herein, the term "transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance.

Host organisms containing the transformed nucleic acid fragments are referred to as "recombinant" or "transformed" organisms.

[0073] The terms "plasmid," "vector," and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

[0074] As used herein, "native" refers to the form of a polynucleotide, gene, or polypeptide as found in nature with its own regulatory sequences, if present.

[0075] The term "endogenous," when used in reference to a polynucleotide, a gene, or a polypeptide refers to a native polynucleotide or gene in its natural location in the genome of an organism, or for a native polypeptide, is transcribed and translated from this location in the genome.

[0076] The term "heterologous" when used in reference to a polynucleotide, a gene, or a polypeptide refers to a polynucleotide, gene, or polypeptide not normally found in the host organism. "Heterologous" also includes a native coding region, or portion thereof, that is reintroduced into the source organism in a form that is different from the corresponding native gene, *e.g.*, not in its natural location in the organism's genome. The heterologous polynucleotide or gene may be introduced into the host organism by, *e.g.*, gene transfer. A heterologous gene may include a native coding region with non-native regulatory regions that is reintroduced into the native host. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

[0077] "Regulatory sequences" refers to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or

translation of the associated coding sequence. Regulatory sequences may include promoters, enhancers, operators, repressors, transcription termination signals, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector binding site and stem-loop structure.

[0078] The term "promoter" refers to a nucleic acid sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleic acid segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". "Inducible promoters," on the other hand, cause a gene to be expressed when the promoter is induced or turned on by a promoter-specific signal or molecule. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity. For example, it will be understood that "FBA1 promoter" can be used to refer to a fragment derived from the promoter region of the FBA1 gene.

[0079] The term "terminator" as used herein refers to DNA sequences located downstream of a coding sequence. This includes polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The 3' region can influence the transcription, RNA processing or stability, or translation of the associated coding sequence. It is recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical terminator activity. For example, it will be understood that "CYC1 terminator" can be used to refer to a fragment derived from the terminator region of the CYC1 gene.

[0080] The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of

effecting the expression of that coding sequence (*i.e.*, that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0081] The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide.

[0082] Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook *et al.* (Sambrook, Fritsch, and Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) (hereinafter "Maniatis"); and by Silhavy *et al.* (Silhavy *et al., Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY, 1984); and by Ausubel, F. M. *et al.,* (Ausubel *et al., Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience, 1987).

Butanol Biosynthetic Pathways

[0083] Carbohydrate utilizing microorganisms employ the Embden-Meyerhof-Parnas (EMP) pathway, the Entner-Doudoroff pathway and the pentose phosphate cycle as the central, metabolic routes to provide energy and cellular precursors for growth and maintenance. These pathways have in common the intermediate glyceraldehyde-3-phosphate and, ultimately, pyruvate is formed directly or in combination with the EMP pathway. Subsequently, pyruvate is transformed to acetyl-coenzyme A (acetyl-CoA) via a variety of means. Acetyl-CoA serves as a key intermediate, for example, in generating fatty acids, amino acids and secondary metabolites. The combined reactions of sugar conversion to pyruvate produce energy (*e.g.*, adenosine-5'-triphosphate, ATP) and reducing equivalents (*e.g.*, reduced nicotinamide adenine dinucleotide, NADH, and reduced nicotinamide adenine dinucleotide phosphate, NADPH). NADH and NADPH must be recycled to their oxidized forms (NAD^+ and $NADP^+$, respectively). In the presence of inorganic electron acceptors (*e.g.*, O_2, NO_3^- and SO_4^{2-}), the reducing equivalents may be used to augment the energy pool; alternatively, a reduced carbon by-product may be formed.

[0084] Engineered biosynthetic pathways for the production of butanol isomers from a fermentable carbon source that may be used in the present invention are, for example,

described in U.S. Pat. Nos. 7,851,188 and 7,993,889, which are incorporated herein by reference. In one embodiment, the engineered butanol biosynthetic pathway is an isobutanol biosynthetic pathway, which comprises the following substrate to product conversions:

a) pyruvate to acetolactate, which may be catalyzed, for example, by acetolactate synthase;

b) acetolactate to 2,3-dihydroxyisovalerate, which may be catalyzed, for example, by acetohydroxy acid isomeroreductase;

c) 2,3-dihydroxyisovalerate to α-ketoisovalerate, which may be catalyzed, for example, by dihydroxyacid dehydratase;

d) α-ketoisovalerate to isobutyraldehyde, which may be catalyzed, for example, by an α-keto acid decarboxylase; and,

e) isobutyraldehyde to isobutanol, which may be catalyzed, for example, by an alcohol dehydrogenase.

[0085] In another embodiment, the isobutanol biosynthetic pathway comprises the following substrate to product conversions:

a) pyruvate to acetolactate, which may be catalyzed, for example, by acetolactate synthase;

b) acetolactate to 2,3-dihydroxyisovalerate, which may be catalyzed, for example, by acetohydroxy acid isomeroreductase;

c) 2,3-dihydroxyisovalerate to α-ketoisovalerate, which may be catalyzed, for example, by dihydroxyacid dehydratase;

d) α-ketoisovalerate to isobutyryl-CoA, which may be catalyzed, for example, by branched-chain keto acid dehydrogenase;

e) isobutyryl-CoA to isobutyraldehyde, which may be catalyzed, for example, by acelylating aldehyde dehydrogenase; and,

f) isobutyraldehyde to isobutanol, which may be catalyzed, for example, by a alcohol dehydrogenase.

[0086] Engineered biosynthetic pathways for the production of 1-butanol that may be used include those described in U.S. Appl. Pub. No. 20080182308, which is incorporated herein by reference. In one embodiment, the 1-butanol biosynthetic pathway comprises the following substrate to product conversions:

a) acetyl-CoA to acetoacetyl-CoA, which may be catalyzed, for example, by acetyl-CoA acetyl transferase;

b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which may be catalyzed, for example, by 3-hydroxybutyryl-CoA dehydrogenase;

c) 3-hydroxybutyryl-CoA to crotonyl-CoA, which may be catalyzed, for example, by crotonase;

d) crotonyl-CoA to butyryl-CoA, which may be catalyzed, for example, by butyryl-CoA dehydrogenase;

e) butyryl-CoA to butyraldehyde, which may be catalyzed, for example, by butyraldehyde dehydrogenase; and,

f) butyraldehyde to 1-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

[0087] In one embodiment, the invention produces butanol from plant derived carbon sources, avoiding the negative environmental impact associated with the standard petrochemical processes for butanol production. In one embodiment, the invention provides a method for the production of butanol using recombinant industrial host cells comprising an engineered butanol biosynthetic pathway.

[0088] In some embodiments, the butanol biosynthetic pathway comprises at least one polynucleotide, at least two polynucleotides, at least three polynucleotides, or at least four polynucleotides that is/are heterologous to the host cell. In some embodiments, each substrate to product conversion of a butanol biosynthetic pathway in a recombinant host cell is catalyzed by a heterologous polypeptide. In embodiments, the polypeptide catalyzing the substrate to product conversions of acetolactate to 2,3-dihydroxyisovalerate and/or the polypeptide catalyzing the substrate to product conversion of isobutyraldehyde to isobutanol are capable of utilizing NADH as a cofactor.

[0089] In some embodiments, the engineered butanol pathway of the butanologen comprises at least one polypeptide selected from the group of enzymes having the following Enzyme Commission Numbers: EC 2.2.1.6, EC 1.1.1.86, EC 4.2.1.9, EC 4.1.1.72, EC 1.1.1.1, EC 1.1.1.265, EC 1.1.1.2, EC 1.2.4.4, EC 1.3.99.2, EC 1.2.1.10, EC 2.3.1.9, EC 2.3.1.16, EC 1.1.1.35, EC 1.1.1.157, EC 1.1.1.36, EC 4.2.1.17, EC 4.2.1.55, EC 1.3.1.44, EC 1.3.1.38, and EC 1.2.1.57.

[0090] In some embodiments, the engineered butanol pathway of the butanologen comprises at least one polypeptide selected from the following group of enzymes:

acetolactate synthase, acetohydroxy acid isomeroreductase, dihydroxyacid dehydratase, branched-chain alpha-keto acid decarboxylase, branched-chain alcohol dehydrogenase, acylating aldehyde dehydrogenase, branched-chain keto acid dehydrogenase, butyryl-CoA dehydrogenase, butyraldehyde dehydrogenase, acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase, butanol dehydrogenase, and butyraldehyde dehydrogenase .

[0091] The terms "acetohydroxyacid synthase," "acetolactate synthase" and "acetolactate synthetase" (abbreviated "ALS") are used interchangeably herein to refer to any polypeptide having a biological function of an acetolactate synthase. Such polypeptides include a polypeptide that catalyzes the conversion of pyruvate to acetolactate and CO_2. Example acetolactate synthases are known by the EC number 2.2.1.6 (Enzyme Nomenclature 1992, Academic Press, San Diego). These unmodified enzymes are available from a number of sources, including, but not limited to, *Bacillus subtilis* (GenBank Nos: CAB15618 and Z99122, NCBI (National Center for Biotechnology Information) amino acid sequence, NCBI nucleotide sequence, respectively), *Klebsiella pneumoniae* (GenBank Nos: AAA25079 and M73842), and *Lactococcus lactis* (GenBank Nos: AAA25161 and L16975).

[0092] The terms "ketol-acid reductoisomerase" ("KARI"),"acetohydroxy acid isomeroreductase" and "acetohydroxy acid reductoisomerase" will be used interchangeably and refer to any polypeptide having a biological function of a ketol-acid reductoisomerase. Such polypeptides include a polypeptide capable of catalyzing the reaction of (S)-acetolactate to 2,3-dihydroxyisovalerate. Example KARI enzymes may be classified as EC number EC 1.1.1.86 (Enzyme Nomenclature 1992, Academic Press, San Diego), and are available from a vast array of microorganisms, including, but not limited to, *Escherichia coli* (SEQ ID NO: 1) (GenBank Nos: NP_418222 and NC_000913), *Saccharomyces cerevisiae* (GenBank Nos: NP_013459 and NC_001144), *Methanococcus maripaludis* (GenBank Nos: CAF30210 and BX957220), *Pseudomonas fluorescens* (SEQ ID NO: 2) and *Bacillus subtilis* (GenBank Nos: CAB14789 and Z99118). KARIs include *Anaerostipes caccae* KARI variants "K9G9" (SEQ ID NO: 132), "K9D3" (SEQ ID NO: 133), “K9JBP4P” (SEQ ID NO: 130), and “K9SB2-SH” (SEQ ID NO: 126). Ketol-acid reductoisomerase (KARI) enzymes are described in U.S. Patent Nos. 7,910,342, and 8,129,162; U.S. Patent Appl. Pub. No. 20100197519; and International Appl. Pub. No. WO/2011/041415, which are incorporated herein by reference. Examples of KARIs

disclosed therein are those from *Lactococcus lactis*, *Vibrio cholera, Pseudomonas aeruginosa* PAO1 and *Pseudomonas fluorescens* PF5 mutants. In some embodiments, the KARI utilizes NADH as a co-factor. In some embodiments, the KARI utilizes NADPH as a co-factor. PCT Patent Appl. Pub. No. WO2012/129555further describes KARI mutants useful in the present invention, and is incorporated herein by reference.

[0093] The terms "acetohydroxy acid dehydratase" and "dihydroxyacid dehydratase" ("DHAD") refer to any polypeptide having a biological function of a dihydroxyacid dehydratase. Such polypeptides include a polypeptide that catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate. Example dihydroxyacid dehydratases are known by the EC number 4.2.1.9. Such enzymes are available from a vast array of microorganisms, including, but not limited to, *E. coli* (GenBank Nos: YP_026248 and NC_000913), *S. cerevisiae* (GenBank Nos: NP_012550 and NC_001142), *M. maripaludis* (GenBank Nos: CAF29874 and BX957219), *B. subtilis* (GenBank Nos: CAB14105 and Z99115), *Lactococcus lactis* (SEQ ID NO: 3), *Streptococcus mutans* (SEQ ID NO: 4) and *N. crassa*. US Appl. Pub. No. 20100081154 A1 and U.S. Patent 7,993,889, which are incorporated herein by reference, describe dihydroxyacid dehydratases (DHADs), including a DHAD from *Streptococcus mutans* (SEQ ID NO: 131). Suitable DHADs also include variants of *Streptococcus mutans* such as "L2V4" (SEQ ID NO: 134).

[0094] The term "branched-chain α-keto acid decarboxylase" or "α-ketoacid decarboxylase" or "α-ketoisovalerate decarboxylase" or "2-ketoisovalerate decarboxylase" ("KIVD") refers to any polypeptide having a biological function of a 2-ketoisovalerate decarboxylase. Such polypeptides include a polypeptide that catalyzes the conversion of α-ketoisovalerate to isobutyraldehyde and CO_2. Example branched-chain α-keto acid decarboxylases are known by the EC number 4.1.1.72 and are available from a number of sources, including, but not limited to, *Lactococcus lactis* (GenBank Nos: AAS49166, AY548760, CAG34226 and AJ746364), *Salmonella typhimurium* (GenBank Nos: NP_461346 and NC_003197), *Clostridium acetobutylicum* (GenBank Nos: NP_149189 and NC_001988), *Macrococcus caseolyticus* (SEQ ID NO: 5), and *Listeria grayi* (SEQ ID NO: 6).

[0095] The terms "branched-chain alcohol dehydrogenase" or "alcohol dehydrogenase" ("ADH") refer to any polypeptide having a biological function of an alcohol dehydrogenase. Such polypeptides include a polypeptide that catalyzes the conversion of

isobutyraldehyde to isobutanol. Example branched-chain alcohol dehydrogenases are known by the EC number 1.1.1.265, but may also be classified under other alcohol dehydrogenases (specifically, EC 1.1.1.1 or 1.1.1.2). Alcohol dehydrogenases may use NADPH or NADH as a co-factor. Such enzymes are available from a number of sources, including, but not limited to, *S. cerevisiae* (GenBank Nos: NP_010656, NC_001136, NP_014051 and NC_001145), *E. coli* (GenBank Nos: NP_417484 and NC_000913), *C. acetobutylicum* (GenBank Nos: NP_349892, NC_003030, NP_349891 and NC_003030), *B. indica* (SEQ ID NO: 7) and *A. xylosoxidans* (SEQ ID NO: 8). U.S. Patent Appl. Publ. No. 20090269823 A1, which is incorporated herein by reference, describes SadB, an alcohol dehydrogenase (ADH) from *Achromobacter xylosoxidans.* Alcohol dehydrogenases also include horse liver ADH and *Beijerinkia indica* ADH (as described by U.S. Appl. Publ. No. 20110269199, which is incorporated herein by reference).

[0096] The term "butanol dehydrogenase" refers to any polypeptide having a biological function of a butanol dehydrogenase. Such polypeptides include a polypeptide that catalyzes the conversion of isobutyraldehyde to isobutanol or the conversion of 2-butanone to 2-butanol. Butanol dehydrogenases are a subset of a broad family of alcohol dehydrogenases. Butanol dehydrogenase may be NADH or NADPH dependent. The NADH dependent enzymes are known as EC 1.1.1.1 and are available, for example, from *Rhodococcus ruber* (GenBank Nos: CAD36475 and AJ491307). The NADPH dependent enzymes are known as EC 1.1.1.2 and are available, for example, from *Pyrococcus furiosus* (GenBank Nos: AAC25556 and AF013169). Additionally, a butanol dehydrogenase is available from *Escherichia coli* (GenBank Nos: NP_417484 and NC_000913) and a cyclohexanol dehydrogenase is available from *Acinetobacter sp.* (GenBank Nos: AAG10026 and AF282240). The term "butanol dehydrogenase" also refers to an enzyme that catalyzes the conversion of butyraldehyde to 1-butanol, using either NADH or NADPH as cofactor. Butanol dehydrogenases are available from, for example, C. *acetobutylicum* (GenBank Nos: NP_149325 and NC_001988 (note: this enzyme possesses both aldehyde and alcohol dehydrogenase activity), NP_349891, NC_003030, NP_349892 and NC_003030) and *E. coli* (GenBank Nos: NP_417484 and NC_000913).

[0097] The term "branched-chain keto acid dehydrogenase" refers to any polypeptide having a biological function of a branched-chain keto acid dehydrogenase. Such polypeptides include a polypeptide that catalyzes the conversion of α-ketoisovalerate to

isobutyryl-CoA (isobutyryl-coenzyme A), typically using NAD^+ (nicotinamide adenine dinucleotide) as an electron acceptor. Example branched-chain keto acid dehydrogenases are known by the EC number 1.2.4.4. Such branched-chain keto acid dehydrogenases are comprised of four subunits and sequences from all subunits are available from a vast array of microorganisms, including, but not limited to, *B. subtilis* (GenBank Nos: CAB14336, Z99116, CAB14335, Z99116, CAB14334, Z99116, CAB14337 and Z99116) and *Pseudomonas putida* (GenBank Nos: AAA65614, M57613, AAA65615, M57613, AAA65617, M57613, AAA65618 and M57613).

[0098] The term "acylating aldehyde dehydrogenase" refers to any polypeptide having a biological function of an acylating aldehyde dehydrogenase. Such polypeptides include a polypeptide that catalyzes the conversion of isobutyryl-CoA to isobutyraldehyde, typically using either NADH or NADPH as an electron donor. Example acylating aldehyde dehydrogenases are known by the EC numbers 1.2.1.10 and 1.2.1.57. Such enzymes are available from multiple sources, including, but not limited to, *Clostridium beijerinckii* (GenBank Nos: AAD31841 and AF157306), *C. acetobutylicum* (GenBank Nos: NP_149325, NC_001988, NP_149199 and NC_001988), *P. putida* (GenBank Nos: AAA89106 and U13232), and *Thermus thermophilus* (GenBank Nos: YP_145486 and NC_006461).

[0099] The term "acetyl-CoA acetyltransferase" refers to any polypeptide having a biological function of an acetyl-CoA acetyltransferase. Such polypeptides include a polypeptide that catalyzes the conversion of two molecules of acetyl-CoA to acetoacetyl-CoA and coenzyme A (CoA). Example acetyl-CoA acetyltransferases are acetyl-CoA acetyltransferases with substrate preferences (reaction in the forward direction) for a short chain acyl-CoA and acetyl-CoA and are classified as E.C. 2.3.1.9 [Enzyme Nomenclature 1992, Academic Press, San Diego]; although, enzymes with a broader substrate range (E.C. 2.3.1.16) will be functional as well. Acetyl-CoA acetyltransferases are available from a number of sources, for example, *Escherichia coli* (GenBank Nos: NP_416728 and NC_000913), *Clostridium acetobutylicum* (GenBank Nos: NP_349476.1, NC_003030, NP_149242 and NC_001988, *Bacillus subtilis* (GenBank Nos: NP_390297 and NC_000964), and *Saccharomyces cerevisiae* (GenBank Nos: NP_015297 and NC_001148).

[00100] The term "3-hydroxybutyryl-CoA dehydrogenase" refers to any polypeptide having a biological function of a 3-hydroxybutyryl-CoA dehydrogenase. Such

polypeptides include a polypeptide that catalyzes the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. 3-Example hydroxybutyryl-CoA dehydrogenases may be reduced nicotinamide adenine dinucleotide (NADH)-dependent, with a substrate preference for (S)-3-hydroxybutyryl-CoA or (R)-3-hydroxybutyryl-CoA. Examples may be classified as E.C. 1.1.1.35 and E.C. 1.1.1.30, respectively. Additionally, 3-hydroxybutyryl-CoA dehydrogenases may be reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent, with a substrate preference for (S)-3-hydroxybutyryl-CoA or (R)-3-hydroxybutyryl-CoA and are classified as E.C. 1.1.1.157 and E.C. 1.1.1.36, respectively. 3-Hydroxybutyryl-CoA dehydrogenases are available from a number of sources, for example, *C. acetobutylicum* (GenBank Nos: NP_349314 and NC_003030), *B. subtilis* (GenBank Nos: AAB09614 and U29084), *Ralstonia eutropha* (GenBank Nos: YP_294481 and NC_007347), and *Alcaligenes eutrophus* (GenBank Nos: AAA21973 and J04987).

[00101] The term "crotonase" refers to any polypeptide having a biological function of a crotonase. Such polypeptides include a polypeptide that catalyzes the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA and H_2O. Example crotonases may have a substrate preference for (S)-3-hydroxybutyryl-CoA or (R)-3-hydroxybutyryl-CoA and may be classified as E.C. 4.2.1.17 and E.C. 4.2.1.55, respectively. Crotonases are available from a number of sources, for example, *E. coli* (GenBank Nos: NP_415911 and NC_000913), *C. acetobutylicum* (GenBank Nos: NP_349318 and NC_003030), *B. subtilis* (GenBank Nos: CAB13705 and Z99113), and *Aeromonas caviae* (GenBank Nos: BAA21816 and D88825).

[00102] The term "butyryl-CoA dehydrogenase" refers to any polypeptide having a biological function of a butyryl-CoA dehydrogenase. Such polypeptides include a polypeptide that catalyzes the conversion of crotonyl-CoA to butyryl-CoA. Example butyryl-CoA dehydrogenases may be NADH-dependent, NADPH-dependent, or flavin-dependent and may be classified as E.C. 1.3.1.44, E.C. 1.3.1.38, and E.C. 1.3.99.2, respectively. Butyryl-CoA dehydrogenases are available from a number of sources, for example, *C. acetobutylicum* (GenBank Nos: NP_347102 and NC_ 003030), *Euglena gracilis* (GenBank Nos: Q5EU90 and AY741582), *Streptomyces collinus* (GenBank Nos: AAA92890 and U37135), and *Streptomyces coelicolor* (GenBank Nos: CAA22721 and AL939127).

[00103] The term "butyraldehyde dehydrogenase" refers to any polypeptide having a biological function of a butyraldehyde dehydrogenase. Such polypeptides include a polypeptide that catalyzes the conversion of butyryl-CoA to butyraldehyde, using NADH or NADPH as cofactor. Butyraldehyde dehydrogenases with a preference for NADH are known as E.C. 1.2.1.57 and are available from, for example, *Clostridium beijerinckii* (GenBank Nos: AAD31841 and AF157306) and *C. acetobutylicum* (GenBank Nos: NP_149325 and NC_001988).

Host Cells

[00104] Host cells for butanol production may be selected from bacteria and yeast. In embodiments, suitable host cells include any bacterial or yeast cell useful for genetic modification and recombinant gene expression. The criteria for selection of suitable microbial hosts include the following: intrinsic tolerance to the butanol isomer being produced, high rate of glucose utilization, availability of genetic tools for gene manipulation, and the ability to generate stable chromosomal alterations.

[00105] The ability to genetically modify the host is essential for the production of any recombinant microorganism. The mode of gene transfer technology may be by electroporation, conjugation, transduction or natural transformation. A broad range of host conjugative plasmids and drug resistance markers are available. The cloning vectors are tailored to the host organisms based on the nature of antibiotic resistance markers that can function in that host.

[00106] The microbial host also has to be manipulated in order to inactivate competing pathways for carbon flow by deleting various genes. This requires the availability of either transposons to direct inactivation or chromosomal integration vectors. Additionally, the production host should be amenable to chemical mutagenesis so that mutations to improve intrinsic butanol tolerance may be obtained.

[0101] The microbial host cell used for the production butanol isomers is preferably tolerant to the butanol isomer that is being produced so that the yield of the butanol isomer is not limited by the toxicity of the butanol isomer. In one embodiment, the host used for the isobutanol production is tolerant to isobutanol. Suitable host strains with a tolerance for isobutanol may be identified by a screening method based on the intrinsic tolerance of the strain as described in U.S. Pat. No. 7,993,889 (incorporated herein by reference).

[0102] The microbial host for isobutanol production should also utilize carbohydrates, including monosaccharides, oligosaccharides and polysaccharides, at a high rate. Most microbes are capable of utilizing carbohydrates. However, certain environmental microbes cannot utilize carbohydrates to high efficiency, and therefore would not be suitable hosts.

[0103] Based on the criteria described above, suitable microbial hosts for the production of butanol include, but are not limited to, members of the genera *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*, *Pichia*, *Candida*, *Hansenula, Schizosaccharomyces, Issatchenkia, Kluyveromyces, Yarrowia, Pichia, Candida, Hansenula* and *Saccharomyces*. Preferred hosts include: *Escherichia coli*, *Alcaligenes eutrophus*, *Bacillus licheniformis*, *Paenibacillus macerans*, *Rhodococcus erythropolis*, *Pseudomonas putida*, *Lactobacillus plantarum*, *Enterococcus faecium*, *Enterococcus gallinarium*, *Enterococcus faecalis*, *Bacillus subtilis, Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces thermotolerans, Kluyveromyces marxianus,Candida glabrata*, *Candida albicans*, *Pichia stipitis*, *Yarrowia lipolytica*, *E. coli, L. plantarum* and *Saccharomyces cerevisiae*. In some embodiments, the host cell is *Saccharomyces cerevisiae*. *S. cerevisiae* yeast are known in the art and are available from a variety of sources, including, but not limited to, American Type Culture Collection (Rockville, MD); Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre; LeSaffre; Gert Strand AB; Ferm Solutions; North American Bioproducts; Martrex and Lallemand. *S. cerevisiae* include, but are not limited to, BY4741, CEN.PK 113-7D, Ethanol Red® yeast, Ferm Pro™ yeast, Bio-Ferm® XR yeast, Gert Strand Prestige Batch Turbo alcohol yeast, Gert Strand Pot Distillers yeast, Gert Strand Distillers Turbo yeast, FerMax™ Green yeast, FerMax™ Gold yeast, Thermosacc® yeast, BG-1, PE-2, CAT-1, CBS7959, CBS7960, and CBS7961.

Host Cells for Butanol Production

[0104] Recombinant microorganisms containing the genes necessary to encode the enzymatic pathway for conversion of a fermentable carbon substrate to butanol isomers may be constructed using techniques well known in the art. In the present invention, genes encoding the enzymes of one of the butanol biosynthetic pathways, for example,

acetolactate synthase, acetohydroxy acid isomeroreductase, dihydroxyacid dehydratase, branched-chain α-keto acid decarboxylase, and branched-chain alcohol dehydrogenase, may be isolated from various sources, as described, for example, in U.S. Pat. No. 7,993,889.

[0105] Once the relevant pathway genes are identified and isolated, the relevant enzymes of the butanol biosynthetic pathway may be introduced into the host cells or manipulated, as described, for example, in U.S. Pat. No. 7,993,889, to produce butanologens. The butanologens generated comprise an engineered butanol biosynthetic pathway. In some embodiments, the butanologen is an isobutanologen, which comprises an engineered isobutanol biosynthetic pathway.

[0106] In some embodiments, the butanologen is a yeast. In some embodiments, the butanologen is a bacterium. In some embodiments, the butanologen is *Saccharomyces cerevisiae*.

[0107] In some embodiments, the engineered butanologen contains one or more polypeptides selected from a group of enzymes having the following Enzyme Commission Numbers: EC 2.2.1.6, EC 1.1.1.86, EC 4.2.1.9, EC 4.1.1.72, EC 1.1.1.1, EC 1.1.1.265, EC 1.1.1.2, EC 1.2.4.4, EC 1.3.99.2, EC 1.2.1.10, EC 2.3.1.9, EC 2.3.1.16, EC 1.1.1.35, EC 1.1.1.157, EC 1.1.1.36, EC 4.2.1.17, EC 4.2.1.55, EC 1.3.1.44, EC 1.3.1.38, and EC 1.2.1.57.

[0108] In some embodiments, the engineered isobutanologen contains one or more polypeptides selected from acetolactate synthase, acetohydroxy acid isomeroreductase, dihydroxyacid dehydratase, branched-chain alpha-keto acid decarboxylase, branched-chain alcohol dehydrogenase, acylating aldehyde dehydrogenase, branched-chain keto acid dehydrogenase, butyryl-CoA dehydrogenase, butyraldehyde dehydrogenase, acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase, butanol dehydrogenase, and butyraldehyde dehydrogenase.

[0109] In some embodiments, enzymes of the butanol biosynthetic pathway that are usually localized to the mitochondria are not localized to the mitochondria. In some embodiments, enzymes of the engineered butanol biosynthetic pathway are localized to the cytosol. In some embodiments, an enzyme of the biosynthetic pathway is localized to the cytosol by removing the mitochondrial targeting sequence. In some

embodiments, mitochondrial targeting is eliminated by generating new start codons as described in for example, U.S. Pat. No. 7,993,889, incorporated herein by reference. In some embodiments, the enzyme of the biosynthetic pathway that is localized to the cytosol is DHAD. In some embodiments, the enzyme from the biosynthetic pathway that is localized to the cytosol is KARI.

[0110] In some embodiments, the enzymes of the engineered butanol biosynthetic pathway may use NADH or NADPH as a co-factor, wherein NADH or NADPH acts as an electron donor. In some embodiments, one or more enzymes of the butanol biosynthetic pathway use NADH as an electron donor. In some embodiments, one or more enzymes of the the butanol biosynthetic pathway use NADPH as an electron donor.

Additional modifications of butanologens

[0111] The butanologens. as provided herein. may further comprise one or more additional modifications. Such modifications, for example, may include disruption of the activity of the genes involved in the production of by-products during the fermentative production of butanol isomers via the engineered butanol biosynthetic pathway. The disruption of the activity of the genes involved in the production of by-products during the fermentative production of butanol isomers reduces yield loss from the competing pathways for carbon flow and increases butanol production. In some embodiments, such modifications include disruption of the activity of pyruvate decarboxylase, aldehyde dehydrogenase or both.

[0112] The term "pyruvate decarboxylase" refers to any polypeptide having a biological function of a pyruvate decarboxylase. Such polypeptides include a polypeptide that catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide. Pyruvate decarboxylases are known by the EC number 4.1.1.1. Such polypeptides can be determined by methods well known in the art and disclosed in PCT Patent Appl. Pub. No. WO2012/129555. These enzymes are found in a number of yeast, including *Saccharomyces cerevisiae* (GenBank Nos: CAA97575, CAA97705 and CAA97091). Additional examples of PDC are provided in U.S. Appl. Pub. No. 2009035363, which is incorporated herein by reference.

[0113] In some embodiments, a butanologen disclosed herein can comprise a modification or disruption of an endogenous polynucleotide and/or gene encoding a

polypeptide having pyruvate decarboxylase activity and/or an endogenous polypeptide having pyruvate decarboxylase activity. In some embodiments, a butanologen disclosed herein can comprise a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having PDC activity, or in an endogenous polypeptide having PDC activity. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in PDC activity that is reduced or eliminated, resulting, for example, in a PDC knock-out (PDC-KO) phenotype.

[0114] Endogenous pyruvate decarboxylase in yeast converts pyruvate to acetaldehyde, which is then converted to ethanol or to acetyl-CoA via acetate. Yeast may have one or more genes encoding pyruvate decarboxylase. For example, there is one gene encoding pyruvate decarboxylase in *Candida glabrata*, *Schizosaccharomyces pombe* and *Kluyveromyces lactis*, while there are three isozymes of pyruvate decarboxylase encoded by the *PDC1*, *PCD5* and/or *PDC6* genes in *Saccharomyces*. In some embodiments, in the present yeast cells at least one PDC gene is inactivated. If the yeast cell used has more than one expressed (active) PDC gene, then each of the active PDC genes may be modified or inactivated thereby producing a pdc- cell. For example, in *S. cerevisiae* the *PDC1*, *PDC5* and *PDC6* genes may be modified or inactivated. If a PDC gene is not active under the fermentation conditions to be used then such a gene would not need to be modified or inactivated. In some embodiments, the pyruvate decarboxylase that is deleted or downregulated is selected from the group consisting of: *PDC1*, *PDC5*, *PDC6* and combinations thereof. U.S. Patent Appl. Pub. No. 20090305363 and PCT Patent Appl. Pub. No. WO2012/129555 (incorporated herein by reference) further describe the modifications in the endogenous pyruvate decarboxylase, and are incorporated herein by reference. U.S. Appl. Pub. No. 20090305363 (incorporated herein by reference) discloses increased conversion of pyruvate to acetolactate by engineering yeast for expression of a cytosol-localized acetolactate synthase and substantial elimination of pyruvate decarboxylase activity. Yeast having a reduced enzymatic activity can be identified using various methods. For example, yeast having reduced pyruvate decarboxylase activity can be identified using common methods, including, for example, measuring ethanol formation via gas chromatography.

[0115] Other target genes, such as those encoding pyruvate decarboxylase proteins having at least about 70-75%, at least about 75-85%, at least about 80-85%, at least

about 85%-90%, at least about 90%-95%, or at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to the pyruvate decarboxylases may be identified in the literature and in bioinformatics databases well known to the skilled person. The methods for disruption of pyruvate decarboxylase activity along with the methods for identification of butanologens with modified or deleted pyruvate decarboxylase are described in detail in U.S. Patent Appl. Pub. No. 20090305363 and PCT Patent Appl. Pub. No. WO2012/129555.

[0116] In some embodiments, a butanologen comprises modifications to reduce glycerol-3-phosphate dehydrogenase activity and/or disruption of at least one gene encoding a polypeptide having PDC activity or a disruption in at least one gene encoding a regulatory element controlling PDC gene expression as described in U.S. Patent Appl. Pub. No. 20090305363 and PCT Patent Appl. Pub. No. WO2012/129555, the modifications that would provide for an increased carbon flux through Entner-Doudoroff Pathway, or reducing equivalents balance as described in U.S. Patent Appl. Pub. No. 20100120105 (incorporated herein by reference). Yeast cells with inactivated endogenous PDC gene and an engineered biosynthethic pathway having improved growth and product yield when glucose repression was reduced are described in U.S. Appl. Publication No. 20110124060, incorporated herein by reference.

[0117] The term "aldehyde dehydrogenases" refers to any polypeptide having a biological function of an aldehyde dehydrogenase. Such polypeptides include a polypeptide that catalyzes the oxidation (dehydrogenation) of aldehydes (Wang *et al.*, *J. Bacteriol.* 180:822-30, 1998; Navarro-Avino *et al.*, *Yeast* 15:829-42, 1999; and Saint-Prix *et al.*, *Microbiology* 150:2209-20, 2004). Such polypeptides include a polypeptide that catalyzes the conversion of isobutyraldehyde to isobutyric acid. Such polypeptides also include a polypeptide that corresponds to EC Numbers 1.2.1.3, EC 1.2.1.4 or 1.2.1.5. Such polypeptides can be determined by methods well known in the art and are disclosed in PCT Patent Appl. Pub. No. WO2012/129555.

[0118] In some embodiments, a butanologen can comprise deletion, mutation and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase (ALD) and/or aldehyde oxidase activity or deletion, mutation and/or substitution in an endogenous polypeptide having aldehyde dehydrogenase activity and/or aldehyde oxidase activity. In some embodiments, a recombinant host cell of the invention can be *S. cerevisiae,* and a polypeptide having aldehyde

dehydrogenase activity can be ALD2, ALD3, ALD4, ALD5, ALD6, or combinations thereof. In some embodiments, a recombinant host cell can be *Kluyveromyces lactis,* and a polypeptide having aldehyde dehydrogenase activity can be KLLA0F00440, KLLA0E23057, KLLA0D10021, KLLA0D09999G, or combinations thereof. In other embodiments, a recombinant host cell can be *Pichia stipitis*, and a polypeptide having aldehyde dehydrogenase activity can be ALD2, ALD3, ALD4, ALD5, ALD7, or combinations thereof. In other embodiments, a recombinant host cell can be *Lactobacillus plantarum,* and a polypeptide having aldehyde dehydrogenase activity can be AldH. In other embodiments, a recombinant host cell can be *E. coli*, and a polypeptide having aldehyde dehydrogenase activity can be aldA, aldB, aldH, or combinations thereof.

[0119] In some embodiments, the polypeptide having aldehyde dehydrogenase activity is *ALD6* in *Saccharomyces cerevisiae* or a homolog thereof. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in ALD activity that is reduced or eliminated, resulting, for example, in an ALD6 knock-out (ALD6-KO) phenotype. Examples of aldehyde dehydrogenase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell are provided in further detail in PCT Patent Appl. Pub. No. WO2012/129555.

[0120] The disruption of a particular aldehyde dehydrogenase could be confirmed, for example, with PCR screening using primers internal and external to the aldehyde dehydrogenase gene or by Southern blot using a probe designed to the aldehyde dehydrogenase gene sequence. Alternatively, one could utilize gas chromatography-mass spectroscopy or liquid chromatography to screen strains exposed to isobutyraldehyde for decreased formation of isobutyric acid. For example, a method of screening for strains with decreased isobutyric acid formation can comprise: a) providing a strain comprising a modification in a polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity and/or a modification in a polynucleotide encoding a polypeptide having aldehyde oxidase activity; b) contacting the cell with isobutyraldehyde; and c) measuring isobutyric acid formation; wherein isobutyric acid formation is reduced as compared to a control strain without the modification. In some embodiments, the measuring is carried out using gas chromatography-mass spectroscopy. The methods for deletion, mutation and/or substitution of polynucleotide, gene or polypeptide for aldehyde dehydrogenase and methods for identifying disruption

of aldehyde dehydrogenase activity are described in detail, *e.g.*, in PCT Patent Appl. Pub. No. WO2012/129555.

[0121] Other target genes, such as those encoding aldehyde dehydrogenase proteins having at least about 70-75%, at least about 75-85%, at least about 80-85%, at least about 85%-90%, at least about 90%-95%, or at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to the aldehyde dehydrogenase may be identified in the literature and in bioinformatics databases well known to the skilled person.

[0122] In some emobodiments, butanologens described herein can comprise a reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity, as described in PCT Patent Appl. Pub. No. WO2012/129555. In some embodiments, a butnologen with reduced or eliminated aldehyde dehydrogenase activity can produce a butanol isomer via the engineered biosynthetic pathway at a greater yield or amount than the yield or amount of the same isomer produced by a butanologen that does not comprise reduced or eliminated aldehyde dehydrogenase activity.

[0123] In some embodiments, a butanologen as described herein can comprise a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide involved in the pathways for the production of by-products during the fermentative production of butanol isomers. In some embodiments, a butanologen can comprise one or more deletions, mutations, and/or substitutions in an endogenous polypeptide that is involved in the pathways for the production of by-products during the fermentative production of butanol isomers. In some embodiments, these modifications are in genes or polynucleotides encoding FRA2 (iron repressor protein), CCC1 (putative vacuolar Fe2+/Mn2+ transporter) or GPD2 (glycerol-2-phosphate dehydrogenase) or polypeptides having FRA2, CCC1 or GPD2 activity or combinations thereof.

[0124] In other embodiments, modifications include integration of at least one polynucleotide encoding a polypeptide that catalyzes a step in a pyruvate-utilizing biosynthetic pathway. Other modifications include at least one deletion, mutation, and/or substitution in an endogenous polynucleotide encoding a polypeptide having acetolactate reductase activity. In embodiments, the polypeptide having acetolactate reductase activity is YMR226C of *Saccharomyces cerevisae* or a homolog thereof.

[0125] In embodiments, host cells can comprise heterologous polynucleotides encoding a polypeptide with phosphoketolase activity and/or a heterologous polynucleotide encoding a polypeptide with phosphotransacetylase activity such as, for example, those encoded by SEQ ID NOs: 262 and 263, and as described in PCT Appn. Pub. No. WO 2011/159853. As described therein, PDC-KO cells so modified exhibit a reduced or eliminated requirement for exogenous two-carbon substrate supplementation for their growth compared to PDC- KO cells. Accordingly, and as demonstrated in the Examples, methods provided herein provide advantages for recombinant host cells engineered to reduce or eliminate pyruvate decarboxylase (PDC) activity and comprising a reduced or eliminated requirement for exogenous two-carbon substrate supplementation for their growth.

Fermentation Medium

[0126] Fermentation medium in the present invention must contain suitable fermentable carbon substrates. Suitable fermentable carbon substrates may include, but are not limited to, monosaccharides such as glucose, fructose, xylose, or arabinose; oligosaccharides such as lactose, maltose, galactose, or sucrose; polysaccharides such as starch or cellulose; or combinations thereof. Suitable fermentable carbon substrate may include unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Additionally the fermentable carbon substrate may also be one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated. In addition to one and two fermentable carbon substrates methylotrophic organisms are also known to utilize a number of other carbon containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion *et al.*, *Microb. Growth C1 Compd.*, [Int. Symp.], 7th ed., 415-32. Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK (1993)). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter *et al.*, *Arch. Microbiol.*, *153*:485-489 (1990)). Hence it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon containing substrates and will only be limited by the choice of organism. Other carbon substrates may include ethanol, lactate, succinate or glycerol.

[0127] Although it is contemplated that all of the above mentioned fermentable carbon substrates and mixtures thereof are suitable in the present invention, preferred fermentable carbon substrates are glucose, fructose, and sucrose, or mixtures of these with C5 sugars such as xylose and arabinose. Sucrose may be derived from renewable sugar sources such as sugar cane, sugar beets, cassava, sweet sorghum, and mixtures thereof. Glucose and dextrose may be derived from renewable grain sources through saccharification of starch based feedstocks including grains such as corn, wheat, rye, barley, oats, and mixtures thereof. In addition, fermentable sugars may be derived from renewable cellulosic or lignocellulosic biomass through processes of pretreatment and saccharification, as described, for example, in U.S. Pat. No. 7,932,063, which is incorporated herein by reference. Biomass includes materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides. Biomass may also comprise additional components, such as protein and/or lipid. Biomass may be derived from a single source, or biomass can comprise a mixture derived from more than one source; for example, biomass may comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

[0128] In some embodiments, the fermentable carbon substrate is glucose derived from corn. In some embodiments, the fermentable carbon substrate is glucose derived from wheat. In some embodiments, the fermentable carbon substrate is sucrose derived from sugar cane. In some embodiments, the fermentable carbon substrate is xylose.

[0129] In addition to an appropriate carbon source, fermentation medium must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for isobutanol production.

[0130] In some embodiments, the fermentation medium in the present invention contains acetate as an exogenous C2 source, which is added to the fermentation medium as a supplement, in an amount sufficient for the growth of the recombinant host cells. In some embodiments, acetate is added to the fermentation medium in an amount sufficient for improved butanol production. In some embodiments, acetate is added to the fermentation medium in the range of about 0.1mM to about 50mM. In some embodiments, the acetate added to the fermentation medium is 0.1mM, 0.2mM, 0.4mM, 0.5mM, 0.6mM, 0.7 mM, 0.8mM, 0.9mM, 1.0mM, 1.1mM, 1.2mM, 1.3mM, 1.4mM, 1.5mM, 1.6mM, 1.7mM, 1.8mM, 1.9mM, 2.0mM, 5mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM or 50mM. In some embodiments, the sugar to C2 supplement ratio in the fermentation medium is 95:5, 90:10, 85:15, 80:20, 75:25 or 70:30. In some embodiments, the acetate is added in growth phase, production phase, or both.

[0131] In some embodiments, the fermentation medium may further contain butanol. In some embodiments, the butanol is in the range of about 0.01mM to about 500mM. In some embodiments, the butanol is 0.01mM, 1.0mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, 50mM, 55mM, 60mM, 65mM, 70mM, 75mM, 80mM, 85mM, 90mM, 95mM, 100mM, 110mM, 120mM, 130mM, 140mM, 150mM, 160mM, 170mM, 180mM, 190mM, 200mM, 210mM, 220mM, 230mM, 240mM, 250mM, 260mM, 270mM, 280mM, 290mM, 300mM, 310mM, 320mM, 330mM, 340mM, 350mM, 360mM, 370mM, 380mM, 390mM, 400mM, 410mM, 420mM, 430mM, 440mM, 450mM, 460mM, 470mM, 480mM, 490mM or 500mM. In some embodiments, butanol present in the fermentation medium is from about 0.01% to about 100% of the theoretical yield of butanol. In some embodiments, butanol present in the fermentation medium is 0.01%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of the theoretical yield of butanol.

[0132] In some embodiments, improved butanol production is manifest as increased yield, effective rate, effective titer, or specific productivity. In embodiments, at least one of yield, effective rate, effective titer, or specific productivity is increased by at least about 3%, at least about 5%, or at least about 10%.

[0133] In some embodiments, improved butanol production is manifest as decreased by-product yield. In embodiments, yield of a by-product is decreased by at least about

3%, at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, or at least about 50%, or at least about 70%. In embodiments, the by-product is isobutyric acid.

Fermentation Conditions

[0134] Typically cells are grown at a temperature in the range of about 20 °C to about 40 °C in an appropriate medium. In some embodiments, the cells are grown at a temperature of 20 °C, 22 °C, 25 °C, 27 °C, 30 °C, 32 °C, 35 °C, 37 °C or 40 °C. Suitable growth medium in the present invention include common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, Yeast Medium (YM) broth, or broth that includes yeast nitrogen base, ammonium sulfate, and dextrose (as the carbon/energy source) or YPD Medium, a blend of peptone, yeast extract, and dextrose in optimal proportions for growing most *Saccharomyces cerevisiae* strains. Other defined or synthetic growth media may also be used, and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, *e.g.*, cyclic adenosine 2':3'-monophosphate, may also be incorporated into the fermentation medium.

[0135] Suitable pH ranges for the fermentation are from about pH 3.0 to about pH 9.0. In one embodiment, about pH 4.0 to about pH 8.0 is used for the initial condition. In another embodiment, about pH 3.5 to about pH 9.0 is used for the initial condition. In one embodiment, about pH 4.5 to about pH 6.5 is used for the initial condition. In one embodiment, about pH 5.0 to about pH 8.0 is used for the initial condition. Suitable pH ranges for the fermentation of yeast are typically from about pH 3.0 to about pH 9.0. Suitable pH ranges for the fermentation of other microorganisms are from about pH 3.0 to about pH 7.5

[0136] In some embodiments, the contacting of the fermentation medium with the recombinant microorganism is performed under anaerobic or microaerobic conditions.

[0137] In some embodiments, the butanol is produced in one or more of the following growth phases: high growth log phase, moderate through static lag phase, stationary phase, steady state growth phase, and combinations thereof.

Industrial Batch and Continuous Fermentations

[0138] In some embodiments, the butanol isomers may be produced using batch or continuous fermentation. Butanol isomers, such as isobutanol, may be produced using a batch method of fermentation. A classical batch fermentation is a closed system where the composition of the medium is set at the beginning of the fermentation and not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism or organisms, and fermentation is permitted to occur without adding anything to the system. Typically, however, a "batch" fermentation is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the fermentation is stopped. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of production of end product or intermediate.

[0139] A variation on the standard batch system is the fed-batch system. Fed-batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO_2. Batch and fed-batch fermentations are common and well known in the art and examples may be found in Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition Sinauer Associates, Inc., Sunderland, MA. (1989) ("Brock"), or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, *36*:227, (1992), incorporated herein by reference.

[0140] Butanol isomers, such as isobutanol, may also be produced using continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous

fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth. Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by medium turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to the medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock.

[0141] It is contemplated that the production of butanol, including isobutanol, may be practiced using batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells may be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isobutanol production.

[0142]

Methods for Butanol Isolation from the Fermentation Medium (Recovery)

[0143] The bioproduced butanol isomers may be recovered from the fermentation medium using methods known in the art. *See, e.g.*, Durre, *Appl. Microbiol. Biotechnol. 49*:639-648 (1998), Groot *et al.*, *Process. Biochem. 27*:61-75 (1992), and references therein. For example, butanol may be isolated from the fermentation medium using methods such as distillation, liquid-liquid extraction, or membrane-based separation. U.S. Patent Appl. Pub. Nos. 20090305370, 20110312043 and 20110312044, which are incorporated herein by reference, describe liquid-liquid extraction, comprising the step of contacting the fermentation broth with a water-immiscible extractant to form a two-phase mixture comprising an aqueous phase and an organic phase.

[0144] In situ product removal (ISPR) can also be utilized to remove butanol from the fermentation broth. In some embodiments, ISPR includes liquid-liquid extraction.

Typically, the extractant can be an organic extractant selected from the group consisting of saturated, mono-unsaturated, poly-unsaturated (and mixtures thereof) C_{12} to C_{22} fatty alcohols, C_{12} to C_{22} fatty acids, esters of C_{12} to C_{22} fatty acids, C_{12} to C_{22} fatty aldehydes, C_{12} to C_{22} fatty amides, triglycerides, and mixtures thereof, which contacts a fermentation broth and to form a two-phase mixture comprising an aqueous phase and an organic phase. The extractant may also be an organic extractant selected from the group consisting of saturated, mono-unsaturated, poly-unsaturated (and mixtures thereof) C_4 to C_{22} fatty alcohols, C_4 to C_{28} fatty acids, esters of C_4 to C_{28} fatty acids, C_4 to C_{22} fatty aldehydes, C_4 to C_{22} fatty amides, and mixtures thereof, which contacts a fermentation broth and to form a two-phase mixture comprising an aqueous phase and an organic phase. Free fatty acids from slurry can also serve as an ISPR extractant. ISPR extractant (FFA) contacts the fermentation broth and forms a two-phase mixture comprising an aqueous phase and an organic phase. The product alcohol present in the fermentation broth preferentially partitions into the organic phase to form an alcohol-containing organic phase.

[0145] Because butanol isomers form a low boiling point, azeotropic mixture with water, distillation can only be used to separate the mixture up to its azeotropic composition. Distillation may be used in combination with another separation method to obtain separation around the azeotrope. Methods that may be used in combination with distillation to isolate and purify butanol include, but are not limited to, decantation, liquid-liquid extraction, adsorption, and membrane-based techniques. Additionally, butanol isomers may be isolated using azeotropic distillation using an entrainer (*see, for example,* Doherty and Malone, *Conceptual Design of Distillation Systems*, McGraw Hill, New York (2001)).

[0146] When distillation is used in combination with decantation to isolate and purify the butanol, the butanol containing fermentation broth is distilled to near the azeotropic composition. Then, the azeotropic mixture is condensed, and the butanol is separated from the fermentation medium by decantation. The decanted aqueous phase may be returned to the first distillation column as reflux. The butanol-rich decanted organic phase may be further purified by distillation in a second distillation column.

[0147] When distillation is used in combination with liquid-liquid extraction, the butanol is extracted from the fermentation broth using liquid-liquid extraction with a

suitable solvent. The butanol-containing organic phase is then distilled to separate the butanol from the solvent.

[0148] When distillation is used in combination with adsorbtion, the fermentation broth containing the butanol is distilled to near the azeotropic composition and then the remaining water is removed by use of an adsorbent, such as molecular sieves (Aden *et al., Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover*, Report NREL/TP-510-32438, National Renewable Energy Laboratory, June 2002).

[0149] When distillation is used in combination with pervaporation, the fermentation broth containing the butanol is distilled to near the azeotropic composition, and then the remaining water is removed by pervaporation through a hydrophilic membrane (Guo *et al., J. Membr. Sci., 245*:199-210 (2004)).

[0150] Butanol titer in any phase can be determined by methods known in the art, such as via high performance liquid chromatography (HPLC) or gas chromatography, as described, for example in U.S. Patent Appl. Pub. No. US20090305370, incorporated herein by reference.

Methods for Removing Solids

[0151] The dried solid residue (or solids) remaining in the fermentation medium after the fermentation of fermentable carbon substrates may be removed using methods known in the art. These solids comprises of proteins, fiber and oils, and could be of three types: Distiller's Dried Grains (DDG), Distiller's Dried Solubles (DDS), and Distiller's Dried Grains with Solubles (DDGS). Of these solids, only DDGS can be used in the animal feed industry. DDGS has high nutrient value, and is therefore suitable as animal feed.

[0152] Solids may be removed from the fermentation medium by centrifugation, filtration, decantation, or the like. Subsequent to the removal of the solids, butanol may be isolated from the fermentation medium using methods such as distillation, azeotropic distillation, liquid-liquid extraction, adsorption, gas stripping, membrane evaporation, or pervaporation.

EXAMPLES

[0153] The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

General Methods

[0154] Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in *Manual of Methods for General Bacteriology* (Phillipp *et al.,* eds., American Society for Microbiology, Washington, DC., (1994)) or by in Brock, *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Sigma-Aldrich Chemicals (St. Louis, MO), BD Diagnostic Systems (Sparks, MD), Invitrogen (Carlsbad, CA), HiMedia (Mumbai, India), SD Fine chemicals (India), or Takara Bio Inc. (Shiga,, Japan), unless otherwise specified.

Methods for Determining Isobutanol Concentration in Culture Medium

[0155] The concentration of isobutanol in the culture medium can be determined by a number of methods known in the art. For example, a specific high performance liquid chromatography (HPLC) method utilized a Shodex SH-1011 column with a Shodex SH-G guard column, both purchased from Waters Corporation (Milford, Mass.), with refractive index (RI) detection. Chromatographic separation was achieved using 0.01M H_2SO_4 as the mobile phase with a flow rate of 0.5 mL/min and a column temperature of 50 °C. Isobutanol had a retention time of 46.6 min under the conditions used. Alternatively, gas chromatography (GC) methods are available. For example, a specific GC method utilized an HP-INNOWax column (30 m X 0.53 mm id,1 μm film thickness, Agilent Technologies, Wilmington, Del.), with a flame ionization detector (FID). The carrier gas was helium at a flow rate of 4.5 mL/min, measured at 150 °C

with constant head pressure; injector split was 1:25 at 200 °C; oven temperature was 45 °C for 1 min, 45 to 220 °C at 10 °C/min, and 220 °C for 5 min; and FID detection was employed at 240 °C with 26 mL/min helium makeup gas. The retention time of isobutanol was 4.5 min.

[0156] The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "h" means hour(s), "nm" means nanometers, "uL" means microliter(s), "mL" means milliliter(s), "mg/mL" means milligram per milliliter, "L" means liter(s), "nm" means nanometers, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmole" means micromole(s), "kg" means kilogram, "g" means gram(s), "µg" means microgram(s) and "ng" means nanogram(s), "PCR" means polymerase chain reaction, "OD" means optical density, "OD600" means the optical density measured at a wavelength of 600 nm, "kDa" means kilodaltons, "g" can also mean the gravitation constant, "bp" means base pair(s), "kbp" means kilobase pair(s), "kb" means kilobase, "%" means percent, "% w/v" means weight/volume percent, "% v/v" means volume/volume percent, "HPLC" means high performance liquid chromatography, "g/L" means gram per liter, "µg/L" means microgram per liter, "ng/µL" means nanogram per microliter, "pmol/µL" means picomol per microliter, "RPM" means rotation per minute, "µmol/min/mg" means micromole per minute per milligram, "w/v" means weight per volume, "v/v" means volume per volume.

[0157] Microbial strains were obtained from The American Type Culture Collection (ATCC), Manassas, Va., unless otherwise noted.

[0158] Certain oligonucleotide primers used in the following Examples are provided in TABLE 1. All the oligonucleotide primers are synthesized by Sigma-Genosys (Woodlands, Tex.) or Integrated DNA Technologies (IDT) (Coralville, Iowa).

TABLE 1. Oligonucleotide Primers

Primer Name	SEQ ID NO
BK505	10
BK506	11
LA468	12
LA492	13
AK109-1	14

AK109-2	15
AK109-3	16
oBP452	17
oBP453	18
oBP454	19
oBP455	20
oBP456	21
oBP457	22
oBP458	23
oBP459	24
oBP460	25
LA135	26
oBP461	27
LA92	28
LA678	30
LA679	31
LA337	32
LA692	33
LA693	34
LA722	36
LA733	37
LA453	38
LA694	39
LA695	40
oBP594	41
oBP595	42
oBP596	43
oBP597	44
oBP598	45
oBP599	46
oBP600	47
oBP601	48
oBP602	49

oBP603	50
LA811	51
LA817	52
LA812	53
LA818	54
LA512	55
LA513	56
LA516	57
LA514	58
LA515	59
LA829	61
LA834	62
N1257	63
LA830	64
LA850	66
LA851	67
N1262	68
LA740	69
N1263	70
LA855	72
LA856	73
LA414	74
LA749	75
LA413	76
LA860	77
N1093	78
LA681	79

Construction of strains used in the the Examples

Construction of PNY2068

[0159] *Saccharomyces cerevisiae* strain PNY0827 is used as the host cell for further genetic manipulation. PNY0827 refers to a strain derived from *Saccharomyces cerevisiae* which has been deposited at the ATCC under the Budapest Treaty on

September 22, 2011 at the American Type Culture Collection, Patent Depository 10801 University Boulevard, Manassas, VA 20110-2209 and has the patent deposit designation PTA-12105.

Deletion of URA3 and sporulation into haploids

[0160] In order to delete the endogenous URA3 coding region, a deletion cassette was PCR-amplified from pLA54 (SEQ ID NO: 9) which contains a P_{TEF1}-*kanMX4*-*TEF1*t cassette flanked by loxP sites to allow homologous recombination in vivo and subsequent removal of the KANMX4 marker. PCR was done by using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and primers BK505 (SEQ ID NO: 10) and BK506 (SEQ ID NO: 11). The *URA3* portion of each primer was derived from the 5' region 180bp upstream of the *URA3* ATG and 3' region 78bp downstream of the coding region such that integration of the *kanMX4* cassette results in replacement of the *URA3* coding region. The PCR product was transformed into PNY0827 using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on YEP medium supplemented 2% glucose and 100 µg/ml Geneticin at 30°C. Transformants were screened by colony PCR with primers LA468 (SEQ ID NO: 12) and LA492 (SEQ ID NO: 13) to verify presence of the integration cassette. A heterozygous diploid was obtained: NYLA98, which has the genotype MATa/α URA3/*ura3*::loxP-*kanMX4*-loxP. To obtain haploids, NYLA98 was sporulated using standard methods (Codón AC, Gasent-Ramírez JM, Benítez T. Factors which affect the frequency of sporulation and tetrad formation in *Saccharomyces cerevisiae* baker's yeast. Appl Environ Microbiol. 1995 PMID: 7574601). Tetrads were dissected using a micromanipulator and grown on rich YPE medium supplemented with 2% glucose. Tetrads containing four viable spores were patched onto synthetic complete medium lacking uracil supplemented with 2% glucose, and the mating type was verified by multiplex colony PCR using primers AK109-1 (SEQ ID NO: 14), AK109-2 (SEQ ID NO: 15), and AK109-3 (SEQ ID NO: 16). The resulting indentified haploid strain called NYLA103, which has the genotype: MATα *ura3*Δ::loxP-*kanMX4*-loxP, and NYLA106, which has the genotype: MATa *ura3*Δ::loxP-*kanMX4*-loxP.

Deletion of His3

[0161] To delete the endogenous HIS3 coding region, a scarless deletion cassette was used. The four fragments for the PCR cassette for the scarless *HIS3* deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, CA). *HIS3* Fragment A was amplified with primer oBP452 (SEQ ID NO: 17) and primer oBP453 (SEQ ID NO: 18), containing a 5' tail with homology to the 5' end of *HIS3* Fragment B. *HIS3* Fragment B was amplified with primer oBP454 (SEQ ID NO: 19), containing a 5' tail with homology to the 3' end of *HIS3* Fragment A, and primer oBP455 (SEQ ID NO: 20) containing a 5' tail with homology to the 5' end of *HIS3* Fragment U. *HIS3* Fragment U was amplified with primer oBP456 (SEQ ID NO: 21), containing a 5' tail with homology to the 3' end of *HIS3* Fragment B, and primer oBP457 (SEQ ID NO: 22), containing a 5' tail with homology to the 5' end of *HIS3* Fragment C. *HIS3* Fragment C was amplified with primer oBP458 (SEQ ID NO: 23), containing a 5' tail with homology to the 3' end of *HIS3* Fragment U, and primer oBP459 (SEQ ID NO: 24). PCR products were purified with a PCR Purification kit (Qiagen). *HIS3* Fragment AB was created by overlapping PCR by mixing *HIS3* Fragment A and *HIS3* Fragment B and amplifying with primers oBP452 (SEQ ID NO: 17) and oBP455 (SEQ ID NO: 20). *HIS3* Fragment UC was created by overlapping PCR by mixing *HIS3* Fragment U and *HIS3* Fragment C and amplifying with primers oBP456 (SEQ ID NO: 21) and oBP459 (SEQ ID NO: 24). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The *HIS3* ABUC cassette was created by overlapping PCR by mixing *HIS3* Fragment AB and *HIS3* Fragment UC and amplifying with primers oBP452 (SEQ ID NO: 17) and oBP459 (SEQ ID NO: 24). The PCR product was purified with a PCR Purification kit (Qiagen). Competent cells of NYLA106 were transformed with the *HIS3* ABUC PCR cassette and were plated on synthetic complete medium lacking uracil supplemented with 2% glucose at 30 °C. Transformants were screened to verify correct integration by replica plating onto synthetic complete medium lacking histidine and supplemented with 2% glucose at 30°C. Genomic DNA preps were made to verify the integration by PCR using primers oBP460 (SEQ ID NO: 25) and LA135 (SEQ ID NO: 26) for the 5' end and primers oBP461 (SEQ ID NO: 27) and LA92 (SEQ ID NO:

28) for the 3' end. The *URA3* marker was recycled by plating on synthetic complete medium supplemented with 2% glucose and 5-FOA at 30°C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD -URA medium to verify the absence of growth. The resulting identified strain, called PNY2003 has the genotype: MATa *ura3*Δ::loxP-*kanMX4*-loxP *his3*Δ.

Deletion of PDC1

[0162] To delete the endogenous *PDC1* coding region, a deletion cassette was PCR-amplified from pLA59 (SEQ ID NO: 29), which contains a *URA3* marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the *URA3* marker. PCR was done by using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and primers LA678 (SEQ ID NO: 30) and LA679 (SEQ ID NO: 31). The PDC1 portion of each primer was derived from the 5' region 50bp downstream of the *PDC1* start codon and 3' region 50bp upstream of the stop codon such that integration of the *URA3* cassette results in replacement of the *PDC1* coding region but leaves the first 50bp and the last 50bp of the coding region. The PCR product was transformed into PNY2003 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 2% glucose at 30°C. Transformants were screened to verify correct integration by colony PCR using primers LA337 (SEQ ID NO: 32), external to the 5' coding region and LA135 (SEQ ID NO: 26), an internal primer to *URA3*. Positive transformants were then screened by colony PCR using primers LA692 (SEQ ID NO: 33) and LA693 (SEQ ID NO: 34), internal to the *PDC1* coding region. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the *GAL1* promoter and plated on synthetic complete medium lacking histidine and supplemented with 2% glucose at 30°C. Transformants were plated on rich medium supplemented with 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete medium lacking uracil and supplemented with 2% glucose to verify absence of growth. The resulting identified strain, called PNY2008 has the genotype: MATa *ura3*Δ::loxP-*kanMX4*-loxP *his3*Δ *pdc1*Δ::loxP71/66.

Deletion of PDC5

[0163] To delete the endogenous *PDC5* coding region, a deletion cassette was PCR-amplified from pLA59 (SEQ ID NO: 29), which contains a *URA3* marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the *URA3* marker. PCR was done by using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and primers LA722 (SEQ ID NO: 36) and LA733 (SEQ ID NO: 37). The *PDC5* portion of each primer was derived from the 5' region 50bp upstream of the *PDC5* start codon and 3' region 50bp downstream of the stop codon such that integration of the *URA3* cassette results in replacement of the entire PDC5 coding region. The PCR product was transformed into PNY2008 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers LA453 (SEQ ID NO: 38), external to the 5' coding region and LA135 (SEQ ID NO: 26), an internal primer to *URA3*. Positive transformants were then screened by colony PCR using primers LA694 (SEQ ID NO: 39) and LA695 (SEQ ID NO: 40), internal to the *PDC5* coding region. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the *GAL1* promoter and plated on synthetic complete medium lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich YEP medium supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete medium lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, called PNY2009 has the genotype: MATa *ura3*Δ::loxP-*kanMX4*-loxP *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66.

Deletion of FRA2

[0164] The *FRA2* deletion was designed to delete 250 nucleotides from the 3' end of the coding sequence, leaving the first 113 nucleotides of the *FRA2* coding sequence intact. An in-frame stop codon was present 7 nucleotides downstream of the deletion. The four fragments for the PCR cassette for the scarless *FRA2* deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and

CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, CA). *FRA2* Fragment A was amplified with primer oBP594 (SEQ ID NO: 41) and primer oBP595 (SEQ ID NO: 42), containing a 5' tail with homology to the 5' end of *FRA2* Fragment B. *FRA2* Fragment B was amplified with primer oBP596 (SEQ ID NO: 43), containing a 5" tail with homology to the 3' end of *FRA2* Fragment A, and primer oBP597 (SEQ ID NO: 44), containing a 5' tail with homology to the 5' end of *FRA2* Fragment U. *FRA2* Fragment U was amplified with primer oBP598 (SEQ ID NO: 45), containing a 5' tail with homology to the 3' end of *FRA2* Fragment B, and primer oBP599 (SEQ ID NO: 46), containing a 5' tail with homology to the 5' end of *FRA2* Fragment C. *FRA2* Fragment C was amplified with primer oBP600 (SEQ ID NO: 47), containing a 5' tail with homology to the 3' end of *FRA2* Fragment U, and primer oBP601 (SEQ ID NO: 48). PCR products were purified with a PCR Purification kit (Qiagen). *FRA2* Fragment AB was created by overlapping PCR by mixing *FRA2* Fragment A and *FRA2* Fragment B and amplifying with primers oBP594 (SEQ ID NO: 41) and oBP597 (SEQ ID NO: 44). *FRA2* Fragment UC was created by overlapping PCR by mixing *FRA2* Fragment U and *FRA2* Fragment C and amplifying with primers oBP598 (SEQ ID NO: 45) and oBP601 (SEQ ID NO: 48). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The *FRA2* ABUC cassette was created by overlapping PCR by mixing *FRA2* Fragment AB and *FRA2* Fragment UC and amplifying with primers oBP594 (SEQ ID NO: 41) and oBP601 (SEQ ID NO: 48). The PCR product was purified with a PCR Purification kit (Qiagen).

[0165] To delete the endogenous *FRA2* coding region, the scarless deletion cassette obtained above was transformed into PNY2009 using standard techniques and plated on synthetic complete medium lacking uracil and supplemented with 1% ethanol. Genomic DNA preps were made to verify the integration by PCR using primers oBP602 (SEQ ID NO: 49) and LA135 (SEQ ID NO: 26) for the 5' end, and primers oBP602 (SEQ ID NO: 49) and oBP603 (SEQ ID NO: 50) to amplify the whole locus. The *URA3* marker was recycled by plating on synthetic complete medium supplemented with 1% ethanol and 5-FOA (5-Fluoroorotic Acid) at 30°C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto synthetic complete medium lacking uracil and supplemented with 1%

ethanol to verify the absence of growth. The resulting identified strain, PNY2037, has the genotype: MATa *ura3*Δ::loxP-*kanMX4*-loxP *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66 *fra2*Δ.

Addition of 2 micron plasmid

[0166] The loxP71-URA3-loxP66 marker was PCR-amplified using Phusion DNA polymerase (New England BioLabs; Ipswich, MA) from pLA59 (SEQ ID NO: 29), and transformed along with the LA811x817 (SEQ ID NOs: 51, 52) and LA812x818 (SEQ ID NOs: 53, 54) 2-micron plasmid fragments into strain PNY2037 on SE –URA plates at 30°C. The resulting strain PNY2037 2μ::loxP71-URA3-loxP66 was transformed with pLA34 (pRS423::*cre*) (also called, pLA34) (SEQ ID NO: 35) and selected on SE –HIS –URA plates at 30°C. Transformants were patched onto YP-1% galactose plates and allowed to grow for 48 hrs at 30°C to induce Cre recombinase expression. Individual colonies were then patched onto SE –URA, SE –HIS, and YPE plates to confirm URA3 marker removal. The resulting identified strain, PNY2050, has the genotype: MATa *ura3*Δ::loxP-kanMX4-loxP, *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66 *fra2*Δ 2-micron.

Deletion of GPD2

[0167] To delete the endogenous *GPD2* coding region, a deletion cassette was PCR-amplified from pLA59 (SEQ ID NO: 29), which contains a *URA3* marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the *URA3* marker. PCR was done by using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and primers LA512 (SEQ ID NO: 55) and LA513 (SEQ ID NO: 56). The *GPD2* portion of each primer was derived from the 5' region 50bp upstream of the *GPD2* start codon and 3' region 50bp downstream of the stop codon such that integration of the *URA3* cassette results in replacement of the entire *GPD2* coding region. The PCR product was transformed into PNY2050 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers LA516 (SEQ ID NO: 57), external to the 5' coding region and LA135 (SEQ ID NO: 26), internal to

URA3. Positive transformants were then screened by colony PCR using primers LA514 (SEQ ID NO: 58) and LA515 (SEQ ID NO: 59), internal to the *GPD2* coding region. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the *GAL1* promoter and plated on synthetic complete medium lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich medium supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete medium lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, PNY2056, has the genotype: MATa *ura3*Δ::loxP-kanMX4-loxP *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66 *fra2*Δ 2-micron *gpd2Δ*.

Deletion of YMR226 and integration of AlsS

[0168] To delete the endogenous *YMR226C* coding region, an integration cassette was PCR-amplified from pLA71 (SEQ ID NO: 60), which contains the gene acetolactate synthase from the species *Bacillus subtilis* with a *FBA1* promoter and a *CYC1* terminator, and a *URA3* marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the URA3 marker. PCR was done by using KAPA HiFi from Kapa Biosystems, Woburn, MA and primers LA829 (SEQ ID NO: 61) and LA834 (SEQ ID NO: 62). The *YMR226C* portion of each primer was derived from the first 60bp of the coding sequence and 65bp that are 409bp upstream of the stop codon. The PCR product was transformed into PNY2056 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers N1257 (SEQ ID NO: 63), external to the 5' coding region and LA740 (SEQ ID NO: 69), internal to the *FBA1* promoter. Positive transformants were then screened by colony PCR using primers N1257 (SEQ ID NO: 63) and LA830 (SEQ ID NO: 64), internal to the *YMR226C* coding region, and primers LA830 (SEQ ID NO: 64), external to the 3' coding region, and LA92 (SEQ ID NO: 28), internal to the *URA3* marker. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the *GAL1* promoter and plated on synthetic complete medium

lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich medium supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete medium lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, PNY2061, has the genotype: MATa *ura3*Δ::loxP-kanMX4-loxP *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66 *fra2*Δ 2-micron *gpd2Δ ymr226c*Δ::P_{FBA1}-*alsS_Bs-CYC1*t-loxP71/66.

Deletion of ALD6 and integration of KivD

[0169] To delete the endogenous *ALD6* coding region, an integration cassette was PCR-amplified from pLA78 (SEQ ID NO: 65), which contains the *kivD* gene from the species *Listeria grayi* with a hybrid *FBA1* promoter and a *TDH3* terminator, and a *URA3* marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the *URA3* marker. PCR was done by using KAPA HiFi from Kapa Biosystems, Woburn, MA and primers LA850 (SEQ ID NO: 66) and LA851 (SEQ ID NO: 67). The *ALD6* portion of each primer was derived from the first 65bp of the coding sequence and the last 63bp of the coding region. The PCR product was transformed into PNY2061 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers N1262 (SEQ ID NO: 68), external to the 5' coding region and LA740 (SEQ ID NO: 69), internal to the *FBA1* promoter. Positive transformants were then screened by colony PCR using primers N1263 (SEQ ID NO: 70), external to the 3' coding region, and LA92 (SEQ ID NO: 28), internal to the *URA3* marker. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the *GAL1* promoter and plated on synthetic complete medium lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich medium supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete medium lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, PNY2065, has the genotype: MATa *ura3*Δ::loxP-kanMX4-loxP *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66 *fra2*Δ 2-micron

gpd2Δ *ymr226c*Δ::P_{FBA1}-*alsS_Bs-CYC1*t-loxP71/66 *ald6*Δ::(UAS)*PGK1*-P_{FBA1}-*kivD_Lg-TDH3*t-loxP71.

Deletion of ADH1 and integration of ADH

[0170] ADH1 is the endogenous alcohol dehydrogenase present in *Saccharomyces cerevisiae*. As described below, the endogenous ADH1 was replaced with alchol dehydrogenase (ADH) from *Beijerinckii indica*.

[0171] To delete the endogenous ADH1 coding region, an integration cassette was PCR-amplified from pLA65 (SEQ ID NO: 71), which contains the alcohol dehydrogenase from the species *Beijerinckii indica* with an *ILV5* promoter and a *ADH1* terminator, and a *URA3* marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the *URA3* marker. PCR was done by using KAPA HiFi from Kapa Biosystems, Woburn, MA and primers LA855 (SEQ ID NO: 72) and LA856 (SEQ ID NO: 73). The *ADH1* portion of each primer was derived from the 5' region 50bp upstream of the *ADH1* start codon and the last 50bp of the coding region. The PCR product was transformed into PNY2065 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers LA414 (SEQ ID NO: 74), external to the 5' coding region and LA749 (SEQ ID NO: 75), internal to the *ILV5* promoter. Positive transformants were then screened by colony PCR using primers LA413 (SEQ ID NO: 76), external to the 3' coding region, and LA92 (SEQ ID NO: 28), internal to the *URA3* marker. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the *GAL1* promoter and plated on synthetic complete medium lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich medium supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete medium lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, called PNY2066 has the genotype: MATa *ura3*Δ::loxP-kanMX4-loxP *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66 *fra2*Δ 2-micron *gpd2Δ ymr226c*Δ::P_{FBA1}-*alsS_Bs-*

*CYC1*t-loxP71/66 *ald6*Δ::(UAS)*PGK1*-P_{FBA1}-*kivD_Lg-TDH3*t-loxP71/66 *adh1*Δ::P_{ILV5}-*ADH_Bi*(y)-*ADH1*t-loxP71/66.

Integration of ADH into pdc1Δ locus

[0172] To integrate an additional copy of *ADH* at the *pdc1*Δ region, an integration cassette was PCR-amplified from pLA65 (SEQ ID NO: 71), which contains the alcohol dehydrogenase from the species *Beijerinckii indica* with an *ADH1* terminator, and a *URA3* marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the *URA3* marker. PCR was done by using KAPA HiFi from Kapa Biosystems, Woburn, MA and primers LA860 (SEQ ID NO: 77) and LA679 (SEQ ID NO: 31). The *PDC1* portion of each primer was derived from the 5' region 60bp upstream of the *PDC1* start codon and 50bp that are 103bp upstream of the stop codon. The endogenous *PDC1* promoter was used. The PCR product was transformed into PNY2066 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers LA337 (SEQ ID NO: 32), external to the 5' coding region and N1093 (SEQ ID NO: 78), internal to the BiADH gene. Positive transformants were then screened by colony PCR using primers LA681 (SEQ ID NO: 79), external to the 3' coding region, and LA92 (SEQ ID NO: 28), internal to the *URA3* marker. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the *GAL1* promoter and plated on synthetic complete medium lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich medium supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete medium lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, called PNY2068 has the genotype: MATa *ura3*Δ::loxP-kanMX4-loxP *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66 *fra2*Δ 2-micron *gpd2Δ* *ymr226c*Δ::P_{FBA1}-*alsS_Bs-CYC1*t-loxP71/66 *ald6*Δ::(UAS)*PGK1*-P_{FBA1}-*kivD_Lg-TDH3*t-loxP71/66 *adh1*Δ::P_{ILV5}-*ADH_Bi*(y)-*ADH1*t-loxP71/66 *pdc1*Δ::P_{PDC1}-*ADH_Bi*(y)-*ADH1*t-loxP71/66.

Construction of isobutanologen strain PNY2270

[0173] Strain PNY2270 was created from strain PNY2068 (described above) by transformation of the cells with two plasmids, pHR81-ILV5p-K9SB2 (SEQ ID NO: 80) and pYZ067DkivDDhADH (SEQ ID NO: 81). Plasmids were introduced by lithium acetate transformation method (Methods in Yeast Genetics, 2005, page 113), and transformants were selected on synthetic complete medium, minus histidine and uracil, with 1% ethanol as carbon source. Transformants were then transferred to plates containing synthetic complete medium, minus histidine and uracil, with 2% glucose as carbon source and either ethanol (0.05%) or acetate (2 mM) as a C2 supplement.

[0174] pHR81-ILV5p-K9SB2 (SEQ ID NO: 80) contains *A. caccae* K9SB2 KARI gene driven by ILV5 promoter and ILV5 terminator in pHR81 plasmid backbone. pYZ067DkivDDhADH (SEQ ID NO: 81) contains *S. mutans ilvD* gene driven by the FBA1 promoter and FBA1 terminator in pRS423 plasmid backbone.

Construction of isobutanologen strain PNY2092

[0175] Strain PNY2092 was constructed by plasmid transformation of the base strain PNY2061 (described above) which has genotype: MATa *ura3*Δ::loxP-kanMX4-loxP *his3*Δ *pdc1*Δ::loxP71/66 *pdc5*Δ::loxP71/66 *fra2*Δ 2-micron *gpd2Δ ymr226c*Δ::P_{FBA1}-*alsS_Bs-CYC1*t-loxP71/66 with plasmids: pHR81-ILV5p-R8B2y2 (SEQ ID NO: 82) and pLA84 (SEQ ID NO: 83).

[0176] pHR81-ILV5p-R8B2y2 (SEQ ID NO: 82) contains *P. fluorescens* R8B2 KARI (codon-optimized for yeast) driven by ILV5 promoter and ILV5 terminator in pHR81 plasmid backbone. pLA84 (SEQ ID NO: 83) contains IlvD from *S. mutans* driven by FBA1 promoter and FBA1 terminator, ADH from *B. indica* driven by GPM1 promoter and ADH1 terminator and KivD from *L. grayi* driven by TDH3 promoter and TDH3 terminator in pRS423 plasmid backbone.

Construction of isobutanologen strains PNY2118, PNY2120 and PNY2318

Construction of PNY2115 from PNY2050

[0177] Construction of PNY2115 [MATa ura3Δ::loxP his3Δ pdc5Δ::loxP66/71 fra2Δ 2-micron plasmid (CEN.PK2) pdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66 pdc6Δ::(UAS)PGK1-P[FBA1]-KIVD|Lg(y)-TDH3t-loxP71/66 adh1Δ::P[ADH1]-ADH|Bi(y)-ADHt-loxP71/66 fra2Δ::P[ILV5]-ADH|Bi(y)-ADHt-loxP71/66 gpd2Δ::loxP71/66] from PNY2050 was as follows.

Pdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66

[0178] To integrate alsS into the pdc1Δ::loxP66/71 locus of PNY2050 using the endogenous PDC1 promoter, An integration cassette was PCR-amplified from pLA71 (SEQ ID NO: 60), which contains the gene acetolactate synthase from the species *Bacillus subtilis* with a FBA1 promoter and a CYC1 terminator, and a URA3 marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the URA3 marker. PCR was done by using KAPA HiFi and primers 895 (SEQ ID NO: 84) and 679 (SEQ ID NO: 31). The PDC1 portion of each primer was derived from 60bp of the upstream of the coding sequence and 50bp that are 53bp upstream of the stop codon. The PCR product was transformed into PNY2050 using standard genetic techniques and transformants were selected on synthetic complete media lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers 681 (SEQ ID NO: 79), external to the 3' coding region and LA92 (SEQ ID NO: 28), internal to the URA3 gene. Positive transformants were then prepped for genomic DNA and screened by PCR using primers N245 (SEQ ID NO: 85) and N246 (SEQ ID NO: 86). The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the GAL1 promoter and plated on synthetic complete media lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich media supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete media lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, called PNY2090 has the genotype MATa ura3Δ::loxP, his3Δ, pdc1Δ::loxP71/66, pdc5Δ::loxP71/66 fra2Δ 2-micron pdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66.

Pdc6Δ::(UAS)PGK1-P[FBA1]-KIVD|Lg(y)-TDH3t-loxP71/66

[0179] To delete the endogenous PDC6 coding region, an integration cassette was PCR-amplified from pLA78 (SEQ ID NO: 65), which contains the kivD gene from the species *Listeria grayi* with a hybrid FBA1 promoter and a TDH3 terminator, and a URA3 marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the URA3 marker. PCR was done by using KAPA

HiFi and primers 896 (SEQ ID NO: 87) and 897 (SEQ ID NO: 88). The PDC6 portion of each primer was derived from 60bp upstream of the coding sequence and 59bp downstream of the coding region. The PCR product was transformed into PNY2090 using standard genetic techniques and transformants were selected on synthetic complete media lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers 365 (SEQ ID NO: 89) and 366 (SEQ ID NO: 90), internal primers to the PDC6 gene. Transformants with an absence of product were then screened by colony PCR N638 (SEQ ID NO: 91), external to the 5' end of the gene, and 740 (SEQ ID NO: 69), internal to the FBA1 promoter. Positive transformants were than the prepped for genomic DNA and screened by PCR with two external primers to the PDC6 coding sequence. Positive integrants would yield a 4720bp product, while PDC6 wild type transformants would yield a 2130bp product. The *URA3* marker was recycled by transforming with pLA34 containing the CRE recombinase under the GAL1 promoter and plated on synthetic complete media lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich media supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete media lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain is called PNY2093 and has the genotype MATa ura3Δ::loxP his3Δ pdc5Δ::loxP71/66 fra2Δ 2-micron pdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66 pdc6Δ::(UAS)PGK1-P[FBA1]-KIVD|Lg(y)-TDH3t-loxP71/66.

Adh1Δ::P[ADH1]-ADH|Bi(y)-ADHt-loxP71/66

[0180] To delete the endogenous ADH1 coding region and integrate BiADH using the endogenous ADH1 promoter, an integration cassette was PCR-amplified from pLA65 (SEQ ID NO: 71), which contains the alcohol dehydrogenase from the species *Beijerinckii* with an ILV5 promoter and a ADH1 terminator, and a URA3 marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the URA3 marker. PCR was done by using KAPA HiFi and primers 856 (SEQ ID NO: 73) and 857 (SEQ ID NO: 110). The ADH1 portion of each primer was derived from the 5' region 50 bp upstream of the ADH1 start codon and the last 50 bp of the coding region. The PCR product was transformed into PNY2093 using

standard genetic techniques and transformants were selected on synthetic complete media lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers BK415 (SEQ ID NO: 92), external to the 5' coding region and N1092 (SEQ ID NO: 93), internal to the BiADH gene. Positive transformants were then screened by colony PCR using primers 413 (SEQ ID NO: 76), external to the 3' coding region, and 92 (SEQ ID NO: 28), internal to the URA3 marker. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the GAL1 promoter and plated on synthetic complete media lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich media supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete media lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, called PNY2101 has the genotype MATa ura3Δ::loxP his3Δ pdc5Δ::loxP71/66 fra2Δ 2-micron pdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66 pdc6Δ::(UAS)PGK1-P[FBA1]-KIVD|Lg(y)-TDH3t-loxP71/66 adh1Δ::P[ADH1]-ADH|Bi(y)-ADHt-loxP71/66.

Fra2Δ::P[ILV5]-ADH|Bi(y)-ADHt-loxP71/66

[0181] To integrate BiADH into the fra2Δ locus of PNY2101, an integration cassette was PCR-amplified from pLA65 (SEQ ID NO: 71), which contains the alcohol dehydrogenase from the species *Beijerinckii indica* with an ILV5 promoter and an ADH1 terminator, and a URA3 marker flanked by degenerate loxP sites to allow homologous recombination *in vivo* and subsequent removal of the URA3 marker. PCR was done by using KAPA HiFi and primers 906 (SEQ ID NO: 94) and 907 (SEQ ID NO: 95). The FRA2 portion of each primer was derived from the first 60bp of the coding sequence starting at the ATG and 56bp downstream of the stop codon. The PCR product was transformed into PNY2101 using standard genetic techniques and transformants were selected on synthetic complete media lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers 667 (SEQ ID NO: 96), external to the 5' coding region and 749 (SEQ ID NO: 75), internal to the ILV5 promoter. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the

CRE recombinase under the GAL1 promoter and plated on synthetic complete media lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich media supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete media lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, called PNY2110 has the genotype MATa ura3Δ::loxP his3Δ pdc5Δ::loxP66/71 2-micron pdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66 pdc6Δ::(UAS)PGK1-P[FBA1]-KIVD|Lg(y)-TDH3t-loxP71/66 adh1Δ::P[ADH1]-ADH|Bi(y)-ADHt-loxP71/66 fra2Δ::P[ILV5]-ADH|Bi(y)-ADHt-loxP71/66.

GPD2 deletion

[0182] To delete the endogenous *GPD2* coding region, a deletion cassette was PCR amplified from pLA59 (SEQ ID NO: 29), which contains a *URA3* marker flanked by degenerate loxP sites to allow homologous recombination in vivo and subsequent removal of the *URA3* marker. PCR was done by using KAPA HiFi and primers LA512 (SEQ ID NO: 55) and LA513 (SEQ ID NO: 56). The *GPD2* portion of each primer was derived from the 5'region 50bp upstream of the *GPD2* start codon and 3' region 50bp downstream of the stop codon such that integration of the *URA3* cassette results in replacement of the entire *GPD2* coding region. The PCR product was transformed into PNY2110 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 1% ethanol at 30°C. Transformants were screened to verify correct integration by colony PCR using primers LA516 (SEQ ID NO: 57) external to the 5' coding region and LA135 (SEQ ID NO: 26), internal to *URA3*. Positive transformants were then screened by colony PCR using primers LA514 (SEQ ID NO: 58) and LA515 (SEQ ID NO: 59), internal to the *GPD2* coding region. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO: 35) containing the CRE recombinase under the *GAL1* promoter and plated on synthetic complete medium lacking histidine and supplemented with 1% ethanol at 30°C. Transformants were plated on rich medium supplemented with 1% ethanol and 0.5% galactose to induce the recombinase. Marker removal was confirmed by patching colonies to synthetic complete medium lacking uracil and supplemented with 1% ethanol to verify absence of growth. The resulting identified strain, called PNY2115,

has the genotype MATa ura3Δ::loxP his3Δ pdc5Δ::loxP66/71 fra2Δ 2-micron pdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66 pdc6Δ::(UAS)PGK1-P[FBA1]-KIVD|Lg(y)-TDH3t-loxP71/66 adh1Δ::P[ADH1]-ADH|Bi(y)-ADHt-loxP71/66 fra2Δ::P[ILV5]-ADH|Bi(y)-ADHt-loxP71/66 gpd2Δ::loxP71/66.

Construction of PNY2145 from PNY2115

[0183] PNY2145 was constructed from PNY2115 by the additional integration of a phosphoketolase gene cassette at the pdc5Δ locus and by replacing the native AMN1 gene with a codon optimized verison of the ortholog from CEN.PK. Integration constructs are further described below.

pdc5Δ::FBA(L8)-xpk1-CYC1t-loxP71/66

[0184] The TEF(M4)-xpk1-CYC1t gene from pRS423::TEF(M4)-xpk1+ENO1-eutD (SEQ ID NO: 111) was PCR amplified using primers N1341 and N1338 (SEQ ID Nos. 112 and 113), generating a 3.1 kb product. The loxP-flanked URA3 gene cassette from pLA59 (SEQ ID NO: 29) was amplified with primers N1033c and N1342 (SEQ ID Nos. 114 and 115), generating a 1.6 kb product. The xpk1 and URA3 PCR products were fused by combining them without primers for an additional 10 cycles of PCR using Phusion DNA polymerase. The resulting reaction mix was then used as a template for a PCR reaction with KAPA Hi Fi and primers N1342 and N1364 (SEQ ID Nos. 115 and 116). A 4.2 kb PCR product was recovered by purification from an electrophoresis agarose gel (Zymo kit). FBA promoter variant L8 (SEQ ID No. 117) was amplified using primers N1366 and N1368 (SEQ ID Nos. 118 and 119). The xpk1::URA3 PCR product was combined with the FBA promoter by additional rounds of PCR. The resulting product was phosphorylated with polynucleotide kinase and ligated into pBR322 that had been digested with EcoRV and treated with calf intestinal phosphatase. The ligation reaction was transformed into E. coli cells (Stbl3 competent cells from Invitrogen). The integration cassette was confirmed by sequencing. To prepare DNA for integration, the plasmid was used as a template in a PCR reaction with Kapa HiFi and primers N1371 and N1372 (SEQ ID Nos. 120 and 121). The PCR product was isolated by phenol-chloroform extraction and ethanol precipitation (using standard methods; eg. Maniatas, et al.). Five micrograms of DNA were used to

transform strain PNY2115. Transformants were selected on medium lacking uracil (synthetic complete medium minus uracil with 1% ethanol as the carbon source). Colonies were screened for the integration event using PCR (JumpStart) with primers BK93 and N1114 (SEQ ID Nos. 122 and 123). Two clones were selected to carry forward. The *URA3* marker was recycled by transforming with pJT254 (SEQ ID NO: 97) containing the CRE recombinase under the *GAL1* promoter and plating on synthetic complete medium lacking histidine and supplemented with 1% ethanol at 30 °C. Transformants were grown in rich medium supplemented with 1% ethanol to derepress the recombinase. Marker removal was confirmed for single colony isolates by patching to synthetic complete medium lacking uracil and supplemented with 1% ethanol to verify absence of growth. Loss of the recombinase plasmid, pJT254, was confirmed by patching the colonies to synthetic complete medium lacking histidine and supplemented with 1% ethanol. Proper marker removal was confirmed by PCR (primers N160SeqF5 (SEQ ID NO: 124) and BK380 (SEQ ID NO: 125). One resulting clone was designated PNY2293.

amn1Δ::AMN1(y)-loxP71/66

[0185] To replace the endogenous copy of *AMN1* with a codon-optimized version of the *AMN1* gene from CEN.PK2, an integration cassette containing the CEN.PK *AMN1* promoter, *AMN1(y)* gene (nucleic acid SEQ ID NO: 98; amino acid SEQ ID NO: 99 translation), and CEN.PK *AMN1* terminator was assembled by SOE PCR and subcloned into the shuttle vector pLA59. The *AMN1(y)* gene was ordered from DNA 2.0 with codon-optimization for *S. cerevisiae*. The completed pLA67 plasmid (SEQ ID NO: 100) contained: 1) pUC19 vector backbone sequence containing an *E. coli* replication origin and ampicillin resistance gene; 2) *URA3* selection marker flanked by loxP71 and loxP66 sites; and 3) $P_{AMN1(CEN.PK)}$-*AMN1(y)*-$term_{AMN1(CEN.PK)}$ expression cassette

[0186] PCR amplification of the *AMN1(y)*-loxP71-*URA3*-loxP66 cassette was done by using KAPA HiFi from Kapa Biosystems, Woburn, MA and primers LA712 (SEQ ID NO: 101) and LA746 (SEQ ID NO: 102). The PCR product was transformed into PNY2293 using standard genetic techniques and transformants were selected on synthetic complete medium lacking uracil and supplemented with 1% ethanol at 30 °C. Transformants were observed under magnification for the absence of a clumping

phenotype with respect to the control (PNY2293). The URA3 marker was recycled using the pJT254 Cre recombinase plasmid as described above. After marker recycle, clones were again observed under magnification to confirm absence of the clumping phenotype. A resulting identified strain, PNY2145, has the genotype: MATa ura3Δ::loxP his3Δ pdc5Δ::P[FBA(L8)]-XPK|xpk1_Lp-CYCt-loxP66/71 fra2Δ 2-micron plasmid (CEN.PK2) pdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66 pdc6Δ::(UAS)PGK1-P[FBA1]-KIVD|Lg(y)-TDH3t-loxP71/66 adh1Δ::P[ADH1]-ADH|Bi(y)-ADHt-loxP71/66 fra2Δ::P[ILV5]-ADH|Bi(y)-ADHt-loxP71/66 gpd2Δ::loxP71/66 amn1Δ::AMN1(y)

[0187] PNY2118 and PNY2120 were both constructed by transforming yeast-E. coli shuttle vectors into strain PNY2115. Plasmid transformants were selected by plating cells on synthetic complete medium without uracil or histidine containing 1% ethanol (v/v) as the sole carbon source. PNY2118 is a clone that received plasmids pYZ067ΔkivDΔhADH (SEQ ID NO: 103), described in PCT Pub. No. WO2012/129555 and pHR81-ILV5p-K9JB4P (SEQ ID NO: 104). PNY2120 is a clone that received plasmids pHR81-ILV5p-K9SB2-SH (SEQ ID NO: 105) and pYZ067ΔkivDΔhADH. The pHR81-ILV5p-K9JB4P and pHR81-ILV5p-K9SB2-SH plasmids are based on pHR81 (available from ATCC, #87541, Manassas, VA) and contain a gene for expression of KARI (variants K9JB4P, SEQ ID NO: 106 nt and SEQ ID NO: 107 protein; and K9SB2-SH, SEQ ID NO: 126 respectively). Plasmid pYZ067ΔkivDΔhADH was derived from pRS423 (available from ATCC, # 77104) and contains a gene for expression of DHAD.

[0100] PNY2318 was constructed by transforming PNY2145 with plasmids pLH689-L2V4 (SEQ ID 108) and pRS413::BiADH-kivD (SEQ ID 109). Transformants were obtained as described above for PNY2118 and PNY2120. Plasmid pLH689-L2V4 is based on pHR81 and contains genes for the expression of KARI (K9JB4P variant, amino acid SEQ ID NO: 107; under control of the ILV5 promoter) and DHAD (L2V4 variant comprising C-terminal Lumio® tag, amino acid SEQ ID NO: 127; under control of the TEF(M7) promoter). Plasmid pRS413::BiADH-kivD is based on pRS413 (ATCC#) and contains genes for expression of BiADH (amino acid SEQ ID NO: 128; under control of the PDC1 promoter) and *L. grayi* kivD (amino acid SEQ ID NO: 129, under control of the PGK(UAS)-FBA1 hybrid promoter).

EXAMPLE 1

Isobutanol production by PNY2270

[0101] The purpose of this example is to demonstrate growth and isobutanol production by strain PNY2270 in a growth medium containing either ethanol or acetate as a C2 supplement.

[0102] PNY2270 was cultured aerobically (10 ml medium in a vented 125 ml flask) in synthetic complete medium with 0.3% glucose and either 2 mM acetate or 0.3% (vol/vol) ethanol at 30°C in a platform shaker (220 rpm). The logarithmic growth rate in the acetate-supplemented medium was 40% higher than with ethanol. Cultures in either medium were grown to an optical density of approximately 2 (as measured using an Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany). Culture medium was then used to inoculate synthetic complete medium with 2% glucose, BME vitamins and either 2 mM acetate or 0.05% (vol/vol) ethanol in serum vials (10 ml medium in 15 ml vials) to a starting OD of 0.2. The vials were stoppered, crimped and incubated at 30°C in a platform shaker (220 rpm). After 48h, crimps and stoppers were removed, optical densities were measured, and culture filtrates were analyzed by HPLC for isobutanol production.

[0103] The strains grown in the culture medium containing acetate were found to produce isobutanol in higher concentration than the strains grown in the culture medium without acetate (results shown in TABLE 2, below).

TABLE 2. Isobutanol Production by PNY2270

Preculture medium supplement	**Serum vial medium supplement**	**48h Isobutanol concentration (mM)**
0.3% ethanol	0.05% ethanol	42.1 +/- 0.8
2 mM acetate	0.05% ethanol	19 +/- 1
0.3% ethanol	2 mM acetate	60.1 +/- 0.6
2 mM acetate	2 mM acetate	66.5 +/- 0.1

EXAMPLE 2

Isobutanol production by PNY2092

[0104] The purpose of this example is to demonstrate growth and isobutanol production by strain PNY2092 in a growth medium containing either ethanol or acetate as a C2 supplement.

[0105] PNY2092 was cultured aerobically (10 ml medium in a vented 125 ml flask) in synthetic complete medium with 0.3% glucose and either 2 mM acetate or 0.3% (vol/vol) ethanol at 30°C in a platform shaker (220 rpm). The logarithmic growth rate in the acetate-supplemented medium was higher than with ethanol. Cultures in either medium were grown to an optical density of approximately 2 (as measured using an Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany). Culture medium was then used to inoculate synthetic complete medium with 2% glucose, BME vitamins and either 2 mM acetate or 0.05% (vol/vol) ethanol in serum vials (10 ml medium in 15 ml vials) to a starting OD of 0.2. The vials were stoppered, crimped and incubated at 30°C in a platform shaker (220 rpm). After 48h, crimps and stoppers were removed, optical densities were measured, and culture filtrates were analyzed by HPLC.

[0106] The strains grown in the culture medium containing acetate were found to produce isobutanol in higher concentration than the strains grown in the culture medium without acetate (results shown in TABLE 3, below).

TABLE 3. Isobutanol Production by PNY2092

Pre-culture medium supplement	**Serum vial medium supplement**	**48h Isobutanol concentration (mM)**
0.3% ethanol	0.05% ethanol	41.5 +/- 0.4
2 mM acetate	2 mM acetate	45.90 +/- 0.02

EXAMPLE 4

Isobutanol production by strains PNY2118 and PNY2120

[0107] The purpose of this example is to demonstrate isobutanol production by strains PNY2118 and PNY2120 in a medium containing either ethanol or acetate as a C2 supplement.

[0108] Strains were cultured and then evaluated for isobutanol production in serum vials as described above in Example 2 except that samples were collected for analyses at 47.3 hours.

Strain	Pre-culture medium supplement	Serum vial medium supplement	48h Isobutanol concentration (mM)
PNY2118	0.3% ethanol	0.05% ethanol	53.7 ± 0.5
PNY2118	2 mM acetate	2 mM acetate	62.6 ± 0.6
PNY2120	0.3% ethanol	0.05% ethanol	40.8 ± 0.7
PNY2120	2 mM acetate	2 mM acetate	63.7 ± 0.2

[0109]

EXAMPLE 5

Isobutanol production by PNY2318

[0110] The purpose of this example is to demonstrate isobutanol production by strain PNY2318 in a medium containing either ethanol or acetate as a C2 supplement.

[0111] Strains were cultured and then evaluated for isobutanol production in serum vials similar the experiments described above (Examples 2 and 3). In this case, PNY2318 does not require C2-supplementation for growth. Thus strains were cultured with or without C2 and then inoculated into serum vials, again with or without C2. Samples were collected for analyses at 36 hours.

Pre-culture medium supplement	Serum vial medium supplement	36h Isobutanol concentration (mM)	Isobutyric acid yield (mole/mole)
none	none	59.6 ± 0.6	0.021 ± 0.000
none	2 mM acetate	57.3 ± 1.7	0.009 ± 0.001
none	0.05% ethanol	60.2 ± 0.4	0.015 ± 0.001
2 mM acetate	2 mM acetate	60.1 ± 5.3	0.009 ± 0.003
2 mM acetate	none	63.4 ± 1.2	0.019 ± 0.000
0.3% ethanol	0.05% ethanol	58.2 ± 0.7	0.014 ± 0.001
0.3% ethanol	none	59.6 ± 1.0	0.017 ± 0.02

WHAT IS CLAIMED IS:

1. A method for producing butanol comprising:

a) providing a recombinant host cell comprising:

i) an engineered butanol biosynthetic pathway; and

b) contacting the host cell of a) with a fermentation medium comprising:

i) a fermentable carbon substrate; and

ii) acetate in an amount sufficient for at least one of improved growth of the host cell or for improved butanol production, wherein the acetate is added to the fermentation medium;

wherein said recombinant host cell has been engineered to reduce or eliminate pyruvate decarboxylase (PDC) activity; and

whereby butanol is produced directly from the fermentable carbon substrate via the engineered butanol biosynthetic pathway.

2. The method according to claim 1, wherein at least one endogenous gene encoding a pyruvate decarboxylase enzyme is inactivated.

3. The method according to claim 2, wherein said endogenous gene is PDC1, PDC5, PDC6 or a combination thereof.

4. The method of any one of the previous claims wherein the host cell has been engineered or evolved to comprise a reduced or eliminated requirement for exogenous two-carbon substrate supplementation for their growth.

5. The method of any one of the previous claims wherein the host cell comprises a heterologous polynucleotide encoding a polypeptide with phosphoketolase activity and a heterologous polynucleotide encoding a polypeptide with phosphotransacetylase activity.

6. The method according to claim 1, wherein said recombinant host cell has been engineered to reduce or eliminate aldehyde dehydrogenase activity.

7. The method according to claim 6, wherein an endogenous gene encoding an aldehyde dehydrogenase enzyme is inactivated.

8. The method according to claim 7, wherein said endogenous gene is ALD2, ALD3, ALD4, ALD5, ALD6 or a combination thereof.

9. The method according to any one of the previous claims wherein butanol production is improved.

10. The method according to claim 9 wherein butyric acid production is decreased.

11. The method according to claim 9 wherein butanol yield or effective titer is increased.

12. The method according to any one of claims 1-11, wherein the butanol is isobutanol or 1-butanol, or combinations thereof.

13. The method according to any one of claims 1-11, wherein the butanol biosynthetic pathway comprises the following substrate to product conversions:

    a) pyruvate to acetolactate (pathway step a);

    b) the acetolactate from a) to 2,3-dihydroxyisovalerate (pathway step b);

    c) the 2,3-dihydroxyisovalerate from b) to α-ketoisovalerate (pathway step c);

    d) the α-ketoisovalerate from c) to isobutyraldehyde (pathway step d); and

    e) the isobutyraldehyde from d) to isobutanol (pathway step e);

    and wherein

        i) the substrate to product conversion of step a) is performed by an acetolactate synthase enzyme;

        ii) the substrate to product conversion of step b) is performed by an acetohydroxy acid isomeroreductase enzyme;

        iii) the substrate to product conversion of step c) is performed by a dihydroxyacid dehydratase enzyme;

iv) the substrate to product conversion of step d) is performed by an α-ketoacid decarboxylase enzyme; and

v) the substrate to product conversion of step e) is performed by an alcohol dehydrogenase enzyme;

whereby isobutanol is produced directly from pyruvate via the engineered butanol biosynthetic pathway.

14. The method according to any one of claims 1-11, wherein the butanol biosynthetic pathway comprises the following substrate to product conversions:

a) pyruvate to acetolactate (pathway step a);

b) the acetolactate from a) to 2,3-dihydroxyisovalerate (pathway step b);

c) the 2,3-dihydroxyisovalerate from b) to α-ketoisovalerate (pathway step c);

d) the α-ketoisovalerate from c) to isobutyryl-CoA (pathway step f); and

e) the isobutyryl-CoA from d) to isobutyraldehyde (pathway step g);

f) the isobutyraldehyde from e) to isobutanol (pathway step e); and wherein

i) the substrate to product conversion of step a) is performed by an acetolactate synthase enzyme;

ii) the substrate to product conversion of step b) is performed by an acetohydroxy acid isomeroreductase enzyme;

iii) the substrate to product conversion of step c) is performed by a dihydroxyacid dehydratase enzyme;

iv) the substrate to product conversion of step d) is performed by a branched-chain keto acid dehydrogenase enzyme;

v) the substrate to product conversion of step e) is performed by an acelylating aldehyde dehydrogenase enzyme; and

vi) the substrate to product conversion of step f) is performed by an alcohol dehydrogenase enzyme;

whereby isobutanol is produced directly from pyruvate via the engineered biosynthetic pathway.

15. The method according to any one of claims 1-11, wherein the recombinant host cell is a bacterium or a yeast.

16. The method according to claim 15, wherein the recombinant host cell is a yeast and wherein two or more enzymes that perform the substrate to product conversions of the engineered butanol biosynthetic pathway are not localized to mitochondria.

17. The method according to any one of claims 1-16, wherein the fermentation medium further comprises butanol.

18. The method according to any one of claims 1-17, wherein at least a portion of the contacting the fermentation medium with the recombinant host cell is performed under anaerobic or microaerobic conditions.

19. The method according to any one of claims 1-18, wherein the contacting of the fermentation medium with the recombinant host cell is performed as a batch or continuous fermentation.

20. The method according to any one of claims 1-19, further comprising c) recovering the butanol.

21. The method according to claim 20, wherein the recovering is by distillation, liquid-liquid extraction, adsorption, decantation, pervaporation or combinations thereof.

22. Butanol produced from the method according to any one of claims 1-21.

23. A composition comprising:

a) a recombinant host cell comprising:

i) an engineered butanol biosynthetic pathway; and

b) a fermentation medium comprising:

i) a fermentable carbon substrate; and

ii) acetate in an amount sufficient for improved growth of the host cell of a) or for improved butanol production, wherein the acetate is added to the fermentation medium

wherein said recombinant host cell has been engineered to reduce or eliminate pyruvate decarboxylase (PDC) activity and, optionally, aldehyde dehydrogenase activity.

24. The composition according to claim 23, wherein at least one endogenous gene encoding a pyruvate decarboxylase enzyme is inactivated.

25. The composition according to claim 23, wherein an endogenous gene encoding an aldehyde dehydrogenase enzyme is inactivated.

26. The composition according to claim 25, wherein said endogenous gene is ALD2, ALD3, ALD4, ALD5, ALD6 or a combination thereof.

27. The composition according to claim 26, wherein said endogenous gene is PDC1, PDC5, PDC6 or a combination thereof.

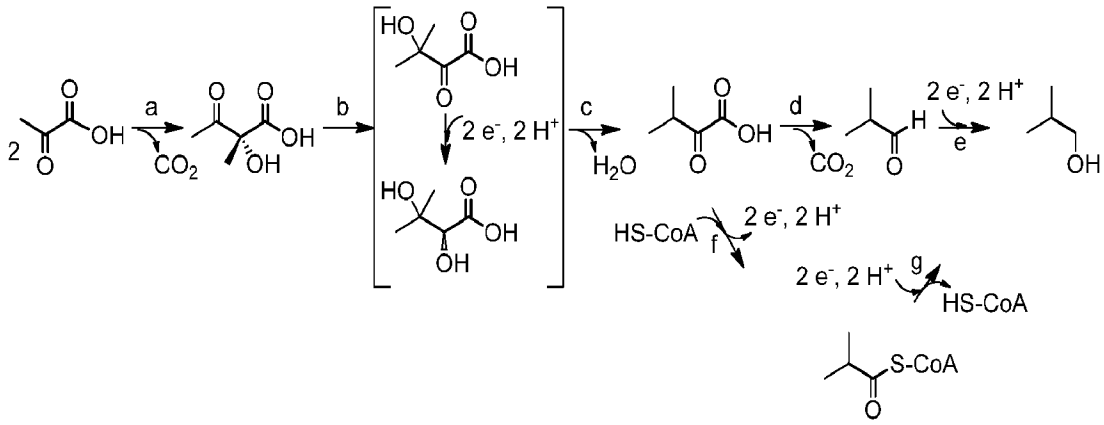
28. The composition according to any one of claims 23-27, wherein the recombinant host cell is a bacteria or a yeast.

29. The composition according to any one of claims 23-27, wherein the fermentation medium further comprises butanol.

30. The composition according to claim 29, wherein the butanol is isobutanol or 1-butanol, or combinations thereof.

31. The composition according to claim 29 or 30, wherein the fermentation medium comprises butanol in the range of about is 0.01mM to about 500mM.

32. The composition according to any one of claims 23-31, wherein the fermentation medium comprises acetate in the range of about 0.1mM to about 50mM.


2
OH
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a
CO2
OH
b
HO
OH
2 e-, 2 H+
c
H2O
d
CO2
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e
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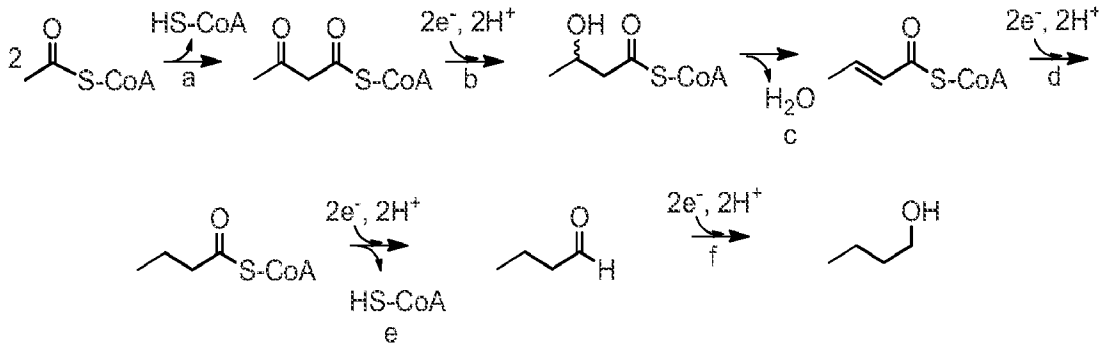
OH
HS-CoA
f
2 e-, 2 H+
2 e-, 2 H+
g
HS-CoA
S-CoA

FIG. 1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HS-CoA
a
S-CoA
2e⁻, 2H⁺
b
OH
S-CoA
H2O
c
S-CoA
2e⁻, 2H⁺
d
S-CoA
2e⁻, 2H⁺
HS-CoA
e
H
2e⁻, 2H⁺
f
OH

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/032159

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/16 C12N1/18 C12N9/04 C12N9/02 C12N9/88
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2010/151525 A1 (GEVO INC [US]; FELDMAN REID M RENNY [US]; GUNAWARDENA UVINI [US]; URAN) 29 December 2010 (2010-12-29) the whole document paragraph [00211] - paragraph [00213] ----- -/--	1-4,9, 11-24, 27-32 5-8,10, 25,26

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 May 2013	06/06/2013

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO 2011/159853 A1 (BUTAMAX TM ADVANCED BIOFUELS [US]; DAUNER MICHAEL [US]; MAGGIO-HALL LO) 22 December 2011 (2011-12-22) abstract paragraphs [0015], [0018], [0171], [0177], [0187]	5-8,10, 25,26
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A	HAZELWOOD LUCIE A ET AL: "The ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces cerevisiae metabolism", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 74, no. 8, April 2008 (2008-04), pages 2259-2266, XP002697280, ISSN: 0099-2240 figure 1 table 1	1-21

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Information on patent family members

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