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(54) **FERMENTIVE PRODUCTION OF FOUR CARBON ALCOHOLS**

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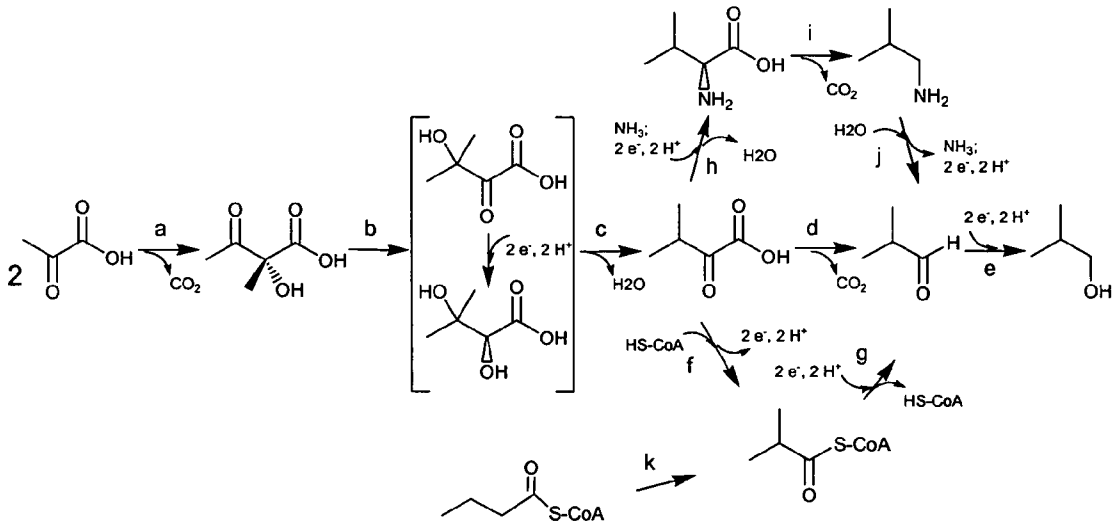
(57) **ABSTRACT**

Methods for the fermentative production of four carbon alcohols is provided. Specifically, butanol, preferably isobutanol is produced by the fermentative growth of a recombinant bacterium expressing an isobutanol biosynthetic pathway.



FERMENTIVE PRODUCTION OF FOUR CARBON ALCOHOLS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority under 35 U.S.C. §119 from U.S. Provisional Application Ser. No. 60/730,290, filed Oct. 26, 2005.

FIELD OF THE INVENTION

[0002] The invention relates to the field of industrial microbiology and the production of alcohols. More specifically, isobutanol is produced via industrial fermentation of a recombinant microorganism.

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 foodgrade extractant in the food and flavor industry. Each year 10 to 12 billion pounds of butanol are produced by petrochemical means and the need for this commodity chemical will likely increase.

[0004] Methods for the chemical synthesis of isobutanol are known, such as oxo synthesis, catalytic hydrogenation of carbon monoxide (*Ullmann's Encyclopedia of Industrial Chemistry,* 6th edition, 2003, Wiley-VCHVerlag GmbH and Co., Weinheim, Germany, Vol. 5, pp. 716-719) and Guerbet condensation of methanol with n-propanol (Carlini et al., *J. Mol. Catal. A: Chem.* 220:215-220 (2004)). These processes use starting materials derived from petrochemicals and are generally expensive and are not environmentally friendly. The production of isobutanol from plant-derived raw materials would minimize green house gas emissions and would represent an advance in the art.

[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 incomplete metabolism of amino acids by this group of fungi. Isobutanol is specifically produced from catabolism of L-valine. After the amine group of L-valine is harvested as a nitrogen source, the resulting α-keto acid is decarboxylated and reduced to isobutanol by enzymes of the so-called Ehrlich pathway (Dickinson et al., *J. Biol. Chem.* 273(40):25752-25756 (1998)). Yields of fusel oil and/or its components achieved during beverage fermentation are typically low. For example, the concentration of isobutanol produced in beer fermentation is reported to be less than 16 parts per million (Garcia et al., *Process Biochemistry* 29:303-309 (1994)). Addition of exogenous L-valine to the fermentation increases the yield of isobutanol, as described by Dickinson et al., supra, wherein it is reported that a yield of isobutanol of 3 g/L is obtained by providing L-valine at a concentration of 20 g/L in the fermentation. However, the use of valine as a feed-stock would be cost prohibitive for industrial scale isobutanol production. The biosynthesis of isobutanol directly from sugars would be economically viable and would represent an advance in the art. There have been no reports of a recombinant microorganism designed to produce isobutanol.

[0006] There is a need, therefore, for an environmentally [illegible] addresses this need by providing a recombinant microbial production host that expresses an isobutanol biosynthetic pathway.

SUMMARY OF THE INVENTION

[0007] The invention provides a recombinant microorganism having an engineered isobutanol biosynthetic pathway. The engineered microorganism may be used for the commercial production of isobutanol. Accordingly, in one embodiment the invention provides a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

[0008] i) pyruvate to acetolactate (pathway step a)

[0009] ii) acetolactate to 2,3-dihydroxyisovalerate (pathway step b)

[0010] iii) 2,3-dihydroxyisovalerate to α-ketoisovalerate (pathway step c)

[0011] iv) α-ketoisovalerate to isobutyraldehyde, (pathway step d), and

[0012] v) isobutyraldehyde to isobutanol; (pathway step e)

wherein the at least one DNA molecule is heterologous to said microbial host cell and wherein said microbial host cell produces isobutanol.

[0013] In another embodiment, the invention provides a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

[0014] i) pyruvate to acetolactate, (pathway step a)

[0015] ii) acetolactate to 2,3-dihydroxyisovalerate, (pathway step b)

[0016] iii) 2,3-dihydroxyisovalerate to α-ketoisovalerate, (pathway step c)

[0017] iv) α-ketoisovalerate to isobutyryl-CoA, (pathway step f)

[0018] v) isobutyryl-CoA to isobutyraldehyde, (pathway step g), and

[0019] vi) isobutyraldehyde to isobutanol; (pathway step e)

wherein the at least one DNA molecule is heterologous to said microbial host cell and wherein said microbial host cell produces isobutanol.

[0020] In another embodiment, the invention provides a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

[0021] i) pyruvate to acetolactate, (pathway step a)

[0022] ii) acetolactate to 2,3-dihydroxyisovalerate, (pathway step b)

[0023] iii) 2,3-dihydroxyisovalerate to α-ketoisovalerate, (pathway step c)

[0025] v) valine to isobutylamine, (pathway step i)

[0026] vi) isobutylamine to isobutyraldehyde, (pathway step j), and

[0027] vii) isobutyraldehyde to isobutanol: (pathway step e)

wherein the at least one DNA molecule is heterologous to said microbial host cell and wherein said microbial host cell produces isobutanol.

[0028] In another embodiment, the invention provides a method for the production of isobutanol comprising:

[0029] 1) providing a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

[0030] i) pyruvate to acetolactate (pathway step a)

[0031] ii) acetolactate to 2,3-dihydroxyisovalerate (pathway step b)

[0032] iii) 2,3-dihydroxyisovalerate to α-ketoisovalerate (pathway step c)

[0033] iv) α-ketoisovalerate to isobutyraldehyde, (pathway step d), and

[0034] v) isobutyraldehyde to isobutanol; (pathway step e)

wherein the at least one DNA molecule is heterologous to said microbial host cell; and

[0035] 2) contacting the host cell of (i) with a fermentable carbon substrate in a fermentation medium under conditions whereby isobutanol is produced.

[0036] In another embodiment, the invention provides a method for the production of isobutanol comprising:

[0037] 1) providing a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

[0038] i) pyruvate to acetolactate, (pathway step a)

[0039] ii) acetolactate to 2,3-dihydroxyisovalerate, (pathway step b)

[0040] iii) 2,3-dihydroxyisovalerate to α-ketoisovalerate, (pathway step c)

[0041] iv) α-ketoisovalerate to isobutyryl-CoA, (pathway step f)

[0042] v) isobutyryl-CoA to isobutyraldehyde, (pathway step g), and

[0043] vi) isobutyraldehyde to isobutanol; (pathway step e)

wherein the at least one DNA molecule is heterologous to said microbial host cell; and

[0044] 2) contacting the host cell of (i) with a fermentable carbon substrate in a fermentation medium under conditions whereby isobutanol is produced.

[0045] In another embodiment, the invention provides a method for the production of isobutanol comprising:

[0046] 1) providing a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

[0047] i) pyruvate to acetolactate, (pathway step a)

[0048] ii) acetolactate to 2,3-dihydroxyisovalerate, (pathway step b)

[0049] iii) 2,3-dihydroxyisovalerate to α-ketoisovalerate, (pathway step c)

[0050] iv) α-ketoisovalerate to valine, (pathway step h)

[0051] v) valine to isobutylamine, (pathway step i)

[0052] vi) isobutylamine to isobutyraldehyde, (pathway step j), and

[0053] vii) isobutyraldehyde to isobutanol: (pathway step e)

wherein the at least one DNA molecule is heterologous to said microbial host cell; and

[0054] 2) contacting the host cell of (i) with a fermentable carbon substrate in a fermentation medium under conditions whereby isobutanol is produced.

[0055] In an alternate embodiment the invention provides an isobutanol constraining fermentation medium produced by the methods of the invention.

BRIEF DESCRIPTION OF THE FIGURES AND SEQUENCE DESCRIPTIONS

[0056] The invention can be more fully understood from the following detailed description, figure, and the accompanying sequence descriptions, which form a part of this application.

[0057] FIG. **1** shows four different isobutanol biosynthetic pathways. The steps labeled "a", "b", "c", "d", "e", "f", "g", "h", "i", "j" and "k" represent the substrate to product conversions described below.

[0058] The following sequences conform with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures—the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

[0059] A Sequence Listing is provided herewith on Compact Disk. The contents of the Compact Disk containing the Sequence Listing are hereby incorporated by reference in compliance with 37 CFR 1.52(e). The Compact Disks are submitted in triplicate and are identical to one another. The disks are labeled "Copy 1—Sequence Listing", "Copy 2—Sequence Listing", and CRF. The disks contain the following file: CL3243 Seq List Conv.ST25 having the

TABLE 1

Summary of Gene and Protein SEQ ID Numbers

Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Peptide
Klebsiella pneumoniae budB (acetolactate synthase)	1	2
Bacillus subtilis alsS (acetolactate synthase)	78	178
Lactococcus lactis als (acetolactate synthase)	179	180
E. coli ilvC (acetohydroxy acid reductoisomerase)	3	4
S. cerevisiae ILV5 (acetohydroxy acid reductoisomerase)	80	181
M. maripaludis ilvC (Ketol-acid reductoisomerase)	182	183
B. subtilis ilvC (acetohydroxy acid reductoisomerase)	184	185
E. coli ilvD (acetohydroxy acid dehydratase)	5	6
S. cerevisiae ILV3 (Dihydroxyacid dehydratase)	83	186
M. maripaludis ilvD (Dihydroxy-acid dehydratase)	187	188
B. subtilis ilvD (dihydroxy-acid dehydratase)	189	190
Lactococcus lactis kivD (branched-chain α-keto acid decarboxylase), codon optimized	7	8
Lactococcus lactis kivD (branched-chain α-keto acid decarboxylase),	191	8
Lactococcus lactis kdcA (branched-chain alpha-ketoacid decarboxylase)	192	193
Salmonella typhimurium (indolepyruvate decarboxylase)	194	195
Clostridium acetobutylicum pdc (Pyruvate decarboxylase)	196	197
E. coli yqhD (branched-chain alcohol dehydrogenase)	9	10
S. cerevisiae YPR1 (2-methylbutyraldehyde reductase)	198	199
S. cerevisiae ADH6 (NADPH-dependent cinnamyl alcohol dehydrogenase)	200	201
Clostridium acetobutylicum bdhA (NADH-dependent butanol dehydrogenase A)	202	203
Clostridium acetobutylicum bdhB Butanol dehydrogenase	158	204
B. subtilis bkdAA (branched-chain keto acid dehydrogenase E1 subunit)	205	206
B. subtilis bkdAB (branched-chain alpha-keto acid dehydrogenase E1 subunit)	207	208
B. subtilis bkdB (branched-chain alpha-keto acid dehydrogenase E2 subunit)	209	210
B. subtilis lpdV (branched-chain alpha-keto acid dehydrogenase E3 subunit)	211	212
P. putida bkdA1 (keto acid dehydrogenase E1-alpha subunit)	213	214
P. putida bkdA2 (keto acid dehydrogenase E1-beta subunit)	215	216
P. putida bkdB	217	218

TABLE 1-continued

Summary of Gene and Protein SEQ ID Numbers

Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Peptide
P. putida 1pdV (lipoamide dehydrogenase)	219	220
C. beijerinckii ald (coenzyme A acylating aldehyde dehydrogenase)	221	222
C. acetobutylicum adhe1 (aldehyde dehydrogenase)	223	224
C. acetobutylicum adhe (alcohol-aldehyde dehydrogenase)	225	226
P. putida nahO (acetaldehyde dehydrogenase)	227	228
T. thermophilus (acetaldehyde dehydrogenase)	229	230
E. coli avtA (valine-pyruvate transaminase)	231	232
B. licheniformis avtA (valine-pyruvate transaminase)	233	234
E. coli ilvE (branched chain amino acid aminotransferase)	235	236
S. cerevisiae BAT2 (branched chain amino acid aminotransferase)	237	238
M. thermoautotrophicum (branched chain amino acid aminotransferase)	239	240
S. coelicolor (valine dehydrogenase)	241	242
B.. subtilis bcd (leucine dehydrogenase)	243	244
S. viridifaciens (valine decarboxyase)	245	246
A. denitrificans aptA (omega-amino acid:pyruvate transaminase)	247	248
R. eutropha (alanine-pyruvate transaminase)	249	250
S. oneidensis (beta alanine-pyruvate transaminase)	251	252
P. putida (beta alanine-pyruvate transaminase)	253	254
S. cinnamonensis icm (isobutyrl-CoA mutase)	255	256
S. cinnamonensis icmB (isobutyrl-CoA mutase)	257	258
S. coelicolor SCO5415 (isobutyrl-CoA mutase)	259	260
S. coelicolor SCO4800 (isobutyrl-CoA mutase)	261	262
S. avermitilis icmA (isobutyrl-COA mutase)	263	264
S. avermitilis icmB (isobutyrl-CoA mutase)	265	266

[0060] SEQ ID NOs:11-38, 40-69, 72-75, 85-138, 144, 145, 147-157, 159-176 are the nucleotide sequences of oligonucleotide cloning, screening or sequencing primers used in the Examples described herein.

[0061] SEQ ID NO:39 is the nucleotide sequence of the cscBKA gene cluster described in Example 16.

[0062] SEQ ID NO:70 is the nucleotide sequence of the glucose isomerase promoter 1.6GI described in Example 13.

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