



- (51) **International Patent Classification:**
C12P 7/10 (2006.01)
- (21) **International Application Number:**
PCT/US2014/028519
- (22) **International Filing Date:**
14 March 2014 (14.03.2014)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
61/801,239 15 March 2013 (15.03.2013) US
- (71) **Applicant:** BUTAMAX ADVANCED BIOFUELS LLC
[US/US]; Route 141 & Henry Clay, Dupont Experimental
Station, Building 356, Wilmington, Delaware 19880-0356
(US).
- (72) **Inventors:** ANTHONY, Larry Cameron; 501 Cory Lane,
Aston, Pennsylvania 19014 (US). DAUNER, Michael; 16
River Way, Wilmington, Delaware 19809 (US).
- (74) **Agents:** LHULIER, Christine M. et al.; Butamax Ad-
vanced Biofuels LLC, Legal Patents Records Center, 974
Centre Road, P.O. Box 2915, Wilmington, Delaware
19805 (US).
- (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, IR, IS, JP, KE, KG, 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, SA,
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,
KM, ML, MR, NE, SN, TD, TG).

Published:

- *without international search report and to be republished
upon receipt of that report (Rule 48.2(g))*
- *with sequence listing part of description (Rule 5.2(a))*

(54) **Title:** COMPETITIVE GROWTH AND/OR PRODUCTION ADVANTAGE FOR BUTANOLOGEN MICROORGANISM

(57) **Abstract:** Provided herein are recombinant yeast host cells and methods for their use for production of fermentation products. Host cells provided herein comprise a pyruvate-utilizing pathway and a competitive growth advantage over other microorganisms in solution.



COMPETITIVE GROWTH AND/OR PRODUCTION ADVANTAGE FOR BUTANOLOGEN MICROORGANISM

REFERENCE TO A SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

[0001] The content of the electronically submitted sequence listing (Name: 20140314_CL5075WOPCT_SequenceListing.txt; Size: 498,299 bytes; and Date of Creation: March 14, 2014) is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The invention relates to the fields of industrial microbiology and alcohol production. The invention also relates to the development of an industrial microorganism capable of producing fermentation products via an engineered pyruvate-utilizing pathway in the microorganism. The invention also relates to the development and use of a butanologen. The invention also relates to the use of inhibitors, antibiotics, and mixtures thereof to give the butanologen a competitive growth and/or production advantage over other organisms in culture in order to increase the yield of fermentation products.

BACKGROUND OF THE INVENTION

[0003] Butanol is an important industrial chemical, useful as a fuel additive, 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.

[0004] Methods for the chemical synthesis of the butanol isomer, isobutanol, are known, such as oxo synthesis, catalytic hydrogenation of carbon monoxide (Ullmann's Encyclopedia of Industrial Chemistry, 6th edition, 2003, Wiley-VCH Verlag GmbH and Co., Weinheim, Germany, Vol. 5, pp. 716-719) 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. The production of isobutanol from plant-derived raw materials could minimize the use of fossil fuels and would represent an advance in the art. Microorganisms capable of fermentative production of isobutanol have been described (for example, in U.S. Patent Nos. 7,851,188 and 7,993,889).

[0005] Isobutanol is produced biologically as a by-product of yeast fermentation. It is a component of "fusel oil" that forms as a result of the incomplete metabolism of amino acids by this group of fungi. Isobutanol may be 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). Microorganisms expressing engineered biosynthetic pathways for producing butanol isomers, including isobutanol, are also described (see U.S. Patent Nos. 7,851,188 and 7,993,889, which are incorporated herein by reference).

SUMMARY OF THE INVENTION

[0006] In some embodiments, the invention is directed to a method for production of a fermentation product in a fermentation process comprising contacting a fermentation mix comprising a recombinant production microorganism which comprises a pyruvate-utilizing pathway with at least one compound which preferentially inhibits at least one contaminant yeast microorganism.

[0007] In some embodiments, the specific growth rate of the at least one contaminant microorganism is reduced more than the specific growth rate of the recombinant production microorganism.

[0008] In some embodiments, production of the fermentation product of the at least one contaminant microorganism is reduced more than production of the fermentation product of the recombinant production microorganism.

[0009] In some embodiments, both the production microorganism and the at least one contaminant microorganism are yeast microorganisms. In some embodiments, the contaminant yeast microorganism is *S. cerevisiae*.

[0010] In some embodiments, the pyruvate utilizing pathway is a butanol biosynthetic pathway. In some embodiments, the pyruvate utilizing pathway is an isobutanol biosynthetic pathway. In some embodiments, the fermentation product of the at least one contaminant microorganism is ethanol.

[0011] In some embodiments, the mechanism of action of the compound that inhibits is heavy metal toxicity, inhibition of amino acid biosynthesis, sulfitolysis, cross-linking, inhibition of ethanol dehydrogenase or inhibition of pyruvate decarboxylase.

[0012] In some embodiments, the inhibitor is an inhibitor of an ethanol biosynthesis pathway. In some embodiments, the inhibitor inhibits pyruvate decarboxylase and/or ethanol dehydrogenase. In some embodiments, the inhibitor comprises a member of the $\text{XC}_6\text{H}_4\text{CH}=\text{CHCOCOOH}$ class of inhibitors/substrate analogues, cinnamaldehydes, glyoxalic acid, ketomalonate, regulatory site inhibitors, p-chloromercuribonzoic acid, 5,5'-dithiobis(2-nitrobenzoic acid), pyrazole, 4-pyrazolecarboxylic acid, 1-H-pyrazole-1-carboxamidinium-HCl, 4-methylpyrazole, 1-bromo-2-butanone, pyrazole-3,5-dicarboxylic acid monohydrate and mixtures thereof. In some embodiments, the inhibitor is selected from the group consisting of fluoroacetate, formaldehyde, sulfite, and mixtures thereof. In some embodiments, the inhibitor is an inhibitor of an amino acid biosynthesis pathway. In some embodiments, the inhibitor is inhibiting at least one enzyme selected from the group consisting of 5-enolpyruvyl-shikimate-3-phosphate synthetase, α -isopropyl malate synthase, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase and mixtures thereof. In some embodiments, the inhibitor is selected from the group consisting of imidazolinone, triazolopyrimidine, pyrimidinyl oxybenzoate, sulfonylurea, sulfonylamino carbonyl triazolinone, glyphosate, trifluoroleucine, fluorophenylalanine and mixtures thereof. In some embodiments, the inhibitor is glyphosate. In some embodiments, the inhibitor is selected from a group consisting of nicosulfuron methyl, metsulfuron methyl, chlorimuron ethyl, sulfometuron methyl, chlorsulfuron, thifensulfuron methyl, and mixtures thereof. In some embodiments, the inhibitor is selected from a group consisting of aureobasiden A, bialaphos, cerulenine, cerulenin, chloramphenicol, cyclohexamide, geneticin, hygromycin B, methotrexate, nourseothricin, phleomycin, triazole, and mixtures thereof. In some embodiments, the inhibitor is selected from a group consisting of bismuth (III), copper (II), and mixtures thereof.

[0013] In some embodiments, the recombinant production microorganism is engineered to express a polypeptide that increases tolerance of the host cell to the at least one compound which preferentially inhibits at least one contaminant microorganism. In some embodiments, the polypeptide comprises an amino acid sequence of at least about 80% identity to SEQ ID NO:9, or an active variant, fragment or derivative of SEQ ID NO:9. In some embodiments, the polypeptide comprises an amino acid sequence of at least about 80% identity to formaldehyde dehydrogenase (SEQ ID NO:7). In some embodiments, the polypeptide is selected from a group consisting of an amino acid sequence of at least about 80% identity to SEQ ID NO:6, an amino acid sequence of at least about 80% identity to SEQ ID NO:7, and mixtures thereof. In some

embodiments, the polypeptide is selected from a group consisting of an amino acid sequence of at least about 80% identity to SEQ ID NO:11, an amino acid sequence of at least about 80% identity to SEQ ID NO:12, and mixtures thereof. In some embodiments, the polypeptide has 3-phosphoshikimate 1-carboxylvinyltransferase activity. In some embodiments, the polypeptide comprises an amino acid sequence of at least about 80% identity to 3-phosphoshikimate 1-carboxylvinyltransferase. In some embodiments, the polypeptide comprises an amino acid sequence of at least about 80% identity to SEQ ID NO:13. In some embodiments, the polypeptide is selected from a group consisting of a polypeptide that has 5-enolpyruvoyl-shikimate-3-phosphate synthetase (ESPS) activity and confers resistance to glyphosate, a polypeptide that has glyphosate N-acetyltransferase activity and confers resistance to glyphosate, and mixtures thereof.

[0014] In some embodiments, the polypeptide is from a bacteria of the family *Enterobacteriaceae*. In some embodiments, the polypeptide is from a bacterial genus selected from the group consisting of: *Alishewanella*, *Alterococcus*, *Aquamonas*, *Aranicola*, *Arsenophonus*, *Azotivirga*, *Blochmannia*, *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Cronobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Ewingella*, *Grimontella*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Leminorella*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Pantoea*, *Candidatus Phlomobacter*, *Photorhabdus*, *Poodoomaamaana*, *Plesiomonas*, *Pragia*, *Proteus*, *Providencia*, *Rahnella*, *Raoultella*, *Salmonella*, *Samsonia*, *Serratia*, *Shigella*, *Sodalis*, *Tatumella*, *Trabulsiella*, *Wigglesworthia*, *Xenorhabdus*, *Yersinia*, and *Yokenella*. In some embodiments, the polypeptide is from a microorganism of the genus *Saccharomyces*.

[0015] In some embodiments, the polypeptide is selected from a group consisting of: a polypeptide that has 5-enolpyruvoyl-shikimate-3-phosphate synthetase (ESPS) activity and confers resistance to glyphosate and a polypeptide that has glyphosate N-acetyltransferase activity and confers resistance to glyphosate. In some embodiments, the polypeptide is encoded by a heterologous polynucleotide.

[0016] In some embodiments, the invention is directed to a genetically modified recombinant production microorganism comprising an engineered pyruvate-utilizing pathway; and a polypeptide that increases tolerance of the host cell to inhibitors, antibiotics, or a combination thereof, wherein the production microorganism has a growth advantage over

contaminant microorganisms that do not produce a desired fermentation product and do not contain said polypeptide.

[0017] In some embodiments, the recombinant production microorganism is selected from the group consisting of bacteria, cyanobacteria, filamentous fungi and yeasts. In some embodiments, the microorganism is a bacterial or cyanobacterial cell. In some embodiments, the genus of the microorganism is selected from the group consisting of *Salmonella*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Clostridium*, *Corynebacterium*, *Gluconobacter*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Streptomyces*, *Zymomonas*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Xanthomonas*, *Saccharomyces*, *Pichia*, *Hansenula*, *Yarrowia*, *Aspergillus*, *Kluyveromyces*, *Pachysolen*, *Rhodotorula*, *Zygosaccharomyces*, *Galactomyces*, *Schizosaccharomyces*, *Torulaspora*, *Debayomyces*, *Williopsis*, *Dekkera*, *Kloeckera*, *Metschnikowia*, and *Candida*.

[0018] In some embodiments, the recombinant production microorganism further comprises one or more polynucleotides that encode one or more 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 1.2.1.57, EC 1.2.1.10, EC 2.6.1.66, EC 2.6.1.42, EC 1.4.1.9, EC 1.4.1.8, EC 4.1.1.14, EC 2.6.1.18, EC 2.3.1.9, EC 2.3.1.16, EC 1.1.1.130, 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, EC 5.4.99.13, EC 4.1.1.5, EC 2.7.1.29, EC 1.1.1.76, EC 1.2.1.57, and EC 4.2.1.28.

[0019] In some embodiments, the recombinant production microorganism has reduced expression of an enzyme having the following Enzyme Commission Number: EC 4.1.1.1 (pyruvate decarboxylase). In some embodiments, microorganism has reduced expression of an enzyme having the following Enzyme Commission Number: EC 1.1.1.1 (ethanol dehydrogenase).

[0020] Some embodiments are directed to a method for the production of a C3-C6 alcohol comprising the recombinant production microorganisms described herein, wherein said engineered pyruvate-utilizing pathway is a C3-C6 alcohol biosynthetic pathway; contacting said recombinant microorganism with a fermentable carbon substrate in a fermentation medium under conditions whereby a C3-C6 alcohol is produced; and recovering said C3-C6 alcohol.

[0021] In some embodiments, the C3-C6 alcohol is produced at a titer from about 5 g/L to about 100 g/L. In some embodiments, the C3-C6 alcohol is produced at a titer of at least 20

g/L. In some embodiments, the C3-C6 alcohol is selected from the group consisting of butanol, isobutanol, propanol, isopropanol, and mixtures thereof.

[0022] Some embodiments are directed to a method for the production of ethanol comprising: providing any recombinant microorganism described herein, wherein said pyruvate-utilizing pathway is an ethanol producing pathway; contacting said recombinant microorganism with a fermentable carbon substrate in a fermentation medium under conditions whereby the ethanol is produced; and recovering said ethanol.

[0023] In some embodiments, the fermentation medium comprises one or more inhibitors, antibiotics, or combinations thereof.

[0024] In some embodiments, the ethanol is produced at a titer from about 80 g/L to about 120 g/L. In some embodiments, the ethanol is produced at a titer of about 120 g/L.

[0025] Some embodiments are directed to a composition comprising any genetically modified recombinant microorganism of the invention, a fermentation medium, and one or more inhibitors, antibiotics or combinations thereof.

[0026] Some embodiments are directed to a method for reducing microbial contamination in a fermentation mix, wherein said method comprises contacting any genetically modified recombinant microorganism of the invention and a fermentation medium with one or more inhibitors, antibiotics, or mixtures thereof, and wherein the addition of more inhibitors, antibiotics, or mixtures thereof results in from about 1 log to about 10 log reduction in contamination. In some embodiments, the fermentation mix is in a propagation tank. In some embodiments, the fermentation mix is in a fermenter.

[0027] In some embodiments, reduction in contamination is measured by standard plating assays, qPCR/RT-PCR, or by measuring improved fermentation yields of desired product.

[0028] Some embodiments are directed to a method for reducing microbial contamination in a fermentation mix, wherein said method comprises contacting any genetically modified recombinant microorganism of the invention and a fermentation medium with one or more inhibitors, antibiotics, or combinations thereof, and wherein the addition of inhibitors, antibiotics, or combinations thereof results in from about 1 log to about 10 log reduction in contamination.

[0029] In some embodiments, the addition of inhibitors, antibiotics, or combinations thereof results in the death of between about 10% and about 100% of the microbial contaminants in the fermentation mix.

[0030] Some embodiments of the invention are directed to a method for reducing microbial contamination in a fermentation mix, wherein said method comprises contacting any genetically modified recombinant microorganism of the invention and a fermentation medium comprising one or more inhibitors, antibiotics, or combinations thereof, and wherein the reduction in contamination is associated with a decrease in ethanol production. Some embodiments are directed to any composition of the invention, wherein the ethanol titer is less than about 5 g/L, or less than about 1 g/L.

[0031] Some embodiments of the invention are directed to a method for reducing microbial contamination in a fermentation mix, wherein said method comprises contacting any genetically modified recombinant microorganism of the invention and a fermentation medium comprising one or more inhibitors, antibiotics, or combinations thereof, and wherein the reduction in contamination is associated with an increase in ethanol production.

[0032] Some embodiments are directed to a method for reducing microbial contamination in a fermentation mix, wherein said method comprises contacting any genetically modified recombinant microorganism of the invention and a fermentation medium comprising one or more inhibitors, antibiotics, or combinations thereof, and wherein the addition of said one or more inhibitors, antibiotics, or combinations thereof results in less than an about 20% loss in the yield of a lower alkyl alcohol produced by said host cell due to the presence of microbial contaminants.

[0033] In some embodiments, the addition of said one or more inhibitors, antibiotics, or combinations thereof results in less than an about 10% loss in the yield of a lower alkyl alcohol produced by said host cell due to the presence of microbial contaminants. In some embodiments, the C3-C6 alcohol or ethanol produced is a gasoline fuel component.

[0034] Some embodiments are directed to a gasoline blend comprising about 5 to about 20% of the C3-C6 alcohol produced by the recombinant microorganisms described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] Figure 1 depicts different isobutanol biosynthetic pathways. The steps labeled "a", "b", "c", "d", "e", "f", "g", "h", "i", "j", and "k" represent substrate to product conversions described below. "a" may be catalyzed, for example, by acetolactate synthase. "b" may be catalyzed, for example, by ketol-acid reductoisomerase. "c" may be catalyzed, for example, by acetohydroxy acid dehydratase. "d" may be catalyzed, for example, by branched-chain keto acid

decarboxylase. "e" may be catalyzed, for example, by branched chain alcohol dehydrogenase. "f" may be catalyzed, for example, by branched chain keto acid dehydrogenase. "g" may be catalyzed, for example, by acetylating aldehyde dehydrogenase. "h" may be catalyzed, for example, by transaminase or valine dehydrogenase. "i" may be catalyzed, for example, by valine decarboxylase. "j" may be catalyzed, for example, by omega transaminase. "k" may be catalyzed, for example by isobutyryl-CoA mutase.

[0036] Figure 2 depicts a growth inhibition assay measuring the ability of *S. cerevisiae* PNY0860-1A, PNY 827, and CEN.PK113-7D to grow in the presence of AHAS inhibitors.

[0037] Figure 3 depicts the production of isobutanol and isobutyric acid as a function of time for the strain NYLA84.

[0038] Figure 4 depicts the μ_{max} of PNY 827 in dependence on concentration of copper(2+) in the medium.

[0039] Figure 5 depicts the μ_{max} of PNY 827 in dependence on concentration of sulfometuron methyl in the medium.

[0040] Figure 6 depicts the μ_{max} of PNY 827 in dependence on concentration of sulfite in the medium.

[0041] Figure 7 depicts the μ_{max} of PNY 827 in dependence on concentration of formaldehyde in the medium.

[0042] Figure 8 depicts μ_{max} of PNY 827 in dependence on concentration of 4-pyrazolecarboxylic acid in the medium.

[0043] Figure 9 depicts μ_{max} of PNY 827 in dependence on concentration of 4-methylpyrazole hydrochloride in the medium.

[0044] Figure 10 depicts μ_{max} of PNY 827 in dependence on concentration of glyoxylic acid in the medium.

[0045] Figure 11 depicts μ_{max} of PNY 827 in dependence on concentration of pyrazole in the medium.

[0046] Figure 12 depicts μ_{max} of PNY 827 in dependence on concentration of cinnamaldehyde in the medium.

[0047] Figure 13 depicts μ_{max} of PNY 827 in dependence on concentration of 1-bromo-2-butanone in the medium.

[0048] Figure 14 depicts the ratio of the produced molar butanol to ethanol concentration at EPT = 8 h in mixed cultures inoculated in an OD600 ratio of 1 ethanologen strain PNY 827 to

11 butanologen strain PNY 2129 in cultures without addition of an inhibitor ("Ctrl") and trans-cinnamaldehyde concentrations of 250 μ M (A) and 25 mM (B), 1-bromo-2-butanone concentrations of 2 μ M (A) and 200 μ M (B), and pyrazole concentrations of 3 mM (A) and 30 mM (B).

[0049] Figure 15 depicts the ratio of the produced molar butanol to ethanol concentration at EPT = 8 h in mixed cultures inoculated in an OD600 ratio of 1 ethanologen strain PNY 827 to 1 butanologen strain PNY 2129 in cultures without addition of an inhibitor ("Ctrl") and trans-cinnamaldehyde at concentrations of 250 μ M (A) and 25 mM (B), 1-bromo-2-butanone at concentrations of 2 μ M (A) and 200 μ M (B), and pyrazole at concentrations of 3 mM (A) and 30 mM (B).

[0050] Figure 16 depicts the ratio of the produced molar butanol to ethanol concentration at EPT = 48 h in mixed cultures inoculated in an OD600 ratio of 1 ethanologen strain PNY 827 to 11 butanologen strain PNY 2129 in cultures without addition of an inhibitor ("Ctrl") and trans-cinnamaldehyde at concentrations of 250 μ M (A) and 25 mM (B), 1-bromo-2-butanone at concentrations of 2 μ M (A) and 200 μ M (B), and pyrazole at concentrations of 3 mM (A) and 30 mM (B).

[0051] Figure 17 depicts the ratio of the produced molar butanol to ethanol concentration at EPT = 48 h in mixed cultures inoculated in an OD600 ratio of 1 ethanologen strain PNY 827 to 1 butanologen strain PNY 2129 in cultures without addition of an inhibitor ("Ctrl") and trans-cinnamaldehyde at concentrations of 250 μ M (A) and 25 mM (B), 1-bromo-2-butanone at concentrations of 2 μ M (A) and 200 μ M (B), and pyrazole at concentrations of 3 mM (A) and 30 mM (B).

[0052] Figure 18 depicts simulated growth curves of strains A and B growing in a mixed culture at a maximum specific growth rate of 0.16 1/h and 0.61 1/h, respectively. The ratio of the biomass of strains A vs. strain B is continuously decreasing during the cultivation and is below 3% at the end of the run.

[0053] Figure 19 depicts the predicted effect of an inhibitor c (compound) on the maximum specific growth rate of a hypothetical strain with a $\mu_{\max}$ without inhibitor addition ($\mu_{\max}^0$) of 1.00 1/h, a K_I -value of 5 mM, and its behavior according a squared inhibition kinetics as described by equation (2)

DETAILED DESCRIPTION

[0054] Competition for carbon substrates in a butanologen fermentation process between the butanologen and contaminant microorganisms, such as, for example ethanol-producing yeast strains. A competitive advantage and/or selective pressure in favor of the butanologen could thus favor high yields of butanol. Such an advantage for a butanologen system may be extended to any organisms competing for the carbon substrate. The same competitive advantage may be desirable for any other recombinant production microorganism, particularly yeast competing with wildtype, ethanologen yeast and/or other microbial communities.

[0055] This invention is directed to methods employing engineered microorganisms that produce fermentation products for industrial uses, and to optimizations for producing such fermentation products at high rates and titers with advantaged economic process conditions.

[0056] Contamination by ethanologen yeast and other microbes can be problematic and can quickly lead to takeover of the fermentation, particularly when the butanologen has a slower growth rate or is otherwise less fit than the ethanologen yeast or microbe.

[0057] Applicants have solved the problem of microbial contamination by ethanologen yeast and other microbes through the use of inhibitors, antibiotics, and mixtures thereof. Butanologen yeasts either have resistance to the inhibitors, antibiotics and mixtures thereof employed, or are engineered to have resistance to the inhibitors, antibiotics, and mixtures thereof employed. The yield of the butanol process when contacted with a carbon substrate may be increased without a buildup of microbial contamination.

[0058] 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.

[0059] In order to further define this invention, the following terms and definitions are herein provided.

[0060] As used herein, the terms "comprises," "comprising," "includes," "including," "has," "having," "contains" or "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).

[0061] 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.

[0062] 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.

[0063] 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.

[0064] 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.

[0065] As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention employed 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

embodiments, the term "about" means within 10% of the reported numerical value, preferably within 5% of the reported numerical value.

[0066] 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).

[0067] The term "fermentation product" includes any desired product of interest, including, but not limited to lactic acid, 3-hydroxy-propionic acid, acrylic acid, acetic acid, succinic acid, citric acid, fumaric acid, malic acid, itaconic acid, 1,3-propane-diol, ethylene, glycerol, isobutyrate, butanol and other lower alkyl alcohols etc.

[0068] The term "fermentation process" refers to any process by which a desired fermentation product is produced.

[0069] The term "specific growth rate", often also referred to as " μ " or "mu", is defined as $\mu = 1/cx * dcx/dt$, representing the change of the biomass concentration cx in an infinitesimal short time interval dt , divided by the biomass concentration at this time.

[0070] The term "maximum specific growth rate", often also referred to as " μ_{max} " or "mumax", refers to the "specific growth rate" ("mu") during the exponential growth phase of a culture. Usually during the exponential growth phase, mu is approximately constant as the substrates are not limiting as well as the produced by-products are still not exerting a significant inhibition on growth.

[0071] The term "lower alkyl alcohol" refers to any straight-chain or branched, saturated or unsaturated, alcohol molecule with 3-6 carbon atoms.

[0072] The term "butanol" refers to 1-butanol, 2-butanol, 2-butanone, isobutanol, or mixtures thereof. Isobutanol is also known as 2-methyl-1-propanol.

[0073] The term "C3-C6 alcohol" refers to any alcohol with 3, 4, 5 or 6 carbons.

[0074] The term "butanol biosynthetic pathway" as used herein refers to an enzyme pathway to produce 1-butanol, 2-butanol, 2-butanone or isobutanol. For example, isobutanol biosynthetic pathways are disclosed in U.S. Patent No. 7,851,188, which is incorporated by reference herein.

[0075] 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" (see U.S. Patent Nos. 7,851,188 and 7,993,889, which are herein incorporated herein by reference).

[0076] The term "1-butanol biosynthetic pathway" refers to an enzymatic pathway to produce 1-butanol. A "1-butanol biosynthetic pathway" can refer to an enzyme pathway to produce 1-butanol from acetyl-coenzyme A (acetyl-CoA). For example, 1-butanol biosynthetic pathways are disclosed in U.S. Patent Application Publication No. 2008/0182308 and International Publication No. WO 2007/041269, which are herein incorporated by reference in their entireties.

[0077] The term "2-butanol biosynthetic pathway" refers to an enzymatic pathway to produce 2-butanol. A "2-butanol biosynthetic pathway" can refer to an enzyme pathway to produce 2-butanol from pyruvate. For example, 2-butanol biosynthetic pathways are disclosed in U.S. Patent No. 8,206,970, U.S. Patent Application Publication No. 2007/0292927, International Publication Nos. WO 2007/130518 and WO 2007/130521, which are herein incorporated by reference in their entireties.

[0078] The term "2-butanone biosynthetic pathway" as used herein refers to an enzymatic pathway to produce 2-butanone (see U.S. Appl. Pub. No. 2007/0259410 and U.S. Appl. Pub. No. 2009/0155870, which are incorporated herein by reference).

[0079] The term "engineered" as used herein refers to an enzymatic pathway that is not present endogenously in a microorganism and is deliberately constructed to produce a fermentation product from a starting substrate through a series of specific substrate to product conversions.

[0080] A "recombinant microbial host cell" or a "recombinant microorganism" 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. A "production microorganism" is any microorganism that produces a desired fermentation product. A "contaminant microorganism" is any microorganism that either does not produce a desired fermentation product or does produce a desired fermentation product, but at lower efficiency (for example, with lower specific productivity, rate, titer or yield) than a production microorganism. It will be appreciated that microorganisms may produce measureable amounts of more than one product, however, for the purposes herein, "product" typically refers to the major product produced by a microorganism.

[0081] 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, monosaccharides, such as glucose or fructose; disaccharides, such as lactose or sucrose; oligosaccharides; polysaccharides, such as starch, cellulose, or lignocellulose, hemicellulose; one-carbon substrates, fatty acids; and any combination of these.

[0082] "Fermentation medium" as used herein means the mixture of water, sugars (fermentable carbon substrates), dissolved solids, fermentation product and all other constituents of the material in which the fermentation product is being made by the reaction of fermentable carbon substrates to fermentation products, water and carbon dioxide (CO₂) by the microorganisms present. From time to time, as used herein the term "fermentation broth", "fermentation mix" and "fermentation mixture" can be used synonymously with "fermentation medium."

[0083] The term "aerobic conditions" as used herein means growth conditions in the presence of oxygen.

[0084] 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.

[0085] The term "anaerobic conditions" as used herein means growth conditions in the absence of oxygen.

[0086] 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, methanol, glucose, fructose, sucrose, xylose, arabinose, dextrose, amino acids, and mixtures thereof.

[0087] The term "sucrose utilizing butanologen" as used herein refers to a microorganism capable of producing butanol from sucrose. Such microorganisms are typically recombinant microorganisms comprising an engineered butanol biosynthetic pathway. "Sucrose utilizing isobutanologen" as used herein refers to a microorganism capable of producing isobutanol from sucrose. Such microorganisms are typically recombinant microorganisms comprising an engineered isobutanol biosynthetic pathway.

[0088] 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 29.7 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.

[0089] The term "effective titer" as used herein, refers to the total amount of C3-C6 alcohol produced by fermentation per liter of fermentation medium. The total amount of C3-C6 alcohol includes: (i) the amount of C3-C6 alcohol in the fermentation medium; (ii) the amount of C3-C6 alcohol recovered from the organic extractant; and (iii) the amount of C3-C6 alcohol recovered from the gas phase, if gas stripping is used.

[0090] The term "effective rate" as used herein, refers to the total amount of C3-C6 alcohol produced by fermentation per liter of fermentation medium per hour of fermentation.

[0091] The term "specific productivity" as used herein, refers to the g of C3-C6 alcohol produced per g of dry cell weight of cells per unit time.

[0092] As used herein the term "coding sequence" refers to a DNA sequence that encodes for a specific amino acid sequence. "Suitable regulatory sequences" refer 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, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector binding site and stem-loop structure.

[0093] The terms "derivative" and "analog" refer to a polypeptide differing from the enzymes of the invention, but retaining essential properties thereof. The term "derivative" may also refer to a host cells differing from the host cells of the invention, but retaining essential properties thereof. Generally, derivatives and analogs are overall closely similar, and, in many regions, identical to the enzymes of the invention. The terms "derived-from", "derivative" and "analog" when referring to enzymes of the invention include any polypeptides which retain at

least some of the activity of the corresponding native polypeptide or the activity of its catalytic domain.

[0094] Derivatives of enzymes disclosed herein are polypeptides which may have been altered so as to exhibit features not found on the native polypeptide. Derivatives can be covalently modified by substitution (e.g. amino acid substitution), chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (e.g., a detectable moiety such as an enzyme or radioisotope). Examples of derivatives include fusion proteins, or proteins which are based on a naturally occurring protein sequence, but which have been altered. For example, proteins can be designed by knowledge of a particular amino acid sequence, and/or a particular secondary, tertiary, and/or quaternary structure. Derivatives include proteins that are modified based on the knowledge of a previous sequence, natural or synthetic, which is then optionally modified, often, but not necessarily to confer some improved function. These sequences, or proteins, are then said to be derived from a particular protein or amino acid sequence. In some embodiments of the invention, a derivative must retain at least 50% identity, at least 60% identity, at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, at least 97% identity, or at least 99% identity to the sequence the derivative is "derived-from." In some embodiments of the invention, an enzyme is said to be derived-from an enzyme naturally found in a particular species if, using molecular genetic techniques, the DNA sequence for part or all of the enzyme is amplified and placed into a new host cell.

Polypeptides and Polynucleotides for Use in the Invention

[0095] 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.

[0096] 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.

[0097] A polypeptide of the invention may be of a size of about 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides may have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides which do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, and are referred to as unfolded.

[0098] Also included as polypeptides of the present invention are derivatives, analogs, or variants of the foregoing polypeptides, and any combination thereof. The terms "active variant," "active fragment," "active derivative," and "analog" refer to polypeptides of the present invention and include any polypeptides that are capable of catalyzing the reduction of a lower alkyl aldehyde. Variants of polypeptides of the present invention include polypeptides with altered amino acid sequences due to amino acid substitutions, deletions, and/or insertions. Variants may occur naturally or be non-naturally occurring. Non-naturally occurring variants may be produced using art-known mutagenesis techniques. Variant polypeptides may comprise conservative or non-conservative amino acid substitutions, deletions and/or additions. Derivatives of polypeptides of the present invention are polypeptides which have been altered so as to exhibit additional features not found on the native polypeptide. Examples include fusion proteins. Variant polypeptides may also be referred to herein as "polypeptide analogs." As used herein a "derivative" of a polypeptide refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Also included as "derivatives" are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For example, 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine.

[0099] A "fragment" is a unique portion of a polypeptide or other enzyme used in the invention which is identical in sequence to but shorter in length than the parent full-length sequence. A fragment may comprise up to the entire length of the defined sequence, minus one amino acid residue. For example, a fragment may comprise from 5 to 1000 contiguous amino acid residues. A fragment may be at least 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 100, 150, 250 or at least 500 contiguous amino acid residues in length. Fragments may be preferentially selected from certain regions of a molecule. For example, a polypeptide fragment may comprise a certain length of contiguous amino acids selected from the first 100 or 200 amino acids of a polypeptide as shown in a certain defined sequence. Clearly these lengths are exemplary, and any length that is supported by the specification, including the Sequence Listing, tables, and figures, may be encompassed by the present embodiments.

[0100] Alternatively, recombinant variants encoding these same or similar polypeptides can be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce various restriction sites, may be introduced to optimize cloning into a plasmid or viral vector or expression in a host cell system.

[0101] Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements, or they can be result of replacing one amino acid with an amino acid having different structural and/or chemical properties, i.e., non-conservative amino acid replacements. "Conservative" amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Alternatively, "non-conservative" amino acid substitutions can be made by selecting the differences in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of any of these amino acids.

"Insertions" or "deletions" are preferably in the range of about 1 to about 20 amino acids, more preferably 1 to 10 amino acids. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide

molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

[0102] By a polypeptide having an amino acid or polypeptide sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

[0103] As a practical matter, whether any particular polypeptide is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to a reference polypeptide can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag *et al.*, *Comp. Appl. Biosci.* 6:237-245 (1990). In a sequence alignment, the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of the global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are:

Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

[0104] If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total

bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

[0105] For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case, the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

[0106] 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.

[0107] 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.

[0108] 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.

[0109] 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.

[0110] 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.

[0111] 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.

[0112] Polynucleotide and nucleic acid coding regions of the present invention may be associated with additional coding regions which encode secretory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide of the present invention.

[0113] 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.

[0114] 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.

[0115] 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.

[0116] The term "artificial" refers to a synthetic, or non-host cell derived composition, e.g., a chemically-synthesized oligonucleotide.

[0117] 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.

[0118] 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.

[0119] 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.

[0120] The term "recombinant genetic expression element" refers to a nucleic acid fragment that expresses one or more specific proteins, including regulatory sequences preceding (5' non-coding sequences) and following (3' termination sequences) coding sequences for the proteins. A chimeric gene is a recombinant genetic expression element. The coding regions of an operon may form a recombinant genetic expression element, along with an operably linked promoter and termination region.

[0121] "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.

[0122] 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.

[0123] 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.

[0124] 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.

[0125] The term "codon-optimized" as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA. Such optimization includes replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of that organism.

[0126] Deviations in the nucleotide sequence that comprise the codons encoding the amino acids of any polypeptide chain allow for variations in the sequence coding for the gene. Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino acids (the remaining three codons encode signals ending translation). The "genetic code" which shows which codons encode which amino acids is reproduced herein as Table 1. As a result, many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six, whereas tryptophan and methionine are coded by just one triplet. This degeneracy allows for DNA base composition to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA.

Table 1. The Standard Genetic Code

	T	C	A	G
T	TTT Phe (F) TTC " TTA Leu (L) TTG "	TCT Ser (S) TCC " TCA " TCG "	TAT Tyr (Y) TAC " TAA Ter TAG Ter	TGT Cys (C) TGC TGA Ter TGG Trp (W)
C	CTT Leu (L) CTC " CTA " CTG "	CCT Pro (P) CCC " CCA " CCG "	CAT His (H) CAC " CAA Gln (Q) CAG "	CGT Arg (R) CGC " CGA " CGG "
A	ATT Ile (I) ATC " ATA " ATG Met (M)	ACT Thr (T) ACC " ACA " ACG "	AAT Asn (N) AAC " AAA Lys (K) AAG "	AGT Ser (S) AGC " AGA Arg (R) AGG "
G	GTT Val (V)	GCT Ala (A)	GAT Asp (D)	GGT Gly (G)

	GTC "	GCC "	GAC "	GGC "
	GTA "	GCA "	GAA Glu (E)	GGA "
	GTG "	GCG "	GAG "	GGG "

[0127] Many organisms display a bias for use of particular codons to code for insertion of a particular amino acid in a growing peptide chain. Codon preference or codon bias, differences in codon usage between organisms, is afforded by degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, *inter alia*, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization.

[0128] Given the large number of gene sequences available for a wide variety of animal, plant and microbial species, it is possible to calculate the relative frequencies of codon usage. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at <http://www.kazusa.or.jp/codon/> (visited June 26, 20012, and these tables can be adapted in a number of ways. See Nakamura, Y., *et al. Nucl. Acids Res.* 28:292 (2000). Codon usage tables for yeast, calculated from GenBank Release 128.0 [15 February 2002], are reproduced below as Table 2. This table uses mRNA nomenclature, and so instead of thymine (T) which is found in DNA, the tables use uracil (U) which is found in RNA. The Table has been adapted so that frequencies are calculated for each amino acid, rather than for all 64 codons.

Table 2. Codon Usage Table for *Saccharomyces cerevisiae* Genes

Amino Acid	Codon	Number	Frequency per thousand
Phe	UUU	170666	26.1
Phe	UUC	120510	18.4
Leu	UUA	170884	26.2
Leu	UUG	177573	27.2
Leu	CUU	80076	12.3
Leu	CUC	35545	5.4
Leu	CUA	87619	13.4
Leu	CUG	68494	10.5
Ile	AUU	196893	30.1
Ile	AUC	112176	17.2

Amino Acid	Codon	Number	Frequency per thousand
Ile	AUA	116254	17.8
Met	AUG	136805	20.9
Val	GUU	144243	22.1
Val	GUC	76947	11.8
Val	GUA	76927	11.8
Val	GUG	70337	10.8
Ser	UCU	153557	23.5
Ser	UCC	92923	14.2
Ser	UCA	122028	18.7
Ser	UCG	55951	8.6
Ser	AGU	92466	14.2
Ser	AGC	63726	9.8
Pro	CCU	88263	13.5
Pro	CCC	44309	6.8
Pro	CCA	119641	18.3
Pro	CCG	34597	5.3
Thr	ACU	132522	20.3
Thr	ACC	83207	12.7
Thr	ACA	116084	17.8
Thr	ACG	52045	8.0
Ala	GCU	138358	21.2
Ala	GCC	82357	12.6
Ala	GCA	105910	16.2
Ala	GCG	40358	6.2
Tyr	UAU	122728	18.8
Tyr	UAC	96596	14.8
His	CAU	89007	13.6
His	CAC	50785	7.8
Gln	CAA	178251	27.3
Gln	CAG	79121	12.1
Asn	AAU	233124	35.7
Asn	AAC	162199	24.8

Amino Acid	Codon	Number	Frequency per thousand
Lys	AAA	273618	41.9
Lys	AAG	201361	30.8
Asp	GAU	245641	37.6
Asp	GAC	132048	20.2
Glu	GAA	297944	45.6
Glu	GAG	125717	19.2
Cys	UGU	52903	8.1
Cys	UGC	31095	4.8
Trp	UGG	67789	10.4
Arg	CGU	41791	6.4
Arg	CGC	16993	2.6
Arg	CGA	19562	3.0
Arg	CGG	11351	1.7
Arg	AGA	139081	21.3
Arg	AGG	60289	9.2
Gly	GGU	156109	23.9
Gly	GGC	63903	9.8
Gly	GGA	71216	10.9
Gly	GGG	39359	6.0
Stop	UAA	6913	1.1
Stop	UAG	3312	0.5
Stop	UGA	4447	0.7

[0129] By utilizing this or similar tables, one of ordinary skill in the art can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide, but which uses codons optimal for a given species.

[0130] Randomly assigning codons at an optimized frequency to encode a given polypeptide sequence can be done manually by calculating codon frequencies for each amino acid, and then assigning the codons to the polypeptide sequence randomly. Additionally, various algorithms and computer software programs are readily available to those of ordinary skill in the art. For example, the "EditSeq" function in the Lasergene Package, available from DNASTar,

Inc., Madison, WI, the backtranslation function in the VectorNTI Suite, available from InforMax, Inc., Bethesda, MD, and the "backtranslate" function in the GCG--Wisconsin Package, available from Accelrys, Inc., San Diego, CA. In addition, various resources are publicly available to codon-optimize coding region sequences, e.g., the "JAVA Codon Adaptation Tool" at <http://www.jcat.de/> (visited June 25, 2012) and the "Codon optimization tool" available at <http://www.entelechon.com/2008/10/backtranslation-tool/> (visited June 25, 2012).

[0131] By a nucleic acid or polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence.

[0132] As a practical matter, whether any particular nucleic acid molecule or polynucleotide is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to a nucleotide sequence or polypeptide sequence of the present invention can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag *et al.*, *Comp. Appl. Biosci.* 6:237-245 (1990). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. The result of the global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject nucleotide sequences, whichever is shorter.

[0133] If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject

sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score.

[0134] For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

[0135] Standard recombinant DNA and molecular cloning techniques are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); and by Silhavy, T. J., Bennis, M. L. and Enquist, L. W., *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1984); and by Ausubel, F. M. *et al.*, *Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience (1987). Additional methods used here are in *Methods in Enzymology*, Volume 194, *Guide to Yeast Genetics and Molecular and Cell Biology*

(Part A, 2004, Christine Guthrie and Gerald R. Fink (Eds.), Elsevier Academic Press, San Diego, CA). Other molecular tools and techniques are known in the art and include splicing by overlapping extension polymerase chain reaction (PCR) (Yu, et al. (2004) *Fungal Genet. Biol.* 41:973–981), positive selection for mutations at the *URA3* locus of *Saccharomyces cerevisiae* (Boeke, J.D. et al. (1984) *Mol. Gen. Genet.* 197, 345-346; M A Romanos, et al. *Nucleic Acids Res.* 1991 January 11; 19(1): 187), the cre-lox site-specific recombination system as well as mutant lox sites and FLP substrate mutations (Sauer, B. (1987) *Mol Cell Biol* 7: 2087-2096; Senecoff, et al. (1988) *Journal of Molecular Biology*, Volume 201, Issue 2, Pages 405-421; Albert, et al. (1995) *The Plant Journal*. Volume 7, Issue 4, pages 649–659), "seamless" gene deletion (Akada, et al. (2006) *Yeast*;23(5):399-405), and gap repair methodology (Ma *et al.*, *Genetics* 58:201-216; 1981).

[0136] The genetic manipulations of a recombinant host cell disclosed herein can be performed using standard genetic techniques and screening and can be made in any host cell that is suitable to genetic manipulation (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202). Construction of butanologens is described herein and in the art, for example in in PCT Pub. No. WO/2012/129555, incorporated herein by reference

[0137] "qPCR" or "RT-PCR" is a PCT-based laboratory technique that simultaneously amplifies and quantifies a target gene.

Biosynthetic Pathways

[0138] Biosynthetic pathways for the production of isobutanol that may be used include those described in U.S. Pat. Nos. 7,851,188 and 7,993,889, which are incorporated herein by reference. Isobutanol pathways are referred to with their lettering in Figure 1. In one 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 ketol-acid reductoisomerase;
- 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 a branched-chain keto acid decarboxylase; and,

– e) isobutyraldehyde to isobutanol, which may be catalyzed, for example, by a branched-chain alcohol dehydrogenase.

[0139] 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 ketol-acid reductoisomerase;
- c) 2,3-dihydroxyisovalerate to α -ketoisovalerate, which may be catalyzed, for example, by dihydroxyacid dehydratase;
- h) α -ketoisovalerate to valine, which may be catalyzed, for example, by transaminase or valine dehydrogenase;
- i) valine to isobutylamine, which may be catalyzed, for example, by valine decarboxylase;
- j) isobutylamine to isobutyraldehyde, which may be catalyzed by, for example, omega transaminase; and,
- e) isobutyraldehyde to isobutanol, which may be catalyzed, for example, by a branched-chain alcohol dehydrogenase.

[0140] 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 ketol-acid reductoisomerase;
- c) 2,3-dihydroxyisovalerate to α -ketoisovalerate, which may be catalyzed, for example, by dihydroxyacid dehydratase;
- f) α -ketoisovalerate to isobutyryl-CoA, which may be catalyzed, for example, by branched-chain keto acid dehydrogenase;
- g) isobutyryl-CoA to isobutyraldehyde, which may be catalyzed, for example, by acetylating aldehyde dehydrogenase; and,
- e) isobutyraldehyde to isobutanol, which may be catalyzed, for example, by a branched-chain alcohol dehydrogenase.

[0141] In another embodiment, the isobutanol biosynthetic pathway comprises the substrate to product conversions shown as steps k, g, and e in Figure 1.

[0142] Biosynthetic pathways for the production of 1-butanol that may be used include those described in U.S. Appl. Pub. No. 2008/0182308, 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.

[0143] Biosynthetic pathways for the production of 2-butanol that may be used include those described in U.S. Appl. Pub. No. 2007/0259410 and U.S. Appl. Pub. No. 2009/0155870, which are incorporated herein by reference. In one embodiment, the 2-butanol biosynthetic pathway comprises the following substrate to product conversions:

- a) pyruvate to alpha-acetolactate, which may be catalyzed, for example, by acetolactate synthase;
- b) alpha-acetolactate to acetoin, which may be catalyzed, for example, by acetolactate decarboxylase;
- c) acetoin to 3-amino-2-butanol, which may be catalyzed, for example, acetoin aminase;
- d) 3-amino-2-butanol to 3-amino-2-butanol phosphate, which may be catalyzed, for example, by aminobutanol kinase;
- e) 3-amino-2-butanol phosphate to 2-butanone, which may be catalyzed, for example, by aminobutanol phosphate phosphorylase; and,
- f) 2-butanone to 2-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

[0144] In another embodiment, the 2-butanol biosynthetic pathway comprises the following substrate to product conversions:

- a) pyruvate to alpha-acetolactate, which may be catalyzed, for example, by acetolactate synthase;
- b) alpha-acetolactate to acetoin, which may be catalyzed, for example, by acetolactate decarboxylase;
- c) acetoin to 2,3-butanediol, which may be catalyzed, for example, by butanediol dehydrogenase;
- d) 2,3-butanediol to 2-butanone, which may be catalyzed, for example, by dial dehydratase; and,
- e) 2-butanone to 2-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

[0145] Biosynthetic pathways for the production of 2-butanone that may be used include those described in U.S. Appl. Pub. No. 2007/0259410 and U.S. Appl. Pub. No. 2009/0155870, which are incorporated herein by reference. In one embodiment, the 2-butanone biosynthetic pathway comprises the following substrate to product conversions:

- a) pyruvate to alpha-acetolactate, which may be catalyzed, for example, by acetolactate synthase;
- b) alpha-acetolactate to acetoin, which may be catalyzed, for example, by acetolactate decarboxylase;
- c) acetoin to 3-amino-2-butanol, which may be catalyzed, for example, acetoin aminase;
- d) 3-amino-2-butanol to 3-amino-2-butanol phosphate, which may be catalyzed, for example, by aminobutanol kinase; and,
- e) 3-amino-2-butanol phosphate to 2-butanone, which may be catalyzed, for example, by aminobutanol phosphate phosphorylase.

[0146] In another embodiment, the 2-butanone biosynthetic pathway comprises the following substrate to product conversions:

- a) pyruvate to alpha-acetolactate, which may be catalyzed, for example, by acetolactate synthase;
- b) alpha-acetolactate to acetoin which may be catalyzed, for example, by acetolactate decarboxylase;

- c) acetoin to 2,3-butanediol, which may be catalyzed, for example, by butanediol dehydrogenase;
- d) 2,3-butanediol to 2-butanone, which may be catalyzed, for example, by dial dehydratase.

[0147] In one embodiment, the invention produces butanol from plant derived carbon sources, avoiding the negative environmental impact associated with 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 a butanol pathway.

[0148] In some embodiments, the isobutanol 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 embodiments, each substrate to product conversion of an isobutanol 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.

[0149] The terms "acetohydroxyacid synthase," "acetolactate synthase" and "acetolactate synthetase" (abbreviated "ALS") are used interchangeably herein to refer to an enzyme that catalyzes the conversion of pyruvate to acetolactate and CO₂. 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: CAB07802.1, Z99122 (SEQ ID NO:16), NCBI (National Center for Biotechnology Information)), CAB15618), *Klebsiella pneumoniae* (GenBank Nos: AAA25079, M73842, *Lactococcus lactis* (GenBank Nos: AAA25161, L16975), *S. cerevisiae* (SEQ ID NOs:130 and 131), *E. coli* K12 (SEQ ID NOs:132 and 133).

[0150] The term "ketol-acid reductoisomerase" ("KARI"), "acetohydroxy acid reductoisomerase" and "acetohydroxy acid isomeroreductase" will be used interchangeably and refer to enzymes 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* (GenBank Nos: NP_418222, NC_000913), *Saccharomyces cerevisiae* (GenBank Nos: NP_013459, NC_001144), *Methanococcus maripaludis* (GenBank Nos: CAF30210, BX957220), and *Bacillus subtilis*

(GenBank Nos: CAB14789, Z99118). KARIs include *Anaerostipes caccae* KARI variants "K9G9," "K9D3" and "K9JB4P" (SEQ ID NOs:37, 38, and 182 respectively). Ketol-acid reductoisomerase (KARI) enzymes are described in U.S. Patent Appl. Pub. Nos. 20080261230 A1, 20090163376 A1, 20100197519 A1, PCT Appl. Pub. Nos. WO/2011/041415, and WO/2012/129555, 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. *Pseudomonas fluorescens* KARIs include SEQ ID NO:134. In some embodiments, the KARI utilizes NADH. In some embodiments, the KARI utilizes NADPH. In some embodiments, the KARI utilizes NADH or NADPH.

[0151] The term "acetohydroxy acid dehydratase" and "dihydroxyacid dehydratase" ("DHAD") refers to an enzyme that catalyzes the conversion of 2,3-dihydroxyisovalerate to α -ketoisovalerate. Example acetohydroxy acid 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, NC_000913), *S. cerevisiae* (GenBank Nos: NP_012550, NC_001142), *M. maripaludis* (GenBank Nos: CAF29874, BX957219), *B. subtilis* (GenBank Nos: CAB14105, Z99115), *L. lactis* (SEQ ID NO:108), and *N. crassa*. US Appl. Pub. No. 20100081154 A1, and U.S. Patent 7,851,188, which are incorporated herein by reference, describe dihydroxyacid dehydratases (DHADs), including a DHAD from *Streptococcus mutans* (SEQ ID NO:135). Example DHADs include variants of *S. mutans* DHAD, for example "L2V4" (SEQ ID NO:183).

[0152] The term "branched-chain α -keto acid decarboxylase" or " α -ketoacid decarboxylase" or " α -ketoisovalerate decarboxylase" or "2-ketoisovalerate decarboxylase" ("KIVD") refers to an enzyme that catalyzes the conversion of α -ketoisovalerate to isobutyraldehyde and CO₂. 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 (SEQ ID NO:141), AY548760; CAG34226, AJ746364, *Salmonella typhimurium* (GenBank Nos: NP_461346, NC_003197), *Clostridium acetobutylicum* (GenBank Nos: NP_149189, NC_001988), *M. caseolyticus* (SEQ ID NOs:118, 137), and *L. grayi* (SEQ ID NO:136).

[0153] The term "branched-chain alcohol dehydrogenase" ("ADH") refers to an enzyme 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 be NADPH dependent or NADH dependent. Such enzymes are available from a number of sources, including, but not limited to, *S. cerevisiae* (GenBank Nos: NP_010656, NC_001136; NP_014051, NC_001145), *E. coli* (GenBank Nos: NP_417484, NC_000913), *C. acetobutylicum* (GenBank Nos: NP_349892, NC_003030; NP_349891, NC_003030). U.S. Patent No. 8,188,250, which is incorporated herein by reference, describes SadB, an alcohol dehydrogenase (ADH) from *Achromobacter xylosoxidans* (SEQ ID NO:139). Alcohol dehydrogenases also include horse liver ADH (SEQ ID NO:142) and *Beijerinckia indica* ADH (SEQ ID NO:138) (as described by U.S. Appl. Publ. No. 20110269199, which is incorporated herein by reference).

[0154] The term "butanol dehydrogenase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of isobutyraldehyde to isobutanol or the conversion of 2-butanone and 2-butanol. Butanol dehydrogenases are a subset of a broad family of alcohol dehydrogenases. Butanol dehydrogenase may be NAD- or NADP-dependent. The NAD-dependent enzymes are known as EC 1.1.1.1 and are available, for example, from *Rhodococcus ruber* (GenBank Nos: CAD36475, AJ491307). The NADP dependent enzymes are known as EC 1.1.1.2 and are available, for example, from *Pyrococcus furiosus* (GenBank Nos: AAC25556, AF013169). Additionally, a butanol dehydrogenase is available from *Escherichia coli* (GenBank Nos: NP_417484, NC_000913) and a cyclohexanol dehydrogenase is available from *Acinetobacter sp.* (GenBank Nos: AAG10026, 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, NC_001988; note: this enzyme possesses both aldehyde and alcohol dehydrogenase activity); NP_349891, NC_003030; and NP_349892, NC_003030), *E. coli* (GenBank NOs: NP_417-484, NC_000913), and *A. xylosoxidans* (SEQ ID NOs:47 and 48, as described in U.S. Patent No. 8,188,250, which is incorporated herein by reference in its entirety).

[0155] The term "branched-chain keto acid dehydrogenase" refers to an enzyme 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; and CAB14337, Z99116) and *Pseudomonas putida* (GenBank Nos: AAA65614, M57613; AAA65615, M57613; AAA65617, M57613; and AAA65618, M57613).

[0156] The term "acylating aldehyde dehydrogenase" refers to an enzyme 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, AF157306), *C. acetobutylicum* (GenBank Nos: NP_149325, NC_001988; NP_149199, NC_001988), *P. putida* (GenBank Nos: AAA89106, U13232), and *Thermus thermophilus* (GenBank Nos: YP_145486, NC_006461).

[0157] The term "transaminase" refers to an enzyme that catalyzes the conversion of α -ketoisovalerate to L-valine, using either alanine or glutamate as an amine donor. Example transaminases are known by the EC numbers 2.6.1.42 and 2.6.1.66. Such enzymes are available from a number of sources. Examples of sources for alanine-dependent enzymes include, but are not limited to, *E. coli* (GenBank Nos: YP_026231, NC_000913) and *Bacillus licheniformis* (GenBank Nos: YP_093743, NC_006322). Examples of sources for glutamate-dependent enzymes include, but are not limited to, *E. coli* (GenBank Nos: YP_026247, NC_000913), *S. cerevisiae* (GenBank Nos: NP_012682, NC_001142) and *Methanobacterium thermoautotrophicum* (GenBank Nos: NP_276546, NC_000916).

[0158] The term "valine dehydrogenase" refers to an enzyme that catalyzes the conversion of α -ketoisovalerate to L-valine, typically using NAD(P)H as an electron donor and ammonia as an amine donor. Example valine dehydrogenases are known by the EC numbers 1.4.1.8 and 1.4.1.9 and such enzymes are available from a number of sources, including, but not limited to, *Streptomyces coelicolor* (GenBank Nos: NP_628270, NC_003888) and *B. subtilis* (GenBank Nos: CAB14339, Z99116).

[0159] The term "valine decarboxylase" refers to an enzyme that catalyzes the conversion of L-valine to isobutylamine and CO₂. Example valine decarboxylases are known by the EC number 4.1.1.14. Such enzymes are found in *Streptomyces*, such as for example, *Streptomyces viridifaciens* (GenBank Nos: AAN10242, AY116644).

[0160] The term "omega transaminase" refers to an enzyme that catalyzes the conversion of isobutylamine to isobutyraldehyde using a suitable amino acid as an amine donor. Example

omega transaminases are known by the EC number 2.6.1.18 and are available from a number of sources, including, but not limited to, *Alcaligenes denitrificans* (AAP92672, AY330220), *Ralstonia eutropha* (GenBank Nos: YP_294474, NC_007347), *Shewanella oneidensis* (GenBank Nos: NP_719046, NC_004347), and *P. putida* (GenBank Nos: AAN66223, AE016776).

[0161] The term "acetyl-CoA acetyltransferase" refers to an enzyme 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, NC_000913; NCBI (National Center for Biotechnology Information) amino acid sequence, NCBI nucleotide sequence), *Clostridium acetobutylicum* (GenBank Nos: NP_349476.1, NC_003030; NP_149242, NC_001988, *Bacillus subtilis* (GenBank Nos: NP_390297, NC_000964), and *Saccharomyces cerevisiae* (GenBank Nos: NP_015297, NC_001148).

[0162] The term "3-hydroxybutyryl-CoA dehydrogenase" refers to an enzyme that catalyzes the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. 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, NC_003030), *B. subtilis* (GenBank NOs: AAB09614, U29084), *Ralstonia eutropha* (GenBank NOs: YP_294481, NC_007347), and *Alcaligenes eutrophus* (GenBank NOs: AAA21973, J04987).

[0163] The term "crotonase" refers to an enzyme that catalyzes the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA and H₂O. 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, NC_000913), *C. acetobutylicum* (GenBank NOs: NP_349318, NC_003030), *B. subtilis* (GenBank NOs: CAB13705, Z99113), and *Aeromonas caviae* (GenBank NOs: BAA21816, D88825).

[0164] The term "butyryl-CoA dehydrogenase" refers to an enzyme 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, NC_003030), *Euglena gracilis* (GenBank NOs: Q5EU90, AY741582), *Streptomyces collinus* (GenBank NOs: AAA92890, U37135), and *Streptomyces coelicolor* (GenBank NOs: CAA22721, AL939127).

[0165] The term "butyraldehyde dehydrogenase" refers to an enzyme 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, AF157306) and *C. acetobutylicum* (GenBank NOs: NP_149325, NC_001988).

[0166] The term "isobutyryl-CoA mutase" refers to an enzyme that catalyzes the conversion of butyryl-CoA to isobutyryl-CoA. This enzyme uses coenzyme B₁₂ as cofactor. Example isobutyryl-CoA mutases are known by the EC number 5.4.99.13. These enzymes are found in a number of *Streptomyces*, including, but not limited to, *Streptomyces cinnamonensis* (GenBank Nos: AAC08713, U67612; CAB59633, AJ246005), *S. coelicolor* (GenBank Nos: CAB70645, AL939123; CAB92663, AL939121), and *Streptomyces avermitilis* (GenBank Nos: NP_824008, NC_003155; NP_824637, NC_003155).

[0167] The term "acetolactate decarboxylase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of alpha-acetolactate to acetoin. Example acetolactate decarboxylases are known as EC 4.1.1.5 and are available, for example, from *Bacillus subtilis* (GenBank Nos: AAA22223, L04470), *Klebsiella terrigena* (GenBank Nos: AAA25054, L04507) and *Klebsiella pneumoniae* (GenBank Nos: AAU43774, AY722056).

[0168] The term "acetoin aminase" or "acetoin transaminase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of acetoin to 3-amino-2-butanol. Acetoin aminase may utilize the cofactor pyridoxal 5'-phosphate or NADH (reduced nicotinamide adenine dinucleotide) or NADPH (reduced nicotinamide adenine dinucleotide).

phosphate). The resulting product may have (R) or (S) stereochemistry at the 3-position. The pyridoxal phosphate-dependent enzyme may use an amino acid such as alanine or glutamate as the amino donor. The NADH- and NADPH-dependent enzymes may use ammonia as a second substrate. A suitable example of an NADH dependent acetoin aminase, also known as amino alcohol dehydrogenase, is described by Ito *et al.* (U.S. Pat. No. 6,432,688). An example of a pyridoxal-dependent acetoin aminase is the amine:pyruvate aminotransferase (also called amine:pyruvate transaminase) described by Shin and Kim (*J. Org. Chem.* 67:2848-2853 (2002)).

[0169] The term "acetoin kinase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of acetoin to phosphoacetoin. Acetoin kinase may utilize ATP (adenosine triphosphate) or phosphoenolpyruvate as the phosphate donor in the reaction. Enzymes that catalyze the analogous reaction on the similar substrate dihydroxyacetone, for example, include enzymes known as EC 2.7.1.29 (Garcia-Alles *et al.* (2004) *Biochemistry* 43:13037-13046).

[0170] The term "acetoin phosphate aminase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of phosphoacetoin to 3-amino-2-butanol O-phosphate. Acetoin phosphate aminase may use the cofactor pyridoxal 5'-phosphate, NADH or NADPH. The resulting product may have (R) or (S) stereochemistry at the 3-position. The pyridoxal phosphate-dependent enzyme may use an amino acid such as alanine or glutamate. The NADH and NADPH-dependent enzymes may use ammonia as a second substrate. Although there are no reports of enzymes catalyzing this reaction on phosphoacetoin, there is a pyridoxal phosphate-dependent enzyme that is proposed to carry out the analogous reaction on the similar substrate serinol phosphate (Yasuta *et al.* (2001) *Appl. Environ. Microbial.* 67:4999-5009).

[0171] The term "aminobutanol phosphate phospholyase", also called "amino alcohol O-phosphate lyase", refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of 3-amino-2-butanol O-phosphate to 2-butanone. Amino butanol phosphate phospho-lyase may utilize the cofactor pyridoxal 5'-phosphate. There are reports of enzymes that catalyze the analogous reaction on the similar substrate 1-amino-2-propanol phosphate (Jones *et al.* (1973) *Biochem J.* 134:167-182). U.S. Appl. Pub. No. 2007/0259410 describes an aminobutanol phosphate phospho-lyase from the organism *Erwinia carotovora*.

[0172] The term "aminobutanol kinase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of 3-amino-2-butanol to 3-amino-2butanol O-phosphate. Amino butanol kinase may utilize ATP as the phosphate donor. Although there are

no reports of enzymes catalyzing this reaction on 3-amino-2-butanol, there are reports of enzymes that catalyze the analogous reaction on the similar substrates ethanolamine and 1-amino-2-propanol (Jones *et al.*, supra). U.S. Appl. Pub. No. 2009/0155870 describes, in Example 14, an amino alcohol kinase of *Erwinia carotovora subsp. Atroseptica*.

[0173] The term "butanediol dehydrogenase" also known as "acetoin reductase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of acetoin to 2,3-butanediol. Butanediol dehydrogenases are a subset of the broad family of alcohol dehydrogenases. Butanediol dehydrogenase enzymes may have specificity for production of (R)- or (S)-stereochemistry in the alcohol product. (S)-specific butanediol dehydrogenases are known as EC 1.1.1.76 and are available, for example, from *Klebsiella pneumoniae* (GenBank Nos: BBA13085, D86412). (R)-specific butanediol dehydrogenases are known as EC 1.1.1.4 and are available, for example, from *Bacillus cereus* (GenBank Nos. NP 830481, NC_004722; AAP07682, AE017000), and *Lactococcus lactis* (GenBank Nos. AAK04995, AE006323).

[0174] The term "butanediol dehydratase", also known as "dial dehydratase" or "propanediol dehydratase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of 2,3-butanediol to 2-butanone. Butanediol dehydratase may utilize the cofactor adenosyl cobalamin (also known as coenzyme B₁₂ or vitamin B₁₂; although vitamin B₁₂ may refer also to other forms of cobalamin that are not coenzyme B₁₂). Adenosyl cobalamin-dependent enzymes are known as EC 4.2.1.28 and are available, for example, from *Klebsiella oxytoca* (GenBank Nos: AA08099 (alpha subunit), D45071; BAA08100 (beta subunit), D45071; and BBA08101 (gamma subunit), D45071 (Note all three subunits are required for activity), and *Klebsiella pneumonia* (GenBank Nos: AAC98384 (alpha subunit), AF102064; GenBank Nos: AAC98385 (beta subunit), AF102064, GenBank Nos: AAC98386 (gamma subunit), AF102064). Other suitable dial dehydratases include, but are not limited to, B₁₂-dependent dial dehydratases available from *Salmonella typhimurium* (GenBank Nos: AAB84102 (large subunit), AF026270; GenBank Nos: AAB84103 (medium subunit), AF026270; GenBank Nos: AAB84104 (small subunit), AF026270); and *Lactobacillus collinoides* (GenBank Nos: CAC82541 (large subunit), AJ297723; GenBank Nos: CAC82542 (medium subunit); AJ297723; GenBank Nos: CAD01091 (small subunit), AJ297723); and enzymes from *Lactobacillus brevis* (particularly strains CNRZ 734 and CNRZ 735, Speranza et al., J. Agric. Food Chem. (1997) 45:3476-3480), and nucleotide sequences that encode the

corresponding enzymes. Methods of dial dehydratase gene isolation are well known in the art (e.g., U.S. Pat. No. 5,686,276).

[0175] The term "pyruvate decarboxylase" refers to an enzyme that catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide. Pyruvate decarboxylases are known by the EC number 4.1.1.1. These enzymes are found in a number of yeast, including *Saccharomyces cerevisiae* (GenBank Nos: CAA97575 (SEQ ID NO:1), CAA97705 (SEQ ID NO:2), CAA97091 (SEQ ID NO:3)).

[0176] The term "ethanol dehydrogenase" or "alcohol dehydrogenase" refers to an enzyme that catalyze the interconversion between aldehydes or ketones and alcohols, frequently using either NADH and/or NADPH as cofactors. Ethanol dehydrogenases comprise the EC numbers 1.1.1.1., 1.1.99.8., 1.1.1.244., 1.1.2.B1., 1.1.2.B2., 1.1.2.B3.

[0177] It will be appreciated that host cells comprising an isobutanol biosynthetic pathway as provided herein may further comprise one or more additional modifications. U.S. Appl. Pub. No. 20090305363 (incorporated 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. In some embodiments, the host cells comprise modifications to reduce glycerol-3-phosphate dehydrogenase activity and/or disruption in at least one gene encoding a polypeptide having pyruvate decarboxylase activity or a disruption in at least one gene encoding a regulatory element controlling pyruvate decarboxylase gene expression as described in U.S. Patent Appl. Pub. No. 20090305363 (incorporated herein by reference), modifications to a host cell that provide for increased carbon flux through an Entner-Doudoroff Pathway or reducing equivalents balance as described in U.S. Patent Appl. Pub. No. 20100120105 (incorporated herein by reference). Other modifications are described in PCT Pub. No. WO/2012/129555, incorporated herein by reference, and 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 (SEQ ID NOs:5,6) of *Saccharomyces cerevisiae* or a homolog thereof. Additional modifications include a deletion, mutation, and/or substitution in an endogenous polynucleotide encoding a polypeptide having aldehyde dehydrogenase and/or aldehyde oxidase activity. In embodiments, the polypeptide having aldehyde dehydrogenase activity is *ALD6* from

Saccharomyces cerevisiae or a homolog thereof. A genetic modification which has the effect of reducing glucose repression wherein the yeast production host cell is *pdc-* is described in U.S. Appl. Publication No. 20110124060, incorporated herein by reference. In some embodiments, the pyruvate decarboxylase that is deleted or downregulated is selected from the group consisting of: *PDC1*, *PDC5*, *PDC6*, and combinations thereof. In some embodiments, the pyruvate decarboxylase is selected from those enzymes described in U.S. Patent Appl. Pub. No. 20090305363. In some embodiments, host cells contain a deletion or downregulation of a polynucleotide encoding a polypeptide that catalyzes the conversion of glyceraldehyde-3-phosphate to glycerate 1,3, biphosphate. In some embodiments, the enzyme that catalyzes this reaction is glyceraldehyde-3-phosphate dehydrogenase.

[0178] Yeasts may have one or more genes encoding pyruvate decarboxylase. For example, there is one gene encoding pyruvate decarboxylase in *Candida glabrata* and *Schizosaccharomyces pombe*, while there are three isozymes of pyruvate decarboxylase encoded by the *PDC1*, *PCD5*, and *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.

[0179] Other target genes, such as those encoding pyruvate decarboxylase proteins having at least 70-75%, at least 75-80%, at least 80-85%, at least 85%-90%, at least 90%-95%, or at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the pyruvate decarboxylases described in U.S. Patent Appl. Pub. No. 20090305363 may be identified in the literature and in bioinformatics databases well known to the skilled person.

[0180] Recombinant host cells may further comprise (a) at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity; and (b)(i) at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis; and/or (ii) at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis described in U.S. Patent Appl. Pub. No. US20120064561, incorporated herein by reference. In embodiments, the polypeptide affecting Fe-S cluster biosynthesis is encoded by *AFT1*, *AFT2*, *FRA2*, *GRX3* or *CCC1*. *AFT1* and *AFT2* are described by WO/2001/103300, which is incorporated herein by reference. In embodiments,

the polypeptide affecting Fe-S cluster biosynthesis is constitutive mutant *AFTI* L99A, *AFTI* L102A, *AFTI* C291F, or *AFTI* C293F.

[0181] In some embodiments, the host cell further comprises one or more polynucleotides that encode one or more enzymes having the following Enzyme Commission Numbers: EC 4.1.1.1 (PDC1, 5, and 6) (SEQ ID NOs:1, 2, and 3) and EC 1.1.1.1 (alcohol dehydrogenase).

[0182] In some embodiments of the invention, there are one or more inhibitors, antibiotics, or combinations thereof in the fermentation medium.

[0183] In some embodiments, the inhibitor is an inhibitor of an ethanol biosynthesis pathway. In some embodiments, the inhibitor inhibits pyruvate decarboxylase and/or alcohol dehydrogenase. In some embodiments, the inhibitor is selected from the group consisting of: the XC₆H₄CH=CHCOCOOH class of inhibitors/substrate analogues, cinnamaldehydes, glyoxalic acid, ketomalonate, regulatory site inhibitors, p-chloromercuribonzoic acid (pCMB), 5,5'-dithiobis(2-nitrobenzoic acid) (DNTB), pyrazole, 4-pyrazolecarboxylic acid, 1-H-pyrazole-1-carboxamidinium-HCl, 4-methylpyrazole, 1-bromo-2-butanone, pyrazole-3,5-dicarboxylic acid monohydrate, and mixtures thereof. In some embodiments, the XC₆H₄CH=CHCOCOOH inhibitors/substrate analogue is CPB((E)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid). In some embodiments the cinnamaldehyde is p-nitrocinnamaldehyde (NA). In some embodiments, the regulatory site inhibitors are iodoacetate, 1,3-dibromoacetone, 1-bromo-2-butanone.

"Cinnamaldehyde" includes both trans-cinnamaldehydes and 4-nitrocinnamaldehydes. In some embodiments, copper (II) is added at a concentration of at least about 1.1 mM, at least about 11 mM, at least about 33 mM. In some embodiments, sulfometuron methyl is added at a concentration of at least about 0.001 mM, at least about 0.01 mM, at least about 0.1 mM. In some embodiments, sulfite is added at a concentration of at least about 0.6 mM, at least about 6.2 mM, at least about 62 mM. In some embodiments, formaldehyde is added at a concentration of at least about 0.09 mM, at least about 0.9 mM, at least about 2.7 mM. In some embodiments, pyrazole is added at a concentration of at least about 0.3 mM, at least about 3 mM, at least about 30 mM. In some embodiments, 4-methylpyrazole hydrochloride is added at a concentration of at least about 4.1 mM, at least about 41 mM, at least about 123 mM. In some embodiments, 4-pyrazolecarboxylic acid is added at a concentration of at least about 10 mM, at least about 100 mM, at least about 300 mM. In some embodiments, 1-bromo-2-butanone is added at a concentration of at least about 0.0002 mM, at least about 0.002 mM, at least about 0.006 mM. In some embodiments, trans-cinnamaldehyde is added at a concentration of at least about 0.025

mM, at least about 0.25 mM, at least about 0.75 mM. In some embodiments, glyoxylic acid is added at a concentration of at least about 16.8 mM, at least about 168 mM, at least about 504 mM.

[0184] In some embodiments, the inhibitor is a chemical. In some embodiments, the chemical is selected from the group consisting of: fluoroacetate (dehH1), fluorophenylalanine, formaldehyde (SFA1), sulfite (FZF1-4), and trifluoroleucine (LEU4-1).

[0185] In some embodiments, the inhibitor is an inhibitor of an amino acid biosynthesis pathway. In some embodiments, the inhibitor is an acetohydroxy acid synthase (AHAS) inhibitor. In some embodiments, the inhibitor is a sulfonylurea herbicide. In some embodiments, the sulfonylurea herbicide is selected from the group consisting of: imidazolinones, triazolopyrimidines, pyrimidinyl oxybenzoates, sulfonylureas, sulfonylamino carbonyl triazolinones, and mixtures thereof. In some embodiments, the inhibitor is selected from the group consisting of: nicosulfuron methyl, metsulfuron methyl, chlorimuron ethyl, sulfometuron methyl, chlorsulfuron, thifensulfuron methyl, and mixtures thereof. In some embodiments, the sulfonylurea herbicide is an acetohydroxyacid synthase (AHAS) inhibitor).

[0186] In some embodiments, resistance to the sulfonyl urea is conferred by a polypeptide encoded by a heterologous polynucleotide. In some embodiments, the heterologous polynucleotide provides resistance to AHAS inhibitors and comprises a sequence having at least 80% identity to a sequence selected from the group consisting of: SEQ ID NO:130 (ILV2 gene from *S. cerevisiae* BY4700) and SEQ ID NO:132 (ALS I gene from *E. coli* K12). In some embodiments, the heterologous polypeptide provides resistance to AHAS inhibitors and comprises an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO:131 (ILV2 from *S. cerevisiae* BY4700) and SEQ ID NO:133 (ALS I from *E. coli* K12). In some embodiments, the polypeptide provides resistance to AHAS inhibitors and comprises an amino acid sequence selected from the group consisting of: SEQ ID NO:131 (ILV2 from *S. cerevisiae* BY4700) and SEQ ID NO:133 (ALS I gene from *E. coli* K12) or an active variant, fragment or derivative thereof. In some embodiments, the polypeptide is from a bacteria of the family *Enterobacteriaceae*. In some embodiments, the polypeptide is from a bacterial genus selected from the group consisting of: *Alishewanella*, *Alterococcus*, *Aquamonas*, *Aranicola*, *Arsenophonus*, *Azotivirga*, *Blochmannia*, *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Cronobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Ewingella*, *Grimontella*, *Hafnia*, *Klebsiella*,

Kluyvera, *Leclercia*, *Leminorella*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Pantoea*, *Candidatus Phlomobacter*, *Photorhabdus*, *Poodoomaamaana*, *Plesiomonas*, *Pragia*, *Proteus*, *Providencia*, *Rahnella*, *Raoultella*, *Salmonella*, *Samsonia*, *Serratia*, *Shigella*, *Sodalis*, *Tatumella*, *Trabulsiella*, *Wigglesworthia*, *Xenorhabdus*, *Yersinia*, and *Yokenella*. In some embodiments, the polypeptide is from a microorganism of the genus *Saccharomyces*. In some embodiments, the AHAS enzymes can be mutated to confer sulfonyl urea resistance. In some embodiments, the *B. subtilis* AlsS enzyme is mutated to increase its sulfonyl urea resistance.

[0187] In some embodiments, the inhibitor of amino acid synthesis is glyphosate. In some embodiments, resistance to the glyphosate is conferred by a polypeptide that has 5-enolpyruvoyl-shikimate-3-phosphate synthetase (EPSPS) activity. In some embodiments, the polypeptide is encoded by a heterologous polynucleotide. In some embodiments, the inhibitor is a 5-enolpyruvoyl-shikimate-3-phosphate synthetase (EPSPS) inhibitor. In some embodiments, the inhibitor is a glyphosate derivative. In some embodiments, resistance to the glyphosate is conferred by a polypeptide that has glyphosate N-acetyltransferase activity. In some embodiments, the polypeptide is encoded by a heterologous polynucleotide. Sequences describing polypeptides with glyphosate N-acetyltransferase activity are described in, for example, U.S. Patent No. 7,863,503, which is incorporated herein by reference.

[0188] In some embodiments, the antibiotic is selected from the group consisting of: aureobasiden A, bialaphos, cerulenine, cerulenin, chloramphenicol, cyclohexamide, geneticin/G418, hygromycin B, methotrexate, nourseothricin, phleomycin, triazole, and mixtures thereof. In some embodiments, a polypeptide confers resistance to one or more antibiotics. In some embodiments, the polypeptide is encoded by a heterologous polynucleotide.

[0189] In some embodiments a polypeptide confers resistance to the inhibitor or antibiotic. In some embodiments, the polypeptide is encoded by a polynucleotide. In some embodiments, the polypeptide conferring resistance to the inhibitor or antibiotic has one or more amino acid deletions when compared with the amino acid sequence of the corresponding native polypeptide. In some embodiments, the amino acid sequence of the polypeptide has one or more amino acid substitutions when compared with the amino acid sequence of the corresponding native polypeptide.

[0190] In some embodiments, the inhibitor is an α -isopropyl malate (α -IPM) synthase inhibitor. In some embodiments, the inhibitor is trifluoroleucine or a trifluoroleucine derivative. In some embodiments, the inhibitor is a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase

(DAHPS) inhibitor. In some embodiments, the inhibitor is fluorophenylalanine or a fluorophenylalanine derivative. In some embodiments, the inhibitor is bismuth (III) or copper (II).

[0191] In some embodiments, the polypeptide confers tolerance to fluoroacetate. In some embodiments, the polypeptide confers tolerance to formaldehyde. In some embodiments, the polypeptide confers tolerance to sulfite.

[0192] In some embodiments, the polypeptide confers tolerance to an α -isopropyl malate (a-IPM) synthase inhibitor. In some embodiments, the polypeptide confers tolerance to trifluoroleucine or a trifluoroleucine derivative (isopropyl malate resistance). In some embodiments, the polynucleotide sequence encoding the polypeptide providing resistance to trifluoroleucine comprises a sequence having at least 80% identity to a sequence disclosed by: Chianelli, M.S., *et al.*, *Cell. Mol. Biol.* 42(6):847-57 (1996) or Oba, T., *et al.*, *Biosci. Biotechnol. Biochem.* 70(7):1776-9 (2006) and incorporated by reference. In some embodiments, the polypeptide confers tolerance to a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS) inhibitor. In some embodiments, the polynucleotide sequence encoding the polypeptide providing resistance to DAHPS comprises a sequence having at least 80% identity to a sequence disclosed by: Fukada, K., *et al.*, *Agric. Biol. Chem.* 54:3151-3156 (1990); Meuris, P. 1974. *Genetics* 76:735-744 (1974); Shimura, K., *et al.*, 1993. *Enzyme Microbiol. Technol.* 15:874-876 (1993) and incorporated by reference.

[0193] In some embodiments, the polypeptide confers tolerance to an antibiotic. In some embodiments, the polypeptide confers tolerance to an antibiotic selected from the group consisting of: aureobasidin A, bialaphos, cerulenin, chloramphenicol, cyclohexamide, geneticin, hygromycin B, methotrexate, nourseothricin, phleomycin, triazole, and mixtures thereof. In some embodiments, the polynucleotide sequence encoding the polypeptide comprises a sequence having at least 80% identity to a sequence selected from the group consisting of: SEQ ID NOs: 92 and 143-157 (Aureobasidin A resistance (AUR1-C) (SEQ ID NOs:143 and 144); bialaphos resistance protein (SEQ ID NOs:145 and 146); cerulenin resistance YML007W Chr 13 (SEQ ID NOs:147 and 148); Geneticin resistance (kanMX) (SEQ ID NOs:149 and 150); Hygromycin B resistance (HygR) (SEQ ID NOs:151 and 152); *Streptomyces noursei* nourseothricin resistance (nat1) (SEQ ID NOs:153 and 154); phleomycin/zeocin binding protein (SEQ ID NOs:155 and 156); and Triazole resistance (cyp51A) (SEQ ID NOs:157 and 92).

[0194] In some embodiments, the inhibitor is inhibiting at least one enzyme selected from the group consisting of: 5-enolpyruvoyl-shikimate-3-phosphate synthetase, α -isopropyl malate synthase, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase and mixtures thereof. In some embodiments, the polynucleotide sequence encoding the polypeptide providing resistance to 5-enolpyruvoyl-shikimate-3-phosphate synthetase comprises a sequence having at least 80% identity to a sequence disclosed by: Cao G, *et al.*, (2012) *PLoS ONE* 7(6): e38718 (2012) incorporated by reference. In some embodiments, the polynucleotide sequence encoding the polypeptide with glyphosate N-acetyltransferase activity comprises a sequence having at least 80% identity to a sequence disclosed by U.S. Pat. No. 7,666,644, which is incorporated herein by reference in its entirety and Siehl D.L., *et al.*, *Pest Manag Sci.* 61(3):235-40 (2005) incorporated by reference. In some embodiments, the polynucleotide sequence encoding the polypeptide providing resistance to 3-phosphoshikimate 1-carboxylvinyltransferase comprises a sequence having at least 80% identity to a sequence disclosed by: Vande Berg B.J., *et al.*, *Pest Manag Sci.* 64(4):340-5 (2008) incorporated by reference. In some embodiments, the polypeptide that provides resistance to the inhibitor is a formaldehyde dehydrogenase. In some embodiments, the polypeptide comprises an amino acid sequence of at least about 80% identity to SEQ ID NO:6 or 7.

[0195] In some embodiments, the polypeptide that confers resistance comprises an amino acid sequence of at least about 80% identity to SEQ ID NO:11 or SEQ ID NO:12. In some embodiments, the polypeptide that confers resistance is a 3-phosphoshikimate 1-carboxylvinyltransferase. In some embodiments, the polypeptide comprises an amino acid sequence of at least about 80% identity to SEQ ID NO:13.

[0196] In some embodiments, one or more AHAS inhibitors is present at a concentration from about 0.1 $\mu\text{g/mL}$ to about 2 g/mL, about 1.0 $\mu\text{g/mL}$ to about 0.1 g/mL, about 1 mg/mL to about 0.1 g/mL, or about 10 mg/mL to about 100 mg/mL. In some embodiments, one or more AHAS inhibitors is present at a concentration of 0.0125 mg/mL. In some embodiments, one or more AHAS inhibitors is present at a concentration of 1 mg/mL. In some embodiments, one or more AHAS inhibitors is present at a concentration of 2 mg/mL.

[0197] In some embodiments, glyphosate is at a concentration from about 0.1 $\mu\text{g/mL}$ to about 2 g/mL, for example about 10 $\mu\text{g/mL}$, about 100 $\mu\text{g/mL}$, about 1 mg/mL, about 10 mg/mL, about 100 mg/mL, about 1 g/mL, or about 2g/mL.

[0198] In some embodiments, the antibiotic is present at a concentration from about 2 ppm to about 500 ppm, for example about 5ppm, about 20 ppm, about 50 ppm, about 100 ppm, about 150 ppm, about 200 ppm, about 300 ppm, about 400 ppm, or about 500 ppm.

[0199] In some embodiments, the addition inhibitors of amino acid synthesis, antibiotics, or combinations thereof results in death of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the microbial contaminants in the fermentation mix.

[0200] In embodiments, the effective concentration of inhibitor for use in methods provided herein can be determined empirically for a given production strain, contaminant strain, and production process. Alternatively, minimal data may be obtained for a given system and used to determine appropriate concentrations for inhibitors. Such determination is disclosed and demonstrated herein (see Examples), and is readily available to one of skill in the art, equipped with this disclosure.

[0201] Briefly, as described herein, growth and/or production competitiveness may be improved by, for example, i) adding a genetic trait that provides growth and/or production competitiveness, or ii) by providing growth conditions that increase growth and/or production competitiveness, e.g. through the addition of selective inhibitors. While not wishing to be bound by theory, in the first case, increased growth competitiveness of a strain A carrying a genetic trait that provides growth competitiveness will have a higher biomass ratio after a growth phase compared to a competing strain B than an strain C isogenic to strain A without the genetic trait, i.e. $cx_{A_mod}(t)/cx_{B_mod}(t) > cx_C(t)/cx_B(t)$. In the later case, strain A will have a higher biomass ratio after a growth phase compared to a competing strain B under the conditions promoting growth competitiveness, i.e. $cx_A(t, c(inhibitor) > 0 \text{ g/L})/cx_B(t, c(inhibitor) > 0 \text{ g/L}) > cx_A(t, c(inhibitor) = 0 \text{ g/L})/cx_B(t, c(inhibitor) = 0 \text{ g/L})$. After a growth phase indicates that the biomass of strain A had to increase during the respective time interval, i.e. $cx_A(t) > cx_A(t_{start})$. While not wishing to be bound by theory, in the first case, increased production competitiveness of a strain A carrying a genetic trait that provides production competitiveness will have a higher product ratio compared to a product of a competing strain B than an strain C isogenic to strain A without the genetic trait, i.e. $cp_A(t)/cp_B(t) > cp_C(t)/cp_B(t)$. In the later case, strain A will have a higher product ratio compared to a product of a competing strain B, i.e. $cp_A(t, c(inhibitor) > 0 \text{ g/L})/cp_B(t, c(inhibitor) > 0 \text{ g/L}) > cp_A(t, c(inhibitor) = 0 \text{ g/L})/cp_B(t, c(inhibitor) = 0 \text{ g/L})$.

[0202] Under situations where substrates are not limiting, e.g. under glucose excess conditions, maximum specific growth rate of the strains under the given cultivation conditions

(medium, temperature, etc.) will be a component for determining growth competitiveness. Changes in the given conditions (e.g. changing concentrations of products, substrates, signaling molecules, etc.) may result in different values of maximum specific growth rate, and the maximum specific growth rate of strains may be different in a given condition. Considering such factors and assuming a constant $\mu_{\max}$ for an exponential growth phase, the biomass concentration during the exponential growth phase that started at t_{lag} can be approximately described according to

$$c_X(t) = c_X(t_{\text{lag}}) \cdot e^{\mu_{\max} \cdot (t - t_{\text{lag}})} \quad \text{Eq. (1)}$$

[0203] Under aerobic, glucose-excess conditions and acetic acid in the medium, maximum specific growth rate of an exemplary ethanologen *S. cerevisiae* strain (PNY 827) was determined to be 0.61 1/h. In contrast, an isobutanologen *S. cerevisiae* strain (PNY 2129, constructed using PNY827) exhibited a maximum specific growth rate of 0.16 1/h. If an aerobic batch cultivation with a mixed culture consisting of both strains with a biomass ratio of 1:1, i.e. with a cell dry weight concentration of 1 g/L each, would be started, and both strains would be growing for 8 hours at $\mu_{\max}$ without any lag phase, at the end of the process PNY 827 would account for approximately 131.6 g/L cell dry weight in the mixed culture, while PNY 2129 would account for only about 3.6 g/L. The ratio of biomass PNY 2129/PNY 827 would be below 3%. This phenomenon is illustrated in Figure 18 where PNY 2129 is represented by strain A and PNY 827 by strain B.

[0204] In order to describe growth performance of a strain according to Eq. (1) under the influence of different inhibitor/mixture of inhibitors concentrations in a batch experiment, $\mu_{\max}$ was determined in a way to incorporate effect of the inhibitor/mixture of inhibitors. According to the observed inhibition kinetics, usually a "squared" inhibition term according to Eq. (2) was applied,

$$\mu_{\max} = \frac{\mu_{\max}^0}{\left(1 + \frac{c(I)^2}{K_I^2}\right)} \quad \text{Eq. (2),}$$

with $\mu_{\max}$ denoting a strain characteristic maximum specific growth rate at the inhibitor concentration $c(I)$, and $\mu_{\max}^0$ the maximum specific growth rate of the strain under the same conditions, but without inhibitor ($c(\text{inhibitor}) = 0$ g/L). Finally K_I represents an inhibitory constant K_I .

[0205] In some occasions, inhibition of a cellular process significantly reduces but not completely abolishes growth of the organism. This effect can sometimes be explained by the action of alternative cellular processes available to the organism. In this particular situation, the "squared" inhibition term according to Eq. (2) is insufficient to describe growth of the strain, and a hybrid modeling approach according to Eq. (3) was used instead according to

$$\mu_{\max} = \frac{\mu_{\max 1}^{\circ}}{\left(1 + \frac{c(I)^2}{K_I^2}\right)} + \mu_{\max 2}^{\circ} \quad \text{Eq. (3),}$$

with $\mu_{\max}$ denoting a strain characteristic maximum specific growth rate at the inhibitor concentration $c(I)$, and the sum of $\mu_{\max 1}^{\circ}$ and $\mu_{\max 2}^{\circ}$ the maximum specific growth rate of the strain under the same conditions, but without inhibitor ($c(\text{inhibitor}) = 0 \text{ g/L}$). Finally K_I represents an inhibitory constant K_I . Using such equations to fit minimal data collected for a given system allows for determination of strain-specific parameters, i.e. of a maximum specific growth rate without inhibitor ($\mu_{\max}^{\circ}$ or sum of $\mu_{\max 1}^{\circ}$ and $\mu_{\max 2}^{\circ}$) and an inhibitory constant K_I . Based on these parameters, effect of inhibitor concentrations on maximum specific growth rate $\mu_{\max}$ of a given production or contaminant strain can be made, as well as the IC50 value of the inhibitor on their growth be estimated. Equipped with this disclosure, one of skill in the art will be able to utilize parameters such as the IC50 to determine suitable concentrations of compounds for methods provided herein.

[0206] One embodiment is directed to a method for improving production competitiveness of a butanologen in a fermentation mix, wherein the method comprises contacting a genetically modified host cell and a fermentation medium comprising one or more inhibitors, antibiotics, or combinations thereof, as well as a contaminating organism, and wherein the improved production competitiveness is associated with a higher butanol yield on the consumed substrate.

[0207] One embodiment is directed to a method for improving production competitiveness of a butanologen in a fermentation mix, wherein the method comprises contacting a genetically modified host cell and a fermentation medium comprising one or more inhibitors, antibiotics, or combinations thereof, as well as an ethanologen yeast, and wherein the improved production competitiveness is associated with a higher butanol-to-ethanol ratio as compared to a cultivation without addition of one or more inhibitors, antibiotics, or combinations thereof.

[0208] One embodiment is directed to a method for improved production competitiveness of a butanologen in a fermentation mix, wherein the method comprises contacting a genetically modified host cell and a fermentation medium comprising one or more inhibitors, antibiotics, or combinations thereof, and wherein the addition of the one or more inhibitors, antibiotics, or combinations thereof results in less than a 20% loss in the yield of a lower alkyl alcohol produced by the host cell due to the presence of microbial contaminants. In some embodiments, the addition of the one or more inhibitors of amino acid synthesis, antibiotics, or combinations thereof results in less than a 10% loss in the yield of a lower alkyl alcohol produced by the host cell due to the presence of microbial contaminants.

[0209] It will be appreciated that compounds such as an inhibitor, antibiotic, or combinations thereof can be incorporated into a fermentation mix using any method known in the art. In embodiments, compounds are introduced by incorporation into a fermentation feed. In embodiments, compounds are introduced as a bolus or over the course of a fermentation process or a portion of the process as suitable for the compound and production process.

Alcohol production

[0210] Disclosed herein are processes suitable for production of fermentation products from a carbon substrate. In one embodiment a lower alcohol is produced. In one embodiment, butanol is produced, and a butanologen is employed. In another embodiment, isobutanol is produced, and an isobutanologen is employed. In some embodiments, isobutanologens may comprise an isobutanol biosynthetic pathway, such as, but not limited to isobutanol biosynthetic pathways disclosed elsewhere herein. The ability to utilize carbon substrates to produce isobutanol can be confirmed using methods known in the art, including, but not limited to those described in U.S. Pat. Nos. 7,851,188 and 7,993,889 which are incorporated herein by reference. For example, to confirm isobutanol production, the concentration of isobutanol in the culture media 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 0.01 M H₂SO₄ 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 mx0.53 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.

[0211] In some embodiments, the butanologen comprises an engineered butanol pathway. In some embodiments, the butanologen is an isobutanologen. In some embodiments, the butanologen is a yeast. In some embodiments, the butanologen is a member of a genus of *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Issatchenkia*, or *Pichia*. In some embodiments, the butanologen is *Saccharomyces cerevisiae*.

[0212] In some embodiments, the engineered isobutanologen 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 1.2.1.57, EC 1.2.1.10, EC 2.6.1.66, EC 2.6.1.42, EC 1.4.1.9, EC 1.4.1.8, EC 4.1.1.14, EC 2.6.1.18, EC 2.3.1.9, EC 2.3.1.16, EC 1.1.130, 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, EC 5.4.99.13, EC 4.1.1.5, EC 2.7.1.29, EC 1.1.1.76, EC 1.2.1.57, and EC 4.2.1.28.

[0213] In some embodiments, the engineered isobutanologen contains one or more polypeptides selected from acetolactate synthase, acetohydroxy acid isomeroreductase, acetohydroxy acid dehydratase, branched-chain alpha-keto acid decarboxylase, branched-chain alcohol dehydrogenase, acylating aldehyde dehydrogenase, branched-chain keto acid dehydrogenase, butyryl-CoA dehydrogenase, transaminase, valine dehydrogenase, valine decarboxylase, omega transaminase, acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase, isobutyryl-CoA mutase, acetolactate decarboxylase, acetoin aminase, butanol dehydrogenase, butyraldehyde dehydrogenase, acetoin kinase, acetoin phosphate aminase, aminobutanol phosphate phospholyase, aminobutanol kinase, butanediol dehydrogenase, and butanediol dehydratase.

[0214] In some embodiments, the carbon substrate is selected from the group consisting of: oligosaccharides, polysaccharides, monosaccharides, and mixtures thereof. In some embodiments, the carbon substrate is selected from the group consisting of: fructose, glucose, lactose, maltose, galactose, sucrose, starch, cellulose, feedstocks, ethanol, lactate, succinate,

glycerol, corn mash, sugar cane, biomass, a C5 sugar, such as xylose and arabinose, and mixtures thereof.

[0215] In some embodiments, the engineered isobutanol pathway comprises the following substrate to product conversions:

- a. pyruvate to acetolactate
- b. acetolactate to 2,3-dihydroxyisovalerate
- c. 2,3-dihydroxyisovalerate to α -ketoisovalerate
- d. α -ketoisovalerate to isobutyraldehyde, and
- e. isobutyraldehyde to isobutanol.

[0216] In some embodiments, one or more of the substrate to product conversions utilizes NADH or NADPH as a cofactor.

[0217] In some embodiments, enzymes from the biosynthetic pathway are localized to the cytosol. In some embodiments, enzymes from the biosynthetic pathway that are usually localized to the mitochondria are localized to the cytosol. In some embodiments, an enzyme from 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 *e.g.*, U.S. Pat. Nos. 7,851,188 and 7,993,889, which are incorporated herein by reference in its entirety. In some embodiments, the enzyme from 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.

[0218] In some embodiments, the butanologen produces butanol at least 90% of effective yield, at least 91% of effective yield, at least 92% of effective yield, at least 93% of effective yield, at least 94% of effective yield, at least 95% of effective yield, at least 96% of effective yield, at least 97% of effective yield, at least 98% of effective yield, or at least 99% of effective yield. In some embodiments, the butanologen produces butanol at least 55% to at least 75% of effective yield, at least 50% to at least 80% of effective yield, at least 45% to at least 85% of effective yield, at least 40% to at least 90% of effective yield, at least 35% to at least 95% of effective yield, at least 30% to at least 99% of effective yield, at least 25% to at least 99% of effective yield, at least 10% to at least 99% of effective yield or at least 10% to at least 100% of effective yield.

[0219] In some embodiments, the host cell produces ethanol at least 90% of effective yield, at least 91% of effective yield, at least 92% of effective yield, at least 93% of effective

yield, at least 94% of effective yield, at least 95% of effective yield, at least 96% of effective yield, at least 97% of effective yield, at least 98% of effective yield, or at least 99% of effective yield. In some embodiments, the host cell produces ethanol at least 55% to at least 75% of effective yield, at least 50% to at least 80% of effective yield, at least 45% to at least 85% of effective yield, at least 40% to at least 90% of effective yield, at least 35% to at least 95% of effective yield, at least 30% to at least 99% of effective yield, at least 25% to at least 99% of effective yield, at least 10% to at least 99% of effective yield or at least 10% to at least 100% of effective yield.

[0220] In some embodiments, the host cell produces a C3-C6 alcohol at least 90% of effective yield, at least 91% of effective yield, at least 92% of effective yield, at least 93% of effective yield, at least 94% of effective yield, at least 95% of effective yield, at least 96% of effective yield, at least 97% of effective yield, at least 98% of effective yield, or at least 99% of effective yield. In some embodiments, the host cell produces a C3-C6 alcohol at least 55% to at least 75% of effective yield, at least 50% to at least 80% of effective yield, at least 45% to at least 85% of effective yield, at least 40% to at least 90% of effective yield, at least 35% to at least 95% of effective yield, at least 30% to at least 99% of effective yield, at least 25% to at least 99% of effective yield, at least 10% to at least 99% of effective yield or at least 10% to at least 100% of effective yield.

[0221] One embodiment of this invention is directed to a method for the production of a C3-C6 alcohol comprising:

- a. providing a host cell with an engineered pyruvate-utilizing pathway and a polypeptide conferring resistance to one or more inhibitors, antibiotics or combinations thereof, wherein the engineered pyruvate-utilizing pathway is a C3-C6 alcohol biosynthetic pathway;
- b. contacting the host cell with a fermentable carbon substrate in a fermentation medium under conditions whereby the C3-C6 alcohol is produced; and
- c. recovering the C3-C6 alcohol.

[0222] In some embodiments, the fermentation medium comprises one or more inhibitors, antibiotics or combinations thereof.

[0223] In some embodiments, the C3-C6 alcohol is produced at a titer from about 5 g/L to about 100 g/L. In some embodiments, the C3-C6 alcohol is produced at a titer of at least 20 g/L. In some embodiments, the C3-C6 alcohol is selected from the group consisting of: butanol, isobutanol, propanol, and isopropanol.

[0224] One embodiment is a method for the production of ethanol comprising:

- a. providing a host cell with a pyruvate-utilizing pathway and a polypeptide conferring resistance to one or more inhibitors, antibiotics or combinations thereof, wherein the pyruvate-utilizing pathway is an ethanol producing pathway;
- b. contacting the host cell with a fermentable carbon substrate in a fermentation medium under conditions whereby the ethanol is produced; and
- c. recovering the ethanol.

[0225] In some embodiments, the invention provides a method for production of a fermentation product in a fermentation process comprising contacting a fermentation mix comprising a recombinant production microorganism which comprises a pyruvate-utilizing pathway with at least one compound which preferentially inhibits at least one contaminant microorganism. In some embodiments the inhibition is measured through a reduction in the specific growth rate. In some embodiments the inhibition is measured through a reduced specific product formation rate of the contaminant. In some embodiments, the specific growth rate of the at least one contaminant microorganism is reduced more than the specific growth rate of the recombinant production microorganism. In some embodiments, the production of the fermentation product of the at least one contaminant microorganism is reduced more than production of the fermentation product of the recombinant production microorganism.

[0226] In some embodiments, the major product of a production microorganism is ethanol. In some embodiments, the titer of ethanol that is produced may be at least about 80 g/L to at least about 120 g/L. In some embodiments, the titer of ethanol that is produced is least about 50g/L, at least about 60 g/L, at least about 70 g/L, at least about 80 g/L, at least about 90 g/L, at least about 95 g/L, at least about 100 g/L, at least about 105 g/L, at least about 110 g/L, at least about 115 g/L, or at least about 120 g/L.

[0227] In some embodiments, the major product of a production microorganism is butanol. In some embodiments, the titer of butanol that is produced may be at least about 80 g/L to at least about 120 g/L. In some embodiments, the titer of butanol that is produced is least about 50g/L, at least about 60 g/L, at least about 70 g/L, at least about 80 g/L, at least about 90 g/L, at least about 95 g/L, at least about 100 g/L, at least about 105 g/L, at least about 110 g/L, at least about 115 g/L, or at least about 120 g/L.

[0228] In some embodiments, the major product of a production microorganism is butanol, for example, isobutanol. In some embodiments, the major product of a contaminant

microorganism is ethanol. The titer of ethanol may be less than that of butanol. In some embodiments, the titer of ethanol is less than about 20g/L, 10 g/L, less than about 5 g/L, or less than about 2 g/L.

[0229] In embodiments, the major product of a production microorganism is butanol, for example isobutanol, and the major product of a contaminant microorganism is ethanol. In embodiments, the percentage of ethanol produced as a fraction of the amount of butanol produced is less than about 25%, less than about 20%, less than about 10%, less than about 5%, less than about 2%, or less than about 1%.

[0230] In some embodiments, butanol is contacted with a fatty acid and a lipase producing a fatty acid butyl ester ("FABE"), which may be used as a biodiesel fuel.

[0231] The reduction in contamination can be measured through any assay known in the art, including, but not limited to, standard plating assays, qPCR/RT-PCR, or by measuring fermentation titer, yield, or specific growth rate of a production microorganism in relation to a contaminant microorganism.

[0232] In some embodiments, reduction in contamination and increased production competitiveness of the butanologen is observed through measurement of the ratio of the desired fermentation product to the contaminant fermentation production (*e.g.* butanol to ethanol). As the contaminant microorganism is inhibited to a greater degree than the production microorganism is inhibited, by either specific inhibitors or different concentrations of those inhibitors, the ratio of the desired fermentation product to the contaminant fermentation product will increase. The production of fermentation product in the aqueous phase can be quantified by HPLC, as described in the General Methods Section.

[0233] In some embodiments, the reduction in contamination is observed through measurement of the specific growth rate of samples treated with varying concentrations of inhibitors in a cell suspension, and measuring the OD of the samples at designated time points.

[0234] In some embodiments, the reduction in contamination is seen through the use of plating assays. In some embodiments, early stationary phase cultures are used to inoculate top agar media which is poured onto petri plates. Filter disks containing different concentrations of inhibitor can be applied to the plate surface, and, after a period of incubation, zones of growth inhibition can be observed.

Host cells and microorganisms

[0235] The terms "host cell" and "microorganism" are synonymous and used interchangeably throughout. In embodiments, suitable host cells include any yeast host useful for genetic modification and recombinant gene expression. In some embodiments, the host cell is a butanologen. In some embodiments, the host cell is an isobutanologen. In some embodiments, the isobutanologen host cell can be a member of the genera *Schizosaccharomyces*, *Issatchenkia*, *Kluyveromyces*, *Yarrowia*, *Pichia*, *Candida*, *Hansenula*, *Aspergillus*, *Pachysolen*, *Rhodotorula*, *Zygosaccharomyces*, *Galactomyces*, *Torulaspora*, *Debayomyces*, *Williopsis*, *Dekkera*, *Kloeckera*, *Metschnikowia* or *Saccharomyces*. In other embodiments, the host cell can be *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces thermotolerans*, *Kluyveromyces marxianus*, *Candida glabrata*, *Candida albicans*, *Pichia stipitis*, *Yarrowia lipolytica*, *E. coli*, or *L. plantarum*. In still other embodiments, the host cell is a yeast host cell. In some embodiments, the host cell is a member of the genera *Saccharomyces*. In some embodiments, the host cell is *Kluyveromyces lactis*, *Candida glabrata* or *Schizosaccharomyces pombe*. 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.

[0236] *Saccharomyces cerevisiae* PNY860 (or PNY0860), described in Example 4, was deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia, 20110 on July 21, 2011, and assigned ATCC Accession No. PTA-12007.

[0237] *Saccharomyces cerevisiae* PNY827, described in Examples 3 and 13, was deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia, 20110 on September 22, 2011, and assigned ATCC Accession No. PTA-12105.

[0238] In some embodiments, the host cell expresses an engineered butanol biosynthetic pathway. From time to time, such a host cell is referred to as a "butanologen". In some

embodiments, the butanologen is an isobutanologen expressing an engineered isobutanol biosynthetic pathway. In some embodiments, the butanologen is a bacteria, cyanobacteria or filamentous fungi. In some embodiments, the genus of the host cell is selected from the group consisting of: *Salmonella*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Clostridium*, *Corynebacterium*, *Gluconobacter*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Streptomyces*, *Zymomonas*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, and *Xanthomonas*.

[0239] Some embodiments comprise a genetically modified host cell comprising:

- a. an engineered C3-C6 alcohol biosynthetic pathway; and,
- b. a polypeptide that is resistant to inhibitors, antibiotics, or a combination thereof.

Carbon substrates

[0240] Suitable carbon substrates may include, but are not limited to, monosaccharides such as fructose or glucose, oligosaccharides such as lactose, maltose, galactose, or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Other carbon substrates may include ethanol, lactate, succinate, or glycerol.

[0241] "Sugar" includes monosaccharides such as fructose or glucose, oligosaccharides such as lactose, maltose, galactose, or sucrose, polysaccharides such as starch or cellulose, C5 sugars such as xylose and arabinose, and mixtures thereof.

[0242] Additionally the 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 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 yeasts are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion *et al.*, *Microb. Growth C1 Compd.*, [Int. Symp.], 7th (1993), 415-32, Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). 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.

[0243] Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, in some embodiments, the carbon

substrates are glucose, fructose, and sucrose, or mixtures of these with C5 sugars such as xylose and arabinose for yeasts cells modified to use C5 sugars. 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. Patent Application Publication No. 20070031918 A1, 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.

[0244] In some embodiments, the carbon substrate is glucose derived from corn. In some embodiments, the carbon substrate is glucose derived from wheat. In some embodiments, the carbon substrate is sucrose derived from sugar cane.

[0245] In addition to an appropriate carbon source, fermentation media 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 an enzymatic pathway described herein.

Fermentation Conditions

[0246] Typically cells are grown at a temperature in the range of about 20°C to about 40°C in an appropriate medium. Suitable growth media in the present invention include common commercially prepared media such as 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.

[0247] Suitable pH ranges for the fermentation are from about pH 5.0 to about pH 9.0. In one embodiment, about pH 6.0 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. 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 other microorganisms are from about pH 3.0 to about pH 7.5. In one embodiment, about pH 4.5 to about pH 6.5 is used for the initial condition.

[0248] Fermentations may be performed under aerobic or anaerobic conditions. In one embodiment, anaerobic or microaerobic conditions are used for fermentations.

Industrial Batch and Continuous Fermentations

[0249] Isobutanol, or other products, 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. 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 media. 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 (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36:227, (1992).

[0250] Isobutanol, or other products, 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 media 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. 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, *supra*.

[0251] It is contemplated that the production of isobutanol, or other products, 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 or encapsulated within porous material (e.g. alginate beads) and subjected to fermentation conditions for isobutanol production.

Methods for Isobutanol Isolation from the Fermentation Medium

[0252] Bioproducted isobutanol may be isolated from the fermentation medium using methods known in the art for ABE fermentations (*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, solids may be removed from the fermentation medium by centrifugation, filtration, decantation, or the like. Then, the isobutanol may be isolated from the fermentation medium using methods such as distillation, azeotropic distillation, liquid-liquid extraction, adsorption, gas stripping, membrane evaporation, or pervaporation.

[0253] Because isobutanol forms a low boiling point, azeotropic mixture with water, distillation can 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 may be isolated using azeotropic distillation using an entrainer (*see, e.g., Doherty and Malone, Conceptual Design of Distillation Systems*, McGraw Hill, New York, 2001).

[0254] The butanol-water mixture forms a heterogeneous azeotrope so that distillation may be used in combination with decantation to isolate and purify the isobutanol. In this method, the isobutanol containing fermentation broth is distilled to near the azeotropic composition. Then, the azeotropic mixture is condensed, and the isobutanol is separated from the fermentation medium by decantation. The decanted aqueous phase may be returned to the first distillation

column as reflux. The isobutanol-rich decanted organic phase may be further purified by distillation in a second distillation column.

[0255] The isobutanol can also be isolated from the fermentation medium using liquid-liquid extraction in combination with distillation. In this method, the isobutanol is extracted from the fermentation broth using liquid-liquid extraction with a suitable solvent. The isobutanol-containing organic phase is then distilled to separate the butanol from the solvent.

[0256] Distillation in combination with adsorption can also be used to isolate isobutanol from the fermentation medium. In this method, the fermentation broth containing the isobutanol 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).

[0257] Additionally, distillation in combination with pervaporation may be used to isolate and purify the isobutanol from the fermentation medium. In this method, the fermentation broth containing the isobutanol 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)).

[0258] *In situ* product removal (ISPR) (also referred to as extractive fermentation) can be used to remove butanol (or other fermentative alcohol) from the fermentation vessel as it is produced, thereby allowing the microorganism to produce butanol at high yields. One method for ISPR for removing fermentative alcohol that has been described in the art is liquid-liquid extraction. In general, with regard to butanol fermentation, for example, the fermentation medium, which includes the microorganism, is contacted with an organic extractant at a time before the butanol concentration reaches a toxic level. The organic extractant and the fermentation medium form a biphasic mixture. The butanol partitions into the organic extractant phase, decreasing the concentration in the aqueous phase containing the microorganism, thereby limiting the exposure of the microorganism to the inhibitory butanol.

[0259] Liquid-liquid extraction can be performed, for example, according to the processes described in U.S. Patent Appl. Pub. No. 2009/0305370, the disclosure of which is hereby incorporated in its entirety. U.S. Patent Appl. Pub. No. 2009/0305370 describes methods for producing and recovering butanol from a fermentation broth using liquid-liquid extraction,

the methods 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. Typically, the extractant can be an organic extractant selected from the group consisting of saturated, mono-unsaturated, poly-unsaturated (and mixtures thereof) C₁₂ to C₂₂ fatty alcohols, C₁₂ to C₂₂ fatty acids, esters of C₁₂ to C₂₂ fatty acids, C₁₂ to C₂₂ fatty aldehydes, and mixtures thereof. The extractant(s) for ISPR can be non-alcohol extractants. The ISPR extractant can be an exogenous organic extractant such as oleyl alcohol, behenyl alcohol, cetyl alcohol, lauryl alcohol, myristyl alcohol, stearyl alcohol, 1-undecanol, oleic acid, lauric acid, myristic acid, stearic acid, methyl myristate, methyl oleate, undecanal, lauric aldehyde, 20-methylundecanal, and mixtures thereof.

[0260] In some embodiments, an ester can be formed by contacting the alcohol in a fermentation medium with an organic acid (e.g., fatty acids) and a catalyst capable of esterifying the alcohol with the organic acid. In such embodiments, the organic acid can serve as an ISPR extractant into which the alcohol esters partition. The organic acid can be supplied to the fermentation vessel and/or derived from the biomass supplying fermentable carbon fed to the fermentation vessel. Lipids present in the feedstock can be catalytically hydrolyzed to organic acid, and the same catalyst (e.g., enzymes) can esterify the organic acid with the alcohol. The catalyst can be supplied to the feedstock prior to fermentation, or can be supplied to the fermentation vessel before or contemporaneously with the supplying of the feedstock. When the catalyst is supplied to the fermentation vessel, alcohol esters can be obtained by hydrolysis of the lipids into organic acid and substantially simultaneous esterification of the organic acid with butanol present in the fermentation vessel. Organic acid and/or native oil not derived from the feedstock can also be fed to the fermentation vessel, with the native oil being hydrolyzed into organic acid. Any organic acid not esterified with the alcohol can serve as part of the ISPR extractant. The extractant containing alcohol esters can be separated from the fermentation medium, and the alcohol can be recovered from the extractant. The extractant can be recycled to the fermentation vessel. Thus, in the case of butanol production, for example, the conversion of the butanol to an ester reduces the free butanol concentration in the fermentation medium, shielding the microorganism from the toxic effect of increasing butanol concentration. In addition, unfractionated grain can be used as feedstock without separation of lipids therein, since the lipids can be catalytically hydrolyzed to organic acid, thereby decreasing the rate of build-up of lipids in the ISPR extractant. Other butanol product recovery and/or ISPR methods may be

employed, including those described in U.S. Patent No. 8,101,808, incorporated herein by reference.

[0261] *In situ* product removal can be carried out in a batch mode or a continuous mode. In a continuous mode of *in situ* product removal, product is continually removed from the reactor. In a batchwise mode of *in situ* product removal, a volume of organic extractant is added to the fermentation vessel and the extractant is not removed during the process. For *in situ* product removal, the organic extractant can contact the fermentation medium at the start of the fermentation forming a biphasic fermentation medium. Alternatively, the organic extractant can contact the fermentation medium after the microorganism has achieved a desired amount of growth, which can be determined by measuring the optical density of the culture. Further, the organic extractant can contact the fermentation medium at a time at which the product alcohol level in the fermentation medium reaches a preselected level. In the case of butanol production according to some embodiments of the present invention, the organic acid extractant can contact the fermentation medium at a time before the butanol concentration reaches a toxic level, so as to esterify the butanol with the organic acid to produce butanol esters and consequently reduce the concentration of butanol in the fermentation vessel. The ester-containing organic phase can then be removed from the fermentation vessel (and separated from the fermentation broth which constitutes the aqueous phase) after a desired effective titer of the butanol esters is achieved. In some embodiments, the ester-containing organic phase is separated from the aqueous phase after fermentation of the available fermentable sugar in the fermentation vessel is substantially complete.

[0262] Isobutanol 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, which is incorporated herein by reference.

EXAMPLES

[0263] 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

[0264] Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook *et al.* (Sambrook, J., Fritsch, E. F. and Maniatis, T. (Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989, here in referred to as Maniatis) and by Ausubel *et al.* (Ausubel *et al.*, Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience, 1987).

[0265] 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 Thomas D. Brock 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.

[0266] The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "h" means hour(s), "nm" means nanometers, "uL" or "μl" 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.

Example 1: Construction of expression vectors for isobutanol pathway gene expression in *S. cerevisiae*.

pLH475-JEA1 construction

[0267] The pLH475-JEA1 plasmid (SEQ ID NO:23) was constructed for expression of ALS and KARI in yeast. pLH475-JEA1 is a pHR81 vector (ATCC #87541) containing the following chimeric genes: (1) the CUP1 promoter (SEQ ID NO:24), acetolactate synthase coding region from *Bacillus subtilis* (AlsS; SEQ ID NO:25; protein SEQ ID NO:36) and CYC1 terminator2 (SEQ ID NO:27); (2) an *ILV5* promoter (SEQ ID NO:28), Pf5.IlvC-JEA1 coding region (SEQ ID NO:29; protein SEQ ID NO:30 and *ILV5* terminator (SEQ ID NO:31); and (3) the FBA1 promoter (SEQ ID NO:32), *S. cerevisiae* KARI coding region (*ILV5*; SEQ ID NO:33; protein SEQ ID NO:34) and CYC1 terminator (SEQ ID NO:35). The Pf5.IlvC-JEA1 coding region is a sequence encoding KARI derived from *Pseudomonas fluorescens* but containing mutations, that was described in commonly owned and co-pending US Patent Application Publication US20100197519A1, which is herein incorporated by reference (Pf5.IlvC-JEA1 encoded KARI: SEQ ID NO:29; protein SEQ ID NO:30)

Expression Vector pLH468

[0268] The pLH468 plasmid (SEQ ID NO:39) was constructed for expression of DHAD, KivD and HADH in yeast. Coding regions for *L. lactis* ketoisovalerate decarboxylase (KivD) (SEQ ID NO:141) and Horse liver alcohol dehydrogenase (HADH) (SEQ ID NO:40 and 142) were synthesized by DNA2.0 based on codons that were optimized for expression in *Saccharomyces cerevisiae* and provided in plasmids pKivDy-DNA2.0 and pHadhy-DNA2.0. Individual expression vectors for KivD and HADH were constructed. To assemble pLH467 (pRS426::P_{TDH3}-kivDy-TDH3t), vector pNY8 (SEQ ID NO:14; also named pRS426.GPD-ald-GPDt, described in commonly owned and co-pending US Patent App. Pub. US2008/0182308, Example 17, which is herein incorporated by reference) was digested with *AscI* and *SfiI* enzymes, thus excising the *GPD* promoter and the ald coding region. A *TDH3* promoter fragment (SEQ ID NO:41) from pNY8 was PCR amplified to add an *AscI* site at the 5' end, and an *SpeI* site at the 3' end, using 5' primer OT1068 and 3' primer OT1067 (SEQ ID NOs:42 and 43). The

AscI/SfiI digested pNY8 vector fragment was ligated with the *TDH3* promoter PCR product digested with *AscI* and *SpeI*, and the *SpeI-SfiI* fragment containing the codon optimized *kivD* coding region isolated from the vector pKivD-DNA2.0. The triple ligation generated vector pLH467 (pRS426::P_{TDH3}-*kivDy*-*TDH3t*). pLH467 (SEQ ID NO:44) was verified by restriction mapping and sequencing.

[0269] pLH435 (pRS425::P_{GPM1}-*Hadhy*-*ADH1t*) (SEQ ID NO:52) was derived from vector pRS425::GPM-sadB (SEQ ID NO:45) which is described in commonly owned and co-pending US Patent App. Pub No. US20090305363 A1, Example 3, which is herein incorporated by reference in its entirety. pRS425::GPM-sadB is the pRS425 vector (ATCC #77106) with a chimeric gene containing the GPM1 promoter (SEQ ID NO: 46), coding region from a butanol dehydrogenase of *Achromobacter xylosoxidans* (*sadB*; DNA SEQ ID NO:47; protein SEQ ID NO:48: disclosed in US Patent No. 8,188,250, which is herein incorporated by reference in its entirety), and *ADH1* terminator (SEQ ID NO:49). pRS425::GPMp-sadB contains *BbvI* and *PacI* sites at the 5' and 3' ends of the *sadB* coding region, respectively. A *NheI* site was added at the 5' end of the *sadB* coding region by site-directed mutagenesis using primers OT1074 and OT1075 (SEQ ID NO:50 and 51) to generate vector pRS425-GPMp-sadB-*NheI*, which was verified by sequencing. pRS425::P_{GPM1}-*sadB*-*NheI* was digested with *NheI* and *PacI* to drop out the *sadB* coding region, and ligated with the *NheI-PacI* fragment containing the codon optimized HADH coding region from vector pHadhy-DNA2.0 to create pLH435.

[0270] To combine *KivD* and HADH expression cassettes in a single vector, yeast vector pRS411 (ATCC # 87474) was digested with *SacI* and *NotI*, and ligated with the *SacI-Sall* fragment from pLH467 that contains the P_{TDH3}-*kivDy*-*TDH3t* cassette together with the *Sall-NotI* fragment from pLH435 that contains the P_{GPM1}-*Hadhy*-*ADH1t* cassette in a triple ligation reaction. This yielded the vector pRS411::P_{TDH3}-*kivDy*-P_{GPM1}-*Hadhy* (pLH441), which was verified by restriction mapping.

[0271] In order to generate a co-expression vector for all three genes in the lower isobutanol pathway: *ilvD*, *kivDy* and *Hadhy*, we used pRS423 FBA *ilvD*(Strep) (SEQ ID NO:53, which is described in commonly owned and co-pending US Patent App. Pub. US 20100081154A1, which is herein incorporated by reference in its entirety, as the source of the *ilvD* gene. This shuttle vector contains an F1 origin of replication (nt 1423 to 1879) for maintenance in *E. coli* and a 2 micron origin (nt 8082 to 9426) for replication in yeast. The vector has an FBA1 promoter (nt 2111 to 3108; SEQ ID NO:32) and FBA terminator (nt 4861 to

5860; SEQ ID NO:54). In addition, it carries the His marker (nt 504 to 1163) for selection in yeast and ampicillin resistance marker (nt 7092 to 7949) for selection in *E. coli*. The *ilvD* coding region (nt 3116 to 4828; SEQ ID NO:55; protein SEQ ID NO:56) from *Streptococcus mutans* UA159 (ATCC #700610) is between the FBA promoter and FBA terminator forming a chimeric gene for expression. In addition there is a lumio tag fused to the *ilvD* coding region (nt 4829-4849).

[0272] The first step was to linearize pRS423 FBA *ilvD*(Strep) (also called pRS423-FBA(SpeI)-*IlvD*(*Streptococcus mutans*)-Lumio) with *SacI* and *SacII* (with *SacII* site blunt ended using T4 DNA polymerase), to give a vector with total length of 9,482 bp. The second step was to isolate the *kivDy-hADHy* cassette from pLH441 with *SacI* and *KpnI* (with *KpnI* site blunt ended using T4 DNA polymerase), which gives a 6,063 bp fragment. This fragment was ligated with the 9,482 bp vector fragment from pRS423-FBA(SpeI)-*IlvD*(*Streptococcus mutans*)-Lumio. This generated vector pLH468 (pRS423::P_{FBAI}-*ilvD*(Strep)Lumio-FBAIt-P_{TDH3}-*kivDy*-TDH3t-P_{GPMI}-*hadhy-ADHIt*), which was confirmed by restriction mapping and sequencing.

Example 2: Construction of *S. cerevisiae* host strain containing disruptions in pyruvate decarboxylase and hexokinase II

[0273] This example describes insertion-inactivation of endogenous PDC1, PDC5, and PDC6 genes of *S. cerevisiae*. PDC1, PDC5, and PDC6 genes encode the three major isozymes of pyruvate decarboxylase. Hexokinase II, which is responsible for phosphorylation of glucose and transcriptional repression, is also inactivated. The resulting PDC/HXK2 inactivation strain (U.S. Publication No: 2011/0124060, which is incorporated herein by reference) was used as a host for expression vectors pLH475-JEA1 and pLH468 that were described in Example 2.

Construction of *pdh6*::P_{GPMI}-*sadB* integration cassette and PDC6 deletion:

[0274] A *pdh6*::P_{GPMI}-*sadB*-ADHIt-URA3r integration cassette was made by joining the *GPM-sadB-ADHIt* segment from pRS425::GPM-*sadB* (described above) to the URA3r gene from pUC19-URA3r. pUC19-URA3r (SEQ ID NO:57) contains the URA3 marker from pRS426 (ATCC # 77107) flanked by 75 bp homologous repeat sequences to allow homologous recombination *in vivo* and removal of the URA3 marker. The two DNA segments were joined by SOE PCR (as described by Horton *et al.* (1989) *Gene* 77:61-68) using as template pRS425::GPM-*sadB* and pUC19-URA3r plasmid DNAs, with Phusion DNA polymerase (New England Biolabs Inc., Beverly, MA; catalog no. F-540S) and primers 114117-11A through

114117-11D (SEQ ID NOs:58, 59, 60 and 61), and 114117-13A and 114117-13B (SEQ ID NOs:62 and 63).

[0275] The outer primers for the SOE PCR (114117-13A and 114117-13B) contained 5' and 3' ~50 bp regions homologous to regions upstream and downstream of the *PDC6* promoter and terminator, respectively. The completed cassette PCR fragment was transformed into BY4700 (ATCC # 200866) and transformants were maintained on synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202). Transformants were screened by PCR using primers 112590-34G and 112590-34H (SEQ ID NOs:64 and 65), and 112590-34F and 112590-49E (SEQ ID NOs:66 and 67) to verify integration at the *PDC6* locus with deletion of the *PDC6* coding region. The *URA3r* marker was recycled by plating on synthetic complete media 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 media to verify the absence of growth. The resulting identified strain has the genotype: BY4700 *pdc6::P_{GPM1}-sadB-ADH1t*.

Construction of *pdcl::P_{PDC1}-ilvD* integration cassette and *PDC1* deletion:

[0276] A *pdcl::P_{PDC1}-ilvD-FBA1t-URA3r* integration cassette was made by joining the *ilvD-FBA1t* segment from pLH468 (described above) to the *URA3r* gene from pUC19-URA3r by SOE PCR (as described by Horton et al. (1989) Gene 77:61-68) using as template pLH468 and pUC19-URA3r plasmid DNAs, with Phusion DNA polymerase (New England Biolabs Inc., Beverly, MA; catalog no. F-540S) and primers 114117-27A through 114117-27D (SEQ ID NOs:68, 69, 70 and 71).

[0277] The outer primers for the SOE PCR (114117-27A and 114117-27D) contained 5' and 3' ~50 bp regions homologous to regions downstream of the *PDC1* promoter and downstream of the *PDC1* coding sequence. The completed cassette PCR fragment was transformed into BY4700 *pdc6::P_{GPM1}-sadB-ADH1t* and transformants were maintained on synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202). Transformants were screened by PCR using primers 114117-36D and 135 (SEQ ID NOs:72 and 73), and primers 112590-49E and 112590-30F (SEQ ID NOs:67 and 74) to verify integration at the *PDC1* locus with deletion of the *PDC1* coding sequence. The *URA3r* marker was recycled by plating on synthetic complete media

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 media to verify the absence of growth. The resulting identified strain "NYLA67" has the genotype: BY4700 *pdv6::P_{GPMI}-sadB-ADH1t pdv1::P_{PDCI}-ilvD-FBA1t*.

HIS3 deletion

[0278] To delete the endogenous HIS3 coding region, a *his3::URA3r2* cassette was PCR-amplified from URA3r2 template DNA (SEQ ID NO:75). URA3r2 contains the *URA3* marker from pRS426 (ATCC # 77107) flanked by 500 bp homologous repeat sequences to allow homologous recombination *in vivo* and removal of the *URA3* marker. PCR was done using Phusion DNA polymerase and primers 114117-45A and 114117-45B (SEQ ID NOs:76 and 77) which generated a ~2.3 kb PCR product. The *HIS3* portion of each primer was derived from the 5' region upstream of the *HIS3* promoter and 3' region downstream of the coding region such that integration of the *URA3r2* marker results in replacement of the *HIS3* coding region. The PCR product was transformed into NYLA67 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 synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C. Transformants were screened to verify correct integration by replica plating of transformants onto synthetic complete media lacking histidine and supplemented with 2% glucose at 30°C. The *URA3r* marker was recycled by plating on synthetic complete media 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 media to verify the absence of growth. The resulting identified strain, called NYLA73, has the genotype: BY4700 *pdv6::P_{GPMI}-sadB-ADH1t pdv1::P_{PDCI}-ilvD-FBA1t Δhis3*.

Deletion of HXK2 (hexokinase II):

[0279] A *hvk2::URA3r* cassette was PCR-amplified from URA3r2 template (described above) using Phusion DNA polymerase and primers 384 and 385 (SEQ ID NOs:78 and 79) which generated a ~2.3 kb PCR product. The *HXK2* portion of each primer was derived from the 5' region upstream of the *HXK2* promoter and 3' region downstream of the coding region such that integration of the *URA3r2* marker results in replacement of the *HXK2* coding region. The PCR product was transformed into NYLA73 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 synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C. Transformants were screened by PCR to verify correct integration at the *HXK2* locus with replacement of the *HXK2* coding region using primers N869 and N871 (SEQ ID NOs:80 and 81). The *URA3r2* marker was recycled by plating on synthetic complete media 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 media to verify the absence of growth, and by PCR to verify correct marker removal using primers N946 and N947 (SEQ ID NOs:82 and 83). The resulting identified strain named NYLA83 has the genotype: BY4700 *pdcc6::P_{GPMI}-sadB-ADH1t pdcc1::P_{PDC1}-ilvD-FBA1t Δhis3 Δhxc2*.

Construction of *pdcc5::kanMX* integration cassette and *PDC5* deletion

[0280] A *pdcc5::kanMX4* cassette was PCR-amplified from strain YLR134W chromosomal DNA (ATCC No. 4034091) using Phusion DNA polymerase and primers *PDC5::KanMXF* and *PDC5::KanMXR* (SEQ ID NOs:84 and 85) which generated a ~2.2 kb PCR product. The *PDC5* portion of each primer was derived from the 5' region upstream of the *PDC5* promoter and 3' region downstream of the coding region such that integration of the *kanMX4* marker results in replacement of the *PDC5* coding region. The PCR product was transformed into NYLA83, and transformants were selected and screened as described above. The identified correct transformants named NYLA84 have the genotype: BY4700 *pdcc6::P_{GPMI}-sadB-ADH1t pdcc1::P_{PDC1}-ilvD-FBA1t Δhis3 Δhxc2 pdcc5::kanMX4*.

[0281] Plasmid vectors pLH468 and pLH475-JEA1 were simultaneously transformed into strain NYLA84 (BY4700 *pdcc6::P_{GPMI}-sadB-ADH1t pdcc1::P_{PDC1}-ilvD-FBA1t Δhis3 Δhxc2 pdcc5::kanMX4*) using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and the resulting strain was maintained on synthetic complete media lacking histidine and uracil, and supplemented with 1% ethanol at 30°C.

Example 3: Construction of *S. cerevisiae* host strain containing disruptions in *URA3*, *HIS3*, and insertion of sulfonyleurea-resistant *ILV2*

[0282] This example describes inactivation of the *URA3* and *HIS3* genes of *S. cerevisiae*, and replacement of the native *ILV2* gene with a variant that is resistant to sulfonyleurea herbicides.

The resulting strain will be used as a host for expression vectors pLH475-JEA1 and pLH468 that were described in Example 1.

URA3 deletion

[0283] To delete the endogenous URA3 coding region, a deletion cassette was PCR-amplified from pLA54 (SEQ ID NO:100) which contains a P_{TEF1} -*kanMX*-TEF1t cassette flanked by loxP sites to allow homologous recombination in vivo and subsequent removal of the KanMX marker. PCR was performed using Phusion DNA polymerase and primers BK505 and BK506 (SEQ ID NOs:101 and 102). 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 KanMX cassette results in replacement of the *URA3* coding region. The PCR product was transformed into PNY827 (ATCC # PTA-12105), 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 rich media supplemented 2% glucose and 100µg/ml Geneticin at 30°C. Transformants were screened by colony PCR with primers BK468 and LA492 (SEQ ID NOs:103 and 104) to verify presence of the integration cassette. A heterozygous *URA3* mutant was obtained; NYLA98 MATa/ α *URA3/ura3::loxP-kanMX-loxP*. To obtain haploids, NYLA98 was sporulated using standard methods (Codón AC, Gasent-Ramírez JM, Benítez T., *Appl Environ Microbiol.* 1995 PMID: 7574601). Tetrads were dissected using a micromanipulator and grown on rich media supplemented with 2% glucose. Tetrads containing four viable spores were patched onto synthetic complete media lacking uracil and supplemented with 2% glucose, and the mating type was verified by multiplex colony PCR using primers AK109-1, AK109-2, and AK109-3 (SEQ ID NOs:105, 106, and 107). The resulting identified haploid strain called NYLA103 has the genotype MATa *ura3Δ::loxP-kanMX-loxP*, and NYLA106 has the genotype MATa *ura3Δ::loxP-kanMX-loxP*.

HIS3 deletion

[0284] 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: 89) and primer oBP453 (SEQ ID NO:109), 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:110),

containing a 5' tail with homology to the 3' end of HIS3 Fragment A, and primer oBP455 (SEQ ID NO:90), 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:91, containing a 5' tail with homology to the 3' end of HIS3 Fragment B, and primer oBP457 (SEQ ID NO:86), 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:87), containing a 5' tail with homology to the 3' end of HIS3 Fragment U, and primer oBP459 (SEQ ID NO:88). 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:89) and oBP455 (SEQ ID NO:90). 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:91) and oBP459 (SEQ ID NO:88). 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:89) and oBP459 (SEQ ID NO:88). The final PCR product was purified with a PCR Purification kit (Qiagen).

[0285] To delete the endogenous *HIS3* coding region, the "scarless" deletion cassette was transformed into NYLA106 using standard techniques and plated on synthetic complete media lacking uracil and supplemented with 2% glucose. Transformants were screened to verify correct integration by replica plating onto synthetic complete media lacking histidine and supplemented with 2% glucose at 30°C. Genomic DNA preps were made to verify the integration by PCR using primers BP460 and LA135 (SEQ ID NOs:93 and 94) for the 5' end and primers BP461 and LA92 (SEQ ID NOs:95 and 96) for the 3' end. The *URA3* marker was recycled by plating on synthetic complete media 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 media to verify the absence of growth. The resulting identified strain, called PNY2003, has the genotype MATa *ura3Δ::loxP-kanMX-loxP his3Δ*.

Deletion of *PDC1*:

[0286] To delete the endogenous *PDC1* coding region, a deletion cassette was PCR-amplified from pLA59 (SEQ ID NO:97), 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 DNA polymerase and primers LA678 and LA679

(SEQ ID NOs:98 and 99). 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 strain PNY2003 using standard genetic techniques with selection on synthetic complete media 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:111), external to the 5' coding region and LA135 (SEQ ID NO:94), an internal to *URA3*. Positive transformants were then screened by colony PCR using primers LA692 and LA693 (SEQ ID NOs:112 and 113), which were internal to the *PDC1* coding region. The *URA3* marker was recycled by transforming with pRS423::P_{GALI}-*cre* (SEQ ID NO:121) and plated on synthetic complete media lacking histidine and supplemented with 2% glucose at 30°C. Transformants were plated on YP supplemented with 0.5% galactose to induce expression of Cre recombinase. Marker removal was confirmed by patching colonies to synthetic complete media lacking uracil and supplemented with 2% glucose to verify absence of growth. The resulting identified strain, called PNY2008, has the genotype MATa *ura3Δ::loxP-kanMX-loxP his3Δ pdc1Δ::loxP71/66*.

Construction of *ILV2-410* integration vector:

[0287] A fragment of the native *ILV2* gene from *S. cerevisiae* BY4700 was PCR-amplified using Phusion DNA polymerase and primers LA684 and LA685 (SEQ ID NOs: 114 and 115). The ~2 kb PCR product was digested with *Bam*HI and *Sph*I and cloned into pUC19, and the resulting vector was named pUC19::ILV2 (SEQ ID No:17). Site-directed mutagenesis (QuickChange XL, Stratagene, CA) was used to introduce a C to T transition at base pair 574, resulting in a proline-to-serine substitution (Yadav *et al.* 1986 *PNAS*. 83:4418-4422). PfuUltra DNA polymerase (Stratagene, CA), primers LA682 and LA683 (SEQ ID NOs:116 and 117), and pUC19::ILV2 template were used to introduce the mutation following standard protocol. The PCR reaction was digested with *Dpn*I to remove parental DNA, and the reaction was transformed into DH5α competent cells on LB-Amp(100 μg/ml). The presence of DNA containing the *ILV2-410* allele was confirmed by DNA sequencing of plasmid DNA isolated from transformants. The resulting vector was named pUC19::ILV2-410.

[0288] The *ILV2-410* fragment was digested from pUC19::ILV2-410 by *Bam*HI *Sph*I digest and subcloned into pLA59. pLA59 (SEQ ID No:97) is a pUC19 cloning vector that contains a *loxP71-URA3-loxP66* cassette. The resulting vector, pLA59::ILV2-410 (SEQ ID

NO:18), was used as template for PCR of the full integration cassette. The *ILV2-410-loxP71-URA3-loxP66* integration cassette was PCR amplified from pLA59::ILV2-410 template using Phusion DNA polymerase and primers LA686 and LA687 (SEQ ID NOs:119 and 120), producing a ~3 kb product. The *ILV2* portion of each primer was derived from the 5' region downstream of the *ILV2* start codon and 3' region downstream of the stop codon such that integration of the *URA3* cassette results in replacement of the native *ILV2* coding region.

[0289] The PCR product was transformed into strain PNY2008 and plated on synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C. The *loxP71-URA3-loxP66* marker was recycled by transformation with pRS423::P_{GALI}-cre (SEQ ID NO:121) and plating on synthetic complete media lacking histidine supplemented with 3% glucose at 30°C. Colonies were patched onto YP (1% galactose) plates at 30°C to induce *URA3* marker excision and were transferred onto YP (2% glucose) plates at 30°C for recovery. Removal of the *URA3* marker were confirmed by patching colonies from the YP (2% glucose) plates onto synthetic complete media lacking uracil supplemented with 2% glucose to verify the absence of growth. The resulting identified strain, called PNY2010, has the genotype MATa *ura3Δ::loxP-kanMX-loxP his3Δ pdc1Δ::loxP71/66 ILV2-410::loxP71/66*.

Example 4: Susceptibility of wildtype *S. cerevisiae* strains to sulfonylurea herbicides

[0290] This example describes experiments that demonstrate yeast strains, expressing wildtype acetolactate synthase, are resistant to many AHAS-inhibiting sulfonylurea herbicides. Strains tested in this experiment included: *S. cerevisiae* yeast strain PNY0860-1A), a haploid derived from sporulation of the yeast strain deposited with ATCC (ATCC #PTA-12007; *S. cerevisiae* yeast strain PNY827 (ATCC # PTA-12105); and *S. cerevisiae* strain CEN.PK 113-7D (Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre #8340).

[0291] The following AHAS inhibitors were resuspended in 10 mM KOH to give final concentrations of 2 mg/ml (w/v).

Accent™	nicosulfuron methyl (V9360)
W4189-128	research sample
Ally™	metsulfuron methyl (T6376)
Classic™	chlorimuron ethyl (F6025)
Oust™	sulfometuron methyl (SM)
Harmony™	thifensulfuron methyl

[0292] The yeast strains were initially grown on synthetic complete liquid media lacking all amino acids and supplemented with 2% glucose at 30°C. Early stationary phase cultures (OD_{600nm} of ~5.0) were used to inoculate 40 ml of top agar media (synthetic complete lacking all amino acids with 0.7% agarose), which were poured into petri plates. Filter disks containing 20 µg AHAS inhibitor (20 µl of a 1 mg/ml stock) or 10 µg AHAS inhibitor (20 µl of a 0.2 mg/ml stock) were added to the plate surface. Plates were incubated for 72 hours at 30°C before visualization of zones of growth inhibition. Clear zones surrounding the AHAS-laced filter disks indicate inhibition of yeast growth. These results suggested that Classic, Ally, and Oust herbicides inhibit growth of the yeast strains. Accent, Harmony, and W4189 did not inhibit the industrial yeast strains at the concentrations used in this experiment. (Figure 2)

Example 5: Resistance of engineered *S. cerevisiae* strains containing an *ILV2* variant gene to sulfonylurea herbicides

[0293] This example describes experiments that demonstrate yeast strains, expressing a resistant variant of acetolactate synthase, are resistant to the AHAS inhibitor sulfometuron methyl.

[0294] *S. cerevisiae* yeast strains PNY2008 and PNY2010 are described in Example 3. PNY2010 contains the *ILV2-410* allele that confers resistance to sulfonylureas.

[0295] The yeast strains were initially grown on synthetic complete media supplemented with 2% glucose at 30°C. The strains were patched onto either synthetic complete media supplemented with 2% glucose at 30°C or synthetic complete media supplemented with 2% glucose and 12.5 µg/ml sulfometuron methyl (prepared in 10 mM KOH as in Example 4). Plates were incubated for 48 hours at 30°C before visualization. Strain PNY2008 was unable to grow on plates containing sulfometuron methyl, whereas strain PNY2010 grew normally due to the presence of the *ILV2-410* allele.

Example 6: Production of isobutanol in recombinant *S. cerevisiae* NYLA84

[0296] The purpose of this example is to describe the production of isobutanol in the yeast strain NYLA84. The yeast strain comprises deletions of *PDC1*, *PDC5*, and *PDC6*, genes encoding three isozymes of pyruvate decarboxylase, and deletion of *HXK2* encoding hexokinase II. The strain also contains constructs for heterologous expression of AlsS (acetolactate synthase), KARI (keto acid reductoisomerase), DHAD (dihydroxy acid dehydratase), KivD (ketoisovalerate decarboxylase), and SadB (secondary alcohol dehydrogenase).

Strain construction

[0297] Plasmids pLH468 and pLH475-JEA1 were introduced into NYLA84, described in Example 3, by standard PEG/lithium acetate-mediated transformation methods. Transformants were selected on synthetic complete medium lacking glucose, histidine and uracil. Ethanol (1% v/v) was used as the carbon source. After three days, transformants were patched to synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources. Freezer vials were made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

Production of isobutanol

[0298] 80 ml of synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources was inoculated with a yeast strain.

[0299] Fermentation conditions:

[0300] *Medium* (final concentration): 6.7 g/L, Yeast Nitrogen Base w/o amino acids (Difco); 2.8 g/L, Yeast Synthetic Drop-out Medium Supplement Without Histidine, Leucine, Tryptophan and Uracil (Sigma Y2001); 20 mL/L of 1% (w/v) L-Leucine; 4 mL/L of 1% (w/v) L-Tryptophan; 1 mL/L ergosterol/tween/ethanol solution; 0.2 mL/L Sigma DF204; 10 g/L glucose

[0301] The fermenter was set to control at pH 5.5 with KOH, 30% dO, temperature 30°C, and airflow of 0.2 SLPM (or, 0.25 vvm). At inoculation, the airflow was set to 0.01 SLPM initially, then increased to 0.2 SLPM once growth was established. Glucose was maintained at 5-15 g/L throughout by manual addition.

[0302] Because efficient production of isobutanol with NYLA84 pLH486/pLH475 requires microaerobic conditions to enable redox balance in the biosynthetic pathway, air was continuously supplied to the fermenter at 0.25 vvm. Continuous aeration led to significant stripping of isobutanol from the aqueous phase of the fermenter. To quantify the loss of isobutanol due to stripping, the off-gas from the fermenter was directly sent to a mass spectrometer (Prima dB mass spectrometer, Thermo Electron Corp., Madison, WI) to quantify the amount of isobutanol in the gas stream. The isobutanol peaks at mass to charge ratios of 74 or 42 were monitored continuously to quantify the amount of isobutanol in the gas stream.

[0303] Glucose and organic acids in the aqueous phase were monitored during the fermentation using HPLC. Glucose was also monitored quickly using a glucose analyzer (YSI,

Inc., Yellow Springs, OH). Isobutanol in the aqueous phase was quantified by HPLC as described in the General Methods Section herein above after the aqueous phase was removed periodically from the fermenter. The effective titer, corrected for the isobutanol lost due to stripping, was 7.5 g/L. The titer of isobutyric acid was 1.28 g/L. (Figure 3)

Example 7 (Prophetic): Resistance of engineered *S. cerevisiae* isobutanologens containing an *ILV2* variant gene to sulfonylurea herbicides

[0304] This example describes experiments that demonstrate yeast strains that contain an engineered isobutanol production pathway which also express a resistant variant of acetolactate synthase, are resistant to the AHAS inhibitor sulfometuron methyl. Construction of strain NYLA84 is shown in Example 2.

[0305] The *ILV2-410-loxP71-URA3-loxP66* integration cassette (described in Example 3) is PCR amplified from pLA59::ILV2-410 template using Phusion DNA polymerase and primers LA686 and LA687 (SEQ ID NOs:119 and 120), producing a ~3 kb product. The PCR product is transformed into strain NYLA84 and plated on synthetic complete media lacking uracil and supplemented with 1% ethanol at 30°C. The *loxP71-URA3-loxP66* marker is recycled by transformation with pRS423::P_{GAL1}-cre (SEQ ID NO:121) and plating on synthetic complete media lacking histidine supplemented with 1% ethanol at 30°C. Colonies are patched onto YP (1% galactose) plates at 30°C to induce *URA3* marker excision and are transferred onto YP (1% ethanol) plates at 30°C for recovery. Removal of the *URA3* marker is confirmed by patching colonies from the YP (1% ethanol) plates onto synthetic complete media lacking uracil supplemented with 1% ethanol to verify the absence of growth. The resulting identified strain has the genotype NYLA84 *ILV2-410::loxP71/66*.

[0306] Plasmid vectors pLH468 and pLH475-JEA1 were simultaneously transformed into strain NYLA84 *ILV2-410::loxP71/66* using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and the resulting strain was maintained on synthetic complete media lacking histidine and uracil, and supplemented with 1% ethanol at 30°C.

[0307] The yeast strains are initially grown on synthetic complete media lacking histidine and uracil, and supplemented with 1% ethanol at 30°C. After three days, transformants are patched to synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources.

[0308] 20 ml of synthetic complete medium lacking histidine and uracil supplemented with both 0.2% glucose and 0.5% ethanol as carbon sources at 30°C is inoculated with the yeast strain. Each strain is diluted to an initial OD of 0.2 in tubes containing fresh synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources. The AHAS inhibitor sulfometuron methyl is added to the tubes at concentrations ranging from 0 µg/ml to 50 µg/ml. The tubes are incubated overnight at 30°C shaking at 220 rpm and are scored the following day for growth. Strains expressing cytosolic acetolactate synthase demonstrate higher resistance to sulfometuron methyl. Isobutanol in the aqueous phase is quantified by HPLC as described in the General Methods Section.

Example 8 (Prophetic): Production of isobutanol in recombinant *S. cerevisiae* NYLA84 in the presence of wildtype yeast competitor and sulfometuron methyl herbicide

[0309] The purpose of this example is to describe the production of isobutanol in the yeast strain NYLA84 *ILV2-410::loxP71/66* pLH468/pLH475-JEA1 when challenged with a wildtype yeast strain. The AHAS inhibitor sulfometuron methyl is added to prevent or retard growth of the wildtype yeast strain.

Strain construction

[0310] Plasmids pLH468 and pLH475-JEA1 are introduced into NYLA84 *ILV2-410::loxP71/66* pLH468/pLH475-JEA1, described in Example 7, by standard PEG/lithium acetate-mediated transformation methods. Transformants are selected on synthetic complete medium lacking glucose, histidine and uracil. Ethanol (1% v/v) is used as the carbon source. After three days, transformants are patched to synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources.

[0311] Wildtype competitor strain Ethanol Red (Fermentis) is grown in synthetic complete medium supplemented with 2% glucose as carbon source.

Production of isobutanol

[0312] 80 ml of synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources is inoculated with the yeast strain.

Fermentation conditions:

[0313] *Medium* (final concentration): 6.7 g/L, Yeast Nitrogen Base w/o amino acids (Difco); 2.8 g/L, Yeast Synthetic Drop-out Medium Supplement Without Histidine, Leucine

Tryptophan and Uracil (Sigma Y2001); 20 mL/L of 1% (w/v) L-Leucine; 4 mL/L of 1% (w/v) L-Tryptophan; 1 mL/L ergosterol/tween/ethanol solution; 0.2 mL/L Sigma DF204; 10 g/L glucose.

[0314] Both fermenters are inoculated with NYLA84 pLH486/pLH475 and Ethanol Red (at 0.5x number of cells as the NYLA84 strain). Sulfometuron methyl is added to one fermenter at a concentration found to be inhibitory (*see* Example 4). The fermenters are set to control at pH 5.5 with KOH, 30% dO, temperature 30°C, and airflow of 0.2 SLPM (or, 0.25 vvm). At inoculation, the airflow is set to 0.01 SLPM initially, then increased to 0.2 SLPM once growth is established. Glucose is maintained at 5-15 g/L throughout by manual addition.

[0315] Because efficient production of isobutanol with NYLA84 pLH486/pLH475 requires microaerobic conditions to enable redox balance in the biosynthetic pathway, air is continuously supplied to the fermenter at 0.25 vvm. Continuous aeration leads to significant stripping of isobutanol from the aqueous phase of the fermenter. To quantify the loss of isobutanol due to stripping, the off-gas from the fermenter is directly sent to a mass spectrometer (Prima dB mass spectrometer, Thermo Electron Corp., Madison, WI) to quantify the amount of isobutanol in the gas stream. The isobutanol peaks at mass to charge ratios of 74 or 42 are monitored continuously to quantify the amount of isobutanol in the gas stream.

[0316] Glucose and organic acids in the aqueous phase are monitored during the fermentation using HPLC. Glucose is also monitored quickly using a glucose analyzer (YSI, Inc., Yellow Springs, OH). Isobutanol in the aqueous phase is quantified by HPLC as described in the General Methods Section herein above, after the aqueous phase is removed periodically from the fermenter.

Example 9 (Prophetic): Isobutanol production in an engineered *S. cerevisiae* isobutanologens containing a heterologous acetolactate synthase that is resistant to sulfonylurea herbicides

[0317] This example describes experiments that demonstrate yeast strains, which contain an engineered isobutanol production pathway and express a heterologous acetolactate synthase that is resistant to sulfonylurea herbicides, are resistant to the AHAS inhibitor sulfometuron methyl. Construction of strain NYLA84 is shown in Example 2.

[0318] The enzyme ALS I (encoded by *ilvB*) from the enterobacteria *Escherichia coli* K12, which is intrinsically resistant to sulfonylurea herbicides, is PCR-amplified from *E. coli* K12 genomic DNA using Phusion DNA polymerase and primers T001 and T002 (SEQ ID

NOs:122 and 123). The *FBAI* promoter is PCR amplified from BY4700 genomic DNA using Phusion DNA polymerase and primers T003 and T004 (SEQ ID NOs:124 and 125). The *FBAI* terminator is PCR amplified from BY4700 genomic DNA using Phusion DNA polymerase and primers T005 and T006 (SEQ ID NOs:126 and 127). The *FBAI* promoter is digested with *SphI* *KpnI*, the *ilvB* gene is digested with *KpnI* *NotI*, and the *FBAI* terminator is digested with *NotI* *BamHI*. The three fragments are ligated together and subcloned into vector pLA59 (described in Example 3) via *SphI* *BamHI* sites, creating vector pLA59::*ilvB* (SEQ ID NO:19).

[0319] The *bdh1*Δ::P_{FBAI}-*ilvB*-*FBAI*t-*loxP71*-*URA3*-*loxP66* integration cassette is PCR amplified from pLA59::*ilvB* template (SEQ ID NO:19) using Phusion DNA polymerase and primers T007 and T008 (SEQ ID NOs:128 and 129). The *BDHI* portion of each primer was derived from the 5' region 50bp downstream of the *BDHI* start codon and 3' region 50bp upstream of the stop codon such that integration of the *URA3* cassette results in replacement of the *BDHI* coding region but leaves the first ~50bp and the last ~50bp of the coding region. The PCR product is transformed into strain NYLA84 and plated on synthetic complete media lacking uracil and supplemented with 1% ethanol at 30°C. The *loxP71*-*URA3*-*loxP66* marker is recycled by transformation with pRS423::P_{GALI}-cre (SEQ ID NO:121) and plating on synthetic complete media lacking histidine supplemented with 1% ethanol at 30°C. Colonies are patched onto YP (1% galactose) plates at 30°C to induce *URA3* marker excision and are transferred onto YP (1% ethanol) plates at 30°C for recovery. Removal of the *URA3* marker is confirmed by patching colonies from the YP (1% ethanol) plates onto synthetic complete media lacking uracil supplemented with 1% ethanol to verify the absence of growth. The resulting identified strain has the genotype NYLA84 *bdh1*::*ilvB*-*loxP71/66*.

[0320] Plasmid vectors pLH468 and pLH475-JEA1 are simultaneously transformed into strain NYLA84 *bdh1*::*ilvB*-*loxP71/66* using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and the resulting strain is maintained on synthetic complete media lacking histidine and uracil, and supplemented with 1% ethanol at 30°C.

[0321] The yeast strains are initially grown on synthetic complete media lacking histidine and uracil, and supplemented with 1% ethanol at 30°C. After three days, transformants are patched to synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources.

[0322] 20 ml of synthetic complete medium lacking histidine and uracil supplemented with both 0.2% glucose and 0.5% ethanol as carbon sources at 30°C is inoculated with the yeast strain. Each strain is diluted to an initial OD of 0.2 in tubes containing fresh synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources. The AHAS inhibitor sulfometuron methyl is added to the tubes at concentrations ranging from 0 µg/ml to 50 µg/ml. The tubes are incubated overnight at 30°C shaking at 220 rpm and are scored the following day for growth. Strains expressing cytosolic acetolactate synthase demonstrate higher resistance to sulfometuron methyl. Isobutanol in the aqueous phase is quantified by HPLC as described in the General Methods Section.

[0323] Enterobacterial ALS enzymes are described in LaRossa and Smul, *J. Bacteriol.* 160(1):391-394 (1984). LaRossa describes ALSI enzymes from *S. typhimurium* and *E. coli* that are resistant to sulfonylurea herbicides.

Materials and methods for examples 10 to 20

[0324] Yeast synthetic medium w/o amino acids, w/o glucose, w/o ethanol/Tween (2x) 13.4 g/l, Yeast Nitrogen Base w/o amino acids (Difco 0919-15-3); 40 mg/L thiamine; 40 mg/L niacin; 200 ml/L 1M MES buffer, pH = 5.5

[0325] Supplement aa sol. without histidine and uracil (SAAS-1 10x): 18.5 g/L, Synthetic complete amino acid dropout (Kaiser) -His, -Ura (Formedium).

[0326] Na-acetate stock solution: 3 M sodium acetate solution

[0327] Glucose stock solution: 250 g/L glucose solution

[0328] Inhibitor stock solutions: (1) copper (II) sulfate pentahydrate: CuSO₄ • 5 H₂O (MW 249.6 g/mol, CAS Number 7758-99-8): 150 mM; (2) formaldehyde solution (SIGMA F8775, 36.5-38% in H₂O, MW 30.03 g/mol, CAS Number 50-00-0): 12.15 M; (3) sodium sulfite (Na₂SO₃, SIGMA-ALDRICH S0505, CAS Number 7757-83-7, MW 126.04 g/mol): 100 mM in SF11, 500 mM in SF12; (4) bismuth(III) citrate (CAS Number 813-93-4, [O₂CCH₂C(OH)(CO₂)CH₂CO₂] Bi, MW 398.08 g/mol): saturated solution; (5) sulfometuron methyl (Fluka #34224, CAS Number 74222-97-2, C₁₅H₁₆N₄O₅S, MW 364.38 g/mol): 10 g/L in DMSO; (6) 4-pyrazolecarboxylic acid (Sigma-Aldrich, # 300713, C₄H₄N₂O₂, MW: 112.09 g/mol, CAS Number: 37718-11-9): 1.0 M in SF12 (= 112mg/ml(DMSO)); (7) 4-methylpyrazole hydrochloride (Sigma, # M1387, C₄H₆N₂ • HCl, MW: 118.56 g/mol, CAS: 56010-88-9): 1.0M in SF12 (= 119 mg/ml(DMSO)); (8) pyrazole (Aldrich, # P56607, C₃H₄N₂, MW: 68.08 g/mol, CAS Number: 288-13-1): 0.5 M in SF 12 (= 34 mg/ml); (9) glyoxylic acid sodium salt

monohydrate ($\text{HC(O)COONa} \cdot \text{H}_2\text{O}$, MW: 114.03, CAS Number: 918149-31-2): 0.5 M in SF12 (= 57 mg/ml); (10) pyrazole (Aldrich, # P56607, $\text{C}_3\text{H}_4\text{N}_2$, MW: 68.08 g/mol, CAS Number: 288-13-1): 0.5 M (= 34 mg/ml) in SF13; (11) trans-cinnamaldehyde (Aldrich #239968, $\text{C}_6\text{H}_5\text{CH}=\text{CHCHO}$, MW: 132.16 g/mol, CAS: 14371-10-9, $d = 1.050 \text{ g/ml}$): SF12 and SF13 = pure liquid, SF14 = 20 mM in DMSO; (12) 1-bromo-2-butanone (Sigma-Aldrich #243299, $\text{C}_2\text{H}_5\text{COCH}_2\text{Br}$, MW: 151.00 g/mol, CAS: 816-40-0, $d = 1.479 \text{ g/l}$): SF12 and SF13 = pure liquid, SF14 = 10 mM in DMSO; (13) 4-nitrocinnamaldehyde (predominantly "trans" form, Aldrich #281670, $\text{O}_2\text{NC}_6\text{H}_4\text{CH}=\text{CHCHO}$, MW: 177.16 g/mol, CAS: 49678-08-2): SF12 = pure substance was weighted and added to the culture

[0329] SEED medium: 10.000 mL Yeast synthetic medium w/o aa, w/o glucose, w/o ethanol/Tween (2x); 2.000 mL Supplement aa sol. without histidine and uracil (SAAS-1 10x); 3.200 mL 250 g/L glucose solution (resulting in 40 g/l glucose); 0.046 mL Na-acetate stock solution; 4.754 mL H_2O .

Example 10: Inhibition of ethanologen yeast PNY 827 by copper(II)

[0330] The inhibitory effect of copper (II) on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without copper or addition of copper at concentrations of 5 mM, 10 mM and 25 mM, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600 \text{ nm}$. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates μ_{max} were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted μ_{max} values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent

vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3). The solution with 25 mM showed precipitation and was not analyzed. At 5 mM of copper $\mu_{\max}$ was determined to be 0.46 1/h. In the medium containing 10 mM of copper, maximum specific growth rate of $\mu_{\max} = 0.33$ 1/h was found. Fitting the data to the "squared inhibition" kinetics yielded parameters of $\mu_{\max}^{\circ} = 0.58$ 1/h and a K_I value of $K_I = 11$ mM (Figure 4).

Decrease in $\mu_{\max}$ with increasing copper (II) concentrations in the medium indicates inhibition of ethanologen yeast PNY 827. Inhibition kinetics were used and fitted parameters predict an IC_{50} value of 11 mM. Data from the samples is seen in Table 3 below.

Table 3. Data for control samples and copper-inhibited experiments. Copper concentrations in the experiments were: F1-ctrl-a: 0 mM; F2-ctrl-b: 0 mM; F5-cu-1: 5 mM; F6-cu-2: 10 mM; F7-cu-3: 25 mM.

F1-ctrl-a

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.157	1	0.090	
2	10:40	135	2.25	0.188	1	0.121	0.121
3	11:40	195	3.25	0.255	1	0.188	0.188
4	12:40	255	4.25	0.372	1	0.305	0.305
5	14:25	360	6.00	0.285	5	1.102	1.102
6	8:05	1420	23.67	0.672	20	12.157	

F2-ctrl-b

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.153	1	0.086	
2	10:40	135	2.25	0.187	1	0.120	0.120
3	11:40	195	3.25	0.251	1	0.184	0.184
4	12:40	255	4.25	0.371	1	0.304	0.304
5	14:25	360	6.00	0.281	5	1.082	1.082
6	8:05	1420	23.67	0.647	20	11.657	

F5-cu-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.336	1	0.097	
1	9:25	60	1.00	0.346	1	0.107	0.107
2	10:40	135	2.25	0.467	1	0.228	0.228
3	11:40	195	3.25	0.538	1	0.299	0.299
4	12:40	255	4.25	0.543	1	0.304	
5	14:25	360	6.00	0.152	5	0.265	
6	18:05	580	9.67	0.163	5	0.320	
6	8:05	1420	23.67	0.171	5	0.360	

F6-cu-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.389	1	0.115	
1	9:25	60	1.00	0.399	1	0.125	
2	10:40	135	2.25	0.401	1	0.127	0.127
3	11:40	195	3.25	0.437	1	0.163	0.163
4	12:40	255	4.25	0.521	1	0.247	0.247
5	14:25	360	6.00	0.158	5	0.260	
6	18:05	580	9.67	0.166	5	0.300	
6	8:05	1420	23.67	0.202	5	0.480	

F7-cu-3

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
precipitation							

Example 11: Inhibition of ethanologen yeast PNY 827 by sulfometuron methyl

[0331] The inhibitory effect of the sulfonylurea sulfometuron methyl on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without sulfometuron methyl or addition of sulfometuron methyl at concentrations of 0.11 mM, 0.16 mM and 0.27, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of

Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0332] At all three applied concentrations of 0.11 mM, 0.16 mM and 0.27 mM of sulfometuron methyl a significant reduction in specific maximum growth rate was found, yielding $\mu_{\max}$ values of 0.04 1/h, 0.05 1/h and 0.04 1/h, down from uninhibited maximum specific growth in the experiment of $\mu^{\circ}_{\max} = 0.59$ 1/h, respectively (5).

[0333] Sulfometuron methyl is poorly water soluble, consequently the compound was administered dissolved in DMSO. In order to make sure the observed inhibition did not result from DMSO, DMSO was added only ad 0.14 mM to a culture and a maximum specific growth rate of 0.56 1/h was found. Follow up experiments with DMSO point to a "squared inhibition" with a K_I value of about 16 mM (data not shown). So while DMSO alone seems to have an inhibitory effect on growth, its inhibitory effects at concentrations of 0.06 mM, 0.08 mM and 0.14 mM, as used in the experiments with sulfometuron methyl, can be neglected. Fitting a "hybrid" inhibition kinetics model to the measurements yields values of $\mu^{\circ}_{\max 1} = 0.55$ 1/h and $\mu^{\circ}_{\max 2} = 0.04$ 1/h. Not sufficient data are available for accurate determination of K_I , but from the curve shape it can be concluded that the K_I value is significantly below 0.1 mM. The "hybrid" inhibition kinetics model predicts an overall observable $\mu^{\circ}_{\max}$ of 0.59 1/h. Due to the underdetermined K_I value, the IC_{50} value cannot reliably be determined, but it can be concluded that it is significantly lower than 0.1 mM. Data from the samples is seen in Table 4 below.

Table 4. Data for control samples and sulfometuron methyl-inhibited experiments.

Sulfometuron methyl concentrations in the experiments were: F1-ctrl-a: 0 mM; F2-ctrl-b: 0 mM; F12-sm-1: 0.11 mM; F13-sm-2: 0.16 mM; F14-sm-3: 0.27 mM.

F1-ctrl-a

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.157	1	0.090	
2	10:40	135	2.25	0.188	1	0.121	0.121
3	11:40	195	3.25	0.255	1	0.188	0.188
4	12:40	255	4.25	0.372	1	0.305	0.305
5	14:25	360	6.00	0.285	5	1.102	1.102
6	8:05	1420	23.67	0.672	20	12.157	

F2-ctrl-b

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.153	1	0.086	
2	10:40	135	2.25	0.187	1	0.120	0.120
3	11:40	195	3.25	0.251	1	0.184	0.184
4	12:40	255	4.25	0.371	1	0.304	0.304
5	14:25	360	6.00	0.281	5	1.082	1.082
6	8:05	1420	23.67	0.647	20	11.657	

F12-sm-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.155	1	0.088	
1	9:25	60	1.00	0.160	1	0.093	0.093
2	10:40	135	2.25	0.165	1	0.098	0.098
3	11:40	195	3.25	0.170	1	0.103	0.103
4	12:40	255	4.25	0.175	1	0.108	0.108
5	14:25	360	6.00	0.182	1	0.115	0.115
6	17:50	565	9.42	0.222	1	0.155	
7	8:05	1420	23.67	0.272	5	1.037	

F13-sm-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.158	1	0.091	
1	9:25	60	1.00	0.160	1	0.093	0.093
2	10:40	135	2.25	0.166	1	0.099	0.099
3	11:40	195	3.25	0.171	1	0.104	0.104
4	12:40	255	4.25	0.175	1	0.108	0.108
5	14:25	360	6.00	0.184	1	0.117	0.117
6	17:50	565	9.42	0.219	1	0.152	
7	8:05	1420	23.67	0.467	1	0.400	

F14-sm-3

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.157	1	0.090	0.090
2	10:40	135	2.25	0.164	1	0.097	0.097
3	11:40	195	3.25	0.164	1	0.097	0.097
4	12:40	255	4.25	0.166	1	0.099	0.099
5	14:25	360	6.00	0.177	1	0.110	0.110
6	17:50	565	9.42	0.201	1	0.134	0.134
7	8:05	1420	23.67	0.306	1	0.239	0.239

Example 12: Inhibition of ethanologen yeast PNY 827 by sulfite

[0334] The inhibitory effect of sulfite on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without sulfite or addition of sulfite at concentrations of 1 mM, 2 mM, 5 mM, 10 mM, 20 mM and 50 mM, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0335] Sulfite concentrations of 1 mM, 2 mM, 5 mM, 10 mM, 20 mM and 50 mM resulted in maximum specific growth rates of 0.59 1/h, 0.54 1/h, 0.33 1/h, 0.23 1/h, 0.18 1/h and 0.14 1/h, respectively, indicating significant inhibitory effect of sulfite on ethanologen yeast PNY 807. Fitting the measured data to the "hybrid" inhibition kinetics model, values of $\mu_{\max 1}^{\circ} = 0.46$ 1/h, $\mu_{\max 2}^{\circ} = 0.15$ 1/h and $KI = 4.4$ mM were determined. The "hybrid" inhibition kinetics

model predicts an overall observable $\mu_{\max}^{\circ} = 0.61$ 1/h and an IC₅₀ value of 6.2 mM. Measured $\mu_{\max}$ values and fitted dependency of $\mu_{\max}$ on the concentration of sulfite in the medium is depicted in Figure 6. Data from the samples is seen in Table 5 below.

Table 5. Data for control samples and sulfite-inhibited experiments. Sulfite concentrations in the experiments were: F1-ctrl-a: 0 mM; F2-ctrl-b: 0 mM; F8-su-1: 1 mM; F9-su-2: 2 mM; F10-su-3: 5 mM; F11-su-4: 10 mM; SF12-F1-CtrlA: 0 mM; SF12-F2-ctrlB: 0M; F3-su-1: 20 mM; F4-su-2: 50 mM.

F1-ctrl-a

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.157	1	0.090	
2	10:40	135	2.25	0.188	1	0.121	0.121
3	11:40	195	3.25	0.255	1	0.188	0.188
4	12:40	255	4.25	0.372	1	0.305	0.305
5	14:25	360	6.00	0.285	5	1.102	1.102
6	8:05	1420	23.67	0.672	20	12.157	

F2-ctrl-b

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.153	1	0.086	
2	10:40	135	2.25	0.187	1	0.120	0.120
3	11:40	195	3.25	0.251	1	0.184	0.184
4	12:40	255	4.25	0.371	1	0.304	0.304
5	14:25	360	6.00	0.281	5	1.082	1.082
6	8:05	1420	23.67	0.647	20	11.657	

F8-su-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.148	1	0.081	
1	9:25	60	1.00	0.149	1	0.082	
2	10:40	135	2.25	0.188	1	0.121	0.121
3	11:40	195	3.25	0.252	1	0.185	0.185
4	12:40	255	4.25	0.374	1	0.307	0.307
5	14:25	360	6.00	0.285	5	1.102	1.102
6	8:05	1420	23.67	0.622	20	11.157	

F9-su-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.153	1	0.086	
2	10:40	135	2.25	0.187	1	0.120	0.120
3	11:40	195	3.25	0.240	1	0.173	0.173
4	12:40	255	4.25	0.332	1	0.265	0.265
5	14:25	360	6.00	0.242	5	0.887	0.887
6	8:05	1420	23.67	0.675	20	12.217	

F10-su-3

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.155	1	0.088	
1	9:25	60	1.00	0.157	1	0.090	
2	10:40	135	2.25	0.183	1	0.116	0.116
3	11:40	195	3.25	0.220	1	0.153	0.153
4	12:40	255	4.25	0.275	1	0.208	0.208
5	14:25	360	6.00	0.459	1	0.392	0.392
6	18:00	575	9.58	0.579	5	2.572	
7	8:05	1420	23.67	0.650	20	11.717	

F11-su-4

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.159	1	0.092	
1	9:25	60	1.00	0.152	1	0.085	0.085
2	10:40	135	2.25	0.178	1	0.111	0.111
3	11:40	195	3.25	0.207	1	0.140	0.140
4	12:40	255	4.25	0.242	1	0.175	0.175
5	14:25	360	6.00	0.335	1	0.268	0.268
6	18:00	575	9.58	0.285	5	1.102	
6	8:05	1420	23.67	0.636	20	11.437	

SF12-F1-Ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.156	1	0.089	
1	10:25	90	1.50	0.170	1	0.103	
2	11:35	160	2.67	0.222	1	0.155	0.155
3	12:55	240	4.00	0.350	1	0.283	0.283
4	14:15	320	5.33	0.220	5	0.777	0.777
6	8:30	1395	23.25	0.648	20	11.677	

SF12-F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.162	1	0.095	
1	10:25	90	1.50	0.172	1	0.105	
2	11:35	160	2.67	0.223	1	0.156	0.156
3	12:55	240	4.00	0.354	1	0.287	0.287
4	14:15	320	5.33	0.228	5	0.817	0.817
5	8:30	1395	23.25	0.667	20	12.057	

F3-su-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.154	1	0.087	
1	10:25	90	1.50	0.166	1	0.099	
2	11:35	160	2.67	0.182	1	0.115	0.115
3	12:55	240	4.00	0.217	1	0.150	0.150
4	14:15	320	5.33	0.249	1	0.182	0.182
5	15:40	405	6.75	0.293	1	0.226	0.226
6	18:20	565	9.42	0.463	1	0.396	0.396
7	8:30	1395	23.25	0.549	20	9.697	

F4-su-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.155	1	0.088	
1	10:25	90	1.50	0.162	1	0.095	
2	11:35	160	2.67	0.183	1	0.116	0.116
3	12:55	240	4.00	0.202	1	0.135	0.135
4	14:15	320	5.33	0.228	1	0.161	0.161
5	15:40	405	6.75	0.259	1	0.192	0.192
6	18:20	565	9.42	0.362	1	0.295	0.295
7	8:30	1395	23.25	0.600	20	10.717	

Example 13: Inhibition of ethanologen yeast PNY 827 by formaldehyde

[0336] The inhibitory effect of formaldehyde on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without formaldehyde or addition of formaldehyde at concentrations of 1 mM, 2 mM, 5 mM and 10 mM, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear

extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0337] With the investigated formaldehyde concentrations of 1 mM, 2 mM, 5 mM and 10 mM, corresponding maximum specific growth rates of PNY827 were 0.28 1/h, 0.08 1/h, 0.00 1/h (no growth), and 0.00 1/h (no growth), respectively. $\mu_{\max}$ values determined without inhibitor addition were 0.59 1/h and 0.59 1/h. Fitting the measured data to the "squared inhibition" kinetics model, a K_I value of $K_I = 904 \mu\text{M}$ ($\mu_{\max}^0 = 0.59 \text{ 1/h}$) was found, indicating a very strong inhibition of *S. cerevisiae* by formaldehyde. The derived IC50 value is 0.9 mM. Measured $\mu_{\max}$ values and fitted dependency of $\mu_{\max}$ on the concentration of formaldehyde in the medium is depicted in Figure 7. Data from the samples is seen in Table 6 below.

Table 6. Data for control samples and formaldehyde-inhibited experiments. Formaldehyde concentrations in the experiments were: F1-ctrl-a: 0 mM; F2-ctrl-b: 0 mM; F16-fa-1: 1 mM; F17-fa-2: 2 mM; F18-fa-3: 5 mM; F19-fa-4: 10 mM.

F1-ctrl-a

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.157	1	0.090	
2	10:40	135	2.25	0.188	1	0.121	0.121
3	11:40	195	3.25	0.255	1	0.188	0.188
4	12:40	255	4.25	0.372	1	0.305	0.305
5	14:25	360	6.00	0.285	5	1.102	1.102
6	8:05	1420	23.67	0.672	20	12.157	

F2-ctrl-b

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.154	1	0.087	
1	9:25	60	1.00	0.153	1	0.086	
2	10:40	135	2.25	0.187	1	0.120	0.120
3	11:40	195	3.25	0.251	1	0.184	0.184
4	12:40	255	4.25	0.371	1	0.304	0.304
5	14:25	360	6.00	0.281	5	1.082	1.082
6	8:05	1420	23.67	0.647	20	11.657	

F16-fa-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:35	0	0.00	0.157	1	0.090	
1	9:35	60	1.00	0.157	1	0.090	
2	10:50	135	2.25	0.165	1	0.098	
3	11:50	195	3.25	0.182	1	0.115	0.115
4	12:50	255	4.25	0.206	1	0.139	0.139
5	14:35	360	6.00	0.285	1	0.218	0.218
6	17:40	545	9.08	0.655	1	0.588	0.588
7	8:20	1425	23.75	0.657	20	11.857	

F17-fa-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:35	0	0.00	0.158	1	0.091	
1	9:35	60	1.00	0.158	1	0.091	
2	10:50	135	2.25	0.159	1	0.092	
3	11:50	195	3.25	0.160	1	0.093	0.093
4	12:50	255	4.25	0.165	1	0.098	0.098
5	14:35	360	6.00	0.177	1	0.110	0.110
6	17:40	545	9.08	0.211	1	0.144	0.144
7	8:20	1425	23.75	0.382	20	6.357	

F18-fa-3

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:35	0	0.00	0.157	1	0.090	
1	9:35	60	1.00	0.162	1	0.095	
2	10:50	135	2.25	0.161	1	0.094	
3	11:50	195	3.25	0.157	1	0.090	0.090
4	12:50	255	4.25	0.155	1	0.088	0.088
5	14:35	360	6.00	0.155	1	0.088	0.088
6	17:40	545	9.08	0.156	1	0.089	0.089
7	8:20	1425	23.75	0.160	1	0.093	

F19-fa-4

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:35	0	0.00	0.162	1	0.095	
1	9:35	60	1.00	0.168	1	0.101	
2	10:50	135	2.25	0.164	1	0.097	
3	11:50	195	3.25	0.163	1	0.096	0.096
4	12:50	255	4.25	0.160	1	0.093	0.093
5	14:35	360	6.00	0.161	1	0.094	0.094
6	17:40	545	9.08	0.162	1	0.095	0.095
7	8:20	1425	23.75	0.168	1	0.101	

Example 14: Inhibition of ethanologen yeast PNY 827 by 4-pyrazolecarboxylic acid

[0338] The inhibitory effect of 4-nitrocinnamaldehyde (predominantly trans) on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of

PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without 4-pyrazolecarboxylic acid or addition of 4-nitrocinnamaldehyde at concentrations of 1 mM and 50 mM, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0339] The inhibitory effect of 4-pyrazolecarboxylic acid (PA) was investigated at 1 mM and 50 mM. 4-pyrazolecarboxylic acid was administered as a DMSO solution, resulting in DMSO concentrations in the cell suspension of 14 mM and 704 mM, respectively. Assuming an additive effect of 4-pyrazolecarboxylic acid and DMSO inhibition, observed maximum specific growth rate of the two cultures was corrected by 0.00 1/h and 0.27 1/h due to the effect of DMSO, resulting in 4-pyrazolecarboxylic acid-based $\mu_{\max}$ values of 0.59 1/h and 0.50 1/h derived from the observed values of 0.59 1/h and 0.23 1/h, respectively. Fitting the data to the "squared inhibition" kinetics (observed maximum specific growth rates without inhibitor addition were 0.59 1/h, 0.59 1/h, 0.60 1/h and 0.62 1/h) yielded parameters of $\mu^{\circ}_{\max} = 0.60$ 1/h and a KI value of KI = 100 mM (Figure 8), indicating only weak inhibitory effects of 4-pyrazolecarboxylic acid. Data from the samples is seen in Table 7 below.

Table 7. Data for control samples and 4-pyrazolecarboxylic acid -inhibited experiments. 4-pyrazolecarboxylic acid concentrations in the experiments were: F1-Ctrl-A: 0 mM; F2-ctrl-B: 0 mM; F12-pa-1: 1 mM; F13-pa-2: 50 mM.

F1-Ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.156	1	0.089	
1	10:25	90	1.50	0.170	1	0.103	
2	11:35	160	2.67	0.222	1	0.155	0.155
3	12:55	240	4.00	0.350	1	0.283	0.283
4	14:15	320	5.33	0.220	5	0.777	0.777
6	8:30	1395	23.25	0.648	20	11.677	

F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.162	1	0.095	
1	10:25	90	1.50	0.172	1	0.105	
2	11:35	160	2.67	0.223	1	0.156	0.156
3	12:55	240	4.00	0.354	1	0.287	0.287
4	14:15	320	5.33	0.228	5	0.817	0.817
5	8:30	1395	23.25	0.667	20	12.057	

F12-pa-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.156	1	0.089	
1	10:25	90	1.50	0.172	1	0.105	
2	11:35	160	2.67	0.227	1	0.160	0.160
3	12:55	240	4.00	0.361	1	0.294	0.294
4	14:15	320	5.33	0.217	5	0.762	0.762
5	8:30	1395	23.25	0.659	20	11.897	

F13-pa-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.157	1	0.090	
1	10:25	90	1.50	0.167	1	0.100	0.100
2	11:35	160	2.67	0.192	1	0.125	0.125
3	12:55	240	4.00	0.234	1	0.167	0.167
4	14:15	320	5.33	0.305	1	0.238	0.238
5	3:40	405	6.75	0.405	1	0.338	0.338
6	8:30	1395	23.25	0.655	20	11.817	

Example 15: Inhibition of ethanologen yeast PNY 827 by 4-methylpyrazole hydrochloride

[0340] The inhibitory effect of 4-methylpyrazole hydrochloride on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without 4-methylpyrazole hydrochloride or addition of 4-methylpyrazole hydrochloride at concentrations of 1 mM and 30

mM, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0341] The inhibitory effect of 4-methylpyrazole hydrochloride was investigated at 1 mM and 30 mM. 4-methylpyrazole hydrochloride was administered as a DMSO solution, resulting in DMSO concentrations in the cell suspension of 14 mM and 423 mM, respectively. Assuming an additive effect of 4-methylpyrazole hydrochloride and DMSO inhibition, observed maximum specific growth rate of the two cultures was corrected by 0.00 1/h and 0.14 1/h due to the effect of DMSO, resulting in 4-methylpyrazole hydrochloride-based $\mu_{\max}$ values of 0.48 1/h and 0.38 1/h derived from the observed values of 0.48 1/h and 0.24 1/h, respectively. Fitting the data to the "squared inhibition" kinetics (observed maximum specific growth rates without inhibitor addition were 0.59 1/h, 0.59 1/h, 0.60 1/h and 0.62 1/h) yielded parameters of $\mu^{\circ}_{\max} = 0.58$ 1/h and a K_I value of $K_I = 41$ mM, indicating inhibitory effects of 4-methylpyrazole hydrochloride (9). Data from the samples is seen in Table 8 below.

Table 8. Data for control samples and 4-methylpyrazole hydrochloride acid-inhibited experiments. 4-methylpyrazole hydrochloride acid concentrations in the experiments were: F1-Ctrl-A: 0 mM; F2-ctrl-B: 0 mM; F14-mp-1: 1 mM; F15-mp-2: 30 mM.

F1-Ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.156	1	0.089	
1	10:25	90	1.50	0.170	1	0.103	
2	11:35	160	2.67	0.222	1	0.155	0.155
3	12:55	240	4.00	0.350	1	0.283	0.283
4	14:15	320	5.33	0.220	5	0.777	0.777
6	8:30	1395	23.25	0.648	20	11.677	

F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.162	1	0.095	
1	10:25	90	1.50	0.172	1	0.105	
2	11:35	160	2.67	0.223	1	0.156	0.156
3	12:55	240	4.00	0.354	1	0.287	0.287
4	14:15	320	5.33	0.228	5	0.817	0.817
5	8:30	1395	23.25	0.667	20	12.057	

F14-mp-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.163	1	0.096	
1	10:25	90	1.50	0.174	1	0.107	0.107
2	11:35	160	2.67	0.221	1	0.154	0.154
3	12:55	240	4.00	0.342	1	0.275	0.275
4	14:15	320	5.33	0.203	5	0.692	0.692
5	8:30	1395	23.25	0.626	20	11.237	

F15-mp-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.161	1	0.094	
1	10:25	90	1.50	0.172	1	0.105	0.105
2	11:35	160	2.67	0.189	1	0.122	0.122
3	12:55	240	4.00	0.237	1	0.170	0.170
4	14:15	320	5.33	0.305	1	0.238	0.238
5	3:40	405	6.75	0.434	1	0.367	0.367
6	8:30	1395	23.25	0.729	20	13.297	

Example 16: Inhibition of ethanologen yeast PNY 827 by glyoxylic acid

[0342] The inhibitory effect of glyoxylic acid on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without glyoxylic acid or addition of glyoxylic acid at concentrations of 10 mM and 50 mM, to give an initial OD of approximately

0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0343] The inhibitory effect of glyoxylic acid was investigated at 10 mM and 50 mM. At the two concentrations, $\mu_{\max}$ values of 0.59 1/h and 0.55 1/h were found, respectively. Fitting the data to the "squared inhibition" kinetics (observed maximum specific growth rates without inhibitor addition were 0.59 1/h, 0.59 1/h, 0.60 1/h and 0.62 1/h) yielded parameters of $\mu_{\max}^0 = 0.60$ 1/h and a K_I value of $K_I = 168$ mM, indicating a weak inhibitory effect of extracellular glyoxylic acid on growth of ethanologen yeast (Figure 10.). Data from the samples is seen in Table 9 below.

Table 9. Data for control samples and glyoxylic acid-inhibited experiments. Glyoxylic acid concentrations in the experiments were: F1-Ctrl-A: 0 mM; F2-ctrl-B: 0 mM; F18-ga-1: 10 mM; F19-ga-2: 50 mM.

F1-Ctrl-A							
sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.156	1	0.089	
1	10:25	90	1.50	0.170	1	0.103	
2	11:35	160	2.67	0.222	1	0.155	0.155
3	12:55	240	4.00	0.350	1	0.283	0.283
4	14:15	320	5.33	0.220	5	0.777	0.777
6	8:30	1395	23.25	0.648	20	11.677	

F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.162	1	0.095	
1	10:25	90	1.50	0.172	1	0.105	
2	11:35	160	2.67	0.223	1	0.156	0.156
3	12:55	240	4.00	0.354	1	0.287	0.287
4	14:15	320	5.33	0.228	5	0.817	0.817
5	8:30	1395	23.25	0.667	20	12.057	

F18-ga-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.161	1	0.094	
1	10:25	90	1.50	0.175	1	0.108	
2	11:35	160	2.67	0.226	1	0.159	0.159
3	12:55	240	4.00	0.361	1	0.294	0.294
4	14:15	320	5.33	0.217	5	0.762	0.762
5	8:30	1395	23.25	0.634	20	11.397	

F19-ga-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.157	1	0.090	
1	10:25	90	1.50	0.173	1	0.106	
2	11:35	160	2.67	0.222	1	0.155	0.155
3	12:55	240	4.00	0.337	1	0.270	0.270
4	14:15	320	5.33	0.199	5	0.672	0.672
5	8:30	1395	23.25	0.655	20	11.817	

Example 17: Inhibition of ethanologen yeast PNY 827 by pyrazole

[0344] The inhibitory effect of pyrazole on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without pyrazole or addition of pyrazole at concentrations of 1 mM, 5 mM, 10 mM, 25 mM and 50 mM, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0345] Pyrazole concentrations of 1 mM, 5 mM, 10 mM, 25 mM and 50 mM were tested, resulting in maximum specific growth rates of 0.54 1/h, 0.21 1/h, 0.12 1/h, 0.09 1/h and 0.08 1/h. $\mu_{\max}$ values determined without inhibitor addition were 0.59 1/h, 0.59 1/h, 0.60 1/h, 0.62 1/h, 0.61 1/h and 0.62 1/h, respectively. Inhibitory effect of pyrazole on growth was best described by the hybrid growth model. If fitted to the "hybrid" inhibition kinetics model, values of $\mu_{\max 1}^{\circ} = 0.52$ 1/h, $\mu_{\max 2}^{\circ} = 0.08$ 1/h and $K_I = 2.8$ mM were determined. The "hybrid" inhibition kinetics model predicts an overall observable $\mu_{\max}^{\circ} = 0.60$ 1/h and an IC_{50} (inhibitor concentration with a specific growth rate of 50% $\mu_{\max}^{\circ}$) value of 3.3 mM. Measured $\mu_{\max}$ values and fitted dependency of $\mu_{\max}$ on the concentration of pyrazole in the medium is depicted in Figure 11. Data from the samples is seen in Table 10 below.

Table 10. Data for control samples and pyrazole-inhibited experiments. Pyrazole concentrations in the experiments were: SF12-F1-Ctrl-A: 0 mM; SF12-F2-ctrl-B: 0 mM; F16-py-1: 1 mM; F17-py-2: 50 mM; F1-ctrl-A: 0 mM; F2-ctrl-B: 0 mM; F8-Py-5: 5 mM; F9- Py-10: 10 mM; F10- Py-25: 25 mM.

SF12-F1-Ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.156	1	0.089	
1	10:25	90	1.50	0.170	1	0.103	
2	11:35	160	2.67	0.222	1	0.155	0.155
3	12:55	240	4.00	0.350	1	0.283	0.283
4	14:15	320	5.33	0.220	5	0.777	0.777
6	8:30	1395	23.25	0.648	20	11.677	

SF12-F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.162	1	0.095	
1	10:25	90	1.50	0.172	1	0.105	
2	11:35	160	2.67	0.223	1	0.156	0.156
3	12:55	240	4.00	0.354	1	0.287	0.287
4	14:15	320	5.33	0.228	5	0.817	0.817
5	8:30	1395	23.25	0.667	20	12.057	

F16-py-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.155	1	0.088	
1	10:25	90	1.50	0.167	1	0.100	
2	11:35	160	2.67	0.199	1	0.132	0.132
3	12:55	240	4.00	0.277	1	0.210	0.210
4	14:15	320	5.33	0.444	1	0.377	0.377
5	3:40	405	6.75	0.262	5	0.987	0.987
6	8:30	1395	23.25	0.645	20	11.617	

F17-py-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.164	1	0.097	
1	10:25	90	1.50	0.166	1	0.099	0.099
2	11:35	160	2.67	0.169	1	0.102	0.102
3	12:55	240	4.00	0.175	1	0.108	0.108
4	14:15	320	5.33	0.186	1	0.119	0.119
5	15:40	405	6.75	0.190	1	0.123	0.123
6	18:20	565	9.42	0.209	1	0.142	0.142
7	8:30	1395	23.25	0.302	1	0.235	0.235

F17-py-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.164	1	0.097	
1	10:25	90	1.50	0.166	1	0.099	0.099
2	11:35	160	2.67	0.169	1	0.102	0.102
3	12:55	240	4.00	0.175	1	0.108	0.108
4	14:15	320	5.33	0.186	1	0.119	0.119
5	15:40	405	6.75	0.190	1	0.123	0.123
6	18:20	565	9.42	0.209	1	0.142	0.142
7	8:30	1395	23.25	0.302	1	0.235	0.235

F17-py-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.164	1	0.097	
1	10:25	90	1.50	0.166	1	0.099	0.099
2	11:35	160	2.67	0.169	1	0.102	0.102
3	12:55	240	4.00	0.175	1	0.108	0.108
4	14:15	320	5.33	0.186	1	0.119	0.119
5	15:40	405	6.75	0.190	1	0.123	0.123
6	18:20	565	9.42	0.209	1	0.142	0.142
7	8:30	1395	23.25	0.302	1	0.235	0.235

F1-ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.160	1	0.093	
1	9:45	90	1.50	0.169	1	0.102	
2	11:05	170	2.83	0.229	1	0.162	0.162
3	12:20	245	4.08	0.368	1	0.301	0.301
4	13:35	320	5.33	0.215	5	0.752	0.752
6	8:30	1455	24.25	0.644	20	11.597	

F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.159	1	0.092	
1	9:45	90	1.50	0.168	1	0.101	
2	11:05	170	2.83	0.228	1	0.161	0.161
3	12:20	245	4.08	0.372	1	0.305	0.305
4	13:35	320	5.33	0.215	5	0.752	0.752
5	8:30	1455	24.25	0.652	20	11.757	

F8-Py-5

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.163	1	0.096	
1	9:45	90	1.50	0.162	1	0.095	0.095
2	11:05	170	2.83	0.177	1	0.110	0.110
3	12:20	245	4.08	0.210	1	0.143	0.143
4	13:35	320	5.33	0.256	1	0.189	0.189
5	3:00	405	6.75	0.362	1	0.295	0.295
6	8:30	1455	24.25	0.551	20	9.737	

F9-Py-10

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.164	1	0.097	
1	9:45	90	1.50	0.166	1	0.099	0.099
2	11:05	170	2.83	0.181	1	0.114	0.114
3	12:20	245	4.08	0.205	1	0.138	0.138
4	13:35	320	5.33	0.238	1	0.171	0.171
5	3:00	405	6.75	0.289	1	0.222	0.222
	8:30	1455	24.25	0.475	20	8.217	

F10-Py-25

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.162	1	0.095	
1	9:45	90	1.50	0.159	1	0.092	0.092
2	11:05	170	2.83	0.167	1	0.100	0.100
3	12:20	245	4.08	0.181	1	0.114	0.114
4	13:35	320	5.33	0.194	1	0.127	0.127
5	3:00	405	6.75	0.211	1	0.144	0.144
	8:30	1455	24.25	0.374	20	6.197	

Example 18: Inhibition of ethanologen yeast PNY 827 by cinnamaldehyde

[0346] The inhibitory effect of cinnamaldehyde on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated over night at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without cinnamaldehyde or addition of cinnamaldehyde at concentrations of 200 mM, 100 mM, 50 mM, 25 mM, 10 mM, 1 mM, 0.1 mM, 0.01 mM and 0.001 mM, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0347] The inhibitory effect of cinnamaldehyde (CA) was investigated at 200 mM, 100 mM, 50 mM, 25 mM, 10 mM, 1 mM, 0.1 mM, 0.01 mM and 0.001 mM. For generating the concentrations of 0.1 mM, 0.01 mM and 0.001 mM, cinnamaldehyde was diluted with DMSO, resulting in DMSO concentrations in the cell suspension of 0.7 mM, 7 mM and 70 mM of DMSO, respectively. Assuming an additive effect of cinnamaldehyde and DMSO inhibition, observed maximum specific growth rates of the two cultures were corrected by 0.000 1/h, 0.000 1/h and 0.005 1/h due to the effect of DMSO, resulting in cinnamaldehyde-based $\mu_{\max}$ values of 0.64 1/h, 0.63 1/h and 0.55 1/h derived from the observed values of 0.64 1/h, 0.63 1/h and 0.55 1/h, respectively. At all the other concentrations, no DMSO was used in the stock solution of cinnamaldehyde. However, in none of the non-DMSO experiments any cell growth was observed. Fitting the data to the "squared inhibition" kinetics (observed maximum specific growth rates without inhibitor addition were 0.59 1/h, 0.59 1/h, 0.60 1/h, 0.62 1/h, 0.62 1/h and

0.62 1/h) yielded parameters of $\mu^{\circ}_{\max} = 0.62$ 1/h and a K_I value of $K_I = 0.25$ mM (Figure 12).

These findings indicate strong inhibition of growth of PNY 827 by trans-cinnamaldehyde with a derived IC₅₀ of 0.25 mM. Data from the samples is seen in Table 11 below.

Table 11. Data for control samples and cinnamaldehyde-inhibited experiments.

Cinnamaldehyde concentrations in the experiments were: SF12-F1-Ctrl-A: 0 mM; SF12-F2-ctrl-B: 0 mM; F7-ca-1: 50 mM; F8-ca-2: 100 mM; F9-ca-3: 200 mM; SF13-F1-ctrl-A: 0 mM; SF13-F2-ctrl-B: 0 mM; F3-Ca-1: 1 mM; F4- Ca-10: 10 mM; F5- Ca-25: 25 mM; F1-Ctrl-A: 0 mM; F2-ctrl-B: 0 mM; F3-Ca-a: 0.001 mM; F4- Ca-b: 0.01 mM; F5- Ca-c: 0.1 mM.

SF12-F1-Ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.156	1	0.089	
1	10:25	90	1.50	0.170	1	0.103	
2	11:35	160	2.67	0.222	1	0.155	0.155
3	12:55	240	4.00	0.350	1	0.283	0.283
4	14:15	320	5.33	0.220	5	0.777	0.777
6	8:30	1395	23.25	0.648	20	11.677	

SF12-F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.162	1	0.095	
1	10:25	90	1.50	0.172	1	0.105	
2	11:35	160	2.67	0.223	1	0.156	0.156
3	12:55	240	4.00	0.354	1	0.287	0.287
4	14:15	320	5.33	0.228	5	0.817	0.817
5	8:30	1395	23.25	0.667	20	12.057	

F7-ca-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.167	1	0.100	
1	10:25	90	1.50	0.168	1	0.101	0.101
2	11:35	160	2.67	0.172	1	0.105	0.105
3	12:55	240	4.00	0.171	1	0.104	0.104
4	14:15	320	5.33	0.171	1	0.104	
5	8:30	1395	23.25	0.144	1	0.077	

F8-ca-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.178	1	0.111	
1	10:25	90	1.50	0.179	1	0.112	0.112
2	11:35	160	2.67	0.175	1	0.108	0.108
3	12:55	240	4.00	0.174	1	0.107	0.107
4	14:15	320	5.33	0.170	1	0.103	0.103
5	8:30	1395	23.25	0.136	1	0.069	

F9-ca-3

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.175	1	0.108	
1	10:25	90	1.50	0.179	1	0.112	0.112
2	11:35	160	2.67	0.173	1	0.106	0.106
3	12:55	240	4.00	0.169	1	0.102	0.102
4	14:15	320	5.33	0.160	1	0.093	0.093
5	8:30	1395	23.25	0.122	1	0.055	

SF13-F1-ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.160	1	0.093	
1	9:45	90	1.50	0.169	1	0.102	
2	11:05	170	2.83	0.229	1	0.162	0.162
3	12:20	245	4.08	0.368	1	0.301	0.301
4	13:35	320	5.33	0.215	5	0.752	0.752
6	8:30	1455	24.25	0.644	20	11.597	

SF13 - F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.159	1	0.092	
1	9:45	90	1.50	0.168	1	0.101	
2	11:05	170	2.83	0.228	1	0.161	0.161
3	12:20	245	4.08	0.372	1	0.305	0.305
4	13:35	320	5.33	0.215	5	0.752	0.752
5	8:30	1455	24.25	0.652	20	11.757	

F3-Ca-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.165	1	0.098	
1	9:45	90	1.50	0.164	1	0.097	0.097
2	11:05	170	2.83	0.165	1	0.098	0.098
3	12:20	245	4.08	0.167	1	0.100	0.100
4	13:35	320	5.33	0.167	1	0.100	0.100
5	8:30	1455	24.25	0.167	1	0.100	0.100

F4-Ca-10

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.167	1	0.100	
1	9:45	90	1.50	0.169	1	0.102	0.102
2	11:05	170	2.83	0.170	1	0.103	0.103
3	12:20	245	4.08	0.174	1	0.107	0.107
4	13:35	320	5.33	0.172	1	0.105	0.105
5	8:30	1455	24.25	0.141	1	0.074	0.074

F5-Ca-25

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.171	1	0.104	
1	9:45	90	1.50	0.173	1	0.106	0.106
2	11:05	170	2.83	0.172	1	0.105	0.105
3	12:20	245	4.08	0.174	1	0.107	0.107
4	13:35	320	5.33	0.168	1	0.101	0.101
5	8:30	1455	24.25	0.140	1	0.073	0.073

F1-Ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.167	1	0.100	
1	9:55	90	1.50	0.190	1	0.123	
2	11:15	170	2.83	0.264	1	0.197	0.197
3	12:35	250	4.17	0.452	1	0.385	0.385
4	13:40	315	5.25	0.246	5	0.907	0.907

F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.165	1	0.098	
1	9:55	90	1.50	0.190	1	0.123	
2	11:15	170	2.83	0.264	1	0.197	0.197
3	12:35	250	4.17	0.460	1	0.393	0.393
4	13:40	315	5.25	0.248	5	0.917	0.917

F3-Ca-a

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.166	1	0.099	
1	9:55	90	1.50	0.192	1	0.125	
2	11:15	170	2.83	0.266	1	0.199	0.199
3	12:35	250	4.17	0.461	1	0.394	0.394
4	13:40	315	5.25	0.256	5	0.957	0.957

F4-Ca-b

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.169	1	0.102	
1	9:55	90	1.50	0.190	1	0.123	
2	11:15	170	2.83	0.262	1	0.195	0.195
3	12:35	250	4.17	0.443	1	0.376	0.376
4	13:40	315	5.25	0.245	5	0.902	0.902

F5-Ca-c

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.164	1	0.097	
1	9:55	90	1.50	0.186	1	0.119	
2	11:15	170	2.83	0.235	1	0.168	0.168
3	12:35	250	4.17	0.364	1	0.297	0.297
4	13:40	315	5.25	0.575	1	0.508	0.508
5	14:55	390	6.50	0.321	5	1.282	1.282

Example 19: Inhibition of ethanologen yeast PNY 827 by 1-bromo-2-butanone

[0348] The inhibitory effect of 1-bromo-2-butanone on ethanologen yeast PNY 827 was investigated. Therefore a 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827. The culture was inoculated overnight at 30°C and 250 rpm in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). Subsequently, a sufficient amount of the seed culture was transferred into shake flasks containing 20 ml of production medium without 1-bromo-2-butanone or addition of 1-bromo-2-butanone at concentrations of 50 mM, 5 mM, 1 mM, 0.5 mM, 0.1 mM, 0.01 mM and 0.001 mM, to give an initial OD of approximately 0.1. The cultures were incubated at 250 rpm for 24h in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) and samples of about 1 ml for OD determination withdrawn at designated hours. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. In case cell dry weight concentrations were needed, an OD-DW-correlation of 0.33 gDW/OD was applied. Maximum specific growth rates $\mu_{\max}$ were determined by applying the exponential regression function of Microsoft Excel (Microsoft Office Excel 2003, SP 3). Outliers were discarded until good fit of the regression curve with measurements was confirmed by visual inspection. Parameters of the inhibition kinetics were determined by least square minimization of the differences between measured and predicted $\mu_{\max}$ values. Employed search algorithm was a quasi-Newton method with linear extrapolation from a tangent vector, as implemented in the solver routine of Microsoft Excel (Microsoft Office Excel 2003, SP 3).

[0349] The inhibitory effect of 1-bromo-2-butanone was investigated at 50 mM, 5 mM, 1 mM, 0.5 mM, 0.1 mM, 0.01 mM and 0.001 mM. For generating the concentrations of 0.1 mM, 0.01 mM and 0.001 mM, 1-bromo-2-butanone was diluted with DMSO, resulting in DMSO concentrations in the cell suspension of 0.7 mM, 7 mM and 70 mM of DMSO, respectively. Assuming an additive effect of 1-bromo-2-butanone and DMSO inhibition, observed maximum specific growth rates of the two cultures were corrected by 0.000 1/h, 0.000 1/h and 0.005 1/h

due to the effect of DMSO, resulting in 1-bromo-2-butanone-based mumax values of 0.54 1/h, 0.00 1/h and 0.00 1/h derived from the observed values of 0.54 1/h, 0.00 1/h and 0.00 1/h, respectively. At all the other concentrations, no DMSO was used for dilution of 1-bromo-2-butanone. However, in all of the non-DMSO experiments no cell growth was observed. Fitting the data to the "squared inhibition" kinetics (observed maximum specific growth rates without inhibitor addition were 0.59 1/h, 0.59 1/h, 0.60 1/h, 0.62 1/h, 0.62 1/h and 0.62 1/h) yielded parameters of $\mu_{\max}^{\circ} = 0.61$ 1/h and a K_I value of $K_I = 0.002$ mM (Figure 13). This corresponds to an IC50 value of 1-bromo-2-butanone on growth of 0.002 mM, indication of strong inhibition of ethanologen yeast by 1-bromo-2-butanone. Data from the samples is seen in Table 12 below.

Table 12. Data for control samples and 1-bromo-2-butanone-inhibited experiments. 1-bromo-2-butanone concentrations in the experiments were: SF12-F1-Ctrl-A: 0 mM; SF12-F2-ctrl-B: 0 mM; F10-bb-1: 5 mM; F11-bb-2: 50 mM; SF13-F1-ctrl-A: 0 mM; SF13-F2-ctrl-B: 0 mM; F6-Bb-0.5: 0.5 mM; F7- Bb-1: 1 mM; F1-Ctrl-A: 0 mM; F2-ctrl-B: 0 mM; F6-Bb-a: 0.001 mM; F7-Bb-b: 0.01 mM; F8- Bb-c: 0.1 mM.

SF12-F1-Ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.156	1	0.089	
1	10:25	90	1.50	0.170	1	0.103	
2	11:35	160	2.67	0.222	1	0.155	0.155
3	12:55	240	4.00	0.350	1	0.283	0.283
4	14:15	320	5.33	0.220	5	0.777	0.777
6	8:30	1395	23.25	0.648	20	11.677	

SF12-F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.162	1	0.095	
1	10:25	90	1.50	0.172	1	0.105	
2	11:35	160	2.67	0.223	1	0.156	0.156
3	12:55	240	4.00	0.354	1	0.287	0.287
4	14:15	320	5.33	0.228	5	0.817	0.817
5	8:30	1395	23.25	0.667	20	12.057	

F10-bb-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.161	1	0.094	
1	10:25	90	1.50	0.176	1	0.109	0.109
2	11:35	160	2.67	0.170	1	0.103	0.103
3	12:55	240	4.00	0.168	1	0.101	0.101
4	14:15	320	5.33	0.170	1	0.103	0.103
5	8:30	1395	23.25	0.171	1	0.104	0.104

F11-bb-2

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:55	0	0.00	0.261	1	0.194	
1	10:25	90	1.50	0.238	1	0.171	0.171
2	11:35	160	2.67	0.275	1	0.208	0.208
3	12:55	240	4.00	0.266	1	0.199	0.199
4	14:15	320	5.33	0.264	1	0.197	0.197
5	8:30	1395	23.25	0.161	1	0.094	0.094

SF13-F1-ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.160	1	0.093	
1	9:45	90	1.50	0.169	1	0.102	
2	11:05	170	2.83	0.229	1	0.162	0.162
3	12:20	245	4.08	0.368	1	0.301	0.301
4	13:35	320	5.33	0.215	5	0.752	0.752
6	8:30	1455	24.25	0.644	20	11.597	

SF13 - F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.159	1	0.092	
1	9:45	90	1.50	0.168	1	0.101	
2	11:05	170	2.83	0.228	1	0.161	0.161
3	12:20	245	4.08	0.372	1	0.305	0.305
4	13:35	320	5.33	0.215	5	0.752	0.752
5	8:30	1455	24.25	0.652	20	11.757	

F6-Bb-0.5

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.165	1	0.098	
1	9:45	90	1.50	0.166	1	0.099	0.099
2	11:05	170	2.83	0.169	1	0.102	0.102
3	12:20	245	4.08	0.169	1	0.102	0.102
4	13:35	320	5.33	0.167	1	0.100	0.100
5	8:30	1455	24.25	0.169	1	0.102	0.102

F7-Bb-1

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:15	0	0.00	0.157	1	0.090	
1	9:45	90	1.50	0.161	1	0.094	0.094
2	11:05	170	2.83	0.160	1	0.093	0.093
3	12:20	245	4.08	0.160	1	0.093	0.093
4	13:35	320	5.33	0.158	1	0.091	0.091
5	8:30	1455	24.25	0.161	1	0.094	0.094

F1-Ctrl-A

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.167	1	0.100	
1	9:55	90	1.50	0.190	1	0.123	
2	11:15	170	2.83	0.264	1	0.197	0.197
3	12:35	250	4.17	0.452	1	0.385	0.385
4	13:40	315	5.25	0.246	5	0.907	0.907

F2-ctrl-B

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.165	1	0.098	
1	9:55	90	1.50	0.190	1	0.123	
2	11:15	170	2.83	0.264	1	0.197	0.197
3	12:35	250	4.17	0.460	1	0.393	0.393
4	13:40	315	5.25	0.248	5	0.917	0.917

F6-Bb-a

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.165	1	0.098	
1	9:55	90	1.50	0.180	1	0.113	
2	11:15	170	2.83	0.219	1	0.152	0.152
3	12:35	250	4.17	0.314	1	0.247	0.247
4	13:40	315	5.25	0.500	1	0.433	0.433
5	14:55	390	6.50	0.287	5	1.112	1.112

F7-Bb-b

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.166	1	0.099	
1	9:55	90	1.50	0.184	1	0.117	0.117
2	11:15	170	2.83	0.183	1	0.116	0.116
3	12:35	250	4.17	0.189	1	0.122	0.122
4	13:40	315	5.25	0.186	1	0.119	0.119
5	14:55	390	6.50	0.191	1	0.124	0.124

F8-Bb-c

sample	time	time [min]	time [h]	OD600	dilution [1:x]	OD600corr []	OD600corr []
0	8:25	0	0.00	0.164	1	0.097	
1	9:55	90	1.50	0.168	1	0.101	0.101
2	11:15	170	2.83	0.166	1	0.099	0.099
3	12:35	250	4.17	0.170	1	0.103	0.103
4	13:40	315	5.25	0.170	1	0.103	0.103
5	14:55	390	6.50	0.170	1	0.103	

Example 20: Effect of ethanol dehydrogenase and pyruvate decarboxylase inhibitors on growth and product formation of mixed cultures of ethanologen and butanologen yeast

[0350] Effects of addition of ethanol dehydrogenase and pyruvate decarboxylase inhibitors on mixed cultures of ethanologen *S. cerevisiae* PNY 827 and the butanologen yeast *S. cerevisiae* PNY 2129 were investigated. Therefore two 125 ml aerobic shake flask were prepared with 20 ml SEED medium and each inoculated with 1 ml of frozen glycerol stock culture of PNY 2129 in the morning. Another 125 ml aerobic shake flask was prepared with 20 ml SEED medium and inoculated with 1 ml of frozen glycerol stock culture of PNY 827 in the afternoon. All cultures were incubated at 30°C and 250 rpm overnight in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ). In the morning, sufficient seed culture volume of each strain to give OD600 of 1.000 in the resuspended solution was separately transferred into 50 mL sterile centrifuge tubes and spun down at 9500 rpm for 20 min in an Eppendorf Centrifuge 5804R (Eppendorf, Hamburg, Germany). Supernatants were discarded and the cell pellets resuspended in 20 ml of 0.9% NaCl solution. Optical density was measured with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at $\lambda = 600$ nm. Subsequently "production" cultures were prepared in 25 ml Balch tubes by adding into each tube 6 ml Yeast synthetic w/o aa, w/o glucose, w/o ethanol, w/o Tween (2x), 1.2 ml supplement amino acid solution without histidine and uracil (SAAS-2 10x), 1.92 ml of 250 g/l glucose (ca. 40g/l glucose) and 2.3 μ l of 3M sodium acetate, as well as a specific amount of inoculum solutions, inhibitor solution and water according to the schema in Table 13:

Table 13. Schema showing strains and inhibitors solutions.

	inoculum solution		stock:	300 mM	2 mM (200 mM)	250 mM (2500 mM)
	PNY2129	PNY827	inhibitor solution			
	[ul]	[ul]	H2O	Py	Bb	Ca
			[ul]	[ul]	[ul]	[ul]
T1-ctrl1 B	1200		1200			
T2-ctrl2 B	1200		1200			
T3-ctrl1 E		1200	1200			
T4-ctrl2 E		1200	1200			
T5-1:1-PyA	600	600	1080	120		
T6-1:1-PyB	600	600		1200		
T7-11:1-PyA	1100	100	1080	120		
T8-11:1-PyB	1100	100		1200		
T9-1:1-BbA	600	600	1188		12	
T10-1:1-BbB	600	600	1188		12	
T11-11:1-BbA	1100	100	1188		12	
T12-11:1-BbB	1100	100	1188		12	
T13-1:1-CaA	600	600	1188			12
T14-1:1-CaB	600	600	1080			120
T15-11:1-CaA	1100	100	1188			12
T16-11:1-CaB	1100	100	1080			120
T17-1:1-ctrl1	600	600	1200			
T18-11:1-ctrl1	1100	100	1200			

[0351] Inhibitor solutions were trans-cinnamaldehyde (Aldrich, # 239968, CAS: 14371-10-9) dissolved in water either at 250 mM or 2500 mM, 1-bromo-2-butanone (Sigma-Aldrich, # 243299, CAS: 816-40-0) dissolved in water either at 2 mM or 200 mM, and pyrazole (Aldrich, # P56607, CAS Number: 288-13-1), dissolved in water at 300 mM. Resulting inhibitor concentrations in the Balch tube cultures were pyrazole (PY): 3 mM (A) and 30 mM (B), 1-bromo-2-butanone (BB): 2 μ M (A) and 200 μ M (B), and trans-cinnamaldehyde (CA): 250 μ M (A) and 25 mM (B), respectively. Each Balch tube was fitted with a butyl rubber septum and crimped to the tube with a sheet metal with circular opening to allow samples withdrawal by syringes. The cultures were mixed by a vial/tube rotator (Glas-Col, Terre-Haute, IN) that was placed in an Innova Laboratory Shaker (New Brunswick Scientific, Edison, NJ) for keeping the temperature at 30°C. Samples of about 1 ml for OD determination and extracellular compound analysis were withdrawn at designated hours. Extracellular compound analysis in supernatant was accomplished by HPLC. An Aminex® HPX-87H column (Bio-Rad, Hercules, CA) was used in an isocratic method with 0.01N sulfuric acid as eluent on an Alliance® 2695 Separations Module (Waters Corp., Milford, MA). Flow rate was 0.60 mL/min, column temperature 40°C, injection volume 10 μ L and run time 58 min. Detection was carried out with a refractive index detector (Waters 2414 RI, Waters Corp., Milford, MA) operated at 40°C and an UV detector

(Waters 2996 PDA, Waters Corp., Milford, MA) at 210 nm. Determined optical densities as well as concentrations of extracellular compounds at selected sampling time points can be found in Table 14.

[0352] Butanol to ethanol formed in the mixed cultures with inhibitors was compared to the ratio of butanol to ethanol formed in the mixed cultures without inhibitor (Ctrl) at 8 hours (EPT = 8 h, Figure 14 and Figure 15) and at 48 hours (EPT = 48 h, Figure 16 and Figure 17) of the experiments inoculated with a butanologen-to-ethanologen ratio of 11:1 (b:e=11:1, Figure 14 and Figure 16) or 1:1 (b:e = 1:1, Figure 15 and Figure 17).

[0353] No growth in both mixed cultures and at both time points was observed at the high concentration of trans-cinnamaldehyde of 25 mM (Figure 14 –Figure 17). At the lower concentration of 250 μ M, both mixed cultures grew and produced alcohols. However, at both sampling times (EPT = 8 h and EPT = 48 h) as well as at both inoculum ratios (1:1 and 1:11), the ratio of butanol vs. ethanol produced was lower with addition of trans-cinnamaldehyde than without addition (Figure 14 –Figure 17).

[0354] With 1-bromo-2-butanone, no growth was observed in the 1:1 culture at the high concentration of 200 μ M until EPT = 8 h, only at EPT = 48 h. At low concentration (2 μ M), cultures with both inoculum ratios showed increased butanol-to-ethanol ratios at EPT = 8 h, but not at EPT = 48 h. The same findings apply to the culture with 1:11 ratio at the high concentration (Figure 14 –Figure 17).

[0355] With pyrazole addition at both concentrations, 3 mM and 30 mM, cultures with inoculum ratios 1:1 as well as 1:11 showed dramatically increased butanol-to-ethanol ratios at EPT = 8 h (Figure 14 and Figure 15). However, at EPT = 48 h cultures with both inoculum ratios maintained significantly increased butanol:ethanol ratios only at the higher pyrazole concentration of 30 mM, but not at the lower concentration of 3 mM (Figure 16 and Figure 17).

Table 14. Optical density (OD) and extracellular compound concentrations at the different sampling time points (EPT = elapsed process time) of different pure and mixed cultures.

Abbreviations used were: EtOH = ethanol, PYR = pyruvate, KIV = ketoisovalerate, DHIV = dihydroisovalerate, DHMB = 2,3-dihydroxy-2-methylbutyrate, GLY = glycerol, ACE = acetate, IBOOH = isobutyric acid, IBOH = isobutanol, m-BDO = meso-butanediol, d/l-BDO = d/l-butanediol, LAC = lactate, SUC = succinate.

Sample []	EPT [h]	OD []	GLC [mM]	EtOH [mM]	PYR [mM]	KIV [mM]	DHIV+D				GLY [mM]	ACE [mM]	IBOOH [mM]	IBOH [mM]	m- BDO [mM]	d/l- BDO [mM]	LAC [mM]	SUC [mM]
							HMB [mM]	HMB [mM]	GLY [mM]	ACE [mM]								
SF-17-T1-ctrl-1-B-0	0.00	0.115	226.0	0.0	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T1-ctrl-1-B-3	3.00	0.159	223.4	0.0	0.0	0.2	0.0	0.0	0.2	6.7	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T1-ctrl-1-B-6	6.00	0.210	221.3	0.0	0.1	0.2	0.0	0.0	0.3	6.7	0.3	1.4	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T1-ctrl-1-B-8	8.00	0.247	222.4	0.0	0.1	0.4	0.0	0.0	0.4	6.5	0.4	2.4	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T1-ctrl-1-B-24	24.00	1.382	178.0	1.2	1.8	3.1	0.6	0.6	2.8	4.4	2.5	24.8	0.0	0.0	0.0	0.1	0.3	0.3
SF-17-T1-ctrl-1-B-31	31.00	1.747	147.9	2.0	2.2	3.8	1.2	1.2	5.2	4.1	3.1	39.7	0.0	0.0	0.5	0.1	0.5	0.5
SF-17-T1-ctrl-1-B-48	48.00	1.917	106.9	3.6	2.9	4.4	1.9	1.9	11.9	3.0	3.3	69.1	0.0	0.0	1.3	0.2	0.5	0.5
SF-17-T1-ctrl-1-B-0	0.00	0.109	226.0	0.0	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T2-ctrl-2-B-3	3.00	0.157	223.4	0.0	0.0	0.1	0.0	0.0	0.2	6.7	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T2-ctrl-2-B-6	6.00	0.209	222.0	0.0	0.1	0.2	0.0	0.0	0.3	6.7	0.2	1.4	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T2-ctrl-2-B-8	8.00	0.239	221.1	0.0	0.1	0.4	0.0	0.0	0.4	6.5	0.3	2.4	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T2-ctrl-2-B-24	24.00	1.197	183.6	0.0	1.6	2.9	0.4	0.4	2.6	4.4	2.9	22.2	0.0	0.0	0.0	0.0	0.2	0.2
SF-17-T2-ctrl-2-B-31	31.00	1.627	152.0	2.1	2.1	3.9	1.1	1.1	4.9	4.0	3.3	38.1	0.0	0.0	0.5	0.0	0.5	0.5
SF-17-T2-ctrl-2-B-48	48.00	1.867	104.7	4.0	3.0	4.6	2.0	2.0	11.2	2.9	3.3	69.7	0.0	0.0	1.4	0.3	0.5	0.5
SF-17-T3-ctrl-1-E-0	0.00	0.088	224.0	0.0	0.0	0.0	0.0	0.0	0.0	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T3-ctrl-1-E-3	3.00	0.222	221.8	4.5	0.0	0.0	0.0	0.0	0.4	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T3-ctrl-1-E-6	6.00	1.317	206.1	30.3	0.1	0.0	0.0	0.0	1.6	6.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T3-ctrl-1-A-8	8.00	3.637	162.9	103.8	0.5	0.0	0.0	0.0	5.7	6.3	0.0	0.0	0.0	0.0	0.2	0.0	0.3	0.3
SF-17-T3-ctrl-1-E-24	24.00	9.497	0.0	378.5	2.5	0.0	0.0	0.0	18.6	9.5	0.0	0.0	0.0	0.0	0.5	0.4	0.5	0.5
SF-17-T3-ctrl-1-E-31	31.00	11.997	0.0	372.7	2.6	0.0	0.0	0.0	18.6	10.8	0.0	0.5	0.0	0.0	0.4	0.0	0.5	0.5
SF-17-T3-ctrl-1-E-48	48.00	11.897	0.0	382.0	2.3	0.0	0.0	0.0	18.6	11.0	0.0	0.0	0.0	0.0	0.3	0.3	0.6	0.6
SF-17-T3-ctrl-1-E-0	0.00	0.088	224.0	0.0	0.0	0.0	0.0	0.0	0.0	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T4-ctrl-2-E-3	3.00	0.225	221.7	4.6	0.0	0.0	0.0	0.0	0.4	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T4-ctrl-2-E-6	6.00	1.352	207.3	30.6	0.1	0.0	0.0	0.0	1.6	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T4-ctrl-1-B-8	8.00	3.727	161.8	104.8	0.5	0.0	0.0	0.0	5.6	6.4	0.0	0.0	0.0	0.0	0.0	0.2	0.3	0.3
SF-17-T4-ctrl-2-E-24	24.00	11.847	0.0	377.1	2.5	0.0	0.0	0.0	17.0	12.1	0.0	0.0	0.0	0.0	0.4	0.4	0.5	0.5

Sample	EPT	OD	GLC	EtOH	PYR	KIV	DHIV+D	GLY	ACE	IBOOH	IBOH	m-BDO	d/l-BDO	LAC	SUC
[]	[h]	[]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
SF-17-T4-ctrl-2-E-31	31.00	11.547	0.0	367.9	2.4	0.0	0.0	17.1	13.0	0.0	0.6	0.0	0.4	0.4	0.5
SF-17-T4-ctrl-2-E-48	48.00	11.747	0.0	378.7	2.3	0.0	0.0	16.9	13.6	0.0	0.5	0.0	0.3	0.3	0.4
SF-17-T5-1:1-Py-A-0	0.00	0.104	224.0	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T5-1:1-Py-A-3	3.00	0.141	222.8	1.3	0.0	0.0	0.0	0.7	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T5-1:1-Py-A-6	6.00	0.273	218.1	5.4	0.1	0.1	0.0	2.5	6.9	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T5-1:1-Py-A-8	8.00	0.504	210.2	14.8	0.2	0.1	0.0	6.4	6.8	0.0	0.6	0.4	0.0	0.0	0.0
SF-17-T5-1:1-Py-A-24	24.00	9.777	0.0	346.4	2.9	0.1	0.0	37.0	10.3	0.1	1.6	0.9	1.5	0.4	0.4
SF-17-T5-1:1-Py-A-31	31.00	10.647	0.0	337.3	2.8	0.1	0.0	37.0	11.7	0.3	1.6	0.9	1.6	0.3	0.4
SF-17-T5-1:1-Py-A-48	48.00	10.797	0.0	347.2	2.7	0.1	0.0	37.1	12.7	0.0	1.6	0.9	1.7	0.3	0.5
SF-17-T5-1:1-Py-A-0	0.00	0.103	224.0	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T6-1:1-Py-B-3	3.00	0.125	222.4	0.0	0.0	0.0	0.0	0.7	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T6-1:1-Py-B-6	6.00	0.149	220.7	1.4	0.0	0.1	0.0	2.0	6.8	0.0	0.0	0.2	0.0	0.0	0.0
SF-17-T6-1:1-Py-B-8	8.00	0.173	218.7	2.4	0.1	0.1	0.0	3.9	7.0	0.0	0.2	0.3	0.0	0.0	0.0
SF-17-T6-1:1-Py-B-24	24.00	0.602	175.8	24.6	2.0	0.3	0.0	32.7	7.5	0.0	2.1	2.5	0.0	0.0	0.0
SF-17-T6-1:1-Py-B-31	31.00	0.727	149.4	46.0	3.8	0.4	0.0	48.4	8.2	0.0	2.5	3.2	0.7	0.0	0.0
SF-17-T6-1:1-Py-B-48	48.00	1.477	68.8	140.3	9.1	0.5	0.0	88.0	10.0	0.0	3.0	3.8	2.0	0.0	0.6
SF-17-T8-11:1-Py-B-0	0.00	0.110	223.6	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T7-11:1-Py-A-3	3.00	0.146	223.6	0.0	0.0	0.1	0.0	0.3	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T7-11:1-Py-A-6	6.00	0.174	224.2	0.9	0.0	0.1	0.0	0.8	6.7	0.3	0.4	0.0	0.0	0.0	0.0
SF-17-T7-11:1-Py-A-8	8.00	0.216	221.3	2.5	0.1	0.2	0.0	1.6	6.6	0.4	0.7	0.0	0.0	0.0	0.0
SF-17-T7-11:1-Py-A-24	24.00	9.517	0.0	345.0	3.0	0.3	0.0	37.3	8.8	0.8	3.4	0.0	1.2	0.4	0.5
SF-17-T7-11:1-Py-A-31	31.00	10.047	0.0	336.0	2.9	0.3	0.0	37.4	10.1	0.8	3.4	0.9	1.4	0.4	0.5
SF-17-T7-11:1-Py-A-48	48.00	11.347		344.2	2.8	0.2	0.0	37.2	11.4	0.6	3.5	0.9	1.4	0.3	0.5
SF-17-T8-11:1-Py-B-0	0.00	0.108	223.6	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T8-11:1-Py-B-3	3.00	0.136	223.2	0.0	0.0	0.1	0.0	0.3	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T8-11:1-Py-B-6	6.00	0.150	224.5	0.4	0.0	0.2	0.0	0.7	6.7	0.3	0.2	0.0	0.0	0.0	0.0
SF-17-T8-11:1-Py-B-8	8.00	0.161	222.1	0.7	0.0	0.2	0.0	1.1	6.8	0.3	0.2	0.0	0.0	0.0	0.0
SF-17-T8-11:1-Py-B-24	24.00	0.277	209.8	6.0	0.4	0.6	0.0	6.6	6.6	0.8	1.8	0.0	0.0	0.0	0.0

Sample	EPT	OD	GLC	EtOH	PYR	KIV	HMB	GLY	ACE	IBOOH	IBOH	m-BDO	d/l-BDO	LAC	SUC
[J]	[h]	[J]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
SF-17-T8-11:1-Py-B-31	31.00	0.327	205.2	10.2	0.6	0.8	0.0	10.7	6.7	1.1	3.6	0.3	0.0	0.0	0.0
SF-17-T8-11:1-Py-B-48	48.00	0.667	164.4	31.3	2.0	1.3	0.0	29.7	7.3	2.0	12.1	1.2	0.0	0.0	0.0
SF-17-T9-1:1-Bb-A-0	0.00	0.104	224.2	0.0	0.0	0.0	0.0	0.1	6.9	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T9-1:1-Bb-A-3	3.00	0.144	223.2	1.4	0.0	0.1	0.0	0.2	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T9-1:1-Bb-A-6	6.00	0.330	219.1	7.3	0.0	0.1	0.0	0.7	6.6	0.0	0.4	0.0	0.0	0.0	0.0
SF-17-T9-1:1-Bb-A-8	8.00	1.057	207.4	26.7	0.1	0.1	0.0	2.0	6.6	0.2	0.7	0.0	0.0	0.0	0.0
SF-17-T9-1:1-Bb-A-24	24.00	9.877	0.0	371.9	2.7	0.2	0.0	20.5	9.9	0.5	2.0	0.0	0.3	0.3	0.5
SF-17-T9-1:1-Bb-A-31	31.00	11.047	0.0	363.4	2.6	0.2	0.0	20.5	10.9	0.7	2.0	0.0	0.3	0.3	0.5
SF-17-T9-1:1-Bb-A-48	48.00	12.247	0.0	375.3	2.5	0.2	0.0	20.4	11.8	0.7	2.0	0.4	0.4	0.3	0.6
SF-17-T9-1:1-Bb-A-0	0.00	0.106	224.2	0.0	0.0	0.0	0.0	0.1	6.9	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T10-1:1-Bb-B-3	3.00	0.114	223.7	0.0	0.0	0.0	0.0	0.2	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T10-1:1-Bb-B-6	6.00	0.139	223.4	1.4	0.0	0.0	0.0	0.3	6.8	0.0	0.2	0.0	0.0	0.0	0.0
SF-17-T10-1:1-Bb-B-8	8.00	0.189	223.8	3.8	0.0	0.1	0.0	0.4	6.8	0.0	0.3	0.0	0.0	0.0	0.0
SF-17-T10-1:1-Bb-B-24	24.00	11.847	0.0	373.8	2.6	0.1	0.0	19.2	9.3	0.2	1.3	0.0	0.2	0.3	0.6
SF-17-T10-1:1-Bb-B-31	31.00	11.447	0.0	364.2	2.6	0.1	0.0	19.4	11.0	0.0	1.3	0.3	0.3	0.3	0.6
SF-17-T10-1:1-Bb-B-48	48.00	11.747	0.0	380.5	2.5	0.1	0.0	19.2	12.0	0.3	1.3	0.0	0.3	0.3	0.6
SF-17-T12-11:1-Bb-B-0	0.00	0.110	223.9	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T11-11:1-Bb-A-3	3.00	0.144	224.0	0.0	0.0	0.1	0.0	0.2	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T11-11:1-Bb-A-6	6.00	0.187	222.0	1.0	0.0	0.0	0.0	0.4	6.6	0.0	0.6	0.0	0.0	0.0	0.0
SF-17-T11-11:1-Bb-A-8	8.00	0.259	220.8	3.4	0.1	0.2	0.0	0.7	6.5	0.0	1.0	0.0	0.2	0.0	0.0
SF-17-T11-11:1-Bb-A-24	24.00	10.797	0.0	363.4	2.8	0.5	0.0	22.3	8.4	1.0	4.4	0.0	0.2	0.3	0.5
SF-17-T11-11:1-Bb-A-31	31.00	11.197	0.0	353.8	2.7	0.5	0.0	22.3	10.0	1.1	4.3	0.6	0.4	0.4	0.5
SF-17-T11-11:1-Bb-A-48	48.00	11.497	0.0	365.6	2.7	0.4	0.0	22.2	11.3	1.4	4.5	0.6	0.5	0.3	0.5
SF-17-T12-11:1-Bb-B-0	0.00	0.107	223.9	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T12-11:1-Bb-B-3	3.00	0.123	222.4	0.0	0.0	0.0	0.0	0.2	6.7	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T12-11:1-Bb-B-6	6.00	0.134	222.4	0.0	0.0	0.1	0.0	0.2	6.6	0.0	0.4	0.0	0.0	0.0	0.0
SF-17-T12-11:1-Bb-B-8	8.00	0.138	225.8	0.0	0.0	0.1	0.0	0.3	6.9	0.0	0.5	0.0	0.0	0.0	0.0
SF-17-T12-11:1-Bb-B-24	24.00	10.317	16.0	344.8	2.7	0.2	0.0	20.4	6.4	0.5	2.8	0.3	0.0	0.4	0.5

Sample	EPT	OD	GLC	EtOH	PYR	KIV	HMB	GLY	ACE	IBOOH	IBOH	m-BDO	d/l-BDO	LAC	SUC
[J]	[h]	[J]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
SF-17-T12-11:1-Bb-B-31	31.00	11.447	0.0	360.8	2.7	0.2	0.0	21.4	9.1	0.5	2.8	0.0	0.2	0.3	0.6
SF-17-T12-11:1-Bb-B-48	48.00	11.897	0.0	372.0	2.6	0.2	0.0	21.1	10.8	0.6	2.8	0.0	0.3	0.4	0.6
SF-17-T14-1:1-Ca-B-0	0.00	0.106	233.7	0.0	0.0	0.0	0.0	0.1	7.2	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T13-1:1-Ca-A-3	3.00	0.142	222.8	1.6	0.0	0.0	0.0	0.3	6.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T13-1:1-Ca-A-6	6.00	0.388	217.9	9.6	0.0	0.0	0.0	0.8	6.6	0.0	0.3	0.0	0.0	0.0	0.0
SF-17-T13-1:1-Ca-A-8	8.00	1.417	201.3	36.8	0.2	0.1	0.0	2.5	6.5	0.3	0.6	0.0	0.0	0.0	0.0
SF-17-T13-1:1-Ca-A-24	24.00	11.347	0.0	374.9	2.7	0.1	0.0	18.4	11.2	0.5	1.7	0.0	0.2	0.4	0.5
SF-17-T13-1:1-Ca-A-31	31.00	10.647	0.0	358.7	2.6	0.1	0.0	18.5	11.9	0.7	1.6	0.0	0.3	0.3	0.5
SF-17-T13-1:1-Ca-A-48	48.00	12.097	0.0	369.5	2.5	0.1	0.0	18.4	13.0	0.7	1.6	0.2	0.2	0.3	0.5
SF-17-T14-1:1-Ca-B-0	0.00	n.d.	233.7	0.0	0.0	0.0	0.0	0.1	7.2	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T14-1:1-Ca-B-3	3.00	n.d.	233.9	0.0	0.0	0.0	0.0	0.1	7.2	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T14-1:1-Ca-B-6	6.00	n.d.	233.4	0.0	0.0	0.0	0.0	0.0	7.1	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T14-1:1-Ca-B-8	8.00	n.d.	237.4	0.0	0.0	0.0	0.0	0.1	7.2	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T14-1:1-Ca-B-24	24.00	n.d.	234.0	0.0	0.0	0.0	0.0	0.0	7.2	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T14-1:1-Ca-B-31	31.00	n.d.	234.0	0.0	0.0	0.0	0.0	0.1	7.1	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T14-1:1-Ca-B-48	48.00	n.d.	234.7	0.0	0.0	0.0	0.0	0.0	7.5	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T15-11:1-Ca-A-0	0.00	0.113	232.1	0.0	0.0	0.0	0.0	0.1	7.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T15-11:1-Ca-A-3	3.00	0.141	231.6	0.0	0.0	0.0	0.0	0.2	7.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T15-11:1-Ca-A-6	6.00	0.199	230.2	1.4	0.0	0.1	0.0	0.5	6.8	0.0	0.4	0.0	0.0	0.0	0.0
SF-17-T15-11:1-Ca-A-8	8.00	0.387	228.2	6.5	0.1	0.2	0.0	1.0	6.8	0.6	0.8	0.0	0.0	0.0	0.0
SF-17-T15-11:1-Ca-A-24	24.00	11.647	0.0	381.6	2.9	0.3	0.0	22.4	9.3	1.1	3.5	0.4	0.3	0.3	0.5
SF-17-T15-11:1-Ca-A-31	31.00	10.397	0.0	366.1	2.9	0.3	0.0	22.6	10.7	1.5	3.5	0.4	0.2	0.4	0.5
SF-17-T15-11:1-Ca-A-48	48.00	12.147	0.0	382.7	2.8	0.3	0.0	22.2	11.9	1.3	3.7	0.0	0.3	0.3	0.5
SF-17-T15-11:1-Ca-A-0	0.00	n.d.	232.1	0.0	0.0	0.0	0.0	0.1	7.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T16-11:1-Ca-B-3	3.00	n.d.	224.4	0.0	0.0	0.0	0.0	0.1	6.9	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T16-11:1-Ca-B-6	6.00	n.d.	223.9	0.0	0.0	0.0	0.0	0.1	6.7	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T16-11:1-Ca-B-8	8.00	n.d.	227.0	0.0	0.0	0.0	0.0	0.1	7.1	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T16-11:1-Ca-B-24	24.00	n.d.	224.1	0.0	0.0	0.0	0.0	0.0	6.8	0.0	0.0	0.0	0.0	0.0	0.0

Sample []	EPT [h]	OD []	GLC [mM]	EtOH [mM]	PYR [mM]	KIV [mM]	DHIV+D				ACE [mM]	IBOOH [mM]	IBOH [mM]	m- d/l-		LAC [mM]	SUC [mM]
							HMB [mM]	GLY [mM]	BDO [mM]	BDO [mM]							
SF-17-T16-11:1-Ca-B-31	31.00	n.d.	226.4	0.0	0.0	0.0	0.0	0.1	6.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T16-11:1-Ca-B-48	48.00	n.d.	225.3	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T17-1:1-ctrl-1-0	0.00	0.101	223.9	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T17-1:1-ctrl-1-3	3.00	0.209	221.6	2.5	0.0	0.1	0.0	0.4	6.8	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T17-1:1-ctrl-1-6	6.00	0.862	211.4	17.9	0.1	0.1	0.0	1.3	6.5	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T17-1:1-ctrl-1-8	8.00	2.787	181.0	67.6	0.4	0.2	0.0	4.7	6.5	0.2	1.2	0.0	0.0	0.0	0.1	0.2	0.2
SF-17-T17-1:1-ctrl-1-24	24.00	12.097	0.0	372.5	2.7	0.2	0.0	19.6	9.7	0.3	2.2	0.0	0.0	0.2	0.4	0.5	0.5
SF-17-T17-1:1-ctrl-1-31	31.00	11.997	0.0	358.7	2.7	0.2	0.0	19.9	10.3	0.0	2.1	0.3	0.4	0.4	0.3	0.6	0.6
SF-17-T17-1:1-ctrl-1-48	48.00	12.297	0.0	374.6	2.6	0.2	0.0	19.4	11.0	0.3	2.3	0.0	0.4	0.4	0.3	0.6	0.6
SF-17-T17-1:1-ctrl-1-0	0.00	0.114	223.9	0.0	0.0	0.0	0.0	0.1	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T18-11:1-ctrl-1-3	3.00	0.177	222.1	0.0	0.0	0.1	0.0	0.2	6.7	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T18-11:1-ctrl-1-6	6.00	0.303	218.8	2.9	0.1	0.3	0.0	0.5	6.4	0.3	1.5	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T18-11:1-ctrl-1-8	8.00	0.857	212.3	12.6	0.2	0.4	0.0	1.4	6.4	0.5	2.5	0.0	0.0	0.0	0.0	0.0	0.0
SF-17-T18-11:1-ctrl-1-24	24.00	11.397	0.0	354.1	3.1	0.6	0.1	21.6	8.4	0.8	7.2	0.0	0.0	0.4	0.4	0.5	0.5
SF-17-T18-11:1-ctrl-1-31	31.00	10.947	0.0	351.4	3.1	0.6	0.1	21.9	9.6	1.0	7.1	0.8	0.8	0.5	0.4	0.5	0.5
SF-17-T18-11:1-ctrl-1-48	48.00	11.797	0.0	355.5	3.0	0.6	0.1	21.4	10.5	0.8	7.3	0.9	0.9	0.7	0.3	0.5	0.5

Materials & Methods for Examples 21-25

[0356] Yeast synthetic medium w/o amino acids, w/o glucose, w/o ethanol/Tween (2x): 13.4 g/l, Yeast Nitrogen Base w/o amino acids (Difco 0919-15-3); 40 mg/L thiamine; 40 mg/L niacin; 200 ml/L 1M MES buffer, pH = 5.5 ; Supplement aa sol.without histidine and uracil (SAAS-1 10x); 18.5 g/L, Synthetic complete amino acid dropout (Kaiser) -His, -Ura (Formedium).

[0357] SEED medium: 10.000 mL Yeast synthetic medium w/o aa, w/o glucose, w/o ethanol/Tween (2x); 2.000 mL Supplement aa sol. without histidine and uracil (SAAS-1 10x); 3.200 mL 250 g/L glucose solution (resulting in 40 g/l glucose); 0.046 mL Na-acetate stock solution; 4.754 mL H₂O

Example 21 (Prophetic): Construction of isobutanologen strains expressing a formaldehyde dehydrogenase

[0358] *P. putida fdhA* (SEQ ID NO:7) (GI:1169603) and *S. cerevisiae SFA1* (SEQ ID NO:6) (van den Berg *et al.*, *Yeast* 13(6): 551-9 (1997)) are used to synthesize genes *in vitro* using codon-optimization algorithms for *S. cerevisiae* (e.g. DNA 2.0). The gene cassettes are designed to place 5' *Bam*HI and 3' *Mlu*I restriction sites for subcloning of the coding sequences into expression plasmid pBTX1 (SEQ ID NO:15). pBTX1 is derived from the pRS413 vector backbone (ATCC #87518) and contains the *FBA1* promoter, multiple cloning site (*Bam*HI, *Mlu*I), and *ADHI* terminator.

[0359] An isobutanologen is constructed by transformation of plasmids pBTX1::*SFA1* and pLH804::L2V4 into the host strain PNY2145. Plasmid pLH804::L2V4 is derived from the pHR81 vector backbone (ATCC # 87541) and contains: the *A. caccae* K9JB4P KARI driven by the *ILV5* promoter and *ILV5* terminator, and the *S. mutans* L2V4 DHAD driven by the *TEF1*(M7) promoter and *FBA1* terminator (SEQ ID NO:22). Plasmids are introduced by lithium acetate transformation method (Methods in Yeast Genetics, 2005, page 113), and transformants are selected on synthetic complete medium, minus histidine and uracil, with 1% ethanol as carbon source. Transformants are then transferred to plates containing synthetic complete medium, minus histidine and uracil, with 2% glucose as carbon source and optionally ethanol (0.05%) or acetate (2 mM) as a C2 supplement. Freezer vials are made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

[0360] An isobutanologen is constructed by transformation of plasmids pBTX1::*fdhA* and pLH804::L2V4 into the host strain PNY2145. Plasmid pLH804::L2V4 is derived from the pHR81 vector backbone (ATCC # 87541) and contains: the *A. caccae* K9JB4P KARI driven by the *ILV5* promoter and *ILV5* terminator, and the *S. mutans* L2V4 DHAD driven by the *TEF1*(M7) promoter and *FBA1* terminator (SEQ ID NO:22). Plasmids are introduced by lithium acetate transformation method (Methods in Yeast Genetics, 2005, page 113), and transformants are selected on synthetic complete medium, minus histidine and uracil, with 1% ethanol as carbon source. Transformants are then transferred to plates containing synthetic complete medium, minus histidine and uracil, with 2% glucose as carbon source and optionally ethanol (0.05%) or acetate (2 mM) as a C2 supplement. Freezer vials are made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

Example 22 (Prophetic) : Construction of isobutanologen strains expressing a sulfonyleurea-resistant ALS (e.g. SMR1-410)

[0361] To construct an expression plasmid, the protein coding sequence for *S. cerevisiae* *SMR1-410* (SEQ ID NO:9; nucleic acid sequence SEQ ID NO:8) is used to synthesize genes *in vitro* using codon-optimization algorithms for *S. cerevisiae* (e.g. DNA 2.0). The *SMR1-410* gene cassette is designed to place 5' *Bam*HI and 3' *Mlu*I restriction sites for subcloning of the coding sequences into expression plasmid pBTX1 (SEQ ID NO:15). pBTX1 is derived from the pRS413 vector backbone (ATCC #87518) and contains the *FBA1* promoter, multiple cloning site (*Bam*HI, *Mlu*I), and *ADH1* terminator.

[0362] An isobutanologen is constructed by transformation of plasmids pBTX1::*SMR1-410* and pLH804::L2V4 into the host strain PNY2145 (referenced in US Pat. Publ. No. 2014/0004526, which is incorporated herein by reference in its entirety, and described in Example 26). Plasmid pLH804::L2V4 is derived from the pHR81 vector backbone (ATCC # 87541) and contains: the *A. caccae* K9JB4P KARI driven by the *ILV5* promoter and *ILV5* terminator, and the *S. mutans* L2V4 DHAD driven by the *TEF1*(M7) promoter and *FBA1* terminator (SEQ ID NO:22). Plasmids are introduced by lithium acetate transformation method (Methods in Yeast Genetics, 2005, page 113), and transformants are selected on synthetic complete medium, minus histidine and uracil, with 1% ethanol as carbon source. Transformants are then transferred to plates containing synthetic complete medium, minus histidine and uracil, with 2% glucose as carbon source and optionally ethanol (0.05%) or acetate (2 mM) as a C2

supplement. Freezer vials are made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

Example 23 (Prophetic): Construction of isobutanologen strains expressing genes conferring sulfite resistance

[0363] To construct expression plasmids, the protein coding sequences for *S. cerevisiae* FZF1-4 (SEQ ID NO:11) (Park, Lopez *et al.* 1999) and *SSU1* (SEQ ID NO:12) are used to synthesize genes *in vitro* using codon-optimization algorithms for *S. cerevisiae* (e.g. DNA 2.0). SEQ ID NO:10 is the wild type protein sequence for FZF1. The gene cassettes are designed to place 5' *Bam*HI and 3' *Mlu*I restriction sites for subcloning of the coding sequences into expression plasmid pBTX1 (SEQ ID NO:15). pBTX1 is derived from the pRS413 vector backbone (ATCC #87518) and contains the *FBA1* promoter, multiple cloning site (*Bam*HI, *Mlu*I), and *ADHI* terminator.

[0364] An isobutanologen is constructed by transformation of plasmids pBTX1::*FZF1-4* and pLH804::L2V4 into the host strain PNY2145 (described herein) that contains a deletion of the chromosomal *FZF1* gene. The *FZF1* deletion in PNY2145 is made using standard yeast deletions using a *kanMX4* cassette (Brachmann, *et al.* Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast. 14, 115-132 (1998). Plasmid pLH804::L2V4 is derived from the pHR81 vector backbone (ATCC # 87541) and contains: the *A. caccae* K9JB4P KARI driven by the *ILV5* promoter and *ILV5* terminator, and the *S. mutans* L2V4 DHAD driven by the *TEF1*(M7) promoter and *FBA1* terminator (SEQ ID NO:22). Plasmids are introduced by lithium acetate transformation method (Methods in Yeast Genetics, 2005, page 113), and transformants are selected on synthetic complete medium, minus histidine and uracil, with 1% ethanol as carbon source. Transformants are then transferred to plates containing synthetic complete medium, minus histidine and uracil, with 2% glucose as carbon source and optionally ethanol (0.05%) or acetate (2 mM) as a C2 supplement. Freezer vials are made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

[0365] An isobutanologen is constructed by transformation of plasmids pBTX1::*SSU1* and pLH804::L2V4 into the host strain PNY2145 (described herein). Plasmid pLH804::L2V4 is derived from the pHR81 vector backbone (ATCC # 87541) and contains: the *A. caccae* K9JB4P KARI driven by the *ILV5* promoter and *ILV5* terminator, and the *S. mutans* L2V4 DHAD driven by the *TEF1*(M7) promoter and *FBA1* terminator (SEQ ID NO:22). Plasmids are introduced by

lithium acetate transformation method (Methods in Yeast Genetics, 2005, page 113), and transformants are selected on synthetic complete medium, minus histidine and uracil, with 1% ethanol as carbon source. Transformants are then transferred to plates containing synthetic complete medium, minus histidine and uracil, with 2% glucose as carbon source and optionally ethanol (0.05%) or acetate (2 mM) as a C2 supplement. Freezer vials are made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

Example 24 (Prophetic): Construction of isobutanologen strains expressing a glyphosate resistance 3-phosphoshikimate 1-carboxyvinyltransferase

[0366] To construct an expression plasmid, the protein coding sequence for *Salmonella typhi* *aroA*^{GLY+} (SEQ ID NO:13) (Stalker, *et al.*, *J Biol Chem* 260(8): 4724-8 (1985)) is used to synthesize genes *in vitro* using codon-optimization algorithms for *S. cerevisiae* (e.g. DNA 2.0). The *aroA*^{GLY+} gene cassette is designed to place 5' *Bam*HI and 3' *Mlu*I restriction sites for subcloning of the coding sequences into expression plasmid pBTX1 (SEQ ID NO:15). pBTX1 is derived from the pRS413 vector backbone (ATCC #87518) and contains the *FBA1* promoter, multiple cloning site (*Bam*HI, *Mlu*I), and *ADH1* terminator.

[0367] An isobutanologen is constructed by transformation of plasmids pBTX1::*aroA*^{GLY+} and pLH804::L2V4 into the host strain PNY2145 (described herein). Plasmid pLH804::L2V4 is derived from the pHR81 vector backbone (ATCC # 87541) and contains: the *A. caccae* K9JB4P KARI driven by the *ILV5* promoter and *ILV5* terminator, and the *S. mutans* L2V4 DHAD driven by the *TEF1*(M7) promoter and *FBA1* terminator (SEQ ID NO:22). Plasmids are introduced by lithium acetate transformation method (Methods in Yeast Genetics, 2005, page 113), and transformants are selected on synthetic complete medium, minus histidine and uracil, with 1% ethanol as carbon source. Transformants are then transferred to plates containing synthetic complete medium, minus histidine and uracil, with 2% glucose as carbon source and optionally ethanol (0.05%) or acetate (2 mM) as a C2 supplement. Freezer vials are made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

Example 25 (Prophetic): Genetic engineering for increased inhibitor tolerance in butanologen yeast

[0368] In some embodiments, the butanologen is engineered for increased inhibitor tolerance by expressing or overexpressing a formaldehyde dehydrogenase. The formaldehyde

dehydrogenase is selected from one of the following EC groups: EC 1.1.1.284, EC 1.1.1.1, EC 1.2.1.46, EC 1.2.1.66, EC 3.1.2.12, EC 1.2.2.B1 and EC 1.2.2.B2. ED 1.2.2.B1 and EC 1.2.2.B2 are no official designators, but are defined by the BRENDA protein database. Especially suited formaldehyde dehydrogenases are:

[0369] SFA1 (YDL168W, ADH5): glutathione-dependent formaldehyde dehydrogenase (van den Berg *et al.*, *Yeast* 13(6): 551-9 (1997)) (SEQ ID NO:6) and *Pseudomonas putida* glutathione-independent formaldehyde dehydrogenase (SEQ ID NO:7).

[0370] In some embodiments, the butanologen is engineered for increased inhibitor tolerance by expressing or overexpressing a sulfonylurea-resistant ALS (e.g. SMR1-410) (Yadav *et al.*, *Proc Natl Acad Sci U S A* 83(12): 4418-22 (1986)) (SEQ ID NO:9).

[0371] In some embodiments, the butanologen is engineered for increased inhibitor tolerance by expressing or overexpressing other sulfonylurea-resistant ALS enzymes that qualify for (over)expression.

[0372] In some embodiments, the butanologen is engineered for increased inhibitor tolerance by expressing or overexpressing sulfite resistance by convert FZF1 (SEQ ID NO:10) to FZF1-4 (SEQ ID NO:11) (Park *et al.*, *Curr Genet* 36(6): 339-44. (1999)) or overexpressing SSU1 (SEQ ID NO:12).

[0373] FZF1 (YGL254W, NRC299, RSU1 2, SUL1) is a transcription factor involved in sulfite metabolism, sole identified regulatory target is SSU1, overexpression suppresses sulfite-sensitivity of many unrelated mutants due to hyperactivation of SSU1.

[0374] Overexpression of SSU1 (YPL092W, LPG16). SSU1 is a plasma membrane sulfite pump involved in sulfite metabolism and required for efficient sulfite efflux. Homolog enzymes may be considered for overexpression as well to confer increased tolerance/improved competitiveness.

[0375] In some embodiments, the butanologen is engineered for increased inhibitor tolerance by expressing or overexpressing a glyphosate resistance 3-phosphoshikimate 1-carboxyvinyltransferase (e.g. *aroA*^{gly+}) (SEQ ID NO: 13) (Stalker *et al.*, *J Biol Chem* 260(8): 4724-8 (1985)).

[0376] All other glyphosate resistant 3-phosphoshikimate 1-carboxyvinyltransferases qualify for expression or overexpression.

Example 26: Strain Construction

Construction of strain PNY2115

[0377] *Saccharomyces cerevisiae* strain PNY0827 is used as the host cell for further genetic manipulation for PNY2115. 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

[0378] In order to delete the endogenous URA3 coding region, a deletion cassette was PCR-amplified from pLA54 (SEQ ID NO:158) which contains a P_{TEF1} -*kanMX4*-*TEF1* 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:101) and BK506 (SEQ ID NO:102). 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:161) and LA492 (SEQ ID NO:104) 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:105), AK109-2 (SEQ ID NO: 106), and AK109-3 (SEQ ID NO:107). The resulting identified haploid strain called NYLA103, which

has the genotype: MAT α *ura3* Δ ::loxP-*kanMX4*-loxP, and NYLA106, which has the genotype: MAT α *ura3* Δ ::loxP-*kanMX4*-loxP.

Deletion of His3

[0379] 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:89) and primer oBP453 (SEQ ID NO:109), 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:110), containing a 5' tail with homology to the 3' end of *HIS3* Fragment A, and primer oBP455 (SEQ ID NO:90) 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:91), containing a 5' tail with homology to the 3' end of *HIS3* Fragment B, and primer oBP457 (SEQ ID NO:86), 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:87), containing a 5' tail with homology to the 3' end of *HIS3* Fragment U, and primer oBP459 (SEQ ID NO:88). 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:89) and oBP455 (SEQ ID NO:90). *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:91) and oBP459 (SEQ ID NO:88). 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:89) and oBP459 (SEQ ID NO:88). 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:93) and LA135 (SEQ ID NO:94) for the 5' end and primers oBP461 (SEQ ID NO:95) and LA92 (SEQ ID NO:96) 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 *ura3Δ::loxP-kanMX4-loxP his3Δ*.

Deletion of PDC1

[0380] To delete the endogenous *PDC1* coding region, a deletion cassette was PCR-amplified from pLA59 (SEQ ID NO:97), 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:98) and LA679 (SEQ ID NO:99). 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:111), external to the 5' coding region and LA135 (SEQ ID NO:94), an internal primer to *URA3*. Positive transformants were then screened by colony PCR using primers LA692 (SEQ ID NO: 112) and LA693 (SEQ ID NO:113), internal to the *PDC1* coding region. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO:184) 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 *ura3Δ::loxP-kanMX4-loxP his3Δ pdc1Δ::loxP71/66*.

Deletion of PDC5

[0381] To delete the endogenous *PDC5* coding region, a deletion cassette was PCR-amplified from pLA59 (SEQ ID NO:97), 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:185) and LA733 (SEQ ID NO:186). 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:187), external to the 5' coding region and LA135 (SEQ ID NO:94), an internal primer to *URA3*. Positive transformants were then screened by colony PCR using primers LA694 (SEQ ID NO:188) and LA695 (SEQ ID NO:189), internal to the *PDC5* coding region. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO:184) 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 *ura3Δ::loxP-kanMX4-loxP his3Δ pdc1Δ::loxP71/66 pdc5Δ::loxP71/66*.

Deletion of FRA2

[0382] 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:190) and primer oBP595 (SEQ ID NO:191), 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:192), containing a 5' tail with homology to the 3' end of *FRA2* Fragment A, and primer oBP597 (SEQ ID NO:193), 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:194), containing a 5' tail with homology to the 3' end of *FRA2* Fragment

B, and primer oBP599 (SEQ ID NO:195), 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:196), containing a 5' tail with homology to the 3' end of *FRA2* Fragment U, and primer oBP601 (SEQ ID NO:197). 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:190) and oBP597 (SEQ ID NO:193). *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:194) and oBP601 (SEQ ID NO:197). 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:190) and oBP601 (SEQ ID NO:197). The PCR product was purified with a PCR Purification kit (Qiagen).

[0383] 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:198) and LA135 (SEQ ID NO:94) for the 5' end, and primers oBP602 (SEQ ID NO:198) and oBP603 (SEQ ID NO:199) 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 *ura3Δ::loxP-kanMX4-loxP his3Δ pdc1Δ::loxP71/66 pdc5Δ::loxP71/66 fra2Δ*.

Addition of native 2 micron plasmid

[0384] The loxP71-URA3-loxP66 marker was PCR-amplified using Phusion DNA polymerase (New England BioLabs; Ipswich, MA) from pLA59 (SEQ ID NO:97), and transformed along with the LA811xLA817 (SEQ ID NOs:200, 201) and LA812xLA818 (SEQ ID NOs:202, 203) 2-micron plasmid fragments (amplified from the native 2-micron plasmid from CEN.PK 113-7D; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre) 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:184) 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 *ura3Δ::loxP*-kanMX4-loxP, *his3Δ pdc1Δ::loxP71/66 pdc5Δ::loxP71/66 fra2Δ* 2-micron.

Construction of PNY2115 from PNY2050

[0385] 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

[0386] 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:209), 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:212) and 679 (SEQ ID NO:213). 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:214), external to the 3' coding region and 92 (SEQ ID NO:215), internal to the URA3 gene. Positive transformants were then prepped for genomic DNA and screened by PCR using primers N245 (SEQ ID NO:216) and N246 (SEQ ID NO:217). The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO:184) 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

[0387] To delete the endogenous PDC6 coding region, an integration cassette was PCR-amplified from pLA78 (SEQ ID NO:210), 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:218) and 897 (SEQ ID NO:219). 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:220) and 366 (SEQ ID NO:221), internal primers to the PDC6 gene.

Transformants with an absence of product were then screened by colony PCR N638 (SEQ ID NO:222), external to the 5' end of the gene, and 740 (SEQ ID NO:223), internal to the FBA1 promoter. Positive transformants were then 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

[0388] 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:211), 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 and primers 856 (SEQ ID NO:224) and 857 (SEQ ID NO:225). 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:226), external to the 5' coding region and N1092 (SEQ ID NO:227), internal to the BiADH gene. Positive transformants were then screened by colony PCR using primers 413 (SEQ ID NO:160), external to the 3' coding region, and 92 (SEQ ID NO:215), internal to the URA3 marker. The URA3 marker was recycled by transforming with pLA34 (SEQ ID NO:184) 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

[0389] To integrate BiADH into the fra2Δ locus of PNY2101, an integration cassette was PCR-amplified from pLA65 (SEQ ID NO:211), 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 performed by using KAPA HiFi and primers 906 (SEQ ID NO:228) and 907 (SEQ ID NO:229). 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:230), external to the 5' coding region and 749 (SEQ ID NO:159), internal to the ILV5 promoter. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO:184) 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* *cdc5Δ::loxP66/71* 2-micron *cdc1Δ::P[PDC1]-ALS|alsS_Bs-CYC1t-loxP71/66* *cdc6Δ:(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

[0390] To delete the endogenous *GPD2* coding region, a deletion cassette was PCR amplified from pLA59 (SEQ ID NO:97), 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:204) and LA513 (SEQ ID NO:205). 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:206) external to the 5' coding region and LA135 (SEQ ID NO:94), internal to *URA3*. Positive transformants were then screened by colony PCR using primers LA514 (SEQ ID NO:207) and LA515 (SEQ ID NO:208), internal to the *GPD2* coding region. The *URA3* marker was recycled by transforming with pLA34 (SEQ ID NO:184) 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* *cdc5Δ::loxP66/71* *fra2Δ* 2-

micron *pdclΔ::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*.

Creation of PNY2145 from PNY2115

[0391] PNY2145 was constructed from PNY2115 by the additional integration of a phosphoketolase gene cassette at the *pdcs5Δ* locus and by replacing the native *AMN1* gene with a codon optimized version of the ortholog from CEN.PK. Integration constructs are further described below.

pdcs5Δ::FBA(L8)-xpk1-CYC1t-loxP71/66

[0392] The *TEF(M4)-xpk1-CYC1t* gene from *pRS423::TEF(M4)-xpk1+ENO1-eutD* (SEQ ID NO:162) was PCR amplified using primers N1341 and N1338 (SEQ ID NOs:163 and 164), generating a 3.1 kb product. The *loxP*-flanked *URA3* gene cassette from *pLA59* (SEQ ID NO:97) was amplified with primers N1033c and N1342 (SEQ ID NOs:165 and 166), 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:166 and 167). A 4.2 kb PCR product was recovered by purification from an electrophoresis agarose gel (Zymo kit). *FBA* promoter variant L8 (SEQ ID NO:168) was amplified using primers N1366 and N1368 (SEQ ID NOs:169 and 170). 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:171 and 172). The PCR product was isolated by phenol-chloroform extraction and ethanol precipitation (using standard methods; *e.g.* Maniatis, 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:173 and 174). Two clones were selected to carry forward. The *URA3* marker was

recycled by transforming with pJT254 (SEQ ID NO:175) containing the CRE recombinase under the *GALI* 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:176) and BK380. One resulting clone was designated PNY2293.

amn1Δ::AMN1(y)-loxP71/66

[0393] 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:177; amino acid SEQ ID NO:178), 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:179) 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

[0394] PCR amplification of the *AMN1(y)*-loxP71-*URA3*-loxP66 cassette was performed by using KAPA HiFi from Kapa Biosystems, Woburn, MA and primers LA712 (SEQ ID NO:180) and LA746 (SEQ ID NO:181). 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).*

INCORPORATION BY REFERENCE

[0395] 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.

EQUIVALENTS

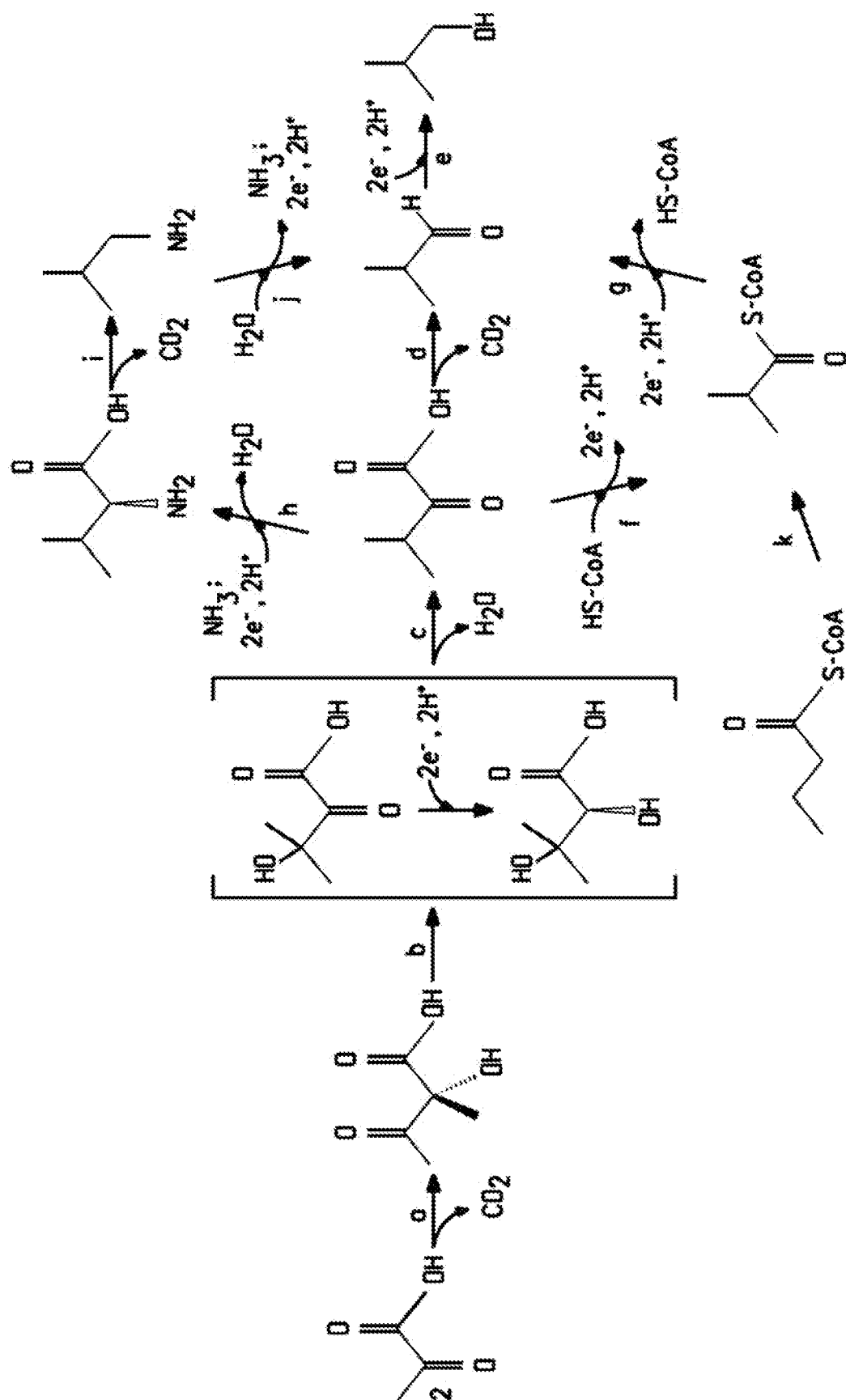
[0396] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

WHAT IS CLAIMED:

1. A method for production of a fermentation product in a fermentation process comprising contacting a fermentation mix comprising a recombinant production microorganism which comprises a pyruvate-utilizing pathway with at least one compound which preferentially inhibits at least one contaminant yeast microorganism.
2. The method of claim 1, wherein the specific growth rate of the at least one contaminant microorganism is reduced more than the specific growth rate of the recombinant production microorganism.
3. The method of claim 1 or claim 2, wherein production of the fermentation product of the at least one contaminant microorganism is reduced more than production of the fermentation product of the recombinant production microorganism.
4. The method of any one of the previous claims, wherein both the production microorganism and the at least one contaminant microorganism are yeast microorganisms.
5. The method of claim 4, wherein the contaminant yeast microorganism is *S. cerevisiae*.
6. The method of any one of claims 1-3, wherein the pyruvate utilizing pathway is a butanol biosynthetic pathway.
7. The method of claim 6, wherein the pyruvate utilizing pathway is an isobutanol biosynthetic pathway.
8. The method of claim 6, wherein the fermentation product of the at least one contaminant microorganism is ethanol.
9. The method of any one of claims 1-3, wherein the mechanism of action of the compound is selected from the group consisting of: heavy metal toxicity, inhibition of amino acid biosynthesis, sulfitolysis, cross-linking, inhibition of ethanol dehydrogenase, inhibition of pyruvate decarboxylase, and mixtures thereof.
10. The method of any one of claims 1-3, wherein said inhibitor is an inhibitor of an ethanol biosynthesis pathway

11. The method of claim 10, wherein said inhibitor inhibits pyruvate decarboxylase and/or ethanol dehydrogenase.
12. The method of claim 11, wherein said inhibitor is selected from a group consisting of: a member of the $\text{XC}_6\text{H}_4\text{CH}=\text{CHCOCOOH}$ class of inhibitors/substrate analogues, cinnamaldehydes, trans-cinnamaldehyde and 4-nitrocinnamaldehyde, glyoxalic acid, ketomalonate, regulatory site inhibitors, p-chloromercuribenzoic acid, 5,5'-dithiobis(2-nitrobenzoic acid), pyrazole, 4-pyrazolecarboxylic acid, 1-H-pyrazole-1-carboxamidinium-HCl, 4-methylpyrazole, 1-bromo-2-butanone pyrazole-3,5-dicarboxylic acid monohydrate, and mixtures thereof.
13. The method of any one of claims 1-3 wherein said inhibitor is selected from the group consisting of: fluoroacetate, formaldehyde, sulfite, and mixtures thereof.
14. The method of any one of claims 1-3 wherein the inhibitor is an inhibitor of an amino acid biosynthesis pathway.
15. The method of claim 14, wherein the inhibitor is inhibiting at least one enzyme selected from the group consisting of: 5-enolpyruvyl-shikimate-3-phosphate synthetase, α -isopropyl malate synthase, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, and mixtures thereof.
16. The method of claim 14, wherein said inhibitor is selected from the group consisting of: imidazolinone, triazolopyrimidine, pyrimidinyl oxybenzoate, sulfonyleurea, sulfonyleamino carbonyl triazolinone, glyphosate, trifluoroleucine, fluorophenylalanine, and mixtures thereof.
17. The method of claim 16, wherein said inhibitor is glyphosate.
18. The method of claim 16, wherein said inhibitor is selected from a group consisting of: nicosulfuron methyl, metsulfuron methyl, chlorimuron ethyl, sulfometuron methyl, chlorsulfuron, thifensulfuron methyl, and mixtures thereof.
19. The method of any one of claims 1-3, wherein said inhibitor is selected from a group consisting of: aureobasiden A, bialaphos, cerulenine, cerulenin, chloramphenicol, cyclohexamide, geneticin, hygromycin B, methotrexate, nourseothricin, phleomycin, triazole, and mixtures thereof.

20. The method of any one of claims 1-3, wherein said inhibitor is selected from a group consisting of: bismuth (III), copper (II), and mixtures thereof.
21. The method of any one of claims 1-3, wherein the recombinant production microorganism is engineered to express a polypeptide that increases tolerance of the host cell to the at least one compound which preferentially inhibits at least one contaminant microorganism.
22. The method of claim 21, wherein said polypeptide comprises an amino acid sequence of at least 80% identity to SEQ ID NO:9 or an active variant, fragment or derivative of SEQ ID NO:9.
23. The method of claim 21, wherein said polypeptide comprises an amino acid sequence of at least 80% identity to formaldehyde dehydrogenase.
24. The method of claim 21, wherein said polypeptide is selected from a group consisting of: an amino acid sequence of at least 80% identity to SEQ ID NO:6, an amino acid sequence of at least 80% identity to SEQ ID NO:7, and mixtures thereof.
25. The method of claim 21, wherein said polypeptide is selected from a group consisting of an amino acid sequence of at least 80% identity to SEQ ID NO:11, an amino acid sequence of at least 80% identity to SEQ ID NO:12, and mixtures thereof.
26. The method of claim 21, wherein said polypeptide has 3-phosphoshikimate 1-carboxylvinyltransferase activity.
27. The method of claim 21, wherein said polypeptide comprises an amino acid sequence of at least 80% identity to SEQ ID NO:13.
28. The method of claim 21, wherein said polypeptide is selected from a group consisting of: a polypeptide that has 5-enolpyruvoyl-shikimate-3-phosphate synthetase (ESPS) activity and confers resistance to glyphosate, a polypeptide that has glyphosate N-acetyltransferase activity and confers resistance to glyphosate and mixtures thereof.





2/19

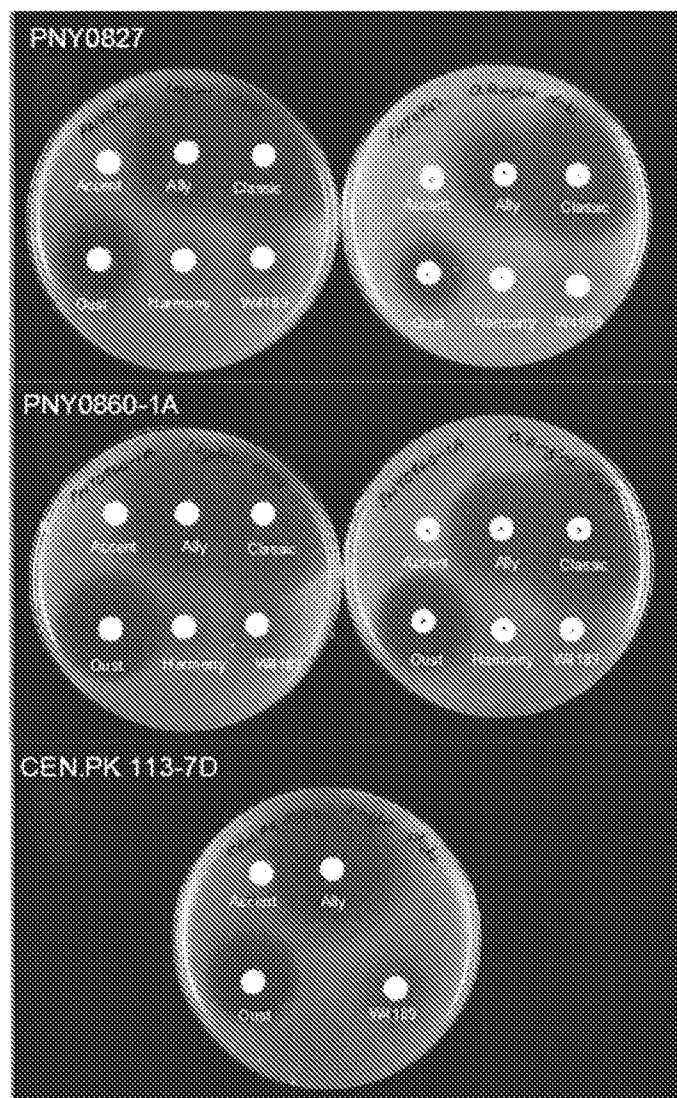


FIG. 2

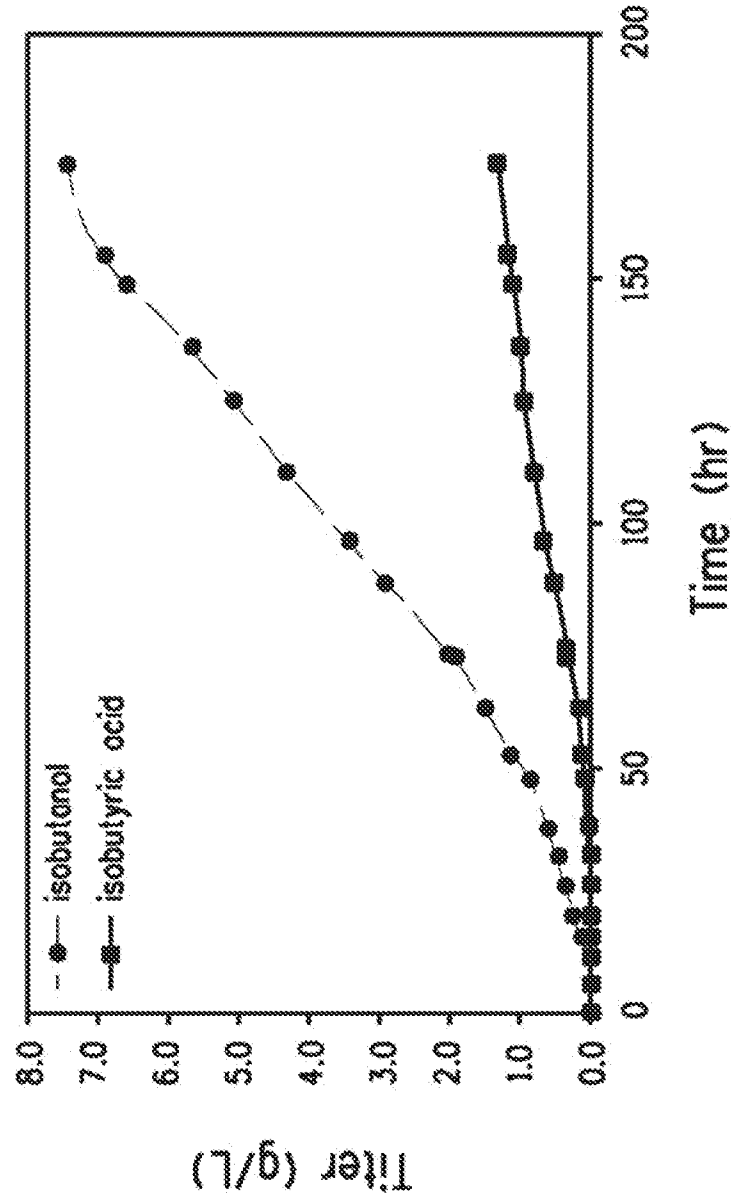


FIG. 3

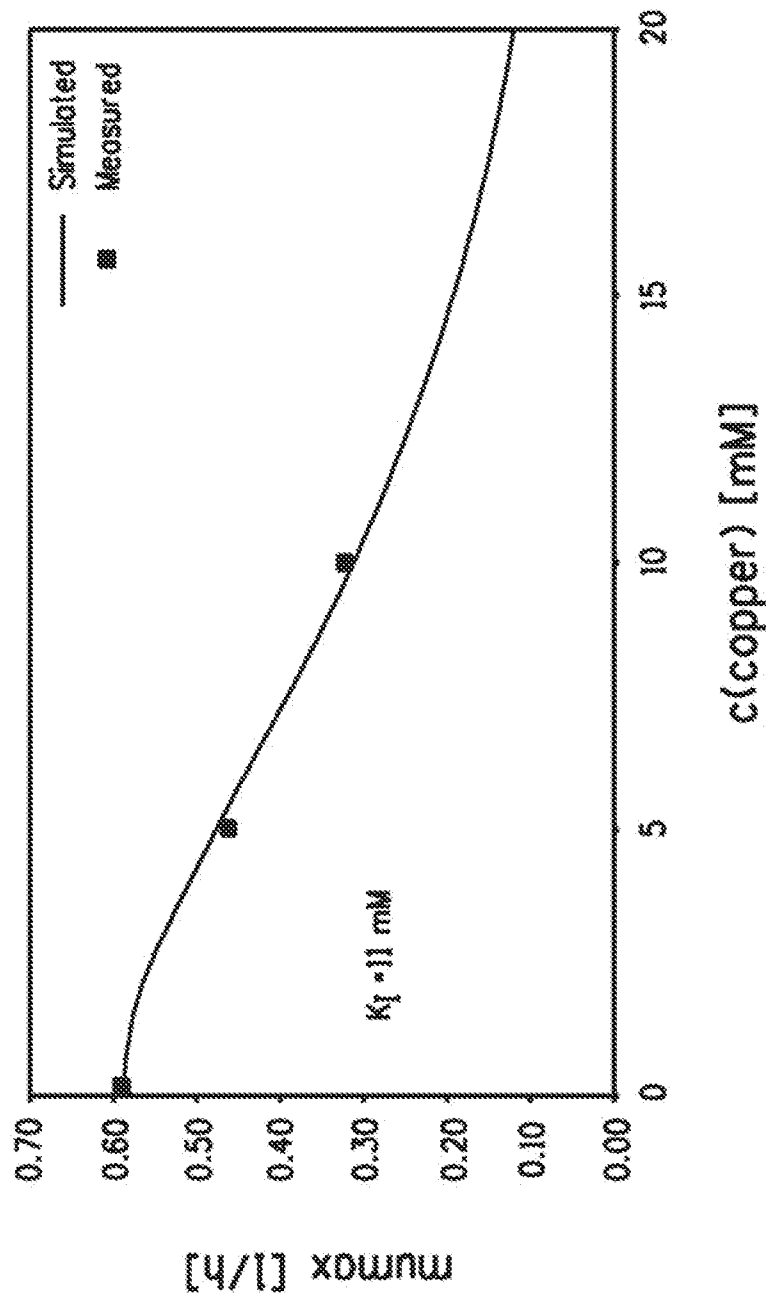


FIG. 4

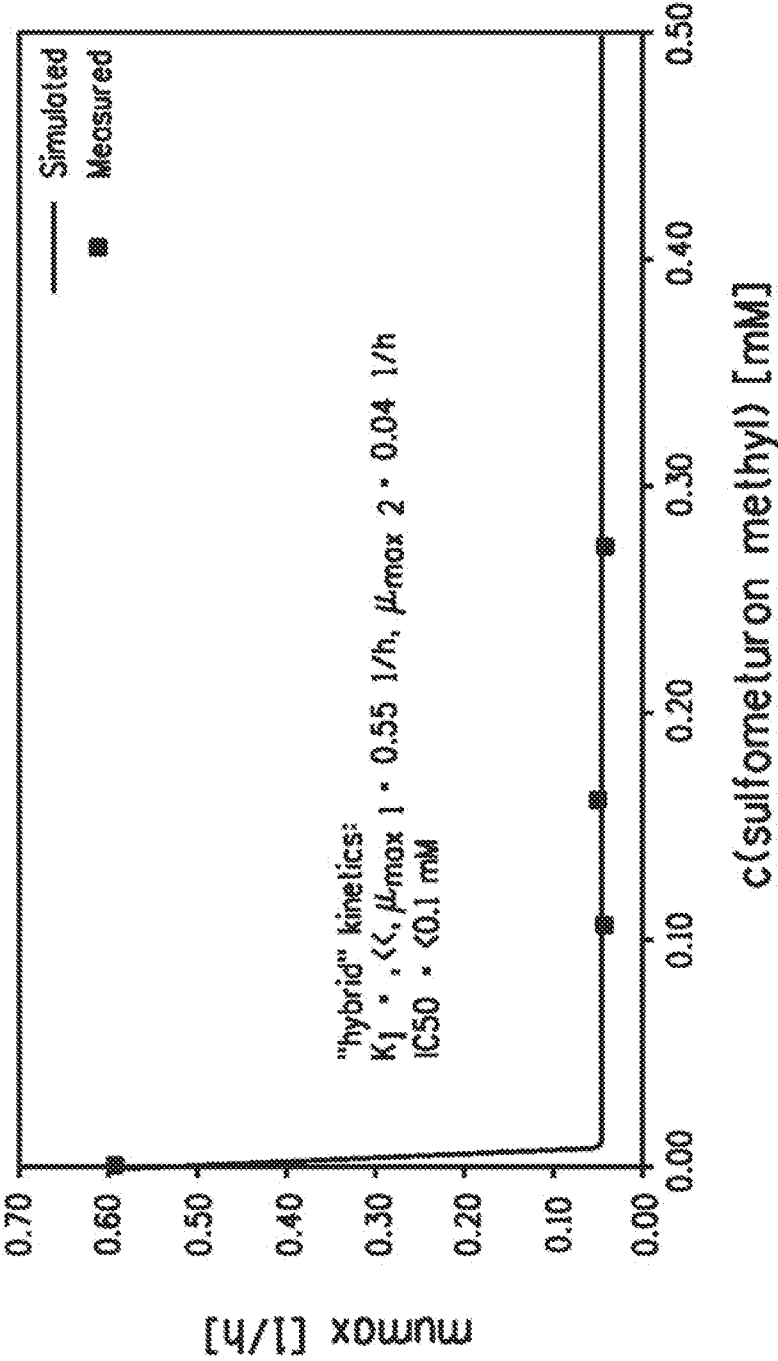


FIG. 5

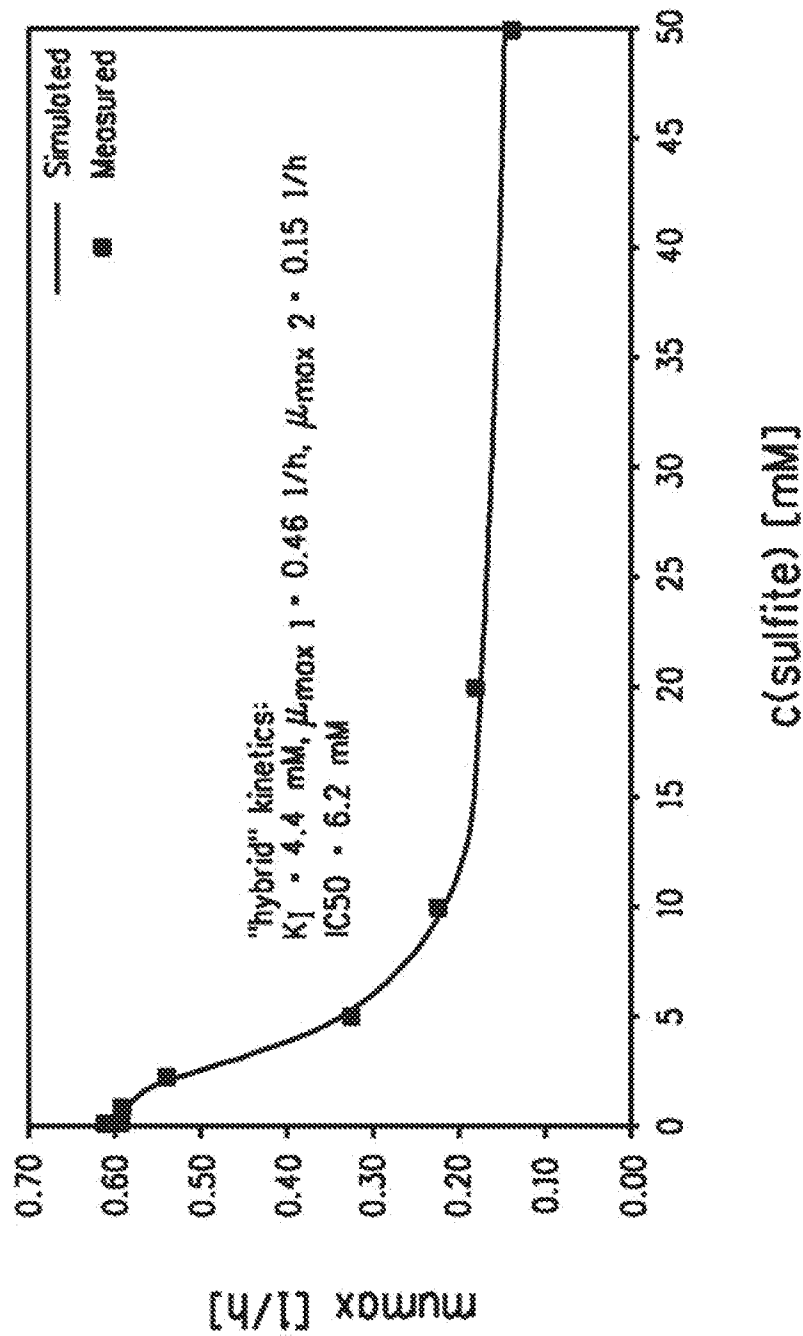


FIG. 6

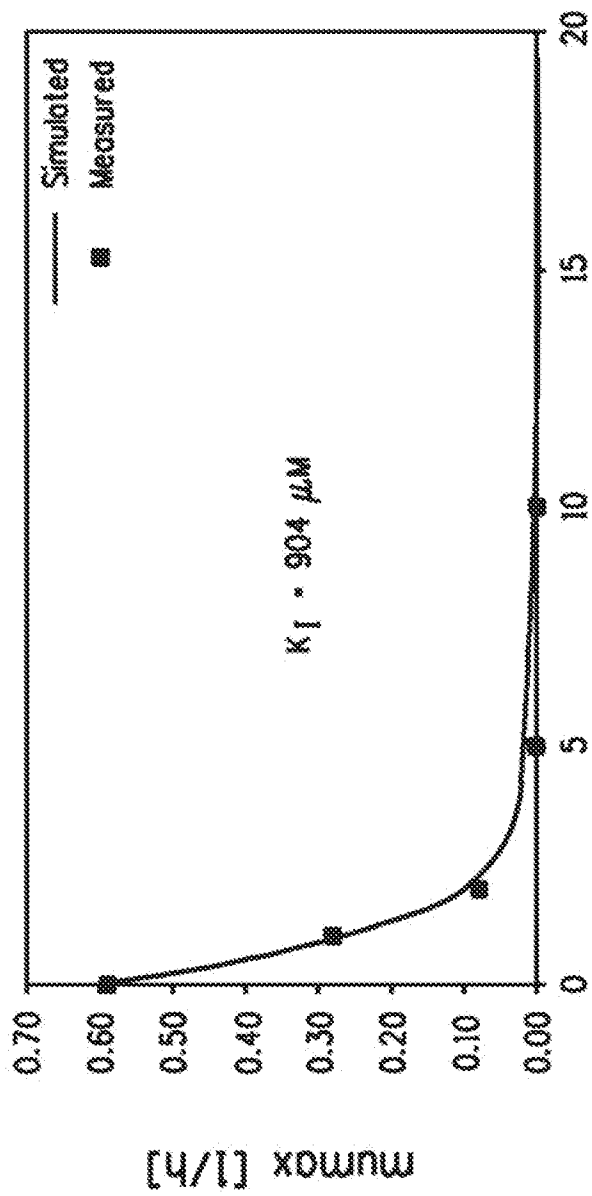


FIG. 7

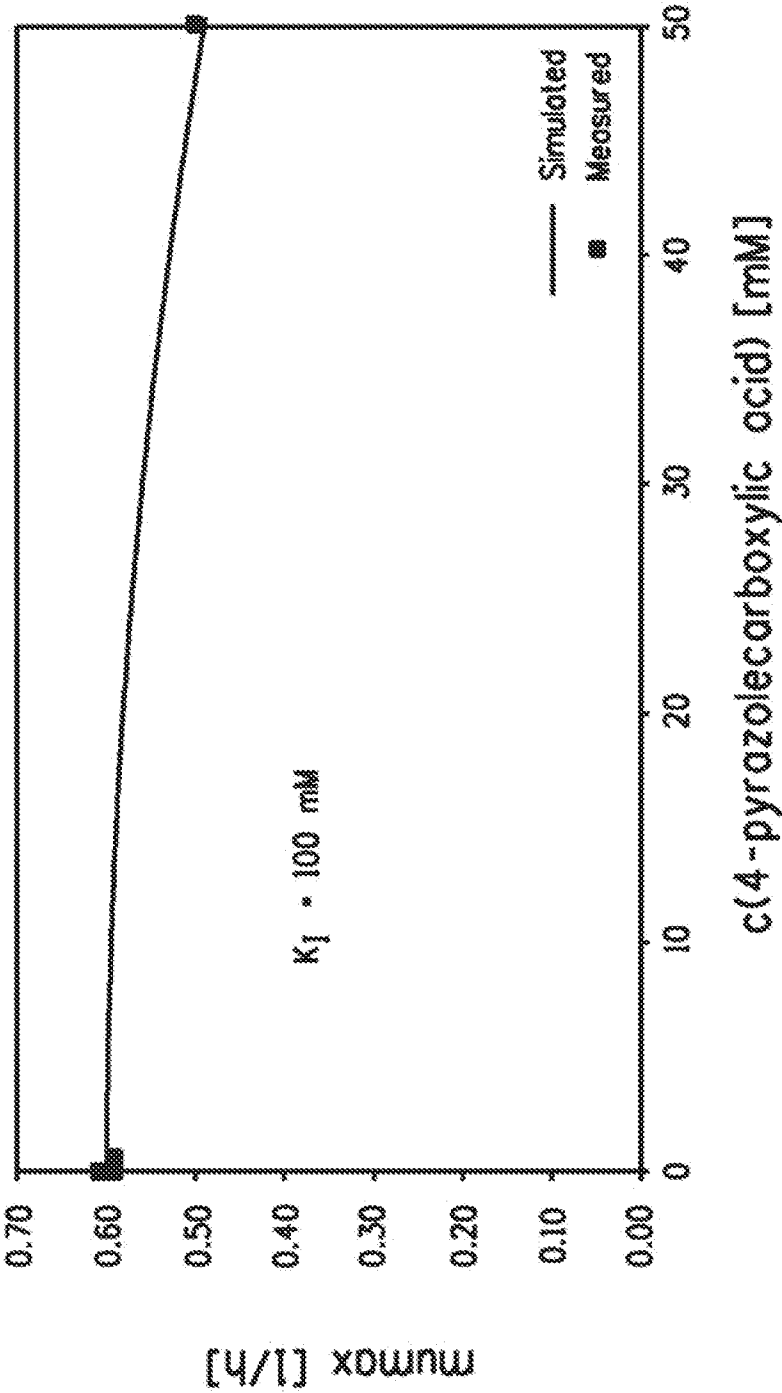


FIG. 8

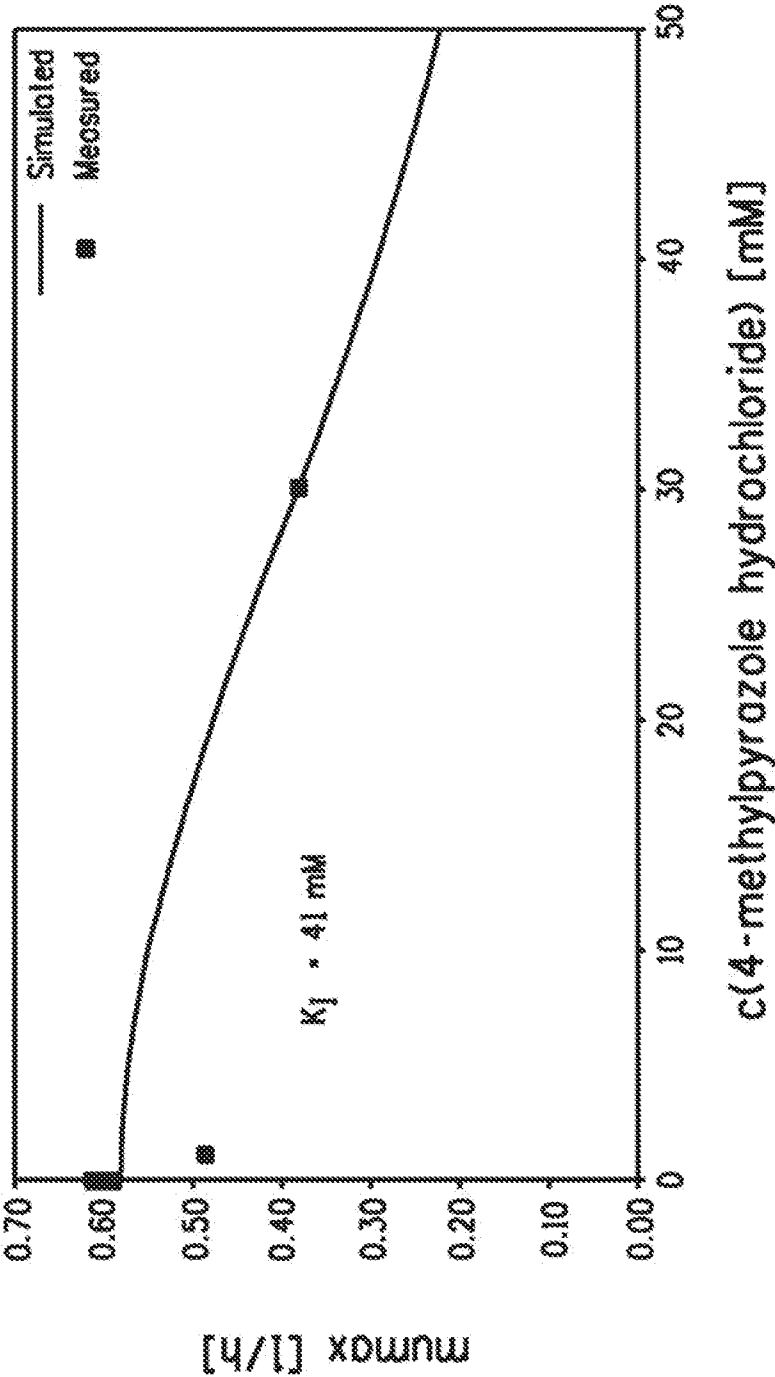


FIG. 9

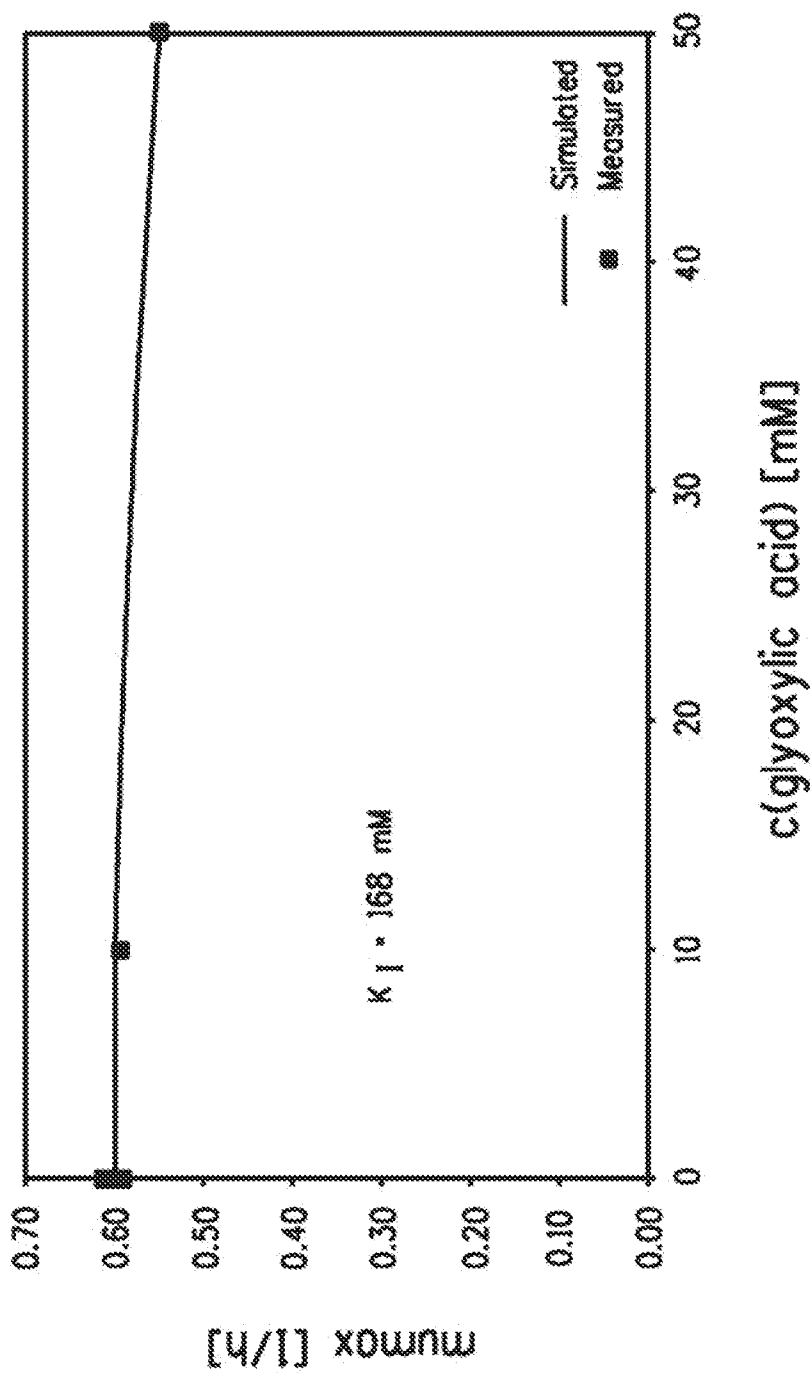


FIG. 10

11/19

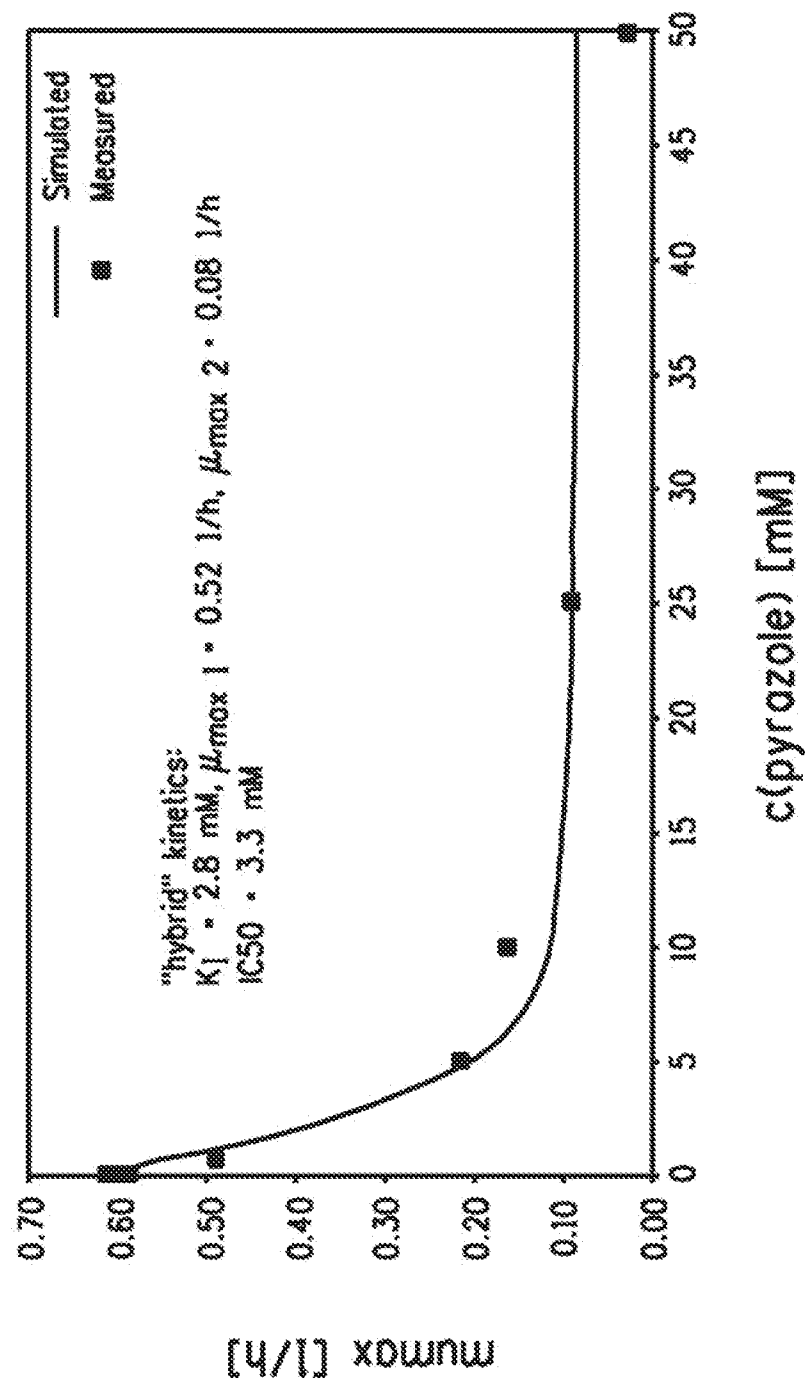


FIG. 11

12/19

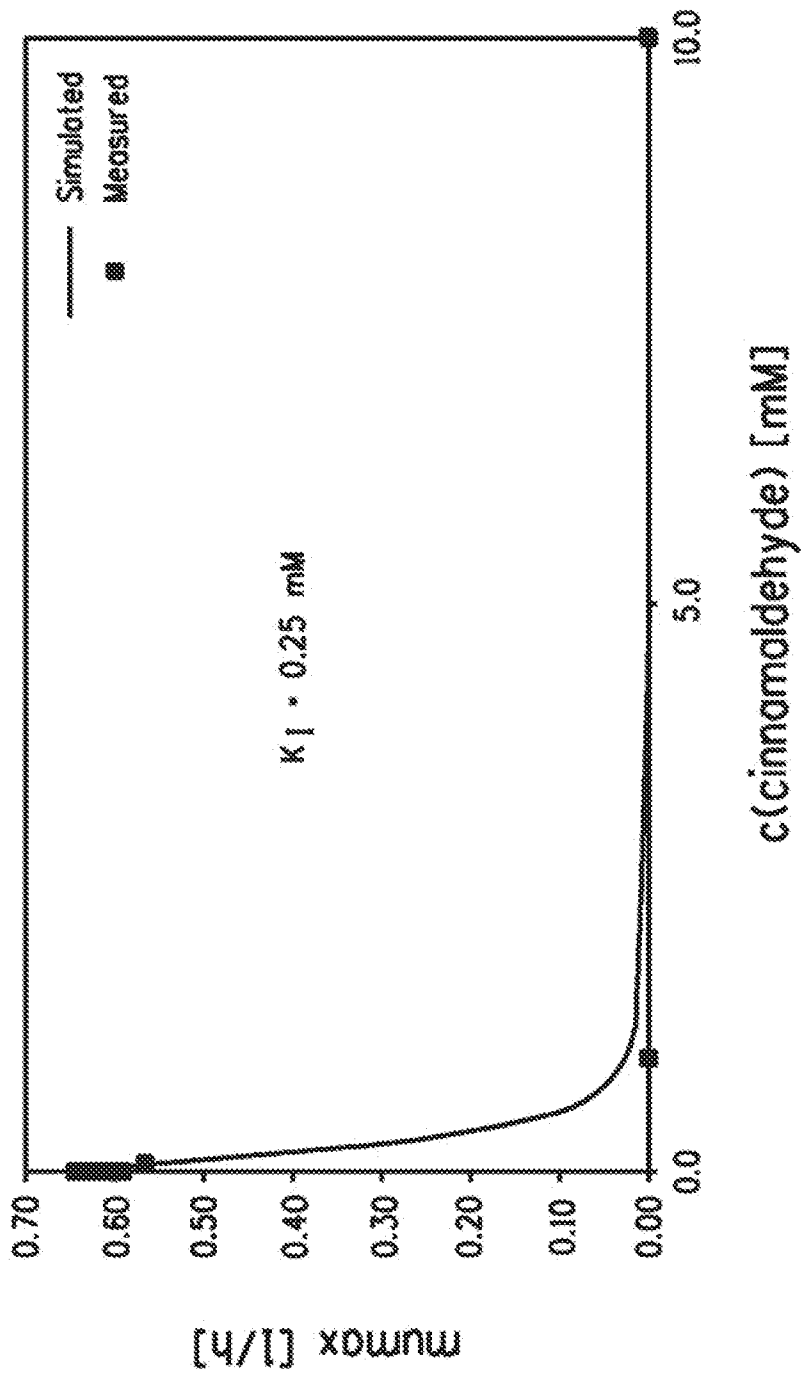


FIG. 12

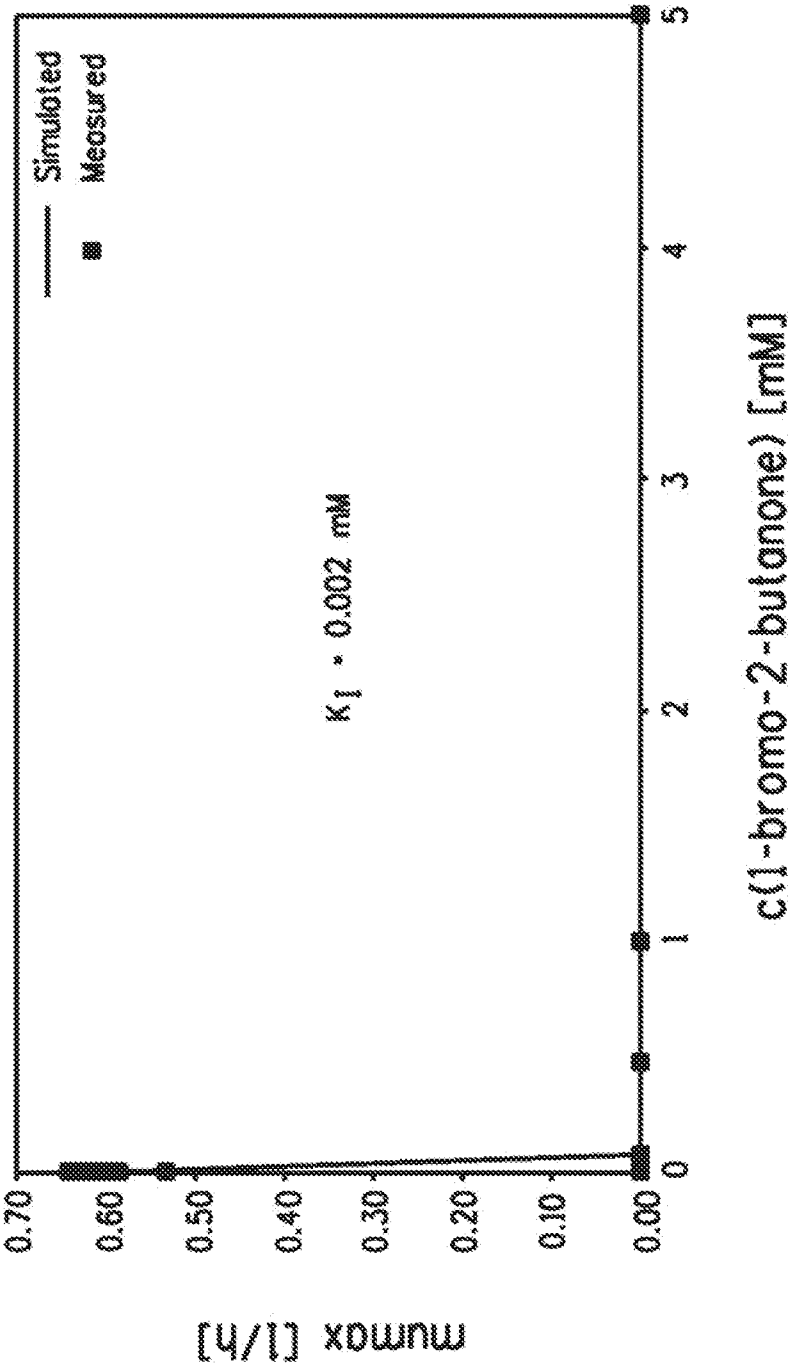


FIG. 13

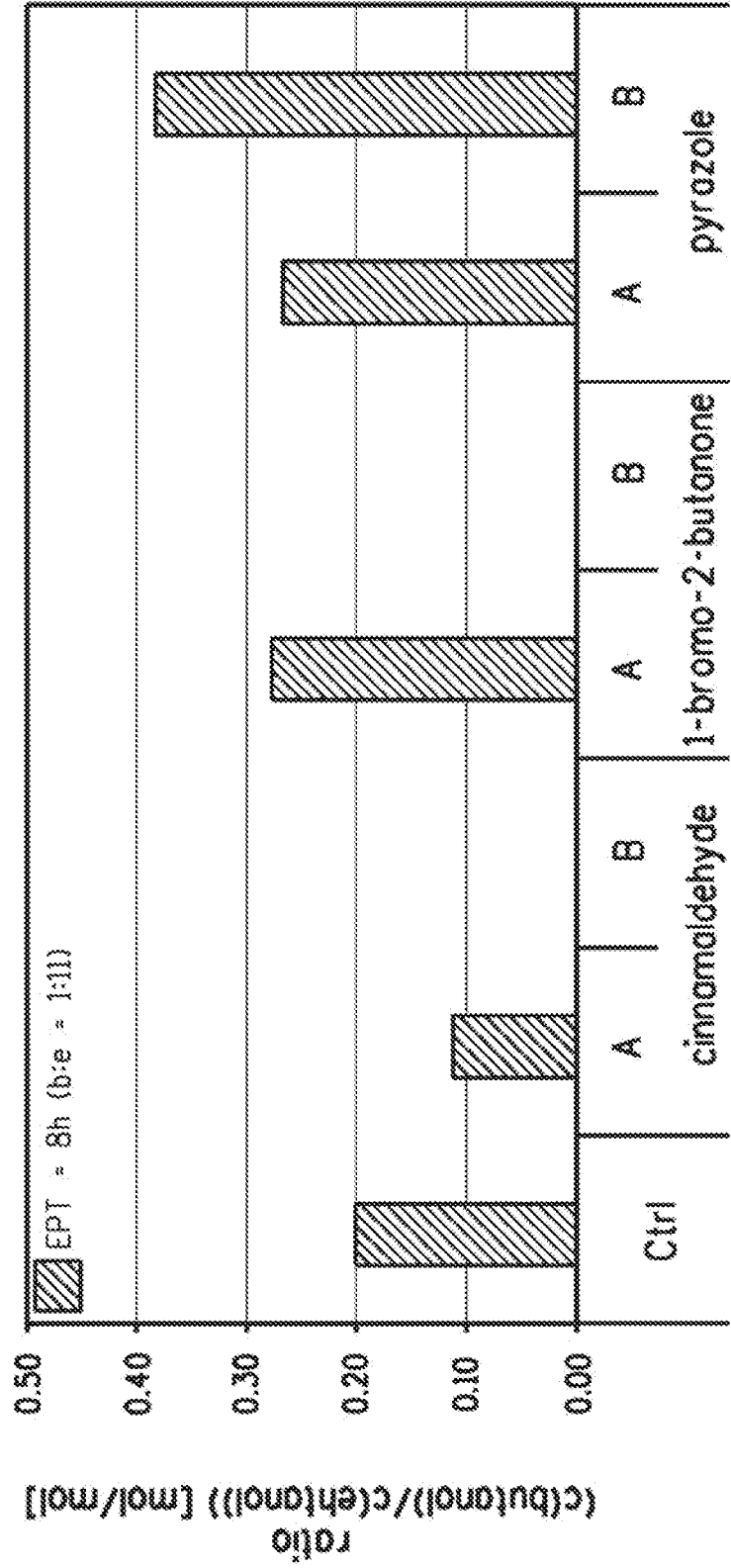


FIG. 14

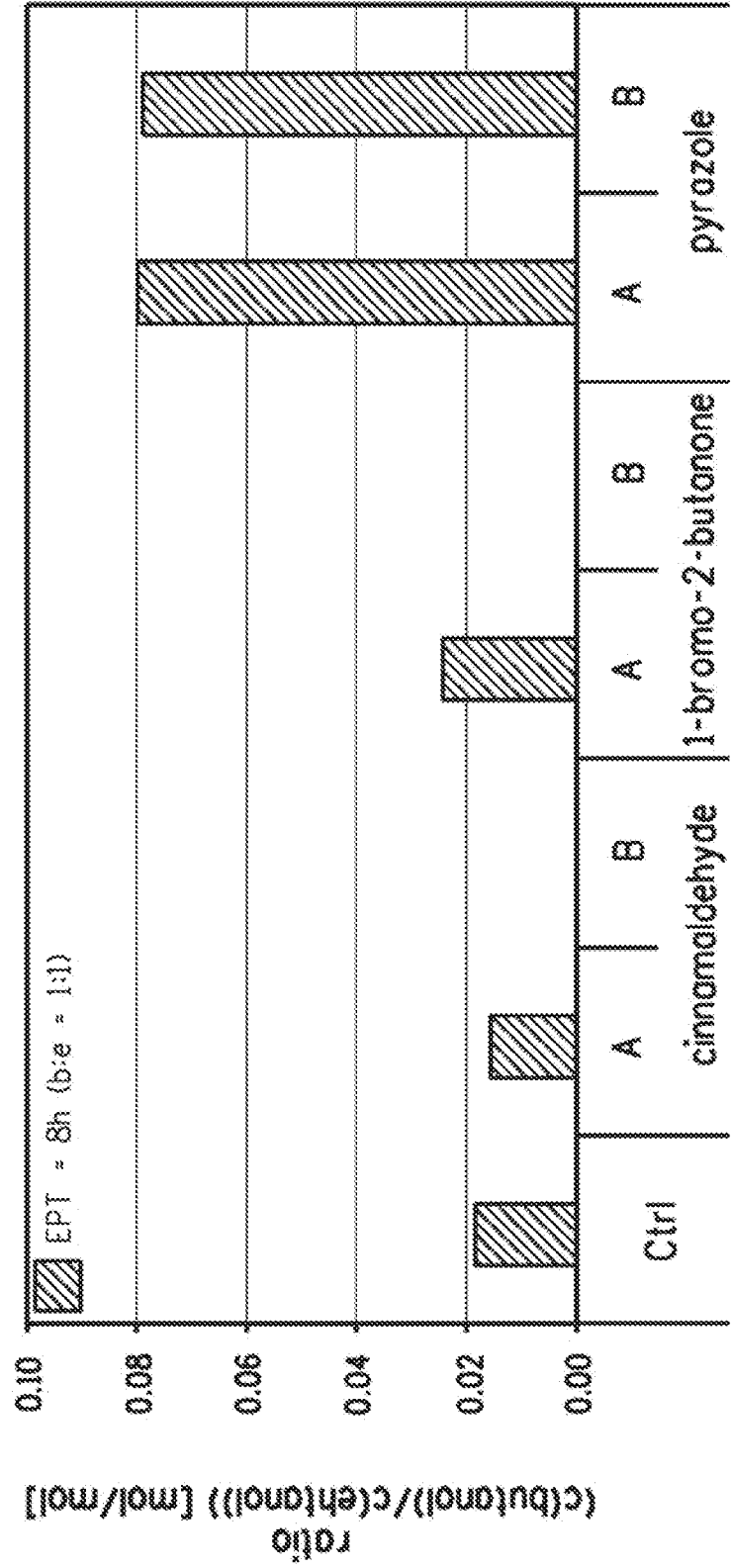


FIG. 15

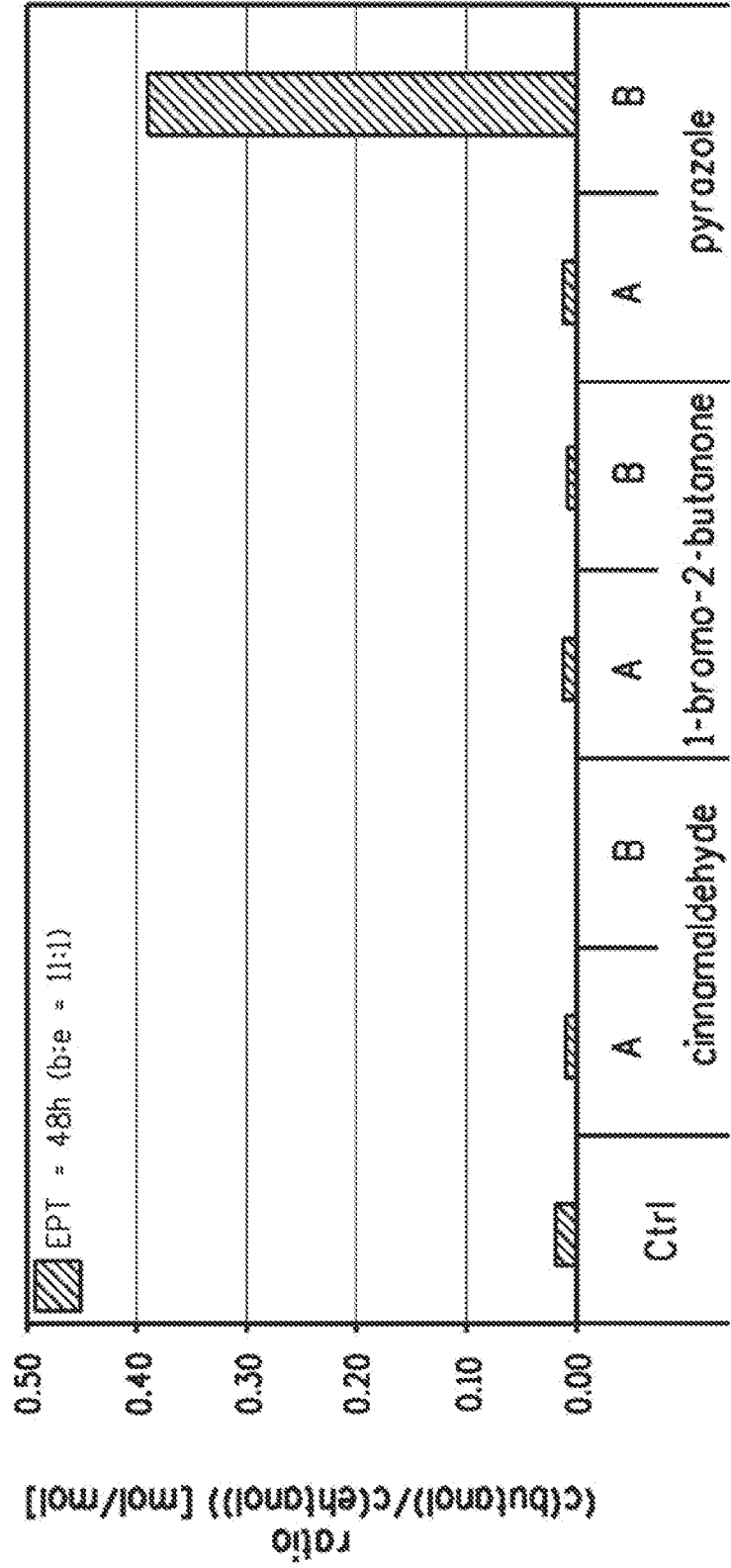


FIG. 16

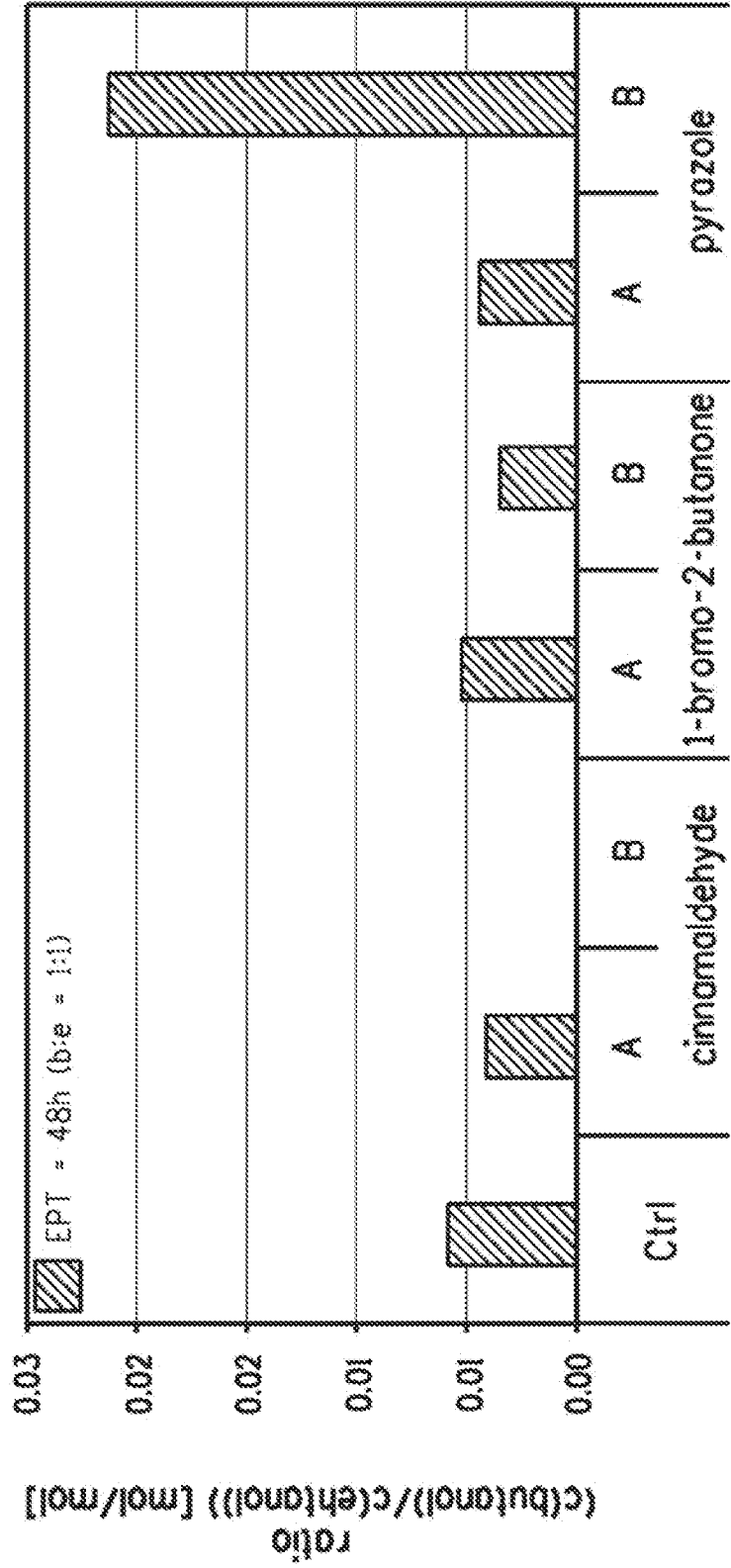


FIG. 17

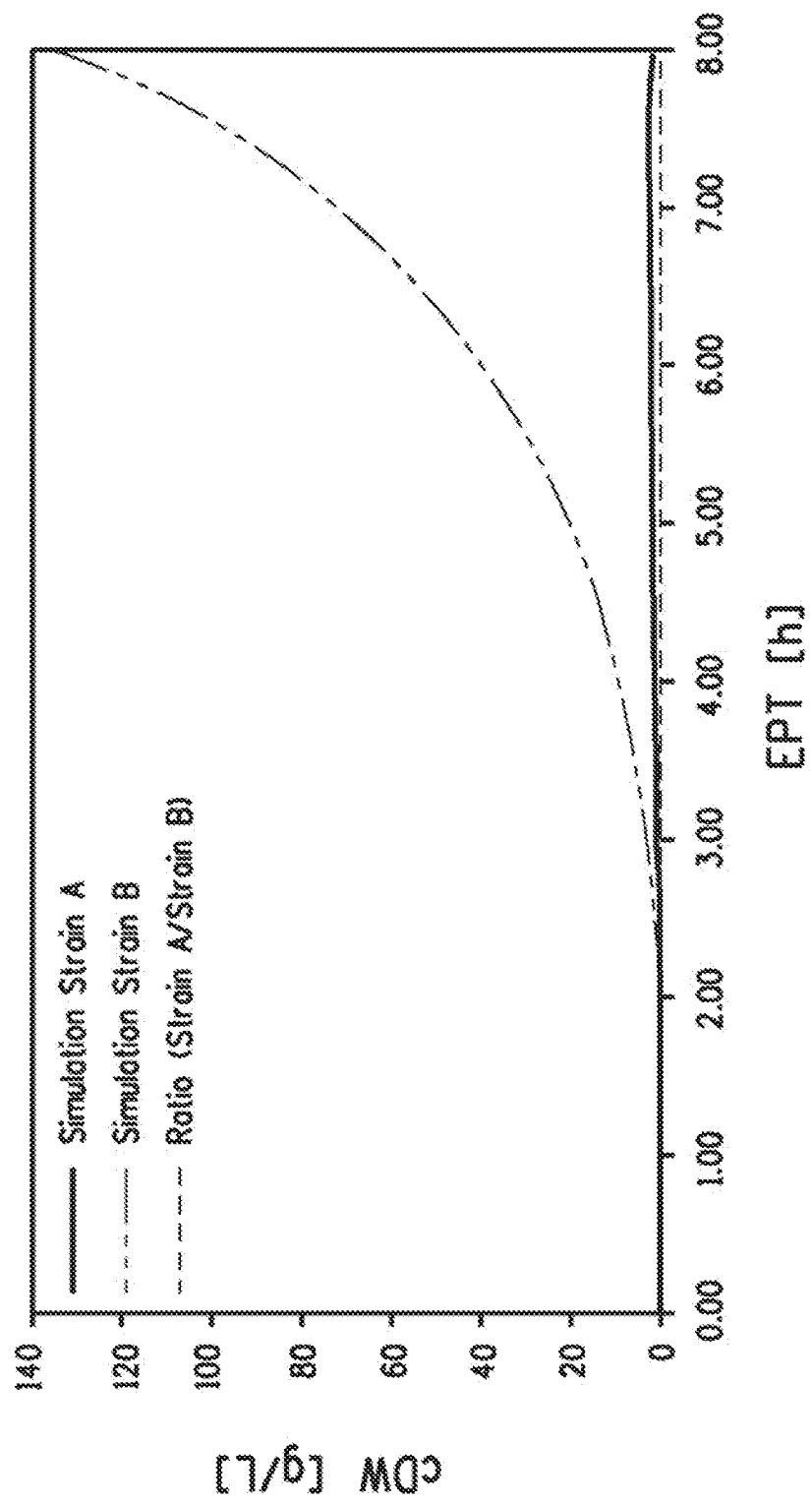


FIG. 18

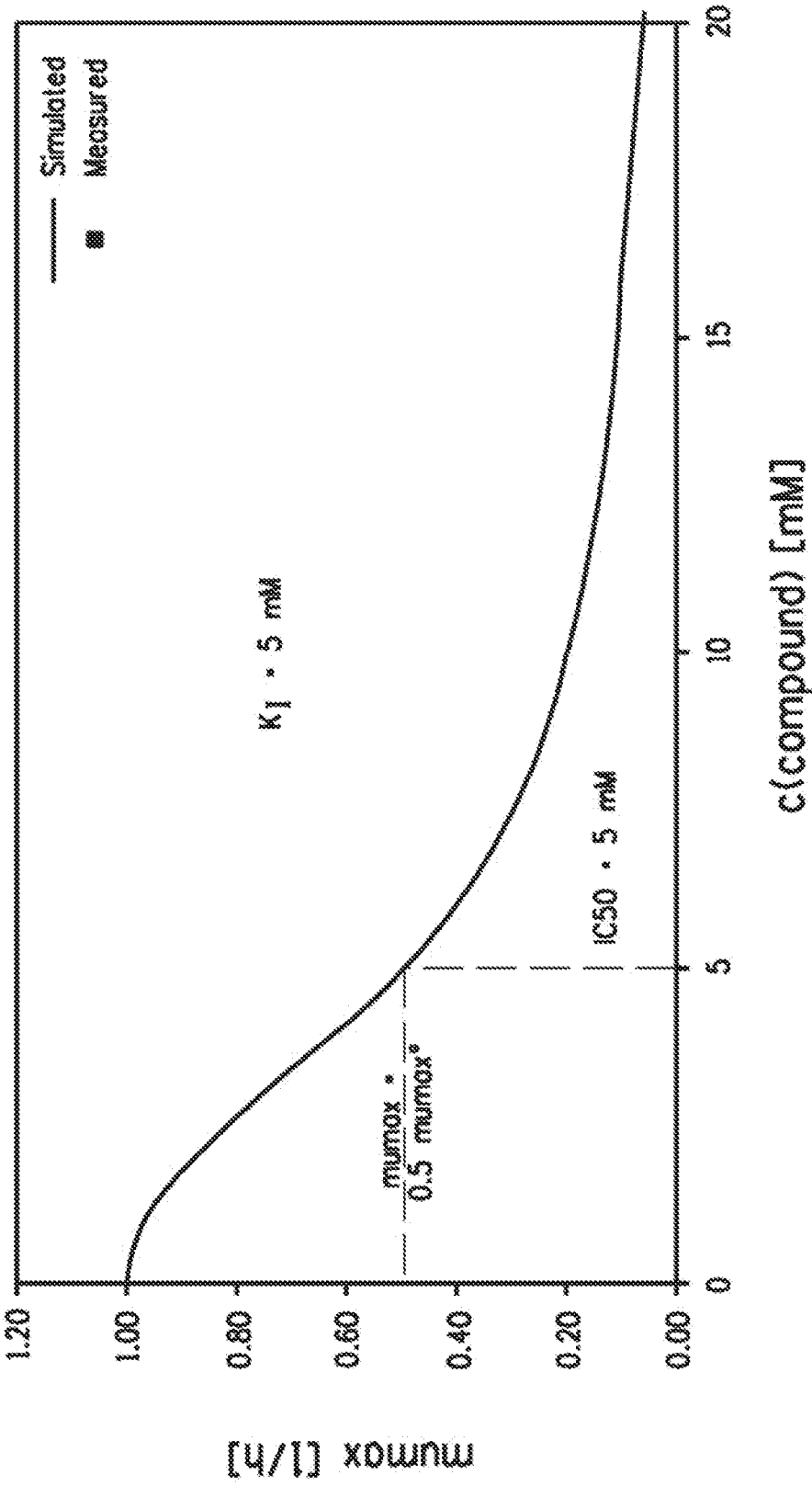


FIG. 19