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(54) Title: SOLVENT TOLERANT MICROORGANISMS AND METHODS OF ISOLATION

(57) Abstract: *Lactobacillus* bacteria having enhanced tolerance to butanols have been isolated. The bacteria are useful for the fermentive production of butanol. New methods for the isolation of butanol tolerant *Lactobacillus* are also provided.

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TITLE

SOLVENT TOLERANT MICROORGANISMS AND METHODS OF ISOLATION

This application claims the benefit of U.S. Provisional Application No 60/813779, filed June 15, 2006.

FIELD OF THE INVENTION

The invention relates to the field of industrial microbiology. Specifically, microorganisms have been isolated that demonstrate high tolerance to alcohols, particularly butanols.

BACKGROUND OF THE INVENTION

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 to12 billion pounds of butanol are produced by petrochemical means and the need for this commodity chemical will likely increase.

Methods for the chemical synthesis of butanols are known. For example, 1-butanol may be produced using the Oxo process, the Reppe process, or the hydrogenation of crotonaldehyde (*Ullmann's Encyclopedia of Industrial Chemistry*, 6th edition, 2003, Wiley-VCHVerlag GmbH and Co., Weinheim, Germany, Vol. 5, pp. 716-719). 2-Butanol may be produced using n-butene hydration (*Ullmann's Encyclopedia of Industrial Chemistry*, 6th edition, 2003, Wiley-VCHVerlag GmbH and Co., Weinheim, Germany, Vol. 5, pp. 716-719). Additionally, isobutanol may be produced using 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) or Guerbet condensation of methanol with n-propanol (Carlini et al., *J. Molec. Catal. A:Chem.* 220:215-220 (2004)). These processes use starting materials derived from petrochemicals, are generally expensive, and are not environmentally friendly.

Methods of producing butanol by fermentation are also known, where the most popular process produces a mixture of acetone, 1-butanol

and ethanol and is referred to as the ABE processes (Blaschek et al., U.S. Patent No. 6,358,717). Acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fermentations, and the pathways and genes responsible for the production of these solvents have been reported (Girbal et al., *Trends in Biotechnology* 16:11-16 (1998)). Additionally, recombinant microbial production hosts expressing a 1-butanol biosynthetic pathway (Donaldson et al., copending and commonly owned U.S. Patent Application No. 11/527995), a 2-butanol biosynthetic pathway (Donaldson et al., copending and commonly owned U.S. Patent Application No. 60/796,816, and an isobutanol biosynthetic pathway (Maggio-Hall et al., copending and commonly owned U.S. Patent Application No. 11/586315) have been described. However, biological production of butanols is believed to be limited by butanol toxicity to the host microorganism used in the fermentation.

Strains of *Clostridium* that are tolerant to 1-butanol have been isolated by chemical mutagenesis (Jain et al. U.S. Patent No. 5,192,673; and Blaschek et al. U.S. Patent No. 6,358,717), overexpression of certain classes of genes such as those that express stress response proteins (Papoutsakis et al. U.S. Patent No. 6,960,465; and Tomas et al., *Appl. Environ. Microbiol.* 69(8):4951-4965 (2003)), and by serial enrichment (Quratulain et al., *Folia Microbiologica* (Prague) 40(5):467-471 (1995); and Soucaille et al., Current Microbiology 14(5):295-299 (1987)). Desmond et al. (*Appl. Environ. Microbiol.* 70(10):5929-5936 (2004)) report that overexpression of GroESL, a stress response protein, in *Lactococcus lactis* and *Lactobacillus paracasei* produced strains that were able to grow in the presence of 0.5% volume/volume (v/v) [0.4% weight/volume (w/v)] 1-butanol. Additionally, the isolation of 1-butanol tolerant strains from estuary sediment (Sardessai et al., *Current Science* 82(6):622-623 (2002)) and from activated sludge (Bieszkiewicz et al., *Acta Microbiologica Polonica* 36(3):259-265 (1987)) have been described. Additionally some *Lactobacillus sp* are known to be tolerant to ethanol (see for example, Couto, Pina and Hogg Biotechnology. Letter 19: 487-490) .Ingram and

Burke (1984) Adv. Micribial. Physiol 25: 253-300. However, for most microorganisms described in the art, growth is totally inhibited at a concentration of less than 2.0% w/v 1-butanol when grown in a liquid medium at 37 °C. Moreover, microbial strains that have a tolerance to 2-butanol and isobutanol are not known in the art. Therefore, identification of microorganisms that have a high tolerance to 1-butanol, 2-butanol, and isobutanol would represent an advance in the art.

In addition, 2-butanone and ethanol are valuable compounds that can be produced by fermentation using microorganisms. 2-Butanone, also referred to as methyl ethyl ketone (MEK), is a widely used solvent and is the most important commercially produced ketone, after acetone. It is used as a solvent for paints, resins, and adhesives, as well as a selective extractant and activator of oxidative reactions. 2-butanone can be made by omitting the last step of the 2-butanol biosynthetic pathway (Donaldson et al., copending and commonly owned U.S. Patent Application No. 60/796816). Ethanol is in high demand as an alternative fuel. Genetically modified strains of *E. coli* have been used as biocatalysts for ethanol production (Underwood et al., (2002) Appl. Environ. Microbiol.68:6263-6272). A genetically modified strain of *Zymomonas mobilis* that has improved production of ethanol is described in US 2003/0162271 A1. Identification of microorganisms with improved tolerance to 2-butanone and ethanol would enhance the production of these compounds.

There is a need, therefore, for microbial host strains that are more tolerant to butanols and may be used for the bioproduction of butanols to high titer. The present invention addresses this need through the discovery of butanol tolerant microorganisms and development of methods for their isolation. In addition, the discovered microorganisms have increased tolerance to 2-butanone and ethanol.

SUMMARY OF THE INVENTION

The invention relates to butanol tolerant microorganisms, particularly members of the genus *Lactobacillus,* and methods for the isolation of the same. Microbial consortia were enriched and selected for tolerance to butanol. Several species of *Lactobacillus* were isolated that

demonstrated tolerance to concentrations of butanol of at least 2.5% w/v 1-butanol when grown on a solid medium at 37 °C.

Accordingly, the invention provides a method for the isolation of a butanol tolerant microorganism comprising:

a) providing a microbial sample comprising a microbial consortium;

b) contacting the microbial consortium with a growth medium comprising a fermentable carbon source until the members of the microbial consortium are growing;

c) contacting the growing microbial consortium of step (b) with butanol; and

d) isolating the viable members of step (c) wherein a butanol tolerant microorganism is isolated.

In another embodiment the invention provides butanol tolerant microorganisms isolated by the methods of the invention, where preferred microorganisms are of the genus *Lactobacillus.*

In an alternate embodiment the invention provides a method for the isolation of a butanol tolerant *Lactobacillus* comprising:

a) providing a microbial sample comprising a microbial consortium;

b) enriching the microbial consortium for the presence of *Lactobacillus* in a medium containing a fermentable carbon source to generate a *Lactobacillus* enriched culture in which members of the *Lactobacillus* enriched culture are growing;

c) contacting the growing *Lactobacillus* enriched culture of step (b) with butanol; and

d) isolating the viable members of step (c) wherein a butanol tolerant *Lactobacillus* is isolated.

In a preferred embodiment the invention provides a butanol tolerant *Lactobacillus* isolated by the process of the invention, where the specific butanol tolerant *Lactobacillus sp* identified as ATCC PTA-8318 (*Lactobacillus plantarum* PN0510), ATCC PTA-8320 (*Lactobacillus*

plantarum PN0511), ATCC PTA-7727 (*Lactobacillus plantarum* PN0512) and ATCC PTA-8319 (*Lactobacillus arizonensis* PN0514) are preferred.

In another embodiment the invention provides a method for the production of butanol comprising:

a) providing a *Lactobacillus* isolated by the process of the invention comprising genetic constructs encoding a butanol biosynthetic pathway; and

b) growing the *Lactobacillus* of step (a) under conditions whereby butanol is produced.

In yet another embodiment the invention provides a method for the production of 2-butanone comprising:

c) providing a *Lactobacillus* isolated by the process of the invention comprising genetic constructs encoding a 2-butanone biosynthetic pathway; and

d) growing the *Lactobacillus* of step (a) under conditions whereby 2-butanone is produced.

BRIEF DESCRIPTION BIOLOGICAL DEPOSITS AND SEQUENCE DESCRIPTIONS

The various embodiments of the invention can be more fully understood from the following detailed description, biological deposits, and the accompanying sequence descriptions, which form a part of this application.

Applicants made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure:

Depositor Identification Reference	International Depository Designation	Date of Deposit
Lactobacillus plantarum PN0510	ATCC: PTA-8318	April 3, 2007

Lactobacillus plantarum PN0511	ATCC: PTA-8320	April 3, 2007
Lactobacillus plantarum PN0512	ATCC: PTA-7727	July 12, 2006
Lactobacillus arizonensis PN0514	ATCC: PTA-8319	April 3, 2007

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.

Table 1

Summary of Gene and Protein SEQ ID Numbers for 1-Butanol Biosynthetic Pathway

Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Peptide
Acetyl-CoA acetyltransferase *thlA* from *Clostridium acetobutylicum* ATCC 824	1	2
Acetyl-CoA acetyltransferase *thlB* from *Clostridium acetobutylicum* ATCC 824	3	4
3-Hydroxybutyryl-CoA dehydrogenase from *Clostridium acetobutylicum* ATCC 824	5	6

Crotonase from *Clostridium acetobutylicum* ATCC 824	7	8
Putative trans-enoyl CoA reductase from *Clostridium acetobutylicum* ATCC 824	9	10
Butyraldehyde dehydrogenase from *Clostridium beijerinckii* NRRL B594	11	12
1-Butanol dehydrogenase *bdhB* from *Clostridium acetobutylicum* ATCC 824	13	14
1-Butanol dehydrogenase *bdhA* from *Clostridium acetobutylicum* ATCC 824	15	16

Table 2

Summary of Gene and Protein SEQ ID Numbers for 2-Butanol Biosynthetic Pathway

Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Peptide
budA, acetolactate decarboxylase from *Klebsiella pneumoniae* ATCC 25955	17	18
budB, acetolactate synthase from *Klebsiella pneumoniae* ATCC 25955	19	20
budC, butanediol dehydrogenase from *Klebsiella pneumoniae* IAM1063	21	22
pddA, butanediol dehydratase alpha subunit from *Klebsiella*	23	24

oxytoca ATCC 8724		
pddB, butanediol dehydratase beta subunit from *Klebsiella oxytoca* ATCC 8724	25	26
pddC, butanediol dehydratase gamma subunit from *Klebsiella oxytoca* ATCC 8724	27	28
sadH, 2-butanol dehydrogenase from *Rhodococcus ruber* 219	29	30

Table 3

Summary of Gene and Protein SEQ ID Numbers for Isobutanol Biosynthetic Pathway

Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Peptide
Klebsiella pneumoniae budB (acetolactate synthase)	19	20
E. coli ilvC (acetohydroxy acid reductoisomerase)	31	32
E. coli ilvD (acetohydroxy acid dehydratase)	33	34
Lactococcus lactis kivD (branched-chain α-keto acid decarboxylase), codon optimized	35	36
E. coli yqhD (branched-chain alcohol dehydrogenase)	37	38

SEQ ID NOs:39 and 40 are the nucleotide sequences of primers used to amplify the16S rRNA genes of butanol tolerant strains, as described in Example 1.

SEQ ID NOs: 41-44 are the nucleotide sequences of the16S rRNA genes of butanol tolerant *Lactobacillus* strains, isolated as described in Example 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides microorganisms that demonstrate high tolerance to alcohols, particularly butanols, as well as to 2-butanone and ethanol. The microorganisms of the invention are able to grow in the presence of 2.5% w/v or greater 1-butanol on a solid medium. Additionally, the invention provides a method for the isolation of butanol tolerant microorganisms. These butanol tolerant microorganisms may be genetically engineered to comprise a butanol biosynthetic pathway or a 2-butanone biosynthetic pathway, and used for the bioproduction of 1-butanol, 2-butanol, isobutanol or 2-butanone to high titer.

The following definitions and abbreviations are to be used for the interpretation of the claims and the specification.

The term "butanol" as used herein, refers to 1-butanol, 2-butanol, isobutanol, or mixtures thereof.

The terms "butanol tolerant microorganism" and "tolerant" when used to describe a microorganism of the invention, refers to a bacterium or yeast that exhibits growth in the presence of 2.5% w/v or greater 1-butanol, 2-butanol, or isobutanol when grown on a solid medium at 37 °C, or in the presence 2.0% w/v or greater 1-butanol, 2-butanol, or isobutanol when grown in a liquid medium at 37 °C.

The term "microbial consortium" refers to a heterogenous group of microbes with different genotypes. By way of example, a microbial consortium may be an environmental sample such as a wastewater sludge or soil or compost or contaminated water sample; a chemically mutagenized microbial population of a pure bacterial strain; a microbial strain containing a multicopy plasmid library; or a population of transposon tagged mutants of a particular strain.

The term "environmental sample" refers to a sample obtained from the environment. In particular, the environmental sample may be wastewater sludge or other sample obtained from an environment where

there has been exposure to butanol and/or other solvents. The environmental sample comprises a microbial consortium.

The term "enriching" as applied to a microbial culture and particularly the culturing of a microbial consortium refers to the practice of supplying the cells of the consortium or microbial culture with an excess of growth nutrients to enhance or encourage the growth of the cells.

The terms "fermentable carbon source", "carbon substrate" or "fermentable carbon substrate" are used interchangeably and refer to a source of carbon that is readily utilized by a microbial consortium. Fermentable carbon sources include, but are not limited to, monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof. A non-limiting list of preferred fermentable carbon sources includes simple sugars, such as glucose, fructose, and sucrose; and carboxylic acids such as fatty acids, butyric acid, and valeric acid.

The term "aerobic conditions" means growth conditions in the presence of oxygen.

The term "anaerobic conditions" means growth conditions in the absence of oxygen.

The term "microaerophilic conditions" means growth conditions with low levels of oxygen (i.e., below normal atmospheric oxygen levels).

The term "butanol biosynthetic pathway" refers to an enzyme pathway to produce 1-butanol, 2-butanol, or isobutanol.

The term "1-butanol biosynthetic pathway" refers to an enzyme pathway to produce 1-butanol from acetyl-coenzyme A (acetyl-CoA).

The term "2-butanol biosynthetic pathway" refers to an enzyme pathway to produce 2-butanol from pyruvate.

The term "isobutanol biosynthetic pathway" refers to an enzyme pathway to produce isobutanol from pyruvate.

The term "2-butanone biosynthetic pathway" refers to an enzyme pathway to produce 2-butanone from pyruvate.

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). Preferred 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 (SEQ ID NO:2), NC_003030; NP_149242 (SEQ ID NO:4), NC_001988), *Bacillus subtilis* (GenBank Nos: NP_390297, NC_000964), and *Saccharomyces cerevisiae* (GenBank Nos: NP_015297, NC_001148).

The term "3-hydroxybutyryl-CoA dehydrogenase" refers to an enzyme that catalyzes the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. 3-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 and are 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 (SEQ ID NO:6), NC_003030), *B. subtilis* (GenBank NOs: AAB09614, U29084), *Ralstonia eutropha* (GenBank NOs: ZP_0017144, NZ_AADY01000001, *Alcaligenes eutrophus* (GenBank NOs: YP_294481, NC_007347), and *A. eutrophus* (GenBank NOs: P14697, J04987).

The term "crotonase" refers to an enzyme that catalyzes the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA and H_2O. Crotonases may have a substrate preference for (S)-3-hydroxybutyryl-CoA or (R)-3-hydroxybutyryl-CoA and are 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 (SEQ ID NO:8), NC_000913), *C. acetobutylicum* (GenBank NOs: NP_349318, NC_003030), *B. subtilis* (GenBank NOs: CAB13705, Z99113), and *Aeromonas caviae* (GenBank NOs: BAA21816, D88825).

The term "butyryl-CoA dehydrogenase", also called trans-enoyl CoA reductase, refers to an enzyme that catalyzes the conversion of crotonyl-CoA to butyryl-CoA. Butyryl-CoA dehydrogenases may be NADH-dependent or NADPH-dependent and are classified as E.C. 1.3.1.44 and E.C. 1.3.1.38, respectively. Butyryl-CoA dehydrogenases are available from a number of sources, for example, *C. acetobutylicum* (GenBank NOs: NP_347102 (SEQ ID NO:10), NC_003030), *Euglena gracilis* (GenBank NOs: Q5EU90, AY741582), *Streptomyces collinus* (GenBank NOs: AAA92890, U37135), and *Streptomyces coelicolor* (GenBank NOs: CAA22721, AL939127).

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 (SEQ ID NO:12), AF157306) and *C. acetobutylicum* (GenBank NOs: NP_149325, NC_001988).

The term "1-butanol dehydrogenase" refers to an enzyme that catalyzes the conversion of butyraldehyde to 1-butanol. 1-butanol dehydrogenases are a subset of the broad family of alcohol dehydrogenases. 1-butanol dehydrogenase may be NADH- or NADPH-dependent. 1-butanol dehydrogenases are available from, for example, *C. acetobutylicum* (GenBank NOs: NP_149325, NC_001988; NP_349891 (SEQ ID NO:14), NC_003030; and NP_349892 (SEQ ID NO:16), NC_003030) and *E. coli* (GenBank NOs: NP_417484, NC_000913).

The term "acetolactate synthase", also known as "acetohydroxy acid synthase", refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of two molecules of pyruvic acid to

one molecule of alpha-acetolactate. Acetolactate synthase, known as EC 2.2.1.6 [formerly 4.1.3.18] (*Enzyme Nomenclature 1992*, Academic Press, San Diego) may be dependent on the cofactor thiamin pyrophosphate for its activity. Suitable acetolactate synthase enzymes are available from a number of sources, for example, *Bacillus subtilis* (GenBank Nos: AAA22222 NCBI (National Center for Biotechnology Information) amino acid sequence, L04470 NCBI nucleotide sequence), *Klebsiella terrigena* (GenBank Nos: AAA25055, L04507), and *Klebsiella pneumoniae* (GenBank Nos: AAA25079 (SEQ ID NO:20), M73842 (SEQ ID NO:19).

The term "acetolactate decarboxylase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of alpha-acetolactate to acetoin. 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* (SEQ ID NO:18 (amino acid) SEQ ID NO:17 (nucleotide)).

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 (SEQ ID NO:22), 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).

The term "butanediol dehydratase", also known as "diol 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, also known as methyl ethyl ketone (MEK).

Butanediol dehydratase may utilize the cofactor adenosyl cobalamin. Adenosyl cobalamin-dependent enzymes are known as EC 4.2.1.28 and are available, for example, from Klebsiella oxytoca (GenBank Nos: BAA08099 (alpha subunit) (SEQ ID NO:24), BAA08100 (beta subunit) (SEQ ID NO:26), and BBA08101 (gamma subunit) (SEQ ID NO:28), (Note all three subunits are required for activity), D45071).

The term "2-butanol dehydrogenase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of 2-butanone to 2-butanol. 2-butanol dehydrogenases are a subset of the broad family of alcohol dehydrogenases. 2-butanol dehydrogenase may be NADH- or NADPH-dependent. The NADH-dependent enzymes are known as EC 1.1.1.1 and are available, for example, from *Rhodococcus ruber* (GenBank Nos: CAD36475 (SEQ ID NO:30), AJ491307 (SEQ ID NO:29)). The NADPH-dependent enzymes are known as EC 1.1.1.2 and are available, for example, from *Pyrococcus furiosus* (GenBank Nos: AAC25556, AF013169).

The term "acetohydroxy acid isomeroreductase" or "acetohydroxy acid reductoisomerase" refers to an enzyme that catalyzes the conversion of acetolactate to 2,3-dihydroxyisovalerate using NADPH (reduced nicotinamide adenine dinucleotide phosphate) as an electron donor. Preferred acetohydroxy acid isomeroreductases are known by the EC number 1.1.1.86 and sequences are available from a vast array of microorganisms, including, but not limited to, *Escherichia coli* (GenBank Nos: NP_418222 (SEQ ID NO:32), NC_000913 (SEQ ID NO:31)), *Saccharomyces cerevisiae* (GenBank Nos: NP_013459, NC_001144), *Methanococcus maripaludis* (GenBank Nos: CAF30210, BX957220), and *Bacillus subtilis* (GenBank Nos: CAB14789, Z99118).

The term "acetohydroxy acid dehydratase" refers to an enzyme that catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate. Preferred acetohydroxy acid dehydratases are known by the EC number 4.2.1.9. These enzymes are available from a vast array of microorganisms, including, but not limited to, *E. coli* (GenBank Nos: YP_026248 (SEQ ID NO:34), NC_000913 (SEQ ID NO:33)), *S. cerevisiae*

(GenBank Nos: NP_012550, NC_001142), *M. maripaludis* (GenBank Nos: CAF29874, BX957219), and *B. subtilis* (GenBank Nos: CAB14105, Z99115).

The term "branched-chain α-keto acid decarboxylase" refers to an enzyme that catalyzes the conversion of α-ketoisovalerate to isobutyraldehyde and CO_2. Preferred branched-chain α-keto acid decarboxylases are known by the EC number 4.1.1.72 and are available from a number of sources, including, but not limited to, *Lactococcus lactis* (GenBank Nos: AAS49166, AY548760; CAG34226 (SEQ ID NO:36), AJ746364, *Salmonella typhimurium* (GenBank Nos: NP_461346, NC_003197), and *Clostridium acetobutylicum* (GenBank Nos: NP_149189, NC_001988).

The term "branched-chain alcohol dehydrogenase" refers to an enzyme that catalyzes the conversion of isobutyraldehyde to isobutanol. Preferred 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). These enzymes utilize NADH (reduced nicotinamide adenine dinucleotide) and/or NADPH as electron donor and 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 (SEQ ID NO:38), NC_000913 (SEQ ID NO:37)), and *C. acetobutylicum* (GenBank Nos: NP_349892, NC_003030).

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. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a

manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

As used herein the term "coding sequence" refers to a DNA sequence that codes 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.

The term "promoter" refers to a DNA 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 DNA 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". 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.

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.

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.

As used herein the term "transformation" refers to the transfer of a nucleic acid fragment into a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The terms "plasmid" and "vector" 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 vector" 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.

As used herein the term "codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is

desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

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.

Standard recombinant DNA and molecular cloning techniques used here 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., Bennan, M. L. and Enquist, L. W., *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Cold Press 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).

The term "invention" or "present invention" as used herein is meant to apply generally to all embodiments of the invention as described in the claims as presented or as later amended and supplemented, or in the specification.

In one embodiment the present invention provides a method for the isolation of butanol tolerant microorganisms. The method comprises enriching a microbial consortium under growth conditions and contacting the enriched consortium with butanol, as described in detail below. Microorganisms identified by the methods of the invention that demonstrate high tolerance to alcohols, particularly butanols, are also provided. These identified microorganisms also have high tolerance to 2-butanone and ethanol. These butanol tolerant microorganisms may be genetically engineered to comprise a butanol biosynthetic pathway or a 2-butanone biosynthetic pathway, and may be used for the bioproduction of 1-butanol, 2-butanol, isobutanol, or 2-butanone to high titer.

Isolation of Butanol Tolerant Microorganisms

Butanol tolerant microorganisms may be isolated from environmental samples such as wastewater sludge and samples from other environments where there is exposure to butanol and/or other solvents. For example, environmental samples may be obtained from wastewater treatment facilities at chemical plants. Industrial wastewater bioreactors are particularly good sources of environmental samples of microorganisms with desirable resistance phenotypes because of the long-term growth in the presence of various organic solvents (Bramucci et al., *Trends Biotechnol.* 18:501-505 (2000)). Butanol tolerant microorganisms may be isolated from other microbial samples as well. For example, the microbial sample may be a chemically mutagenized microbial population of a pure bacterial strain, a microbial strain containing a multicopy plasmid library, or a population of transposon tagged mutants of a particular strain. Any of these microbial samples including a mixed population is said to include a microbial consortium.

In one embodiment of the present invention, the microbial sample is cultured in a growth medium with an excess of growth nutrients thereby enriching the microbial consortium contained therein until the members of the consortium are growing. In one embodiment the cultures are growing in log phase. The growth medium comprises a fermentable carbon source and may include suitable levels of nitrogen, phosphorus, sulfur, and salts. Suitable levels of these nutrients necessary for growth of the microbial consortium are well known to those skilled in the art, and non-limiting examples are provided below. The fermentable carbon source may be any carbon source that is readily metabolized by the members of the microbial consortium, including but not limited to, sucrose, fructose, glucose, and mixtures thereof. The fermentable carbon source may also be a carboxylic acid such as a fatty acid, butyric acid or valeric acid. Typically, the carbon source is present at a concentration from about 0.1 % weight/volume w/v to about 1.5% w/v. The nitrogen source may be any suitable nitrogen source, including but not limited to, ammonium salts or yeast extract. The nitrogen source is typically present in the growth

medium at a concentration of about 10 mM. Phosphorus may be present in the medium in the form of phosphate salts, such as sodium and potassium phosphates, which are typically present in the growth medium at a concentration of about 50 mM. Sulfur may be present in the medium in the form of sulfate salts, such as sodium or ammonium sulfates, which are typically present in the growth medium at a concentration of about 10 mM. Additional salts include, but are not limited to, magnesium chloride, calcium chloride, manganese chloride, ferric chloride, ferrous chloride, zinc chloride, cupric chloride, cobalt chloride, and sodium molybdate. These salts are typically present in the growth medium at a concentration of about 1 μM to about 2 mM. The growth medium may also contain vitamins such as thiamine hydrochloride.

The enrichment culture is grown at a temperature of about 25 °C to about 60 °C for a time sufficient for the members of the microbial consortium in the sample to exhibit growth, typically about 12 hours to about 24 hours. The culture may be grown under anaerobic, microaerophilic, or aerobic conditions, with or without agitation. As is readily understood by the skilled person, anaerobic conditions are those that are devoid of oxygen, aerobic conditions are those that contain oxygen and microaerophilic conditions are those where oxygen is present at a level below that found in air, ie. less than 21%. Growth of the culture may be monitored by measuring the optical density, typically at a wavelength of 600 nm.

The growing enrichment culture is then contacted with butanol. This contacting may be done by diluting the enrichment culture with a fresh growth medium that contains butanol. It is particularly suitable if the enrichment culture is growing in log phase at this point. The butanol concentration used is about 0.8% w/v to about 3.0% w/v, preferably about 0.8% w/v to about 2.0% w/v. In one embodiment, the butanol is predominantly 1-butanol. In another embodiment, the butanol is predominantly 2-butanol. In another embodiment, the butanol is predominantly isobutanol. As used herein, predominantly means at least about 90% by weight of the total butanol. Additionally, mixtures

comprising various combinations of two or more of 1-butanol, 2-butanol, and isobutanol may be used. The culture is grown for a period of time until significant growth is observed. Optionally, the cultures that demonstrate significant growth may be contacted with butanol again one or more times to select for increased tolerance to butanol. Each contacting may be made with progressively higher butanol concentrations.

The microbial consortium that was contacted with butanol is then separated to isolate individual strains. Multiple means of cell isolation are know to those skilled in the art involving either liquid or solid media. For example, the microbial consortium that was contacted with butanol may be plated onto a solid medium, for example nutrient agar, Luria Bertani (LB) agar, modified LB agar (i.e., LB agar supplemented with an fermentable carbon source and salts), or minimal enrichment medium with agar, which may or may not contain butanol. If butanol is present in the solid medium, its concentration is typically about 1.2% w/v to about 3% w/v. The culture is grown until colonies are formed. The colonies are then isolated using methods known in the art to provide a butanol tolerant microorganism. For example, the colonies from the solid medium may be collected and identified using methods known in the art, as described below. Alternatively, the colonies from the solid medium may be inoculated into a growth medium (e.g., minimal enrichment medium), either liquid or solid, that does not contain butanol. After growth, the cells may be collected and identified. Optionally, the cells from the colonies may be grown in the presence of butanol, either in liquid or solid growth medium (e.g., minimal enrichment medium). Typically, the butanol concentration in the medium is about 1.2% w/v to about 3% w/v. The cells that grow in the presence of butanol are collected. The isolated microorganisms may be identified using methods known in the art, such as 16S ribosomal RNA (rRNA) gene sequencing, fatty acid profile analysis, or ribotyping.

The butanol tolerant microorganisms isolated by the method of the present invention are tolerant to at least 2.5% butanol (i.e., 1-butanol, 2-butanol, or isobutanol) when grown on a solid medium at 37 °C, or to at least 2.0% w/v butanol when grown in a liquid medium at 37 °C. It should

be noted that the butanol tolerance of microorganisms is typically higher when grown on a solid medium than when grown on a liquid medium. Additionally, the butanol tolerance of microorganisms is dependent on the growth temperature, typically being higher at lower growth temperatures. Microorganisms isolated by contacting the enriched microbial consortium with one butanol are generally also tolerant to other butanols as well as to 2-butanone and ethanol. For example, microorganisms isolated using 1-butanol are also tolerant to 2-butanol and isobutanol.

The tolerance of strains isolated using the present method may be assessed by determining the IC_{50} values for growth in liquid medium containing added test chemical. The IC_{50} value is the concentration of chemical that causes 50% growth inhibition. As shown in Examples 1 and 2 herein, IC_{50} values of 1.8% w/v for 1-butanol, 2.4% w/v for isobutanol, 3.1% w/v for 2-butanol. 4.5% w/v for 2-butanone and 5.9% w/v for ethanol were determined in a selected strain. Based on the strain's growth on solid medium containing 1-butanol, these IC_{50} values and a correlation seen between tolerance to 1-btuanol and to each of the other tested compounds, the identified tolerant strains are expected to grow on solid medium containing 2.7% w/v isobutanol, 3.9% w/v 2-butanol, 5.0% w/v 2-butanone, or 9.0% w/v ethanol.

The enrichment culture may also be grown and contacted with butanol in a continuous culture in a chemostat bioreactor. The cells in a chemostat bioreactor can be grown at various growth rates by appropriate adjustment of the dilution rate. Chemostat cultures can be precisely controlled for aeration and pH, leading to higher cell densities. Additionally, butanol can be gradually added in increasing concentration by adjusting feed composition. After contacting the enrichment culture with butanol in the bioreactor, the butanol tolerant microorganisms are isolated and identified as described above.

Unexpectedly, many of the butanol tolerant microorganisms identified using the method of the present invention in the examples herein were bacteria belonging to the genus *Lactobacillus*. *Lactobacillus* bacteria are facultatively anaerobic, Gram-positive, non-motile, rod-shaped cells

(*Bergey's Manual of Systematic Bacteriology*, Vol 2, Sneath et al., Eds.; Williams & Wilkins, Baltimore, MD, 1986, pp. 1063-1065). The butanol tolerant *Lactobacillus* strains were further characterized herein by determining the 16S rRNA gene sequences (SEQ ID NOs:41, 42, 43, and 44), which identified them as *Lactobacillus plantarum* and *Lactobacillus arizonensis* strains.

The present method for isolation of butanol tolerant microorganisms may be modified to selectively isolate butanol tolerant *Lactobacillus*. For example *Lactobacillus* may be enriched from a variety of environments using standard methods for culturing *Lactobacilli* using a lactic acid bacteria medium such as Bacto Lactobacilli MRS agar and then screened for tolerance to 1-butanol.

The isolated butanol tolerant *Lactobacillus* strains may be genetically engineered to comprise genetic constructs encoding a butanol biosynthetic pathway or a butanone biosynthetic pathway and grown under suitable conditions to produce butanol or butanone. The butanol biosynthetic pathway may be a 1-butanol, 2-butanol, or isobutanol biosynthetic pathway.

1-Butanol Biosynthetic Pathway

A biosynthetic pathway for the production of 1-butanol is described by Donaldson et al. in co-pending and commonly owned U.S. Patent Application No. 11/527995, which is incorporated herein by reference. This biosynthetic pathway comprises the following substrate to product conversions:

a) acetyl-CoA to acetoacetyl-CoA, as catalyzed for example by acetyl-CoA acetyltransferase encoded by the genes given as SEQ ID NO:1 or 3;

b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA, as catalyzed for example by 3-hydroxybutyryl-CoA dehydrogenase encoded by the gene given as SEQ ID NO:5;

c) 3-hydroxybutyryl-CoA to crotonyl-CoA, as catalyzed for example by crotonase encoded by the gene given as SEQ ID NO:7;

d) crotonyl-CoA to butyryl-CoA, as catalyzed for example by butyryl-CoA dehydrogenase encoded by the gene given as SEQ ID NO:9;

e) butyryl-CoA to butyraldehyde, as catalyzed for example by butyraldehyde dehydrogenase encoded by the gene given as SEQ ID NO:11; and

f) butyraldehyde to 1-butanol, as catalyzed for example by 1-butanol dehydrogenase encoded by the genes given as SEQ ID NO:13 or 15.

The pathway requires no ATP and generates NAD^+ and/or $NADP^+$, thus, it balances with the central, metabolic routes that generate acetyl-CoA.

2-Butanol and 2-Butanone Biosynthetic Pathway

Biosynthetic pathways for the production of 2-butanol and 2-butanone are described by Donaldson et al. in co-pending and commonly owned U.S. Patent Application Nos. 11/741892 and 11/741916, which are incorporated herein by reference. One 2-butanol biosynthetic pathway comprises the following substrate to product conversions:

a) pyruvate to alpha-acetolactate, as catalyzed for example by acetolactate synthase encoded by the gene given as SEQ ID NO:19;

b) alpha-acetolactate to acetoin, as catalyzed for example by acetolactate decarboxylase encoded by the gene given as SEQ ID NO:17;

c) acetoin to 2,3-butanediol, as catalyzed for example by butanediol dehydrogenase encoded by the gene given as SEQ ID NO:21;

d) 2,3-butanediol to 2-butanone, catalyzed for example by butanediol dehydratase encoded by genes given as SEQ ID NOs:23, 25, and 27; and

e) 2-butanone to 2-butanol, as catalyzed for example by 2-butanol dehydrogenase encoded by the gene given as SEQ ID NO:29.

Omitting the last step (e) of the above pathway provides a biosynthetic pathway for production of 2-butanone, also known as methyl ethyl ketone (MEK),

Isobutanol Biosynthetic Pathway

Biosynthetic pathways for the production of isobutanol are described by Maggio-Hall et al. in copending and commonly owned U.S. Patent Application No. 11/586315, which is incorporated herein by reference. One isobutanol biosynthetic pathway comprises the following substrate to product conversions:

a) pyruvate to acetolactate, as catalyzed for example by acetolactate synthase encoded by the gene given as SEQ ID NO:19;

b) acetolactate to 2,3-dihydroxyisovalerate, as catalyzed for example by acetohydroxy acid isomeroreductase encoded by the gene given as SEQ ID NO:31;

c) 2,3-dihydroxyisovalerate to α-ketoisovalerate, as catalyzed for example by acetohydroxy acid dehydratase encoded by the gene given as SEQ ID NO:33;

d) α-ketoisovalerate to isobutyraldehyde, as catalyzed for example by a branched-chain keto acid decarboxylase encoded by the gene given as SEQ ID NO:35; and

e) isobutyraldehyde to isobutanol, as catalyzed for example by a branched-chain alcohol dehydrogenase encoded by the gene given as SEQ ID NO:37.

Construction of *Lactobacillus* Hosts for Butanol or Butanone Production

Recombinant, butanol tolerant *Lactobacillus* strains containing the necessary genes that encode enzymes for one of the enzymatic pathways for the conversion of a fermentable carbon substrate to butanol or butanone may be constructed using techniques well known in the art. The genome sequences of *L. plantarum, L. salivarius, L sakei, L johnsonii, L. acidophilus and L. delbrueckii* are known (National Center for Biotechnology Information (NCBI) database), genbank ™ identification as follows:

- Lactobacillus plantarum WCFS1, complete genome gi|28376974|ref|NC_004567.1|[28376974]
- Lactobacillus salivarius subsp. salivarius UCC118, complete genome gi|90960990|ref|NC_007929.1|[90960990]
- Lactobacillus sakei strain 23K complete genome gi|78609255|emb|CR936503.1|[78609255]
- Lactobacillus johnsonii NCC 533, complete genome gi|42518084|ref|NC_005362.1|[42518084]
- Lactobacillus acidophilus NCFM, complete genome gi|58336354|ref|NC_006814.1|[58336354]
- Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842, complete genomegi|104773257|ref|NC_008054.1|[104773257]

These bacteria have a G+C content ranging from 32% to 49%.

In the present invention, genes encoding the enzymes of one of the butanol or butanone biosynthetic pathways described above may be isolated from various sources (see above). Methods of obtaining desired genes from a bacterial genome are common and well known in the art of molecular biology. For example, if the sequence of the gene is known, primers may be designed and the desired sequence amplified using standard primer-directed amplification methods such as polymerase chain reaction (U.S. Patent No. 4,683,202) to obtain amounts of DNA suitable for cloning into transformation vectors. If a gene that is heterologous to a known sequence is to be isolated, suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes having complementary sequence to the desired gene sequence. Once the sequence is isolated, the DNA may be amplified using standard primer-directed amplification methods such as polymerase chain reaction (U.S. Patent No. 4,683,202) to obtain amounts of DNA suitable for cloning into expression vectors, which are then transformed into appropriate host cells.

In addition, given the amino acid sequence of a protein with desired enzymatic activity, the coding sequence may be ascertained by reverse translating the protein sequence. A DNA fragment containing the coding sequence may be prepared synthetically and cloned into an expression vector, then transformed into the desired host cell.

In preparing a synthetic DNA fragment containing a coding sequence, this sequence may be optimized for expression in the target host cell. Tools for codon optimization for expression in a heterologous host are readily available.

Once the relevant pathway genes are identified and isolated they may be inserted in a vector and transformed into a butanol tolerant *Lactobacillus* host by means well known in the art. Vectors useful for the transformation of *Lactobacillus* are known (see below). Typically the vector or cassette contains sequences directing transcription and translation of the inserted DNA fragment, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the inserted DNA fragment which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. Both control regions may be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions may also be derived from genes that are not native to the specific production host.

Initiation control regions or promoters, which are useful to drive expression of the relevant pathway coding regions in the desired *Lactobacillus* host cell, may be obtained from other lactic acid bacteria or other Gram-positive organisms. A non-limiting example is the *nisA* promoter from *Lactococcus*. Termination control regions may also be derived from various genes native to the preferred hosts or related bacteria. Optionally, a termination site may be unnecessary, however, it is most preferred if included.

The *Lactobacillus* genus belongs to the *Lactobacillales* family and many plasmids and vectors used in the transformation of *Bacillus subtilis* and *Streptococcus* may be used for *Lactobacillus*. Non-limiting examples

of suitable vectors include pAMβ1 and derivatives thereof (Renault et al., *Gene* 183:175-182 (1996); and O'Sullivan et al., *Gene* 137:227-231 (1993)); pMBB1 and pHW800, a derivative of pMBB1 (Wyckoff et al. *Appl. Environ. Microbiol.* 62:1481-1486 (1996)); pMG1, a conjugative plasmid (Tanimoto et al., *J. Bacteriol.* 184:5800-5804 (2002)); pNZ9520 (Kleerebezem et al., *Appl. Environ. Microbiol.* 63:4581-4584 (1997)); pAM401 (Fujimoto et al., *Appl. Environ. Microbiol.* 67:1262-1267 (2001)); and pAT392 (Arthur et al., *Antimicrob. Agents Chemother.* 38:1899-1903 (1994)). Several plasmids from *Lactobacillus plantarum* have also been reported (van Kranenburg R, Golic N, Bongers R, Leer RJ, de Vos WM, Siezen RJ, Kleerebezem M. *Appl. Environ. Microbiol.* 2005 Mar; 71(3): 1223-1230), which may be used for transformation.

The various genes for a butanol or butanone biosynthetic pathway may be assembled into any suitable vector, such as those described above. The codons can be optimized for expression based on the codon index deduced from the genome sequences of the host strain, such as for *Lactobacillus plantarum* or *Lactobacillus arizonensis*. The plasmids may be introduced into the host cell using methods known in the art, such as electroporation, as described in any one of the following references: Cruz-Rodz et al. (*Molecular Genetics and Genomics* 224:1252-154 (1990)), Bringel and Hubert (*Appl. Microbiol. Biotechnol.* 33: 664-670 (1990)), and Teresa Alegre, Rodriguez and Mesas (*FEMS Microbiology letters* 241:73-77(2004)). Plasmids can also be introduced to *Lactobacillus plantatrum* by conjugation (Shrago, Chassy and Dobrogosz *Appl. Environ. Micro.* 52: 574-576 (1986)). The butanol or butanone biosynthetic pathway genes can also be integrated into the chromosome of *Lactobacillus* using integration vectors (Hols et al. *Appl. Environ. Micro.* 60:1401-1403 (1990); Jang et al. *Micro. Lett.* 24:191-195 (2003)).

Fermentation Media

Fermentation media for the production of butanol or butanone must contain suitable carbon substrates. Suitable substrates may include but are not limited to monosaccharides such as glucose and fructose, oligosaccharides such as lactose 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. Sucrose may be obtained from feedstocks such as sugar cane, sugar beets, cassava, and sweet sorghum. Glucose and dextrose may be obtained through saccharification of starch based feedstocks including grains such as corn, wheat, rye, barley, and oats.

In addition, fermentable sugars may be obtained from cellulosic and lignocellulosic biomass through processes of pretreatment and saccharification, as described, for example, in co-owned and co-pending US patent application US20070031918A1, which is herein incorporated by reference. Biomass refers to any cellulosic or lignocellulosic material and 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 could 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 and animal manure.

Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, preferred carbon substrates are glucose, fructose, and sucrose.

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 the enzymatic pathway necessary for butanol or butanone production.

Culture Conditions

Typically cells are grown at a temperature in the range of about 25 °C to about 40 °C in an appropriate medium. Suitable growth media in the present invention are common commercially prepared media such as Bacto Lactobacilli MRS broth or Agar (Difco), Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Medium (YM) broth. 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.

Suitable pH ranges for the fermentation are between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred as the initial condition.

Fermentations may be performed under aerobic or anaerobic conditions, where anaerobic or microaerobic conditions are preferred.

Industrial Batch and Continuous Fermentations

Butanol or butanone 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), herein incorporated by reference.

Butanol or butanone 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*.

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

Methods for Butanol and 2-Butanone Isolation from the Fermentation Medium

Bioproduced butanol may be isolated from the fermentation medium using methods known in the art for ABE fermentations (see for example, 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 butanol may be isolated from the fermentation medium using methods such as distillation, azeotropic distillation, liquid-liquid extraction, adsorption, gas stripping, membrane evaporation, or pervaporation. These same methods may be adapted to isolate bioproduced 2-butanone from the fermentation medium.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred 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.

The meaning of abbreviations used is as follows: "min" means minute(s), "h" means hour(s), "sec' means second(s), "µL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "nm" means nanometer(s), "mm" means millimeter(s), "cm" means centimeter(s), "µm" means micrometer(s), "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmole" means micromole(s), "g" means gram(s), "µg" means microgram(s), "mg" means milligram(s), "rpm" means revolutions per minute, "w/v" means weight/volume, "OD" means optical density, "OD_{600}" means optical density measured at a wavelength of 600 nm, "OD_{595}" means optical density measured at a wavelength of 595 nm, "IC_{50}" means the concentration of butanol that causes a 50% inhibition of growth, "GCMS" means gas chromatography-mass spectrometry, and "HPLC" means high performance liquid chromatography.

GENERAL METHODS:

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, by T. J. Silhavy, M. L. Bennan, and L. W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984, and by Ausubel, F. M. et al., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience, N.Y., 1987.

Materials and methods suitable for the maintenance and growth of bacterial cultures are also well known in the art. Techniques suitable for

use in the following Examples may be found in *Manual of Methods for General Bacteriology,* Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds., American Society for Microbiology, Washington, DC., 1994, or by Thomas D. Brock in *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 Aldrich Chemicals (Milwaukee, WI), BD Diagnostic Systems (Sparks, MD), Life Technologies (Rockville, MD), or Sigma Chemical Company (St. Louis, MO), unless otherwise specified.

EXAMPLE 1

Isolation of Butanol Tolerant Bacterial Strains Using Continuous Culture

The purpose of these Examples was to isolate butanol tolerant bacterial strains. Environmental samples were obtained from several wastewater treatment sites and were grown in the presence of 1-butanol in continuous culture in a chemostat bioreactor. Several 1-butanol tolerant bacterial strains were isolated and identified as *Lactobacillus plantarum or Lactobacillus arizonensis.*

An Appilikon Fermentor (Appilikon Inc., Clinton, NJ) was operated as an anaerobic chemostat. The bioreactor system was composed of a 1-L dished bottom reactor, Controller ADI 1032 P100, and stirrer unit with marine and turbine impellers. Bio Controller ADI 1030 Z510300020 with appropriate sensors monitored pH, dissolved oxygen, and temperature. A Cole Parmer pump and pump head (Cole Parmer Instrument Co., Vernon Hills, IL) were used for addition of acid and base to maintain pH 7.0. The temperature was maintained at 37 °C using a circulating water bath. The culture medium (S20 medium) consisted of 5 mM potassium phosphate buffer, pH 7.0, 10 mM ammonium sulfate, 0.1% yeast extract, 0.1% caseamino acids, 100 mM MOPS, 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.05 mM $MnCl_2$, 0.001 mM $ZnCl_2$, 0.002 mM thiamine hydrochloride, 1.72 μM $CuCl_2$, 2.53 μM $CoCl_2$, 2.42 μM Na_2MoO_4, 25 mM glucose, 12.5 mM sucrose, and 12.5 mM fructose. A volume of 500 mL of this medium was

used in the bioreactor. The bioreactor was operated with a feed rate in the range of 0.1 to 1.0 mL/minute and a stirrer speed of 50 rpm.

The bioreactor was inoculated with a mixture of several wastewater sludge samples obtained from different wastewater treatment facilities at several E.I. du Pont de Nemours and Company sites. After a short period of batch mode operation, the bioreactor was operated in continuous feed mode with 1-butanol gradually added in increasing concentration to the culture medium. The flow rate of the medium was in the range of 0.1 to 1.0 mL/min.

Cell density in the bioreactor was monitored by measuring the optical density at 600 nm. The 1-butanol in the feed and effluent was determined by GCMS using an HP6890 Gas Chromatograph with 5973 Mass Detector (Agilent Technologies, Inc, Wilmington, DE). The GC column was a DB-WAX, 30 m x 0.32 mm ID x 0.25 μm column (J&W Scientific, Inc., Folsom, CA). Alternatively, samples were filtered (Acrodisc CR PTFE 0.2 μm filters) and analyzed by HPLC using a Shodex® SH1011 column (8 mm ID x 300 mm length; Shoko America Inc., Colorado Springs, CO) with a Shodex® SH-G guard column. The mobile phase was 0.01 N sulfuric acid. The column temperature was 50 °C and a flow rate of 0.5 mL/min was used. For detection, a photometric detector at 210 nm and a refractive index detector were used. The sample injection volume was 10 μL.

After an initial adjustment period, the amount of 1-butanol entering the bioreactor through the feed was gradually increased to 2.5% w/v. During this same period, the amount of glucose in the bioreactor effluent was monitored. Increasing the amount of 1-butanol in the feed resulted in a decrease in cell density and a concomitant decrease in glucose utilization. Continued incubation resulted in the cell density and glucose utilization again increasing after adaptation to the higher level of 1-butanol. For example, increasing 1-butanol to 1.6% resulted in the cell density decreasing to less than 1.5 OD_{600} with a corresponding decrease in glucose utilization. However, continued incubation resulted in the cell

density increasing to 2.3 OD_{600} with a corresponding increase in glucose consumption.

Isolation of pure strains of 1-butanol resistant bacteria from this bioreactor were performed as follows. Samples of cells from the bioreactor waste jug were serially diluted, and the serial dilutions were plated on trypticase soy agar (Difco; Bekton Dickinson and Company; San Jose, CA) without 1-butanol. Colonies were then inoculated from the agar media into 1.2 mL of S20 medium without 1-butanol in the wells of a square-well microtiter plate (Beckman Coulter Inc, Fullerton, CA; Catalog No. 069681). The square-well microtiter plate was sealed with an adhesive cover (Beckman Coulter Inc.; Catalog No. 538619) and incubated at 37 °C with shaking for up to 72 h. The square-well microtiter plate was used to make a master plate by dispensing 200 µL of culture from each square well into a corresponding well in a "U-bottom" microtiter plate (VWR Scientific Products, West Chester, PA; Catalog No. 62409-052). Isolates from the master plate were replica-plated onto S20 agar or TSA agar plates containing between 1.2% and 3.4% 1-butanol using the Nunc-TSP transferable solid phase screening system (Nalgene Nunc International, Napersville, IL; Catalog No. 445497). Tolerant isolates were identified by growth at 37 °C after 24 to 72 h. The several isolates that grew on agar medium with 3% 1-butanol were characterized further.

The IC_{50} values of the isolated strains were determined at 37 °C, as follows. The isolates were cultured in S30L medium (i.e., 10 mM ammonium sulfate, 5 mM potassium phosphate buffer, pH 7.0, 50 mM MOPS, pH 7.0, 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 µM $MnCl_2$, 1 µM $FeCl_3$, 1 µM $ZnCl_2$, 1.72 µM $CuCl_2$, 2.53 µM $CoCl_2$, 2.42 µM Na_2MoO_4 , 2 µM thiamine hydrochloride, 0.01 M glucose, and 0.2% yeast extract) at 37 °C in the absence (control) and in the presence of various amounts of 1-butanol, and the doubling time for each culture was calculated from the logarithmic part of the growth curve (doubling time = 0.693/growth rate). The percent growth inhibition caused by 1-butanol in the sample flasks was determined by subtracting the percent growth ([doubling time of the control flask/doubling time of the sample flask] X 100) from 100%. The

IC_{50} was the concentration of butanol that caused 50% growth inhibition and was determined by plotting the concentration of butanol versus percent inhibition. The results are summarized in Table 4.

The isolates were identified by sequencing the product that resulted from polymerase chain reaction (PCR) amplification of the 16S rRNA genes in DNA that was extracted from each isolate. DNA was extracted from each of the 1-butanol tolerant strains. Each isolate was processed using a commercial kit (Ultraclean Microbial Genomic DNA Isolation Kit obtained from Mo Bio Laboratories, Inc, Carlsbad, CA, Part No. 12224-50). The 16S rRNA genes of the isolates were amplified by PCR using HotStar Taq (Qiagen, Valencia, CA; Catalog No. 203446) with primers JCR14 (ACGGGCGGTGTGTAC), given as SEQ ID NO:39 and JCR15 (GCCAGCAGCCGCGGTA), given as SEQ ID NO:40. The PCR conditions were 15 min at 95 °C, followed by 30 cycles at 94 °C for 45 sec, 55 °C for 1 min, and 72 °C for 1 min, followed by 10 min at 72 °C. The PCR products were purified and sequenced. Each sequence was used as the query sequence for a BLAST search of GenBank to determine the most similar previously identified 16S rRNA gene sequence. Three strains selected as being butanol tolerant were identified as *Lactobacillus plantarum* and one strain as *Lactobacillus arizonensis* (see Table 4).

Table 4

Butanol Tolerant Bacterial Strains Isolated from Environmental Samples

Strain	Phylotype	ATCC No.	16S rRNA Sequence	IC_{50} (%) 1-butanol
PN0510	*Lactobacillus plantarum*	PTA-8318	SEQ ID NO:41	1.4
PN0511	*Lactobacillus plantarum*	PTA-8320	SEQ ID NO:42	1.6
PN0512	*Lactobacillus plantarum*	PTA-7727	SEQ ID NO:43	1.8
PN0514	*Lactobacillus arizonensis*	PTA-8319	SEQ ID NO:44	1.7

EXAMPLE 2

Tolerance of 1-Butanol Tolerant *Lactobacillus* to other Compounds

The purpose of this Example was to test the tolerance of a *Lactobacillus* strain isolated based on tolerance to 1-butanol, to the additional compounds 2-butanol, isobutanol, 2-butanone and ethanol. The IC_{50} values of these compounds were determined for the selected 1-butanol tolerant *Lactobacillus plantarum* PN0512 strain as described in Example 1 for 1-butanol. The results are summarized in Table 5.

Based on the IC_{50} values determined for each compound and a correlation seen between tolerance to 1-btuanol and to each of the other tested compounds, the identified tolerant strains are expected to grow on solid medium containing 3.9% w/v 2-butanol, 2.7% w/v isobutanol, 5.0% w/v 2-butanone, or 9.0% w/v ethanol.

Table 5. Tolerance of PN0512 to 2-butanol, isobutanol and 2-butanone.

Compound	IC_{50} (%)
Isobutanol	2.4
2-Butanol	3.1
2-butanone	4.5
Ethanol	5.9

CLAIMS

What is claimed is:

1. A method for the isolation of a butanol tolerant microorganism comprising:

a) providing a microbial sample comprising a microbial consortium;

b) contacting the microbial consortium with a growth medium comprising a fermentable carbon source until the members of the microbial consortium are growing;

c) contacting the growing microbial consortium of step (b) with butanol; and

d) isolating the viable members of step (c) wherein a butanol tolerant microorganism is isolated.

2. A method according to Claim 1 wherein the growing consortium of step (b) are growing in log phase.

3. A method according to Claim 1 wherein after step (c) the consortium is plated on solid medium.

4. A method according to Claim 3 wherein the solid medium contains butanol.

5. A method according to Claim 1 wherein the contacting of step (c) is repeated one or more times.

6. A method according to Claim 1 wherein the butanol concentration of the contacting step (c) is from about 0.8% w/v to about 3.0% w/v.

7. A method according to Claim 1 wherein the isolating of step (d) comprises the steps of:

i) growing the viable members of step (d) in a liquid medium in the absence of butanol; whereby the viable members multiply;

ii) growing the cells of step (i) in the presence of butanol; and

iii) collecting the cells of step (ii) that grow in the presence of

butanol wherein a butanol tolerant microorganism is isolated.

8. A method according to Claim 1 wherein the fermentable carbon source is selected from the group consisting of sucrose, fructose, glucose, butyric acid, valeric acid and mixtures thereof.

9. A method according to Claim 1 wherein the butanol is predominantly 1-butanol.

10. A method according to Claim 1 wherein the butanol is predominantly 2-butanol.

11. A method according to Claim 1 wherein the butanol is predominantly isobutanol.

12. A method according to Claim 1 wherein the consortium is grown under anaerobic conditions.

13. A method according to Claim 1 wherein the consortium is grown under microaerophilic conditions.

14. A method according to Claim 1 wherein the consortium is grown under aerobic conditions.

15. A method according to Claim 1 wherein the viable members of step (d) are tolerant to at least 2.5% w/v 1-butanol when grown on a solid medium at 37 °C.

16. A method according to Claim 1 wherein the viable members of step (d) are tolerant to at least 3.9% w/v 2-butanol when grown on a solid medium at 37 °C.

17. A method according to Claim 1 wherein the viable members of step (d) are tolerant to at least 2.7% w/v isobutanol when grown on a solid medium at 37 °C.

18. A method according to Claim 1 wherein the viable members of step (d) are tolerant to at least 5.0% w/v 2-butanone when grown on a solid medium at 37 °C.

19. A method according to Claim 1 wherein the viable members of step (d) are tolerant to at least 9.0% w/v ethanol when grown on a solid medium at 37 °C.

20. A method according to Claim 1 wherein the microbial sample is an environmental sample.

21. A method according to Claim 1 wherein the butanol tolerant microorganism is a *Lactobacillus sp.*

22. A butanol tolerant microorganism isolated by the process of Claim 1.

23. A butanol tolerant microorganism according to Claim 22 wherein the microorganism is a bacterium of the genus *Lactobacillus*.

24. A method for the isolation of a butanol tolerant *Lactobacillus* comprising:

a) providing a microbial sample comprising a microbial consortium;

b) enriching the microbial consortium for the presence of *Lactobacillus* in a medium containing a fermentable carbon source to generate a *Lactobacillus* enriched culture in which members of the *Lactobacillus* enriched culture are growing;

c) contacting the growing *Lactobacillus* enriched culture of step (b) with butanol; and

d) isolating the viable members of step (c) wherein a butanol tolerant *Lactobacillus* is isolated.

25. A method according to Claim 24 wherein the growing *Lactobacillus* of step (c) are growing in log phase.

26. A method according to Claim 24 wherein the microbial sample is an environmental sample.

27. A method according to Claim 24 wherein after step (c) the members of the consortium are plated on solid medium.

28. A method according to Claim 27 wherein the solid medium contains butanol.

29. A method according to claim 28 wherein the solid medium is a lactic acid bacteria medium containing butanol.

30. A method according to Claim 24 wherein the contacting of step (c) is repeated one or more times.

31. A method according to Claim 24 wherein the contacting of step (c) is done with the butanol at a concentration between about 0.8% w/v to about 3.0% w/v.

32. A method according to Claim 24 wherein the isolating of step (d) comprises the steps of:

i) growing the viable members of step (d) in a liquid medium in the absence of butanol whereby the viable members multiply;

ii) growing the cells of step (i) in the presence of butanol; and

iii) collecting the cells of step (ii) that grow in the presence of butanol wherein a butanol tolerant microorganism is isolated.

33. A method according to Claim 24 wherein the fermentable carbon source is selected from the group consisting of sucrose, fructose, glucose, butyric acid, valeric acid and mixtures thereof.

34. A method according to Claim 24 wherein the butanol is predominantly 1-butanol.

35. A method according to Claim 24 wherein the butanol is predominantly 2-butanol.

36. A method according to Claim 24 wherein the butanol is predominantly isobutanol.

37. A method according to Claim 24 wherein the viable members of step (d) are tolerant to at least 2.5% w/v 1-butanol when grown on a solid medium at 37 °C.

38. A method according to Claim 24 wherein the viable members of step (d) are tolerant to at least 3.9% w/v 2-butanol when grown on a solid medium at 37 °C.

39. A method according to Claim 24 wherein the viable members of step (d) are tolerant to at least 2.7% w/v isobutanol when grown on a solid medium at 37 °C.

40. A method according to Claim 24 wherein the viable members of step (d) are tolerant to at least 5.0% w/v 2-butanone when grown on a solid medium at 37 °C.

41. A method according to Claim 24 wherein the viable members of step (d) are tolerant to at least 9.0% w/v ethanol when grown on a solid medium at 37 °C.

42. A butanol tolerant *Lactobacillus* isolated by the process of Claim 24.

43. A butanol tolerant *Lactobacillus* of Claim 42 which is tolerant to at least 2.5% w/v butanol when grown on a solid medium.

44. A butanol tolerant *Lactobacillus* according to Claim 42 wherein the *Lactobacillus* has the following characteristics:

i) is a nonspore-forming facultative anaerobe; and

ii) is Gram-positive; and

iii) is non-motile; and

iv) has rod shaped cell morphology; and

v) comprises a 16S rRNA gene sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, and SEQ ID NO:44.

45. A butanol tolerant *Lactobacillus* having the following characteristics:

i) is a nonspore-forming facultative anaerobe; and

ii) is Gram-positive; and

iii) is non-motile; and

iv) has rod shaped cell morphology; and

v) is tolerant to at least 2.5% w/v butanol when grown on a solid medium.

46. A butanol tolerant *Lactobacillus* according to Claim 45 comprising a 16S rRNA gene sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, and SEQ ID NO:44.

47. A butanol tolerant *Lactobacillus* selected from the group consisting of ATCC PTA-8318 (*Lactobacillus plantarum* PN0510), ATCC

PTA-8320 (*Lactobacillus plantarum* PN0511), ATCC PTA-7727 (*Lactobacillus plantarum* PN0512) and ATCC PTA-8319 (*Lactobacillus arizonensis* PN0514.

48. A method for the production of butanol comprising:

a) providing a *Lactobacillus* comprising genetic constructs encoding a butanol biosynthetic pathway, having the following characteristics:

i) nonspore-forming facultative anaerobe; and

ii) Gram-positive; and

iii) non-motile; and

iv) rod shaped cell morphology; and

v) tolerant to at least 2.5% w/v butanol when grown on a solid medium at 37 °C and

b) growing the *Lactobacillus* of step (a) under conditions whereby butanol is produced.

49. A method for the production of butanol comprising:

a) providing a *Lactobacillus* isolated by the process of Claim 24 comprising genetic constructs encoding a butanol biosynthetic pathway; and

b) growing the *Lactobacillus* of step (a) under conditions whereby butanol is produced.

50. A method according to Claim 48 or 49 wherein the butanol is predominantly 1-butanol.

51. A method according to Claim 48 or 49 wherein the butanol is predominantly 2-butanol.

52. A method according to Claim 48 or 49 wherein the butanol is predominantly isobutanol.

53. A method according to Claim 48 or 49 wherein the *Lactobacillus* is selected from the group consisting of ATCC PTA-8318 (*Lactobacillus plantarum* PN0510), ATCC PTA-8320 (*Lactobacillus plantarum* PN0511), ATCC PTA-7727 (*Lactobacillus plantarum* PN0512) and ATCC PTA-8319 (*Lactobacillus arizonensis* PN0514.

54. A butanol tolerant *Lactobacillus* for use in the production of butanol.

55. A 2-butanone tolerant *Lactobacillus* for use in the production of 2-butanone.

56. A method for the production of 2-butanone comprising:

a) providing a *Lactobacillus* comprising genetic constructs encoding a 2-butanone biosynthetic pathway, having the following characteristics:

i) is a nonspore-forming facultative anaerobe; and

ii) is Gram-positive; and

iii) is non-motile; and

iv) has rod shaped cell morphology; and

v) is tolerant to at least 5.0% w/v 2-butanone when grown on a solid medium.

b) growing the *Lactobacillus* of step (a) under conditions whereby 2-butanone is produced.

57. A method for the production of 2-butanone comprising:

a) providing a *Lactobacillus* isolated by the process of Claim 24 comprising genetic constructs encoding a 2-butanone biosynthetic pathway; and

b) growing the *Lactobacillus* of step (a) under conditions whereby 2-butanone is produced.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/013955

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 C12P7/16 C12P7/26

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, FSTA, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 757 010 A (HERMANN MONIQUE [FR] ET AL) 12 July 1988 (1988-07-12) abstract column 2, lines 18-36 column 2, lines 53,54; example 1	1-9,12, 22
X	US 6 960 465 B1 (PAPOUTSAKIS ELEFTHERIOS T [US] ET AL) 1 November 2005 (2005-11-01) abstract claims 10,11	22,23, 42,54

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 2007	13/11/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van Voorst, Frank

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/013955

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

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International application No
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