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(54) **Title**: ENZYME-ALTERED METABOLITE ACTIVITY

(57) **Abstract**: The present invention relates to mutant alcohol dehydrogenase enzymes, microorganisms comprising same, and methods for the production of one or more products by microbial fermentation.

ENZYME-ALTERED METABOLITE ACTIVITY

FIELD OF THE INVENTION

0001 The present invention relates to mutant alcohol dehydrogenase enzymes, microorganisms comprising same, and methods for the production of one or more products by microbial fermentation.

BACKGROUND

0002 Production of alcohols such as ethanol or butanol by fermentation with microorganisms is well known and has been industrially used since centuries. Historically, ethanol fermentation is the largest process. Acetone-butanol-ethanol (ABE) fermentation is considered as the second largest fermentation process [Duerre P: Production of solvents. In: Handbook on Clostridia, CRC Press, 2005: 671-696] and current production capacity in countries like China exceeds 1,000,000 tons [Ni Y and Sun Z: Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China. Appl. Microbiol. Biotechnol., 2009, 83: 415-423]. Butanol is usually used as solvent or biofuel, while acetone is considered as an unwanted by-product [Duerre P: Production of solvents. In: Handbook on Clostridia, CRC Press, 2005: 671-696]. A few isolates of *Clostridium beijerinckii* are known to produce isopropanol instead of acetone due to a secondary alcohol dehydrogenase [George HA, Johnson JL, Moore WEC, Holdeman LV, Chen JS: Acetone, isopropanol, and butanol production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutyricum*. *Appl Environ Microbiol* 45: 1160-1163]. Isopropanol has similar properties as butanol and would be beneficial over acetone. However, the reduction is not very efficient and respective *Clostridium beijerinckii* strains don't produce good titers and are not considered as useful production strains.

0003 Recently, *C. acetobutylicum* has been metabolically engineered for isopropanol production using a secondary alcohol dehydrogenase from *C. beijerinckii* [Lee et al, 2012: Metabolic engineering of *Clostridium acetobutylicum* ATCC824 for isopropanol-butanol-ethanol fermentation, *Appl. Environ. Microbiol.* 78: 1416-1423], but a highly efficient alcohol dehydrogenase would be required to optimize this process.

0004 A challenge of the ABE fermentation is that all known organisms rely on sugar or starch based substrates. The cost of many carbohydrate feed stocks suitable for the production of chemical products such as acetone and isoproanol is influenced by their value as human food or animal feed, and the cultivation of starch or sucrose-producing crops for such production is not economically sustainable in all geographies. Therefore, it is of interest to develop technologies to convert lower

cost and/or more abundant carbon resources into useful chemical products such as acetone and isopropanol.

0005 CO is a major free energy-rich by-product of the incomplete combustion of organic materials such as coal or oil and oil derived products. For example, the steel industry in Australia is reported to produce and release into the atmosphere over 500,000 tonnes of CO annually. Acetogenic organisms such as the closely related microorganisms *Clostridium autoethanogenum, C. ljungdahlii, and C. ragsdalei* are able to grow chemoautotrophically on CO or CO_2/H_2 containing gases as sole energy and carbon source and synthesize products such as acetate, ethanol, or 2,3-butanediol, but neither acetone nor isopropanol [Munasinghe PC, Khanal SK: Biomass-derived syngas fermentation into biofuels: Opportunities and challenges. *Bioresource Technol* 2010, 5013-22].

0006 Recently, production of isopropanol was reported in a study on *Clostridium ragsdalei* (*Clostridium* strain P11) in a 100-L pilot scale fermentor from switchgrass derived syngas [Kundiyana DK, Huhnke RL, Wilkins MR: Syngas fermentation in a 100-L pilot scale fermentor: Design and process considerations. *J Biosci Bioeng* 2010, 109: 492-498]. However, related studies from the same lab showed that this was due to a contamination in the used syngas since it was passed through a scrubbing mixture containing 20 % acetone [Ramachandriya KD: Effect of biomass generated producer gas, methane and physical parameters on producer gas fermentations by *Clostridium* strain P11. Masters thesis, Oklahoma State University 2009; Ramachandriya KD, Wilkins MR, Delorme MJM, Zhu X, Kundiyana DK, Atiyeh HK, Huhnke RL: Reduction of acetone to isopropanol using producer gas fermenting microbes. Biofuels Environ Biotechnol, 2011, epub]. The authors however confirmed acetone to isopropanol reduction by *Clostridium ragsdalei* (*Clostridium* strain P11) and speculated about the presence of a secondary alcohol dehydrogenase but couldn't find any evidence.

0007 It is an object of the invention to overcome one or more of the disadvantages of the prior art, or to at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

0008 The inventors have identified a novel primary:secondary alcohol dehydrogenase in *C. autoethanogenum* which could be used for the production of isopropanol and/or one or more other products from CO or to upgrade acetone-butanol-ethanol (ABE) fermentation to isopropanol-butanol-ethanol (IBE) fermentation or for the conversion of MEK to 2-butanol, for example, and have optimized the properties of the enzyme, such as substrate and/or co-factor specificity, by directed mutagenesis.

0009 In one aspect of the present invention there is provided an alcohol dehydrogenase having increased specificity for at least one first substrate over at least one second substrate wherein the at least one first substrate and the at least one second substrate are selected from the group consisting of:
the first substrate is acetone and the second substrate is MEK;
the first substrate is acetone and the second substrate is acetaldehyde;
the first substrate is acetone and the second substrate is acetoin;
the first substrate is MEK and the second substrate is acetaldehyde;
the first substrate is MEK and the second is acetoin;
the first substrate is acetoin and the second acetone;
the first substrate is acetoin and the second substrate is MEK;
the first substrate is acetoin and the second substrate is acetaldehyde
the first substrate is acetaldehyde and the second acetone;
the first substrate is acetaldehyde and the second acetoin; and,
the first substrate is acetaldehyde and the second substrate is MEK; and,
wherein the alcohol dehydrogenase includes at least one or more mutation compared to the corresponding wild type alcohol dehydrogenase.

0010 In one embodiment, the alcohol dehydrogenase has increased specificity for one, two or three first substrates over two or three second substrates. In another embodiment, the alcohol dehydrogenase has increased specificity for two or three first substrates over one, two or three second substrates.

0011 In another aspect, the invention provides an alcohol dehydrogenase having increased specificity for an NADH co-factor over an NADPH co-factor wherein the alcohol dehydrogenase includes at least one or more mutation compared to the corresponding wild type alcohol dehydrogenase.

0012 In another aspect, the invention provides an alcohol dehydrogenase which uses NADH as a co-factor wherein the alcohol dehydrogenase includes at least one or more mutation compared to the corresponding wild type alcohol dehydrogenase which uses NADPH as a co-factor.

0013 In one particular embodiment, the alcohol dehydrogenase has an increased specificity for acetone over MEK and/or acetaldehyde and/or acetoin.

0014 In one particular embodiment, the alcohol dehydrogenase has an increased specificity for MEK over acetone and/or acetaldehyde and/or acetoin.

0015 In one particular embodiment, the alcohol dehydrogenase has an increased specificity for acetaldehyde over acetone and/or MEK and/or acetoin.

0016 In one particular embodiment, the alcohol dehydrogenase has an increased specificity for acetoin over acetone and/or MEK and/or acetaldehyde.

0017 In one particular embodiment, the alcohol dehydrogenase has substantially no ability to use acetoin as a substrate. In one particular embodiment, the alcohol dehydrogenase has substantially no ability to use acetoin as a substrate, but is able to use acetone, MEK and/or acetalydehyde as a substrate.

0018 In one embodiment, the at least one mutation is an amino acid substitution at one or a combination of the amino acids corresponding to position Gly198, Ser199, Arg200, Pro201 and Tyr218 of the alcohol dehydrogenase sequence of SEQ ID 36.

0019 In one embodiment, the alcohol dehydrogenase includes one or more of the following mutations compared to the corresponding wild type alcohol dehydrogenase: Gly198Asp, Gly198Ile, Gly198Leu, Gly198Val, Ser199Asp, Ser199Glu, Ser199Leu, Ser199Val, Arg200Glu, Pro201Asp, Pro201Glu, Tyr218Ala and Tyr218Phe.

0020 In another embodiment, the alcohol dehydrogenase includes one of the following mutations compared to the corresponding wild type alcohol dehydrogenase: Tyr218Gly, Tyr218Ser or Tyr218Val.

0021 In one embodiment, the alcohol dehydrogenase includes a Ser199Asp substitution. In one embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution.

0022 In one embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu. In one embodiment, the alcohol dehydrogenase includes a combination of the following substitutions, Gly198Asp, Ser199Leu, and Pro201Glu. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ala. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Phe. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Gly. In

another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ser. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Val.

0023 In one embodiment, the alcohol dehydrogenase includes a Ser199Asp substitution and has an increased substrate specificity for 1) acetone over MEK and/or acetoin. In one embodiment, the alcohol dehydrogenase includes a Ser199Asp substitution and has an increased substrate specificity for acetone over MEK and acetoin.

0024 In one embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution and has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and/or acetoin; and/or 2) MEK over acetaldehyde and/or acetoin. In one embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution and has an increased substrate specificity for 1) acetone over MEK and acetaldehyde and acetoin. In another embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution and has an increased substrate specificity for MEK over acetaldehyde and acetoin. In one embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution and has an increased substrate specificity for 1) acetone over MEK and acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin.

0025 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, and Pro201Glu and has an increased substrate specificity for 1) acetone over MEK; and/or 2) acetaldehyde over MEK, acetone and/or acetoin; and/or, 3) acetoin over acetone and/or MEK. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for acetaldehyde over MEK and acetone and acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for acetoin over acetone and MEK. In another embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for 1) acetone over MEK; and 2) acetaldehyde over MEK and acetone and acetoin; and 3) acetoin over acetone and MEK.

0026 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ala has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and/or acetoin; and/or 2) MEK over acetaldehyde and/or acetoin; and/or 3) acetoin over acetaldehyde. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for acetone over

MEK and acetaldehyde and acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for MEK over acetaldehyde and acetoin. In another embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for 1) acetone over MEK and acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin; and 3) acetoin over acetaldehyde.

0027 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Phe and has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and/or acetoin; and/or MEK over acetaldehyde and/or acetoin; and/or 3) acetalydehyde over acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for acetone over MEK and acetaldehyde and acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for MEK over acetaldehyde and acetoin. In another embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin; and 3) acetalydehyde over acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has substantially no ability to use acetoin as a substrate.

0028 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ala and is able to use NADH as a co-factor. In one embodiment, the alcohol dehydrogenase includes all of these substitutions and has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin; and 3) acetoin over acetaldehyde; and 4) is able to use NADH as a co-factor.

0029 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Phe and is able to use NADH as a co-factor. In one embodiment, the alcohol dehydrogenase includes all of these substitutions and has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin; and 3) acetalydehyde over acetoin; and 5) is able to use NADH as a co-factor.

0030 In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 38. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 42. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 50.

0031 In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 44. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 46. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 48. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 52. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 54. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 63. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 64. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 65.

0032 In a second aspect, the invention provides a nucleic acid encoding an alcohol dehydrogenase of the first aspect of the invention.

0033 In certain embodiments, the nucleic acid has the sequence of SEQ ID 37, SEQ ID 41, SEQ ID 47, SEQ ID 49, SEQ ID 67, SEQ ID 68, SEQ ID 69, or SEQ ID 70. In other embodiment, the nucleic acid has the sequence of SEQ ID 39, SEQ ID 43, SEQ ID 45, SEQ ID 51, or SEQ ID 53.

0034 In a third aspect, the invention provides a nucleic acid vector comprising a nucleic acid encoding an alcohol dehydrogenase of the first aspect of the invention. In one embodiment, the vector is an expression vector. In one embodiment the nucleic acid encoding an alcohol dehydrogenase of the first aspect is a nucleic acid of the second aspect.

0035 In a fourth aspect, the invention provides a host cell comprising a nucleic acid of the second or third aspects of the invention.

0036 In a fifth aspect, the invention provides a recombinant microorganism which comprises one or more nucleic acid of the second or third aspects of the invention.

0037 In one embodiment, the microorganism is capable of producing one or more products chosen from:
Isopropanol;
2,3-Butanediol;
Ethanol; and,
2-butanol;
and optionally one or more other products by fermentation.

0038 In one embodiment, the microorganism is capable of producing one or more products chosen from:

Acetoin;

Acetalydehyde;

MEK; and,

Acetone;

and optionally one or more other products by fermentation.

0039 In one embodiment, the recombinant microorganism is chosen from the group of microorganisms comprising bacteria, Archae, and fungi.

0040 In one embodiment, the recombinant microorganism is chosen from the genera *Clostridium, Acetobacterium, Moorella, Butyribacterium, Blautia, Oxobacter, Thermoanaerobacter, Escherichia, Klebsiella, Zymomonas, Citrobacter, Enterobacter, Salmonella, Serratia, Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Streptococcus, Saccharomyces, Pichia, Candida Hansenula, Yarrowia, Rhodotorula, Rhizopus, Trichosporon, Lipomyces, Aspergillus, trichoderma, Exophila, Mucor, Cladosporium, Phanerochaete, Cladiophilalophora, Paecilomyces, Scedosporium, Ophistoma, Bacillus, Oligotropha, Pseudomonas, Carbophilus, Hydrogenophaga, Mycobacterium, Zavarzinia, Cupravidus, Senechocystis, Chloroflexus, Methylomonas, Methylobacter, Methylococcus, Methylomicrobium, Methylosphera, Methylocaldum, Methylocystis, Methylosinus, Methanobacterium, Methanococcus, Methanogenium, Methanosarcina, Methanoshera, Methanothermobacter, Methanotrix, Corynebacterium, Acinetobacter, Actinomyces, Bacteriodes, Burkholderia, Brevibacterium, Pyrococcus, Geobacter, Geobacillus, Paenibacillus, Mycobacterium, Rhodopseudomonas, Thermatoga, Thermoanaerobacter, Streptomyces, Rhodobacter, Rhodococcus, Peptococcus, Bifidobacterium, Propionibacterium, Fusobacterium, Campylobacter, Veillonella, Aquincola, Arthrobacter, Moraxella,* and *Psychrobacter.*

0041 In one embodiment the organism is chosen from the group of carboxydotrophic acetogenic microorganisms, the group of ABE microorganisms, the group of Enterobacteria, the group of Lactobacillus, the group of fungi and yeasts, the group of aerobic carboxydotrophes, the group of aerobic CO_2 fixing organisms, the group of methylotrophes, and the group of methanogens.

0042 In one embodiment, the microorganism is a carboxydotrophic acetogen selected from the group comprising *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium scatologenes, Clostridium coskatii, Clostridium aceticum, Clostridium magnum, Clostridium sp.,Butyribacterium limosum,*

Butyribacterium methylotrophicum, Acetobacterium woodii, Alkalibaculum bacchii, Blautia producta, Eubacterium limosum, Moorella thermoacetica, Moorella thermautotrophica, Oxobacter pfennigii, and *Thermoanaerobacter kiuvi.* In one embodiment the microorganism is *Clostridium autoethanogenum* or *Clostridium ljungdahlii.* In one particular embodiment, the microorganism is *Clostridium autoethanogenum* DSM10061 or DSM23693. In another particular embodiment, the microorganism is *Clostridium ljungdahlii* DSM13528 (or ATCC55383).

0043 In one embodiment, the microorganism is an ABE microorganism selected from the group comprising *Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharobutylicum, Clostridium saccharoperbutylacetonicum.* In one embodiment the microorganism is *Clostridium acetobutylicum* or *Clostridium beijerinckii.* In one particular embodiment, the microorganism is *Clostridium acetobutylicum* ATCC824 (or DSM792). In another particular embodiment, the microorganism is *Clostridium beijerinckii* NCIMB8052 (ATCC51743).

0044 In one embodiment, the microorganism is an Enterobacteria selected from the group comprising *Escherichia, Klebsiella, Zymomonas, Citrobacter, Enterobacter, Salmonella, Serratia.* In one embodiment the microorganism is *Eschericia coli, Zymononas mobilis, Klebsiella pneumonia, Klebsiella oxtoca, Enterobacter cloacae* or *Serratia marcescens.*

0045 In one embodiment, the microorganism is a Lactobacillus selected from the group comprising *Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Streptococcus.* In one embodiment the microorganism is *Lactobacillus brevis, Enterococcus faecalis, Lactococcus lactis.*

0046 In one embodiment, the microorganism is a fungi or yeast selected from the group comprising *Saccharomyces, Pichia, Candida Hansenula, Yarrowia, Rhodotorula, Rhizopus, Trichosporon, Lipomyces* and from the group comprising *Aspergillus, Trichoderma, Exophila, Mucor, Cladosporium, Phanerochaete, Cladiophilalophora, Paecilomyces, Scedosporium, Ophistoma.* In one embodiment the microorganism is *Saccharomyces cerevisiae, Candidia tropicalis, Candidia albicans* or *Yarrowia lipolytica.* In one embodiment the microorganism is *Aspargillus niger* or *Trichderma resei.*

0047 In one embodiment, the microorganism is an aerobic carboxydotroph selected from the group comprising *Bacillus, Oligotropha, Pseudomonas, Carbophilus, Hydrogenophaga, Mycobacterium, Zavarzinia.* In one embodiment the microorganism is *Oligotropha carboxydovorans, Carbophilus carboxidus, Hydrogenophaga pseudoflava, Mycobacterium sp., Pseudomonas carboxydohydrogena, Pseudomonas sp., Zavarzinia compransoris* or *Bacillus schlegelii.*

0048 In one embodiment, the microorganism is an aerobic CO_2 fixing organism selected from the group comprising *Cupravidus, Senechocystis, Chloroflexus.* In one embodiment the microorganism is *Cupravidus necator, Senechocystis sp.* or *Chloroflexus auranticus.*

0049 In one embodiment, the microorganism is a methylotroph selected from the group comprising *Methylomonas, Methylobacter, Methylococcus, Methylomicrobium, Methylosphera, Methylocaldum, Methylocystis, Methylosinus.* In one embodiment the microorganism is *Methylococcus capsulatus* or *Methylosinus trichosporium.*

0050 In one embodiment, the microorganism is a methanogen selected from the group comprising *Methanobacterium, Methanococcus, Methanogenium, Methanosarcina, Methanoshera, Methanothermobacter, Methanotrix.* In one embodiment the microorganism is

0051 *Methanothermobacter marburgensis* or *Methanosarcina bakeri.*

0052 In a sixth aspect, invention provides a method for the production of one or more of isopropanol, 2,3-Butanediol, ethanol, and 2-butanol, and optionally one or more other products, by microbial fermentation of a substrate using a microorganism of the fifth aspect of the invention.

0053 In another aspect, the invention provides a method for the production of one or more of acetoin, MEK, acetone and acetaldehyde.

0054 In one embodiment the method comprises the steps of:

(a) providing a substrate to a bioreactor containing a culture of one or more microorganism of the invention; and

(b) fermenting the culture in the bioreactor to produce one or more of isopropanol, 2,3-Butanediol, ethanol, and 2-butanol and, optionally, one or more other products.

0055 In one embodiment, the substrate is a substrate comprising one or more of CO, CO_2 and H_2. In another embodiment, the substrate is a substrate comprising one or more carbohydrate.

0056 In another embodiment, a combination of two or more different substrates may be used. In one embodiment, a combination of a substrate comprising one or more of CO, CO_2 and H_2 and a substrate comprising one or more carbohydrate is used.

0057 In one embodiment the substrate is a substrate comprising CO and the method comprises the steps of:

(a) capturing CO-containing gas produced as a result of the industrial process, before the gas is released into the atmosphere;

(b) the anaerobic fermentation of the CO-containing gas to produce one or more of isopropanol, 2,3-Butanediol, ethanol, and 2-butanol and optionally one or more other products by a culture containing one or more carboxydotrophic acetogenic microorganism of the invention.

0058 The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification, individually or collectively, in any or all combinations of two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which the invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

BRIEF DESCRIPTION OF THE FIGURES

0059 These and other aspects of the present invention, which should be considered in all its novel aspects, will become apparent from the following description, which is given by way of example only, with reference to the accompanying figures, in which:

0060 **Figure 1:** shows that wild-type protein was highly soluble when expressed in *E. coli*, and could be readily purified. Samples (left to right): total cell extract; soluble lysate; molecular weight ladder; column elution fractions 1-7.

0061 **Figure 2:** shows that the Ser199Asp (left panel) and Arg200Gln (right panel) mutant proteins were highly soluble. Ser199Asp samples (left to right): total cell extract; ladder; column wash 1; column wash 2; elution fractions 1-5. Arg200Gln samples (left to right): total cell extract; column wash 1; column wash 2; ladder; elution fractions 1-5.

0062 **Figure 3:** shows the activity of wild-type protein and the Ser199Asp mutant. The Ser199Asp mutant was nearly as active as wild-type with acetone.

0063 **Figure 4:** shows the relative activity of the Ser199Asp mutant.

0064 **Figure 5:** shows that Mutant 2 (panel A), Mutant 7 (panel B), Mutant 10 (panel C), Mutant 11 (panel D) and Mutant 13 (panel E) were soluble. Mutant 2 samples (left to right): total cell extract,

soluble lysate, column wash fractions (1-6), molecular weight ladder, column elution fractions (1-6). Mutant 7 samples (left to right): total cell extract, soluble lysate, column wash fractions (1-5), molecular weight ladder, column elution fractions (1-7). Mutant 10 samples (left to right): total cell extract, soluble lysate, column wash fraction (1), molecular weight ladder, column wash fractions (2-4), column elution fractions (1-5). Mutant 11 samples (left to right): total cell extract, soluble lysate, column wash fractions (1-3), molecular weight ladder, column elution fractions (1-5). Mutant 13 samples (left to right): total cell extract, soluble lysate, column wash fraction (1), molecular weight ladder, column wash fractions (2-3), column elution fractions (1-5), molecular weight ladder.

0065 **Figure 6:** shows the activity of the wild-type protein and Mutants 2, 7, 10, 11 and 13 with NADH as the cofactor and acetone as substrate. Only Mutant 11 has any significant activity using NADH as the cofactor.

0066 **Figure 7:** shows the specific activity of the wild-type and mutant ADH enzymes with different substrates normalized to acetone (acetone set = 1). For each enzyme, the preferred cofactor was used (i.e. NADPH for wild-type ADH, Mutant 2 and Mutant 7; NADH for Mutant 10 and Mutant 11).

0067 **Figure 8:** shows the specific activity of the wild-type and mutant ADH enzymes with different substrates normalized to MEK (2-butanone) (MEK set = 1). For each enzyme, the preferred cofactor was used (i.e. NADPH for wild-type ADH, Mutant 2 and Mutant 7; NADH for Mutant 10 and Mutant 11).

0068 **Figure 9:** shows the specific activity of the wild-type and mutant ADH enzymes with different substrates normalized to Acetaldehyde (acetaldehyde set = 1). For each enzyme, the preferred cofactor was used (i.e. NADPH for wild-type ADH, Mutant 2 and Mutant 7; NADH for Mutant 10 and Mutant 11).

0069 **Figure 10:** shows the specific activity of the wild-type and mutant ADH enzymes with different substrates normalized to acetoin (acetoin set = 1). For each enzyme, the preferred cofactor was used (i.e. NADPH for wild-type ADH, Mutant 2 and Mutant 7; NADH for Mutant 10 and Mutant 11).

0070 **Figure 11:** Activity tests of *C. autoethanogenum* DSM10061 wild-type enzyme with NADH and NADPH as co-factor and acetone as substrate including control (CNTL).

0071 **Figure 12:** Kinetics, KM values and Activity measured for the wild type Adh enzyme with different substrates.

0072 **Figure 13:** Plasmid map of pMTL85147-ThlA-CtfAB-Adc-Adh

0073 **Figure 14:** Fermentation pathways to Isopropanol, 2,3-butanediol, 2-butanol and ethanol and role of the alcohol dehydrogenase (adh). Other reations: acetolactate synthase (als), acetolactate decarboxylase (aldc), diol dehydratase (pdd), aldehyde dehydrogenase (ald), aldehyde:ferredoxin oxidoreductase (aor), phosphotransacetylase (pta), acetate kinase (ack), thiolase (thlA), CoA transferase (ctfAB), acetoacetate decarboxylase (adc).

BRIEF DESCRIPTION OF SEQUENCE LISTING:

0074 This specification is accompanied by a sequence listing in which the following sequences are listed:

0075 **SEQ ID No.s 1 to 34**: are described in Tables 3, 4 and 5 herein after.

0076 **SEQ ID No. 35:** nucleic acid sequence of a wild type ADH studied by the inventors.

0077 **SEQ ID No. 36:** amino acid sequence of a wild type ADH studied by the inventors.

0078 **SEQ ID No. 37:** nucleic acid sequence of a mutant ADH comprising the substitution Ser199Asp generated by the inventors.

0079 **SEQ ID No. 38:** amino acid sequence of a mutant ADH comprising the substitution Ser199Asp generated by the inventors.

0080 **SEQ ID No. 39:** nucleic acid sequence of the Wood Ljungdahl promoter region.

0081 **SEQ ID No. 40:** nucleotide sequence of plasmid pMTL85147-ThlA-CtfAB-Adc-Adh described further herein.

0082 **SEQ ID No. 41:** nucleic acid sequence of mutant 7 described further herein after (codon optimised).

0083 **SEQ ID No. 42:** amino acid sequence of mutant 7 described further herein after.

0084 **SEQ ID No. 43:** nucleic acid sequence of mutant 8 described further herein after (codon optimised).

0085 **SEQ ID No. 44:** amino acid sequence of mutant 8 described further herein after.

0086 **SEQ ID No. 45:** nucleic acid sequence of mutant 9 described further herein after (codon optimised).

0087 **SEQ ID No. 46:** amino acid sequence of mutant 9 described further herein after.

0088 **SEQ ID No. 47:** nucleic acid sequence of mutant 10 described further herein after (codon optimised).

0089 **SEQ ID No. 48:** amino acid sequence of mutant 10 described further herein after.

0090 **SEQ ID No. 49:** nucleic acid sequence of mutant 11 described further herein after (codon optimised).

0091 **SEQ ID No. 50:** amino acid sequence of mutant 11 described further herein after.

0092 **SEQ ID No. 51:** nucleic acid sequence of mutant 12 described further herein after (codon optimised).

0093 **SEQ ID No. 52:** amino acid sequence of mutant 12 described further herein after.

0094 **SEQ ID No. 53:** nucleic acid sequence of mutant 13 described further herein after (codon optimised).

0095 **SEQ ID No. 54:** amino acid sequence of mutant 13 described further herein after.

0096 **SEQ ID No. 55:** nucleotide sequence of 5' homology arm of C. autoethanogenum alcohol dehydrogenase gene.

0097 **SEQ ID No. 56:** nucleotide sequence of 3' homology arm of C. autoethanogenum alcohol dehydrogenase gene.

0098 **SEQ ID No. 57:** primer Sec5f.

0099 **SEQ ID No. 58:** primer Sec5r.

0100 **SEQ ID No. 59:** primer Sec3f.

0101 **SEQ ID No. 60:** primer Sec3r.

0102 **SEQ ID No. 61:** primer SecOf.

0103 **SEQ ID No. 62:** primer SecOr.

0104 **SEQ ID No. 63:** Amino acid sequence of alcohol dehydrogenase enzyme with the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Gly

0105 **SEQ ID No. 64:** Amino acid sequence of alcohol dehydrogenase enzyme with the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ser

0106 **SEQ ID No. 65:** Amino acid sequence of alcohol dehydrogenase enzyme with the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Val

0107 **SEQ ID No. 66**: Amino acid sequence of alcohol dehydrogenase enzyme with the following substitutions Ser199Glu

0108 **SEQ ID No. 67:** Nucleic acid sequence of alcohol dehydrogenase enzyme with the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Gly

0109 **SEQ ID No. 68:** Nucleic acid sequence of alcohol dehydrogenase enzyme with the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ser

0110 **SEQ ID No. 69:** Nucleic acid sequence of alcohol dehydrogenase enzyme with the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Val

0111 **SEQ ID No. 70:** Nucleic acid sequence of alcohol dehydrogenase enzyme with the following substitutions Ser199Glu (codon optimised).

DETAILED DESCRIPTION OF THE INVENTION

0112 The following is a description of the present invention, including preferred embodiments thereof, given in general terms. The invention is further elucidated from the disclosure given under the heading "Examples" herein below, which provides experimental data supporting the invention, specific examples of various aspects of the invention, and means of performing the invention.

0113 The inventor(s) have unexpectedly found that mutation of an alcohol dehydrogenase enzyme from a carboxydotrophic acetogenic microorganism increases its specificity for one or more of the substrates acetoin, methyl ethyl ketone (MEK or 2-butanone), acetone and acetalydehyde relative to one another.

0114 In addition, the inventors have identified mutants that are able to accept NADH as a co-factor instead of or in addition to NADPH. Thus it is not limited by the availability of only one of these co-factors and is able to use the more abundant intracellular NADH pool for increasing the overall efficiency of production formation.

0115 The mutation(s) mean that enzymes of the invention are adapted to preferentially produce one fermentation end product over another and allow one to utilize the typically much bigger NADH pool over the NADPH pool (Bennett & San, 2009). In ABE fermentation, both ethanol and acetone are produced and in IBE fermentation isopropanol is produced (Köpke & Dürre, 2011). The invention may allow one to increase the reduction of acetone to isopropanol or preferentially catalyse this reaction over acetaldehyde to ethanol or vice versa. For example, the invention may help to overcome limitations in isopropanol production, as all strains rely on an unmodified alcohol dehydrogenase that is strictly NADPH dependent and has a high background activity towards acetaldehyde. *E. coli* strains engineered for isopropanol production suffer the same shortcomings and low yields as the same NADPH dependent alcohol dehydrogenase enzyme from *C. beijerinckii* is used without alternatives [Hanai T et al (2007) Engineered synthetic pathway for isopropanol production in Escherichia coli. *Applied and environmental microbiology* 73:7814–8; Inokuma K et al (2010) Improvement of isopropanol production by metabolically engineered Escherichia coli using gas stripping. *Journal of bioscience and bioengineering* 110:696–701; Jojima T et al (2008) Production of isopropanol by metabolically engineered Escherichia coli. *Applied microbiology and biotechnology* 77:1219–24]. Similarly, the invention provides a means for the production of isopropanol from substrates comprising carbon monoxide by carboxydotrophic acetogenic microorganisms previously not able to produce viable levels of isopropanol. Some carboxydotrophic organisms as *C. autoethanogenum, C. ljungdahlii* or *C. ragsdalei* are able to form both ethanol and 2,3-butanediol (Köpke et al., 2011). The invention may also allow one to increase the reduction of acetoin to 2,3-butanediol or preferentially

catalyse this reaction over acetaldehyde to ethanol or vice versa. 2,3-butanediol can be converted to MEK by a diol dehydratase (Toraya et al, 1976, Substrate specificity of coenzyme B12-dependent diol dehydrase - glycerol as both a good substrate and a potent inactivator. *Biochem. Biophys. Res. Commun.*, 69: 475-80). The invention allows effective conversion of MEK into 2-butanol. Accordingly, the invention also provides a solution for producing 2,3-butanediol from acetoin and 2-butanol from MEK with higher specificity.

0116 Accordingly, the invention provides, inter alia, alcohol dehydrogenases having an increased specificity for an acetone substrate over other substrates like acetoin, methyl ethyl ketone (MEK or 2-butanone) and/or an acetaldehyde substrate, an MEK substrate over an acetaldehyde and/or acetoin substrate, an acetaldehyde substrate over an MEK, acetoin and/or acetone substrate, and/or an acetoin substrate over an acetone, MEK and/or acetaldehyde substrate wherein the alcohol dehydrogenase includes at least one or more mutation compared to the corresponding wild type alcohol dehydrogenase, nucleic acids encoding the alcohol dehydrogenases, nucleic acid vectors comprising the nucleic acids, microorganisms which are capable of producing one or more of isopropanol, 2,3-butanediol, ethanol and 2-butanol, and optionally one or more other products, by fermentation of a substrate and which comprise one or more nucleic acid encoding one or more alcohol dehydrogenase of the invention, and methods for the production of one or more of isopropanol, 2,3-butanediol, ethanol and 2-butanol, and optionally one or more other products.

0117 The invention also provides an alcohol dehydrogenase that has substantially no ability to use acetoin as a substrate, nucleic acids and nucleic acid vectors encoding such an alcohol dehydrogenase, microorganisms comprising said nucleic acids or nucleic acid vectors and methods of use of such alcohol dehydrogenase.

0118 The phrase "substrate comprising one or more of CO, CO_2 and H_2" should be understood to include any substrate in which one or more of CO, CO_2 and H_2 is available to one or more strains of microorganisms for growth and/or fermentation, for example. It should be appreciated that the substrate may comprise 100% CO, CO_2 or H_2 or a majority of CO, CO_2 or H_2 compared to the other gases, or may be combined in any ratio of two or more of the gases. In particular embodiments, the substrate comprises a combination of CO and CO_2. In another embodiment the substrate comprises a combination of CO and H_2. In another embodiment, the substrate comprises a combination of CO_2 and H_2. In another embodiment, the substrate comprises a combination of CO, CO_2 and H_2.

0119 In certain embodiments, the substrate may comprise CO_2 and any culture, growth or fermentation performed in the presence of light (photosynthesis) and/or electricity (electrosynthesis). In certain embodiments, the CO_2 is combined with O_2.

0120 In one embodiment, the "substrate comprising CO, CO_2 and H_2" is a "substrate comprising carbon monoxide". A "substrate comprising CO" and like terms should be understood to include any substrate in which carbon monoxide is available to one or more strains of microorganisms for growth and/or fermentation, for example.

0121 The phrase "gaseous substrate comprising carbon monoxide" and like phrases and terms includes any gas which contains a level of carbon monoxide. In certain embodiments the substrate contains at least about 20% to about 100% CO by volume, from 20% to 70% CO by volume, from 30% to 60% CO by volume, and from 40% to 55% CO by volume. In particular embodiments, the substrate comprises about 25%, or about 30%, or about 35%, or about 40%, or about 45%, or about 50% CO, or about 55% CO, or about 60% CO by volume.

0122 While it is not necessary for the substrate comprising CO to contain any hydrogen, the presence of H_2 should not be detrimental to product formation in accordance with methods of the invention. In particular embodiments, the presence of hydrogen results in an improved overall efficiency of alcohol production. For example, in particular embodiments, the substrate may comprise an approx 2:1, or 1:1, or 1:2 ratio of H_2:CO. In one embodiment the substrate comprises about 30% or less H_2 by volume, 20% or less H_2 by volume, about 15% or less H_2 by volume or about 10% or less H_2 by volume. In other embodiments, the substrate stream comprises low concentrations of H_2, for example, less than 5%, or less than 4%, or less than 3%, or less than 2%, or less than 1%, or is substantially hydrogen free. The substrate may also contain some CO_2 for example, such as about 1% to about 80% CO_2 by volume, or 1% to about 30% CO_2 by volume. In one embodiment the substrate comprises less than or equal to about 20% CO_2 by volume. In particular embodiments the substrate comprises less than or equal to about 15% CO_2 by volume, less than or equal to about 10% CO_2 by volume, less than or equal to about 5% CO_2 by volume or substantially no CO_2.

0123 In particular embodiments of the invention, the CO-containing gaseous substrate is an industrial off or waste gas. "Industrial waste or off gases" should be taken broadly to include any gases comprising CO produced by an industrial process and include gases produced as a result of ferrous metal products manufacturing, non-ferrous products manufacturing, petroleum refining processes, gasification of coal, gasification of biomass, electric power production, carbon black production, and coke manufacturing. Further examples may be provided elsewhere herein.

0124 In one embodiment, the "substrate comprising CO, CO_2 and H_2" is a "substrate comprising CO_2 and H_2". A "substrate comprising CO_2 and H_2" and like terms should be understood to include any substrate in which carbon dioxide and hydrogen is available to one or more strains of microorganisms for growth and/or fermentation, for example.

0125 The $C0_2$ and H_2 containing substrate will typically contain a major proportion of H_2, such as at least about 30% H_2 by volume, or at least 40% H_2 by volume, or at least 50% H_2 by volume, or at least 60% H_2 by volume, or at least 70% H_2 by volume, or at least 80% H_2 by volume, or at least 85% H_2 by volume.

0126 The gaseous substrate will typically contain at least about 10% CO_2 by volume, or at least 15% CO_2 by volume, or at least 20%CO_2 by volume, or at least 25% CO_2 by volume, or at least 30% CO_2 by volume, or at least 40% CO_2 by volume.

0127 In certain embodiments, the ratio of H2: CO2 is around 1:1, or around 2:1, or around 3:1.

0128 In certain embodiments the substrate comprising CO_2 and H_2 is a waste gas obtained as a by-product of an industrial process, or from some other source. The largest source of CO_2 emissions globally is from the combustion of fossil fuels such as coal, oil and gas in power plants, industrial facilities and other sources.

0129 The gaseous substrate may be a CO_2 and H_2-containing waste gas obtained as a by-product of an industrial process, or from some another source such as from automobile exhaust fumes. In certain embodiments, the industrial process is selected from the group consisting of hydrogen manufacture, ammonia manufacture, combustion of fuels, gasification of coal, and the production of limestone and cement. The gaseous substrate may be the result of blending one or more gaseous substrates to provide a blended stream. It would be understood to a skilled person that waste gas streams rich in H_2 or rich in CO_2 are more abundant that waste gas streams rich in both H_2 and CO_2. A skilled person would understand that blending one or more gas streams comprising one of the desired components of CO_2 and H_2 would fall within the scope of the present invention.

0130 Hydrogen rich gas streams are produced by a variety of processes including steam reformation of hydrocarbons, and in particular steam reformation of natural gas. The partial oxidation of coal or hydrocarbons is also a source of hydrogen rich gas. Other sources of hydrogen rich gas

include the electrolysis of water, by-products from electrolytic cells used to produce chlorine and from various refinery and chemical streams.

0131 Gas streams typically rich in Carbon dioxide include exhaust gasses from combustion of a hydrocarbon, such as natural gas or oil. Carbon dioxide is also produced as a by-product from the production of ammonia, lime or phosphate.

0132 In the description which follows, embodiments of the invention are described in terms of delivering and fermenting a "gaseous substrate containing CO" or a "gaseous substrate comprising one or more of CO, CO_2 and H_2". However, it should be appreciated that the gaseous substrate may be provided in alternative forms. For example, the gaseous substrate may be provided dissolved in a liquid. Essentially, a liquid is saturated with a carbon monoxide, carbon dioxide and/or hydrogen containing gas and then that liquid is added to the bioreactor. This may be achieved using standard methodology. By way of example, a microbubble dispersion generator (Hensirisak et. al. Scale-up of microbubble dispersion generator for aerobic fermentation; Applied Biochemistry and Biotechnology Volume 101, Number 3 / October, 2002) could be used. By way of further example, the gaseous substrate containing CO may be adsorbed onto a solid support. Such alternative methods are encompassed by use of the term "substrate containing CO", "substrate comprising CO_2 and H_2) and "substrate comprising one or more of CO, CO_2 and H_2" and like phrases.

0133 The phrase "substrate comprising one or more carbohydrates" and like terms should be understood to include any substrate in which one or more carbohydrate is available to one or more strains of microorganisms for growth and/or fermentation, for example. "Carbohydrates" should be taken broadly to include mono-, di-, oligo- and poly-saccharides, simple and complex carbohydrates, including glucose, fructose, molasses and starch.

0134 In one embodiment, the "substrate comprising one or more carbohydrates" may be sourced from biomass. The biomass may be of any nature and includes, for example, residues from forests or other commercial crops (such as trees, branches, stumps, wood chips, saw dust, clippings), municipal solid waste, and crops grown to provide a feedstock for the production of one or more products by microbial fermentation, including, for example, miscanthus, switchgrass, hemp, corn, poplar, willow, sorghum, sugarcane, and bamboo.

0135 Unless the context requires otherwise, the phrases "fermenting", "fermentation process" or "fermentation reaction" and the like, as used herein, are intended to encompass both the growth phase and product biosynthesis phase of the process. As will be described further herein, in some

embodiments the bioreactor may comprise a first growth reactor and a second fermentation reactor. As such, the addition of metals or compositions to a fermentation reaction should be understood to include addition to either or both of these reactors.

0136 The term "bioreactor" includes a fermentation device consisting of one or more vessels and/or towers or piping arrangement, which includes the Continuous Stirred Tank Reactor (CSTR), Immobilized Cell Reactor (ICR), Trickle Bed Reactor (TBR), Bubble Column, Gas Lift Fermenter, Static Mixer, or other vessel or other device suitable for gas-liquid contact. In some embodiments the bioreactor may comprise a first growth reactor and a second fermentation reactor. As such, when referring to the addition of substrate to the bioreactor or fermentation reaction it should be understood to include addition to either or both of these reactors where appropriate.

0137 The terms nucleic acid "constructs" or "vectors" and like terms should be taken broadly to include any nucleic acid (including DNA, cDNA and RNA) suitable for use as a vehicle to transfer genetic material into a cell. The terms should be taken to include plasmids, viruses (including bacteriophage), cosmids and artificial chromosomes. Constructs or vectors may include one or more regulatory elements, an origin of replication, a multicloning site and/or a selectable marker. In one particular embodiment, the constructs or vectors are adapted to allow expression of one or more genes encoded by the construct or vector. Nucleic acid constructs or vectors include naked nucleic acids as well as nucleic acids formulated with one or more agents to facilitate delivery to a cell (for example, liposome-conjugated nucleic acid, an organism in which the nucleic acid is contained). The vectors may be used for cloning or expression of nucleic acids and for transformation of microorganisms to produce recombinant microorganisms. The vectors may include one or more nucleic acids encoding one or more alcohol dehydrogenase enzyme of the invention.

0138 "Exogenous nucleic acids" are nucleic acids which originate outside of the microorganism to which they are introduced. Exogenous nucleic acids may be derived from any appropriate source, including, but not limited to, the microorganism to which they are to be introduced, strains or species of microorganisms which differ from the organism to which they are to be introduced, or they may be created artificially or by recombination. In another embodiment, the exogenous nucleic acids represent nucleic acid sequences not naturally present within the microorganism to which they are to be introduced and allow for the expression of a product not naturally present within the microorganism. The exogenous nucleic acid may be adapted to integrate into the genome of the microorganism to which it is to be introduced or to remain in an extra-chromosomal state.

0139 A "parental microorganism" is a microorganism used to generate a microorganism of the invention. The parental microorganism may be one that occurs in nature (ie a wild type microorganism) or one that has been previously modified but which does not express or over-express one or more of the enzymes the subject of the present invention. Accordingly, the microorganisms of the invention have been modified to express one or more alcohol dehydrogenase of the invention in the parental microorganism.

0140 The alcohol dehydrogenase enzymes of the invention are referred to herein to have "increased specificity" for one substrate over another. This is intended to mean that the alcohol dehydrogenase has increased specificity for one substrate relative to another, compared to the wild type alcohol dehydrogenase. It should not be taken to necessarily infer that an alcohol dehydrogenase of the invention has a higher specificity for a particular substrate compared to the wild type alcohol dehyrodenase, although this may be the case in some embodiments. Additionally, the term should not be taken to mean that an alcohol dehydrogenase of the invention has absolute specificity for a particular substrate over another, although this may be the case in some embodiments, and includes at least a preference for a particular substrate over another substrate.

0141 "Increased specificity", "higher specificity" or like terms, when used in relation to an NADH or NADPH co-factor, refers to the degree of affinity with which a co-factor binds to an alcohol dehydrogenase during a reaction. It should not be taken to mean that an alcohol dehydrogenase and a co-factor have absolute specificity, although this may be the case, and includes at least a preference for the binding between a particular alcohol dehydrogenase and one co-factor over another co-factor.

0142 Reference is made herein to production of "one or more products including isopropanol, 2,3-butanediol, ethanol and 2-butanol". However, it should be appreciated that additional products may also be generated.

0143 Reference may also be made herein to production of "one or more products including acetoin, MEK, acetaldehyde and acetone". While these products are also referred to herein as "substrates", in certain embodiments, where the specificity of a mutant alcohol dehydrogenase of the invention is reduced for a particular substrate compared to another, it will be converted to downstream products at a reduced level, or substantially no conversion will occur, allowing increased levels of acetoin, MEK, acetaldehyde and/or acetone to accumulate. By way of example, in one embodiment an alcohol dehydrogenase of the invention has substantially no ability to use acetoin as a substrate and so acetoin may accumulate.

0144 In addition, in some embodiments, it should be appreciated that one or more of the products referred to herein, including one or more of isopropanol, 2,3-butanediol, ethanol and 2-butanol, may be used as intermediates or precursors which are further converted into downstream products, in the same fermentation reaction, a separate fermentation reaction, or by chemical synthesis. In this case, one may not be able to detect the production of one or more of the products in a particular microorganism or may only be able to detect small levels of production. However, the production of the one or more products may be inferred based on the production of one or more downstream product.

0145 In certain embodiments of the invention, an alcohol dehydrogenase of the invention has "substantially no ability to use acetoin as a substrate". This does not necessarily imply that the enzyme has absolutely no ability to use acetoin as a substrate, although this may be preferred. In one embodiment, the phrase should be taken to include a tolerance of approximately 1% or less of the activity of a wild type enzyme or an enzyme efficiency of kcat/K_M of less than 0.1 sec^{-1} mM^{-1}.

The enzyme

0146 While the inventors have demonstrated that mutation of an alcohol dehydrogenase (SEQ ID 36) from *C. autoethanogenum* increases specificity for various substrates compared to other substrates, and in certain embodiments that co-factor specificity may be altered or optimised, they contemplate that the invention is widely applicable to other alcohol dehydrogenase enzymes from other organisms; in particular, any alcohol dehydrogenase which has activity towards primary or secondary alcohols and uses NADH or NADPH as substrate (EC 1.1.1.1 or EC 1.1.1.2).

0147 Typically, the group of alcohol dehydrogenases to which the invention is applicable will have at least approximately 65% sequence identity to the alcohol dehydrogenase of SEQ ID 36, more particularly at least approximately 70%, 75%, 80%, 85%, 90%, 95%, or 99% sequence identity.

0148 By way of example, the invention may be applied to the following alcohol dehydrogenases: primary: secondary alcohol deheydrogenase of *C. autoethanogenum* (SEQ ID No 36), primary: secondary alcohol deheydrogenase of *C. ljungdahlii* (YP_003780646.1), primary: secondary alcohol deheydrogenase of *C. beijerinckii* (P25984.2), primary: secondary alcohol deheydrogenase of *Thermoanaerobacter ethanolicus* (ABC50090.1), or primary: secondary alcohol deheydrogenase of *Thermoanaerobium brockii* (P14941.1).

0149 An alcohol dehydrogenase of the invention comprises at least one mutation compared to the corresponding wild type alcohol dehydrogenase. In one embodiment, the at least one mutation is an

amino acid substitution at one or a combination of the amino acids corresponding to position Gly198, Ser199, Arg200, Pro201 and Tyr218 of the alcohol dehydrogenase sequence of SEQ ID 36. In one embodiment, the alcohol dehydrogenase comprises at least one mutation wherein the at least one mutation is an amino acid substitution at the position corresponding to position Ser199 of the alcohol dehydrogenase sequence of SEQ ID 36. Skilled persons will readily appreciate the relevant position (corresponding to position 198, 199, 201 and 218 of the ADH of SEQ ID 36) in alternative alcohol dehydrogenases by aligning the sequence with that of the alcohol dehydrogenase of SEQ ID 36 according to standard procedures known in the art.

0150 In one embodiment, the alcohol dehydrogenase includes one or more of the following mutations compared to the corresponding wild type alcohol dehydrogenase: Gly198Asp, Gly198Ile, Gly198Leu, Gly198Val, Ser199Asp, Ser199Glu, Ser199Leu, Ser199Val, Arg200Glu, Pro201Asp, Pro201Glu, Tyr218Ala and Tyr218Phe.

0151 The inventors also envisage alcohol dehydrogenases of the invention including one of the following mutations compared to the corresponding wild type alcohol dehydrogenase: Tyr218Gly, Tyr218Ser and Tyr218Val. These mutations represent substitutions which are all close in size to the Tyr218Ala and Tyr218Phe substitutions exemplified in the examples section herein after.

0152 In one embodiment, the alcohol dehydrogenase includes a Ser199Asp substitution. In one embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution.

0153 In one embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu. In one embodiment, the alcohol dehydrogenase includes a combination of the following substitutions, Gly198Asp, Ser199Leu, and Pro201Glu. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ala. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Phe. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Gly. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ser. In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions: Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Val.

0154 In one embodiment, the alcohol dehydrogenase includes a Ser199Asp substitution and has an increased substrate specificity for 1) acetone over MEK and/or acetoin. In one embodiment, the alcohol dehydrogenase includes a Ser199Asp substitution and has an increased substrate specificity for acetone over MEK and acetoin.

0155 In one embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution and has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and/or acetoin; and/or 2) MEK over acetaldehyde and/or acetoin. In one embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution and has an increased substrate specificity for 1) acetone over MEK and acetaldehyde and acetoin. In another embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution and has an increased substrate specificity for MEK over acetaldehyde and acetoin. In one embodiment, the alcohol dehydrogenase includes a Ser199Glu substitution and has an increased substrate specificity for 1) acetone over MEK and acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin.

0156 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, and Pro201Glu and has an increased substrate specificity for 1) acetone over MEK; and/or 2) acetaldehyde over MEK, acetone and/or acetoin; and/or, 3) acetoin over acetone and/or MEK. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for acetaldehyde over MEK and acetone and acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for acetoin over acetone and MEK. In another embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for 1) acetone over MEK; and 2) acetaldehyde over MEK and acetone and acetoin; and 3) acetoin over acetone and MEK.

0157 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ala has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and/or acetoin; and/or 2) MEK over acetaldehyde and/or acetoin; and/or 3) acetoin over acetaldehyde. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for acetone over MEK and acetaldehyde and acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for MEK over acetaldehyde and acetoin. In another embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for 1) acetone over MEK and acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin; and 3) acetoin over acetaldehyde.

0158 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Phe and has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and/or acetoin; and/or MEK over acetaldehyde and/or acetoin; and/or 3) acetalydehyde over acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for acetone over MEK and acetaldehyde and acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for MEK over acetaldehyde and acetoin. In another embodiment, an alcohol dehydrogenase including this combination of substitutions has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin; and 3) acetalydehyde over acetoin. In one embodiment, an alcohol dehydrogenase including this combination of substitutions has substantially no ability to use acetoin as a substrate.

0159 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ala and is able to use NADH as a co-factor. In one embodiment, the alcohol dehydrogenase includes all of these substitutions and has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin; and 3) acetoin over acetaldehyde; and 4) is able to use NADH as a co-factor.

0160 In another embodiment, the alcohol dehydrogenase includes a combination of the following substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Phe and is able to use NADH as a co-factor. In one embodiment, the alcohol dehydrogenase includes all of these substitutions and has an increased substrate specificity for for 1) acetone over MEK, acetaldehyde and acetoin; and 2) MEK over acetaldehyde and acetoin; and 3) acetalydehyde over acetoin; and 5) is able to use NADH as a co-factor.

0161 In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 38. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 42. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 50.

0162 In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 44. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 46. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 48. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 52. In one

embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 54. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 63. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 64. In one embodiment, the alcohol dehydrogenase has the sequence provided in SEQ ID 65.

0163 The alcohol dehydrogenases of the invention may be made by any appropriate means known in the art including, for example, site directed mutagenesis techniques, random mutagenesis techniques, recombinant methodology and chemical synthesis, as described herein after.

0164 In some cases, mutant alcohol dehydrogenases of the invention may be naturally insoluble. These enzymes may be made soluble using standard techniques. By way of example, techniques involving, co-expression of one or more chaperones, may be used. In one particular embodiment, co-expression of the GroEL and/or GroES chaperones is employed. In one particular embodiment, use of the plasmid pGro7 (Takara Bio, Inc; clontech.com/takara/NZ/Products/Protein_Research/Protein_Folding_and_Expression/Chaperone_Plasmid_Set) may be used. This plasmid facilitates arabinose-inducible expression of the GroEL/ES chaperone proteins. Exemplary techniques are also provided in Example 2 herein after.

0165 One may assess whether an alcohol dehyrogenase of the invention has the appropriate functionality using any number of known methods. However, by way of example, the methods outlined in the Examples herein after may be used. Alternatively, the methods outlined in Ismail et al. [Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*. *J Bacteriol* 1993, 175: 5097-5105], or Khorkin et al [NADP-dependent bacterial alcohol dehydrogenases: crystal structure, cofactor-binding and cofactor specificity of the ADHs of *Clostridium beijerinckii* and *Thermoanaerobacter brockii*. *J Mol Biol.* 1998, 22: 278(5): 967-981] may be used to assess enzyme activity.

0166 Co-factor specificity may be assessed using standard methodology. However, by way of example the methods described in the "Examples" section herein after may be used.

Nucleic acids

0167 In so far as the invention relates to novel alcohol dehydrogenases, it also provides nucleic acids encoding the alcohol dehydrogenases and nucleic acid vectors comprising such nucleic acids.

0168 Skilled persons will readily appreciate the sequence of a nucleic acid encoding an alcohol dehydrogeanse of the invention, having regard to the amino acid sequence of the enzyme and the

degeneracy in the genetic code. However, by way of example only, in one embodiment, the nucleic acid has the sequence of SEQ ID 37. In other embodiment, the nucleic acid has the sequence of SEQ ID 41 or SEQ ID 49. In yet other embodiments, the nucleic acid has the sequence of SEQ ID 39, SEQ ID 43, SEQ ID 45, SEQ ID 47, SEQ ID 51, SEQ ID 53, SEQ ID 67, SEQ ID 68, SEQ ID 69 or SEQ ID 70.

0169 It should be appreciated that the nucleic acids encoding an alcohol dehydrogenase of the invention may be codon optimised for any particular microorganism.

0170 To the extent that nucleic acids, alcohol dehydrogenases and microorganisms of the invention may be made and used using recombinant technology, the invention also provides nucleic acid vectors comprising one or more nucleic acid encoding one or more alcohol dehydrogenase of the invention.

0171 The nucleic acids of the invention may remain extra-chromosomal upon transformation of a microorganism or may be adapted for integration into the genome of the microorganism. Accordingly, nucleic acids of the invention may include additional nucleotide sequences adapted to assist integration (for example, a region which allows for homologous recombination and targeted integration into the host genome) or stable expression and replication of an extrachromosomal construct (for example, origin of replication, promoter and other regulatory sequences).

0172 In one embodiment, the nucleic acids encoding one or more alcohol dehydrogenase of the invention will comprise a promoter adapted to promote expression of the one or more enzymes encoded by the nucleic acids. In one embodiment, the promoter is a constitutive promoter that is preferably highly active under appropriate fermentation conditions. Inducible promoters could also be used. In preferred embodiments, the promoter is selected from the group comprising Wood-Ljungdahl gene cluster or an arabinose inducible pBAD promoter. It will be appreciated by those of skill in the art that other promoters which can direct expression, preferably a high level of expression under appropriate fermentation conditions, would be effective as alternatives to the exemplified embodiments.

0173 Nucleic acids and nucleic acid constructs, including expression constructs/vectors of the invention may be constructed using any number of techniques standard in the art. For example, chemical synthesis, site directed mutagenesis, or recombinant techniques may be used. Such techniques are described, for example, in Sambrook et al (Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Further exemplary techniques are described in the Examples section herein after. Essentially, the individual genes and regulatory elements will be operably linked to one another such that the genes can be expressed to form the desired proteins. Suitable vectors for use in the invention will be appreciated by those of

ordinary skill in the art. However, by way of example, the following vectors may be suitable: pBAD or pMTL80000 vectors, and the plasmids exemplified in the Examples section herein after.

0174 The invention also provides host organisms, particularly microorganisms, and including viruses, bacteria, and yeast, comprising any one or more of the nucleic acids described herein.

Microorganisms

0175 The invention also provides microorganisms which are capable of producing one or more of isopropanol, MEK, 2,3-butanediol and 2-butanol and optionally one or more other products, by fermentation of a substrate and which comprise at least one nucleic acid of the invention.

0176 The microorganisms of the invention may be prepared from a parental microorganism using any number of techniques known in the art, including, for example, site directed mutagenesis techniques to introduce the desired mutation(s) into an alcohol dehydrogenase gene native to a parental microorganism, or other recombinant technologies to introduce one or more nucleic acid encoding one or more alcohol dehydrogeanse of the invention into a parental microorganism.

0177 In one embodiment, one or more exogenous nucleic acid encoding one or more alcohol dehydrogeanse is introduced into a parental microorganism and replaces one or more alcohol dehydrogenase gene native to the parental microorganism. In another embodiment, one or more exogenous nucleic acid encoding one or more alcohol dehydrogeanse of the invention is introduced to a parental microorganism and is supplementary to an alcohol dehydrogenase gene native to the parental microorganism. In other embodiments, one or more exogenous nucleic acid is introduced into a parental microorganism to introduce one or more desired mutation into one or more alcohol dehydrogenase gene native to the parental microorganism. In another embodiment, one or more exogenous nucleic acid encoding one or more alcohol dehydrogeanse is introduced into a parental microorganism, and one or more mutation is introduced to one or more alcohol dehydrogenase gene native to the parental microorganism to reduce or knock out its expression and activity.

0178 In one embodiment, a microorganism of the invention is prepared from a parental microorganism using recombinant technology. For example, a parental microorganism is transformed with one or more exogenous nucleic acid encoding an alcohol dehydrogeanse of the invention, or one or more nucleic acid adapted to introduce a desired mutation to a native alcohol dehydrogenase gene in the parental microorganism. An exogenous nucleic acid may remain extra-chromosomal upon transformation of the parent microorganism or may integrate into the genome of the parent microorganism (in one embodiment to replace a native alcohol dehydrogenase gene, or introduce a

mutation into a native alcohol dehyrogenase gene). Accordingly, they may include additional nucleotide sequences adapted to assist integration (for example, a region which allows for homologous recombination and targeted integration into the host genome) or expression and replication of an extrachromosomal construct (for example, origin of replication, promoter and other regulatory elements or sequences), as described herein before.

0179 By way of example only, transformation (including transduction or transfection) of a microorganism may be achieved by electroporation, ultrasonication, polyethylene glycol-mediated transformation, chemical or natural competence, or conjugation. Suitable transformation techniques are described for example in, Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, 1989.

0180 One or more exogenous nucleic acids may be delivered to a parental microorganism as naked nucleic acids or may be formulated with one or more agents to facilitate the transformation process (for example, liposome-conjugated nucleic acid, an organism in which the nucleic acid is contained). The one or more nucleic acids may be DNA, RNA, or combinations thereof, as is appropriate. Restriction inhibitors may be used in certain embodiments; see, for example Murray, N.E. *et al.* (2000) *Microbial. Molec. Biol. Rev.* 64, 412.)

0181 In one embodiment, the parental microorganism is a bacteria, Archae, and fungi.

0182 In one embodiment, the parental microorganism is chosen from genera *Clostridium, Acetobacterium, Moorella, Butyribacterium, Blautia, Oxobacter, Thermoanaerobacter, Escherichia, Klebsiella, Zymomonas, Citrobacter, Enterobacter, Salmonella, Serratia, Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Streptococcus, Saccharomyces, Pichia, Candida Hansenula, Yarrowia, Rhodotorula, Rhizopus, Trichosporon, Lipomyces, Aspergillus, trichoderma, Exophila, Mucor, Cladosporium, Phanerochaete, Cladiophilalophora, Paecilomyces, Scedosporium, Ophistoma, Bacillus, Oligotropha, Pseudomonas, Carbophilus, Hydrogenophaga, Mycobacterium, Zavarzinia, Cupravidus, Senechocystis, Chloroflexus, Methylomonas, Methylobacter, Methylococcus, Methylomicrobium, Methylosphera, Methylocaldum, Methylocystis, Methylosinus, Methanobacterium, Methanococcus, Methanogenium, Methanosarcina, Methanoshera, Methanothermobacter, Methanotrix, Corynebacterium, Acinetobacter, Actinomyces, Bacteriodes, Burkholderia, Brevibacterium, Pyrococcus, Geobacter, Geobacillus, Paenibacillus, Mycobacterium, Rhodopseudomonas, Thermatoga, Thermoanaerobacter, Streptomyces, Rhodobacter, Rhodococcus, Peptococcus, Bifidobacterium, Propionibacterium, Fusobacterium, Campylobacter, Veillonella, Aquincola, Arthrobacter, Moraxella,* and *Psychrobacter.*

0183 In one embodiment the parental microorganism is chosen from the group of carboxydotrophic acetogenic microorganisms, the group of ABE microorganisms, the group of Enterobacteria, the group of Lactobacillus, the group of fungi and yeasts, the group of aerobic carboxydotrophes, the group of aerobic CO_2 fixing organisms, the group of methylotrophes, and the group of methanogens.

0184 In one embodiment, the parental microorganism is selected from the group of carboxydotrophic acetogenic bacteria. In certain embodiments the microorganism is selected from the group comprising *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium scatologenes, Clostridium coskatii, Clostridium aceticum, Clostridium magnum, Clostridium sp., Butyribacterium limosum, Butyribacterium methylotrophicum, Acetobacterium woodii, Alkalibaculum bacchii, Blautia producta, Eubacterium limosum, Moorella thermoacetica, Moorella thermautotrophica, Oxobacter pfennigii,* and *Thermoanaerobacter kiuvi.*

0185 These carboxydotrophic acetogens are defined by their ability to utilize and grow chemoautotrophically on gaseous one-carbon (C1) sources such as carbon monoxide (CO) and carbon dioxide (CO2) with carbon monoxide (CO) and/or hydrogen (H2) as energy source under anaerobic conditions forming acetyl-CoA, acetate and other products. They share the same mode of fermentation, the Wood-Ljungdahl or reductive acetyl-CoA pathway, and are defined by the presence of the enzyme set consisting of Carbon monoxide dehydrogenase (CODH), Hydrogenase, Formate dehydrogenase, Formyl-tetrahydrofolate synthetase, Methylene-tetrahydrofolate dehydrogenase, Formyl-tetrahydrofolate cyclohydrolase, Methylene-tetrahydrofolate reductase, and Carbon monoxide dehydrogenase/Acetyl-CoA synthase (CODH/ACS), which combination is characteristic and unique to this type of bacteria (Drake, Küsel, Matthies, Wood, & Ljungdahl, 2006).In contrast to chemoheterotrophic growth of sugar-fermenting bacteria that convert the substrate into biomass, secondary metabolites and pyruvate from which products are formed (either via acetyl-CoA or directly), in acetogens the substrate is channelled directly into acetyl-CoA, from which products, biomass, and secondary metabolites are formed.

0186 In a one embodiment, the microorganism is selected from a cluster of carboxydotrophic Clostridia comprising the species *C. autoethanogenum*, *C. ljungdahlii*, and "*C. ragsdalei*" and related isolates. These include but are not limited to strains *C. autoethanogenum* JAI-1^T (DSM10061) (Abrini, Naveau, & Nyns, 1994), *C. autoethanogenum* LBS1560 (DSM19630) (WO/2009/064200), *C. autoethanogenum* LBS1561 (DSM23693), *C. ljungdahlii* PETC^T (DSM13528 = ATCC 55383) (Tanner, Miller, & Yang, 1993), *C. ljungdahlii* ERI-2 (ATCC 55380) (US patent 5,593,886), *C.*

ljungdahlii C-01 (ATCC 55988) (US patent 6,368,819), *C. ljungdahlii* O-52 (ATCC 55989) (US patent 6,368,819), or "*C. ragsdalei* P11[T]" (ATCC BAA-622) (WO 2008/028055), and related isolates such as "*C. coskatii*" (US patent 2011/0229947), *"Clostridium sp.* MT351*"* (Tyurin & Kiriukhin, 2012), and mutant strains thereof such as *C. ljungdahlii* OTA-1 (Tirado-Acevedo O. Production of Bioethanol from Synthesis Gas Using *Clostridium ljungdahlii*. PhD thesis, North Carolina State University, 2010) or *"Clostridium sp.* MT896*"* (Berzin, Kiriukhin, & Tyurin, 2012).

0187 These strains form a subcluster within the Clostridial rRNA cluster I (Collins et al., 1994), having at least 99% identity on 16S rRNA gene level, although being distinct species as determined by DNA-DNA reassociation and DNA fingerprinting experiments (WO 2008/028055, US patent 2011/0229947).

0188 The strains of this cluster are defined by common characteristics, having both a similar genotype and phenotype, and they all share the same mode of energy conservation and fermentative metabolism. The strains of this cluster lack cytochromes and conserve energy via an Rnf complex.

0189 All strains of this cluster have a genome size of around 4.2 MBp (Köpke et al., 2010) and a GC composition of around 32 %mol (Abrini et al., 1994; Köpke et al., 2010; Tanner et al., 1993) (WO 2008/028055; US patent 2011/0229947), and conserved essential key gene operons encoding for enzymes of Wood-Ljungdahl pathway (Carbon monoxide dehydrogenase, Formyl-tetrahydrofolate synthetase, Methylene-tetrahydrofolate dehydrogenase, Formyl-tetrahydrofolate cyclohydrolase, Methylene-tetrahydrofolate reductase, and Carbon monoxide dehydrogenase/Acetyl-CoA synthase), hydrogenase, formate dehydrogenase, Rnf complex (*rnfCDGEAB*), pyruvate:ferredoxin oxidoreductase, aldehyde:ferredoxin oxidoreductase (Köpke et al., 2010, 2011). The organization and number of Wood-Ljungdahl pathway genes, responsible for gas uptake, has been found to be the same in all species, despite differences in nucleic and amino acid sequences (Köpke et al., 2011).

0190 The strains all have a similar morphology and size (logarithmic growing cells are between 0.5-0.7 x 3-5 μm), are mesophilic (optimal growth temperature between 30-37 °C) and strictly anaerobe (Abrini et al., 1994; Tanner et al., 1993)(WO 2008/028055). Moreover, they all share the same major phylogenetic traits, such as same pH range (pH 4-7.5, with an optimal initial pH of 5.5-6), strong autotrophic growth on CO containing gases with similar growth rates, and a metabolic profile with ethanol and acetic acid as main fermentation end product, with small amounts of 2,3-butanediol and lactic acid formed under certain conditions (Abrini et al., 1994; Köpke et al., 2011; Tanner et al., 1993)(WO differentiate in substrate utilization of various sugars (e.g. rhamnose, arabinose), acids (e.g. gluconate, citrate), amino acids (e.g. arginine, histidine), or other substrates (e.g. betaine,

butanol). Some of the species were found to be auxotroph to certain vitamins (e.g. thiamine, biotin) while others were not. Reduction of carboxylic acids into their corresponding alcohols has been shown in a range of these organisms (Perez, Richter, Loftus, & Angenent, 2012).

0191 The traits described are therefore not specific to one organism like *C. autoethanogenum* or *C. ljungdahlii*, but rather general traits for carboxydotrophic, ethanol-synthesizing Clostridia. Thus, the invention can be anticipated to work across these strains, although there may be differences in performance.

0192 In one embodiment, the parental strain uses CO as its sole carbon and energy source.

0193 In certain embodiments, the parental microorganism is selected from the group comprising *Clostridium autoethanogenum, Clostridium ljungdahlii,* and *Clostridium ragsdalei.* In one embodiment, the group also comprises *Clostridium coskatii.* In one particular embodiment, the microorganism is *Clostridium autoethanogenum* DSM10061 or DSM23693. In another particular embodiment, the microorganism is *Clostridium ljungdahlii* DSM13528 (or ATCC55383).

0194 In one embodiment, the parental microorganism is an ABE fermenting microorganism. An "ABE fermenting microorganism" or "ABE microorganism" is a Gram-positive, Clostridial organism which is able to produce the solvents butanol, and ethanol, and acetone or isopropanol. Genera in this group include *Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharobutylicum*, and *Clostridium saccharoperbutylacetonicum*. These organisms are all sporulating, Gram-positive and within the Clostridial rRNA cluster I. This group has been described in detail by Keis et al. (Keis, Shaheen, & Jones, 2001).

0195 In one particular embodiment, the ABE microorganism is selected from the group comprising *Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharobutylicum, Clostridium saccharoperbutylacetonicum.*

0196 In one embodiment the parental microorganism is *Clostridium acetobutylicum* or *Clostridium beijerinckii*. In one particular embodiment, the microorganism is *Clostridium acetobutylicum* ATCC824 (DSM792) or EA 2018 (CCTCC M 94061). In another particular embodiment, the microorganism is *Clostridium beijerinckii* NCIMB8052 (ATCC51743) and NRRL B-593 (DSM 6423).

0197 In one embodiment, the parental microorganism is an Enterobacteria. An Enterobacteria is a rod-shaped Gram-negative bacteria belonging to the order of *Enterobacteriacea* which is able to

fermenting sugars to produce lactic acid, and/or ethanol, and/or acetoin, and/or 2,3-butabediol, and/or other products.

0198 In one particular embodiment, the Enterobacteria is selected from the group comprising *Escherichia, Klebsiella, Zymomonas, Citrobacter, Enterobacter, Salmonella, Serratia.* In one embodiment the parental microorganism is *Eschericia coli, Zymononas mobilis, Klebsiella pneumonia, Klebsiella oxtoca*, *Enterobacter cloacae* or *Serratia marcescens*.

0199 In one embodiment, the parental microorganism is a Lactobacillus. A Lactobacillus is a gram-positive lactic acid bacteria selected from the order of *Lactobacillales* which is able to fermenting sugars to produce lactic acid, and/or 2,3-butabediol, and/or MEK, and/or 2-butanol, and/or other products.

0200 In one particular embodiment, the Lactobacillus is selected from the group comprising *Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Streptococcus.* In one embodiment the parental microorganism is *Lactobacillus brevis, Enterococcus faecalis, Lactococcus lactis*.

0201 In one embodiment, the parental microorganism is a fungi or yeast. Fungi are eukaryotic microorganisms, and yeast are a specific subset thereof, which are able to ferment sugars to ethanol and/or acetoin, and/or other products.

0202 In one particular embodiment, the Fungi is selected from the group comprising *Aspergillus, Trichoderma, Exophila, Mucor, Cladosporium, Phanerochaete, Cladiophilalophora, Paecilomyces, Scedosporium, Ophistoma.* In one embodiment the parental microorganism is *Aspargillus niger* or *Trichderma resei.*

0203 In one particular embodiment, the yeast is selected from the group comprising *Saccharomyces, Pichia, Candida Hansenula, Yarrowia, Rhodotorula, Rhizopus, Trichosporon, Lipomyces* and from the group comprising *Aspergillus, Trichoderma, Exophila, Mucor, Cladosporium, Phanerochaete, Cladiophilalophora, Paecilomyces, Scedosporium, Ophistoma.* In one embodiment the parental microorganism is *Saccharomyces cerevisiae*, *Candidia tropicalis, Candidia albicans* or *Yarrowia lipolytica*. In one embodiment the parental microorganism is *Aspargillus niger* or *Trichderma resei.*

0204 In one embodiment, parental the microorganism is an aerobic carboxydotroph. Aerobic carboxydotrophes are bacteria that can be found ubiquitous in nature and have been isolated from

various environments as well as humans (King and Weber, 2007). On taxonomic level, this physiological group is quite diverse, comprising of different phyla such as α-proteobacteria, firmicutes, or actinobacteria (King and Weber, 2007). All these organisms were shown to grown on CO levels > 1 % in presence of air (King and Weber, 2007). A typical gas mix consists of 50 % CO and 50 % air (Cypionka et al., 1980).

0205 In a particular embodiment, the parental microorganism is selected from the group comprising *Bacillus, Oligotropha, Pseudomonas, Carbophilus, Hydrogenophaga, Mycobacterium, Zavarzinia.* In one embodiment the parental microorganism is *Oligotropha carboxydovorans*, *Carbophilus carboxidus*, *Hydrogenophaga pseudoflava*, *Mycobacterium sp.*, *Pseudomonas carboxydohydrogena*, *Pseudomonas sp.*, *Zavarzinia compransoris* or *Bacillus schlegelii.*

0206 In one embodiment, the parental microorganism is an aerobic CO_2 fixing organism. An aerobic CO2 fixing microorganism is an bacteria able to fix CO2 with H2 or via photosynthesis in present of oxygen. The aerobic CO2 fixing microorganism is selected from the group comprising *Cupravidus, Senechocystis, Chloroflexus.* In one embodiment the parental microorganism is *Cupravidus necator, Senechocystis sp.* or *Chloroflexus auranticus.*

0207 In one embodiment, the parental microorganism is a methylotroph. Methylotrophic microrgansims are able to use reduced one-carbon substrates as methane or methanol as carbon source for growth. The methylotrop is selected from the group comprising *Methylomonas, Methylobacter, Methylococcus, Methylomicrobium, Methylosphera, Methylocaldum, Methylocystis, Methylosinus.* In one embodiment the parental microorganism is *Methylococcus capsulatus* or *Methylosinus trichosporium.*

0208 In one embodiment, the parental microorganism is a methanogen. A methanogen is an Archeae that can reduce CO2 into methane. The methanogen is selected from the group comprising *Methanobacterium, Methanococcus, Methanogenium, Methanosarcina, Methanoshera, Methanothermobacter, Methanotrix.* In one embodiment the parental microorganism is

0209 *Methanothermobacter marburgensis* or *Methanosarcina bakeri.*

Methods

0210 The invention provides a method for the production of isopropanol, ethanol, 2,3-butanediol and/or 2-butanol and optionally one or more other products by microbial fermentation of a substrate using a recombinant microorganism of the invention.

0211 In another embodiment, the invention provides a method for the production of one or more of acetoin, MEK, acetaldehyde and acetone, and optionally one or more other products.

0212 In one embodiment, the substrate is a substrate comprising one or more carbohydrate. In another embodiment, the substrate is a substrate comprising one or a combination of CO, CO_2, and H_2. In certain embodiments, mixed substrates comprising both one or more carbohydrate and a substrate comprising one or more of CO, CO_2, and H_2 may be used.

0213 In one embodiment the method comprises the steps of:

(a) providing a substrate to a bioreactor containing a culture of one or more microorganism of the invention; and

(b) fermenting the culture in the bioreactor to produce one or more of isopropanol, 2,3-butanediol, ethanol and 2-butanol and, optionally, one or more other products.

0214 Preferably, the one or more product includes isopropanol.

0215 In one embodiment the method comprises the steps of:

(c) providing a substrate to a bioreactor containing a culture of one or more microorganism of the invention; and

(d) fermenting the culture in the bioreactor to produce one or more of acetoin, MEK, acetaldehyde and acetone and, optionally, one or more other products.

0216 The method may further comprise the step of recovering one or more products. In certain embodiments, the one or more products are intermediates in the production of one or more downstream products. In this embodiment, the one or more products may be recovered and then used as a substrate in a separate fermentation or in a chemical synthesis reaction, for example. In another embodiment, the one or more products are not recovered and are converted to one or more downstream products in the same fermentation process.

0217 It will be appreciated that for growth of the microorganism and conversion of substrate-to-the one or more product(s) to occur, in addition to the substrate, a suitable liquid nutrient medium will need to be fed to the bioreactor. The substrate and media may be fed to the bioreactor in a continuous, batch or batch fed fashion. A nutrient medium will contain vitamins and minerals sufficient to permit growth of the micro-organism used. Suitable media for fermentation will be known in the art. However, by way of example, for fermentation of a substrate comprising one or more carbohydrate, Luria Broth (LB), Yeast Extract Peptone Dextrose (YEPD) or reinforced

clostridia media (RCM) may be used. In addition, anaerobic media suitable for fermentation using CO are known in the art but by way of example, suitable media are described Biebel (2001). n one embodiment of the invention the media is as described in the Examples section herein after.

0218 The fermentation should desirably be carried out under appropriate conditions for the substrate-to-the one or more product(s), and optionally one or more other product(s), fermentation to occur. Reaction conditions that should be considered include pressure, temperature, gas flow rate, liquid flow rate, media pH, media redox potential, agitation rate (if using a continuous stirred tank reactor), inoculum level, maximum substrate concentrations to ensure that the substrate does not become limiting, and maximum product concentrations to avoid product inhibition.

0219 As noted above, fermentations will be carried out using appropriate media and fermentation conditions. In one embodiment, where gaseous substrates are used, maximum gas substrate concentrations are considered to ensure that CO (and/or CO_2 and/or H_2) in the liquid phase does not become limiting.

0220 In addition, it is often desirable to increase the CO (and/or CO_2 and/or H_2) concentration of a substrate stream (or CO (and/or CO_2 and/or H_2) partial pressure in a gaseous substrate) and thus increase the efficiency of fermentation reactions where CO (and/or CO_2 and/or H_2) is a substrate. Operating at increased pressures allows a significant increase in the rate of CO (and/or CO_2 and/or H_2) transfer from the gas phase to the liquid phase where it can be taken up by the microorganism as a carbon source for the production of one or more products. This in turn means that the retention time (defined as the liquid volume in the bioreactor divided by the input gas flow rate) can be reduced when bioreactors are maintained at elevated pressure rather than atmospheric pressure. The optimum reaction conditions will depend partly on the particular micro-organism of the invention used. However, in general, it is preferred that the fermentation be performed at pressure higher than ambient pressure. Also, since a given CO (and/or CO_2 and/or H_2)-to-the one or more product(s) conversion rate is in part a function of the substrate retention time, and achieving a desired retention time in turn dictates the required volume of a bioreactor, the use of pressurized systems can greatly reduce the volume of the bioreactor required, and consequently the capital cost of the fermentation equipment. According to examples given in US patent no. 5,593,886, reactor volume can be reduced in linear proportion to increases in reactor operating pressure, i.e. bioreactors operated at 10 atmospheres of pressure need only be one tenth the volume of those operated at 1 atmosphere of pressure.

0221 By way of example, the benefits of conducting a gas-to-ethanol fermentation at elevated pressures has been described. For example, WO 02/08438 describes gas-to-ethanol fermentations

performed under pressures of 30 psig and 75 psig, giving ethanol productivities of 150 g/l/day and 369 g/l/day respectively. However, example fermentations performed using similar media and input gas compositions at atmospheric pressure were found to produce between 10 and 20 times less ethanol per litre per day.

0222 It is also desirable that the rate of introduction of the CO (and/or CO_2 and/or H_2)-containing gaseous substrate is such as to ensure that the concentration of CO (and/or CO_2 and/or H_2) in the liquid phase does not become limiting. This is because a consequence of CO (and/or CO_2 and/or H_2)-limited conditions may be that the product(s) is consumed by the culture.

0223 The composition of gas streams used to feed a fermentation reaction can have a significant impact on the efficiency and/or costs of that reaction. For example, O_2 may reduce the efficiency of an anaerobic fermentation process. Processing of unwanted or unnecessary gases in stages of a fermentation process before or after fermentation can increase the burden on such stages (e.g. where the gas stream is compressed before entering a bioreactor, unnecessary energy may be used to compress gases that are not needed in the fermentation). Accordingly, it may be desirable to treat substrate streams, particularly substrate streams derived from industrial sources, to remove unwanted components and increase the concentration of desirable components.

0224 In certain embodiments a culture of a microorganism of the invention is maintained in an aqueous culture medium. Preferably the aqueous culture medium is a minimal anaerobic microbial growth medium. Suitable media are known in the art and described for example in US patent no.s 5,173,429 and 5,593,886 and WO 02/08438, and as described in the Examples section herein after.

0225 In embodiments of the invention comprising fermentation of a substrate comprising CO the fermentation comprises the steps of anaerobically fermenting the substrate in a bioreactor to produce the one or more products using a recombinant microorganism of the invention.

0226 Methods of this embodiment may be used to reduce the total atmospheric carbon emissions from an industrial process.

0227 In one embodiment the method comprises the steps of:

(a) providing a substrate comprising CO to a bioreactor containing a culture of one or more microorganism of the invention; and

(b) anaerobically fermenting the culture in the bioreactor to produce one or more of isopropanol, 2,3-butanediol, ethanol and 2-butanol and, optionally, one or more other products.

0228 In another embodiment, the one or more products in step (b) above are acetoin, MEK, acetaldehyde and acetone, and optionally, one or more other products.

0229 In one embodiment the method comprises the steps of:

0230 capturing CO-containing gas produced as a result of the industrial process, before the gas is released into the atmosphere;

0231 the anaerobic fermentation of the CO-containing gas to produce one or more of isopropanol, 2,3-butanediol, ethanol and 2-butanol and optionally one or more other products by a culture containing one or more microorganism of the invention.

0232 In another embodiment, the one or more products in step (b) above are acetoin, MEK, acetaldehyde and acetone, and optionally, one or more other products.

0233 In an embodiment of the invention, the gaseous substrate fermented by the microorganism is a gaseous substrate containing CO. The gaseous substrate may be a CO-containing waste gas obtained as a by-product of an industrial process, or from some other source such as from automobile exhaust fumes. In certain embodiments, the industrial process is selected from the group consisting of ferrous metal products manufacturing, such as a steel mill, non-ferrous products manufacturing, petroleum refining processes, gasification of coal, electric power production, carbon black production, ammonia production, methanol production and coke manufacturing. In these embodiments, the CO-containing gas may be captured from the industrial process before it is emitted into the atmosphere, using any convenient method. The CO may be a component of syngas (gas comprising carbon monoxide and hydrogen). The CO produced from industrial processes is normally flared off to produce CO_2 and therefore the invention has particular utility in reducing CO_2 greenhouse gas emissions and producing butanol for use as a biofuel. Depending on the composition of the gaseous CO –containing substrate, it may also be desirable to treat it to remove any undesired impurities, such as dust particles before introducing it to the fermentation. For example, the gaseous substrate may be filtered or scrubbed using known methods.

0234 Skilled persons will readily appreciate various methodology of use in fermentations using substrates comprising one or more carbohydrates. However, by way of example, the methodology described in Vogel, H. C., & Todaro, C. C. (2007). Fermentation and Biochemical Engineering Handbook: Principles, process design and equipment (ISBN: 0-8155-1407-7); Vogel, H. C., & Todaro, C. C. (1996). Fermentation and Biochemical Engineering Handbook (ISBN: 978-0-8155-1407-7); Ezeji TC, Qureshi N, Blaschek HP (2005) Industrial relevant fermentations. in *Handbook on Clostridia*, ed Dürre P (CRC Press, Boca Raton, FL), pp 799–814 may be used.

Product recovery

0235 Isopropanol, 2,3-butanediol, ethanol, 2-butanol, acetoin, MEK, acetaldehyde and/or acetone or a mixed stream containing any one or more of these products and acetone and optionally one or more other products, may be recovered from the fermentation broth by methods known in the art, such as fractional distillation or evaporation, pervaporation, gas stripping and extractive fermentation, including for example, liquid-liquid extraction.

0236 In certain preferred embodiments of the invention, the one or more products are recovered from the fermentation broth by continuously removing a portion of the broth from the bioreactor, separating microbial cells from the broth (conveniently by filtration), and recovering one or more products from the broth. Alcohols may conveniently be recovered for example by distillation. Acetone may be recovered for example by distillation. Any acids produced may be recovered for example by adsorption on activated charcoal. The separated microbial cells are preferably returned to the fermentation bioreactor. The cell free permeate remaining after any alcohol(s) and acid(s) have been removed is also preferably returned to the fermentation bioreactor. Additional nutrients (such as B vitamins) may be added to the cell free permeate to replenish the nutrient medium before it is returned to the bioreactor.

0237 Also, if the pH of the broth was adjusted as described above to enhance adsorption of acetic acid to the activated charcoal, the pH should be re-adjusted to a similar pH to that of the broth in the fermentation bioreactor, before being returned to the bioreactor.

EXAMPLES

Example 1 – Characterisation of wild-type alcohol dehydrogenase of *C. autoethanogenum* and range of substrate specificity with single amino acid substitutions

0238 Several species of *Clostridium,* including *C. ljungdahlii* (Köpke, *et al.*, 2010) have been shown to utilise CO as a sole carbon source, with ethanol as the end product. The bacteria are able to fix CO and convert it to acetyl-CoA via the Wood-Ljungdahl pathway. The acetyl moiety of the acetyl CoA can then be used in a variety of metabolic pathways. Of particular interest here the carbonyl group can be reduced to its corresponding alcohol by an alcohol dehydrogenase (ADH) enzyme (Köpke, *et al.*, 2010). This provides a pathway to convert CO into commercially valuable biofuels and biochemicals.

0239 Genome sequencing of a strain of *C. autoethanogenum* DSM10061 identified an ADH that is 86% identical to the previously-characterized enzyme from *C. beijerinckii.* The ADH

from this strain is able to utilise acetaldehyde as its substrate and produce ethanol as the end product. It is also able to catalyse the reduction of acetone to isopropanol.

0240 Isopropanol is a more economically valuable final product than ethanol, as it can be dehydrated to form propylene, which can be polymerised to polypropylene, a commonly used plastic (Inokuma *et al.*, 2010). A microbial route to propylene will also decrease the demand for petroleum, from which most propylene is currently derived.

0241 Mutagenesis studies were completed with the aim of improving the efficiency of isopropanol production via the ADH enzyme. Five mutants were constructed and tested: Ser199Asp, Ser199Glu, Arg200Gln, Arg200Glu and a double mutant Ser199Glu/Arg200Gln.

Materials

Microorganisms and Growth conditions

0242 *E. coli* DH5α-E was obtained from Invitrogen. The genotype of this strain is: F– 80ΔlacZM15 (lacZYA-argF) U169 recA1 endA1 hsdR17(rk–, mk+) gal– phoA supE44 – thi–1 gyrA96 relA1.

0243 *E. coli* LMG194 was obtained from Invitrogen. The genotype of this strain is: F- ΔlacX74 galE thi rpsL ΔphoA (Pvu II) Δara714 leu::Tn10.

0244 *E. coli* MC1061 was obtained from Coli Genetic Stock Centre. The genotype of this strain is araD139 Δ (araA-leu)7697 Δ (lac)X74 galK16 galE15(GalS) lambda- e14- mcrA0 relA1 rpsL150(strR) spoT1 mcrB1 hsdR2.

0245 *Clostridium autoethanogenum* DSM10061 was obtained from DSMZ (The German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7 B, 38124 Braunschweig, Germany).

0246 *C. acetobutylicum* ATCC824 and *C. beijerinckii* NCIMB8052 were obtained from Prof. David Jones (University of Otago) and can also be obtained from public strain collections DSMZ and ATCC under accession numbers ATCC824/DSM792 and ATCC51743 respectively.

0247 All *E. coli* strains were cultivated in aerobic conditions, using Luria Burtani medium supplmented with either ampicillin (100 μg/mL) or carbenicillin (50 μg/mL). [098] Solid media contained 1.5% agar. All strains were grown at 37°C unless otherwise noted.

0248 SOC medium(20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl and 20 mM glucose) was used for recovery of *E. coli* after electroporation.

0249 *Clostridium autoethanogenum* was grown in PETC media with pH5.6 (Tab. 1) and *C. acetobutylicum* and *C. beijerinckii* in RCM media (Tab. 2) using standard anaerobic techniques [Hungate RE: A roll tube method for cultivation of strict anaerobes, in Norris JR and Ribbons DW (eds.), Methods in Microbiology, vol. 3B. Academic Press, New York, 1969: 117-132; Wolfe RS: Microbial formation of methane. *Adv Microb Physiol* 1971, 6: 107-146].

Table 1: PETC medium

Media component	**Concentration per 1.0L of media**
NH_4Cl	1 g
KCl	0.1 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.8 g
KH_2PO_4	0.1 g
$CaCl_2$	0.02 g
Trace metal solution (see below)	10 ml
Wolfe's vitamin solution (see below)	10 ml
Yeast Extract (optional)	1 g
Resazurin (2 g/L stock)	0.5 ml
$NaHCO_3$	2 g
Reducing agent	0.006-0.008 % (v/v)
Fructose (for heterotrophic growth)	5 g
Trace metal solution	**per L of stock**
Nitrilotriacetic Acid	2 g
$MnSO_4.H_2O$	1 g
$Fe(SO_4)_2(NH_4)_2.6H_2O$	0.8 g
$CoCl_2.6H_2O$	0.2 g
$ZnSO_4.7H_2O$	0.2 mg
$CuCl_2.2H_2O$	0.02 g
$NaMoO_4.2H_2O$	0.02 g
Na_2SeO_3	0.02 g

$NiCl_2.6H_2O$	0.02 g
$Na_2WO_4.2H_2O$	0.02 g
Reducing agent stock	**per 100 mL of stock**
NaOH	0.9 g
Cystein.HCl	4 g
Na_2S	4 g

Table 2: Reinforced Clostridial Medium RCM (*C. acetobutylicum, C. beijerinckii*)

Media component	**Concentration per 1.0L of media**
Pancreatic Digest of Casein	5 g
Proteose Peptone No. 3	5 g
Beef Extract	10 g
Yeast Extract	3 g
Dextrose	5 g
NaCl	5 g
Soluble starch	1 g
Cystein.HCl	0.5 g
Sodium Acetate	3 g

ADH gene and protein

0250 The amino acid and nucleic acid sequence of the wild-type ADH of *C. autoethanogenum* DSM10061 are shown in SEQ ID 36 and SEQ ID 35, respectively.

Primers

Table 3 – Oligonucleotides for amplification and site directed mutagenesis.

Primer name	Primer sequence (5' to 3')	SEQ ID No.
ADH_BspHI_for	GGTAATCATGAAAGGTTTTGCAATGTTAGGTATTAAC	1
ADH_HindIII_rev	TCTAGAAGCTTAGAATGTAACTACTGATTTAATTAAATCTTTTGG	2
pBAD_for	ATGCCATAGCATTTTTATCC	3
ADH_TEV_KpnI_for	CAGGTACCGAGAACCTGTATTTCCAAGGAAAAGGTTTTGCAATGTTAGGTATTAAC	4
ADH_Arg200Glu_rev	CACAAACAGGTTCGCTTCCAACACCG	5
ADH_Arg200Glu_for	CGGTGTTGGAAGCGAACCTGTTTGTG	6
ADH_Ser199Glu_rev	CAAACAGGTCTTTCTCCAACACCG	7
ADH_Ser199Glu_for	CGGTGTTGGAGAAAGACCTGTTTG	8
ADH_Ser199Glu_Arg200Gl	CACAAACAGGCTGTTCTCCAACACCG	9

n_rev		
ADH_Ser199Glu_Arg200Gln_for	CGGTGTTGGAGAACAGCCTGTTTGTG	10
ADH_Ser199Asp_rev	CAAACAGGTCTGTCTCCAACACCG	11
ADH_Ser199Asp_for	CGGTGTTGGAGACAGACCTGTTTG	12
ADH_Arg200Gln_for	CGGTGTTGGAAGCCAGCCTGTTTGTG	13
ADH_Arg200Gln_rev	CACAAACAGGCTGGCTTCCAACACCG	14

Plasmids

0251 pMTL85147-ThlA-CtfAB-Adc-Adh (Fig. 8) was used for amplification of *C. autoethanogenum* DSM10061 ADH gene.

0252 Plasmid pMTL85147-ThlA-CtfAB-Adc-Adh has been constructed usingstandard Recombinant DNA and molecular cloning techniques [Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A laboratory Manual, Cold Spring Harbour Labrotary Press, Cold Spring Harbour, 1989; Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: Current protocols in molecular biology. John Wiley & Sons, Ltd., Hoboken, 1987]. *ThlA* gene (NC_003030.1; GI: 1119056) was amplified from genomic DNA of *C. acetobuylicum*, genes *adc-ctfAB-adc* (NC_009617; region: 4,400,524-4,402,656; including GI: 5294994, GI: 5294995, and GI: 5294996) were amplified from *C. beijerinckii*, and *adh* gene (Fig. 5) and Wood-Ljungdahl promoter region (SEQ ID 39) were amplified from *C. autoethanogenum* DSM10061. Oligonucleotide sequences used for amplification are given in Tab. 4. All amplified fragments were subsequently cloned into plasmid pMTL 85141 (FJ797651.1; Nigel Minton, University of Nottingham, UK) [Heap JT, Pennington OJ, Cartman ST, Minton NP. A modular system for Clostridium shuttle plasmids. J Microbiol Methods. 2009, 78: 79-85] using restriction sites NotI, NdeI, EcoRI, KpnI, BamHI, SalI, XhoI. The final plasmid is given in SEQ ID 40 and has been sequenced to ensure it's free of mutations.

0253 Genomic DNA from *Clostridium acetobutylicum* ATCC824, *C. beijerinckii* NCIMB8052 and *C. autoethanogenum* DSM10061 was isolated using a modified method by Bertram and Dürre (Conjugal transfer and expression of streptococcal transposons in *Clostridium acetobutylicum*. *Arch Microbiol* 1989, 151: 551-557). A 100-ml overnight culture was harvested (6,000 x g, 15 min, 4 °C), washed with potassium phosphate buffer (10 mM, pH 7.5) and suspended in 1.9 ml STE buffer (50 mM Tris-HCl, 1 mM EDTA, 200 mM sucrose; pH 8.0). 300 µl lysozyme (~100,000 U) were added and the mixture was incubated at 37 °C for 30 min, followed by addition of 280 µl of a 10 % (w/v) SDS solution and another incubation for 10 min. RNA was digested at room temperature by addition of 240 µl of an EDTA solution (0.5 M, pH 8), 20 µl Tris-HCl (1 M, pH 7.5), and 10 µl RNase A. Then, 100 µl Proteinase K (0.5 U) were added and proteolysis took place for 1-3 h at 37 °C. Finally,

600 µl of sodium perchlorate (5 M) were added, followed by a phenol-chloroform extraction and an isopropanol precipitation. DNA quantity and quality was inspected spectrophotometrically.

Table 4: Oligonucleotides used for amplification of acetone biosynthesis genes and promoter region

Description	Oligonucleotide Name	DNA Sequence (5' to 3')	SEQ ID No.
ThlA	ThlA-Cac-NdeI-F	GTTCATATGAAAGAAGTTGTAATAGC	15
	ThlA-Cac-EcoRI-R	CAAGAATTCCTAGCACTTTTCTAGC	16
CtfA, CtfB, Adc operon	Ctf-adc-cbei-KpnI-F	CTAGGTACCAGGGAGATATTAAAATG	17
	Ctf-adc-cbei-BamHI-R	CGTGGATCCTCTATATTGCTTTTATT	18
Promoter	Pwoodlj-NotI-F	AAGCGGCCGCAGATAGTCATAATAGTTCC	19
	Pwoodlj-NdeI-R	TTCCATATGAATAATTCCCTCCTTAAAGC	20
Adh	SecAdh-SalI-F	TATTTGTCGACTTAGGAGGTTCTATTATGA AAGG	21
	SecAdh-XhoI-R	AAAACTCGAGACATTTTTTTAATGCGACAG	22

0254 The expression vector pBAD(KpnI)-WpiMetC was obtained from Invitrogen. The plasmid used in this study had been modified previously, by the insertion of a fragment encoding a hexahistidine (His_6) tag, a TEV protease cleavage site (for removing His_6 from ADH, if desired, after purification), and the gene encoding the MetC enzyme from an unrelated bacterium (*Wolbachia pipientis*).

Methods

Amplification of Wild Type ADH:

0255 The wild type ADH *C. autoethanogenum* DSM10061 was amplified from a 50ng/µl working stock of the plasmid pMTL85147-ThlA-CtfAB-Adc-Adh(LZ) using the primers ADH_TEV_KpnI_for and ADH_HindIII_Reverse.

0256 A 50µL PCR mixture was made as follows:

Reagent	Initial Conc	Final Conc	Vol/reaction
Phusion Buffer	5x	1x	10µL
dNTPs	2mM	200µM each dNTP	5µL
ADH_TEV_KpnI_for	10mM	0.5µM	2.5µL
ADH_HindIII_Rev	10Mm	0.5µM	2.5µL

Phusion Polymerase	2U/μL	1U/reaction	0.5μL
pMTL85147-ThlA-CtfAB-Adc-Adh(LZ) template DNA	50ng/μL	20ng	0.4μL
Water			29.1

0257 The following cycling conditions were used:

Step	Temperature (°C)	Duration	
Initial Denaturation	98	30 sec	
Denaturation	98	10 sec	30 Cycles
Annealing	60	30 sec	
Extension	72	20 sec	
Final extension	72	5 min	

0258 The product was cleaned up with the Cycle Pure kit (Omega Bio-Tek).

Digestion of Vector and Insert:

0259 The pBAD backbone was prepared by digesting pBAD(KpnI)-WpiMetC with the enzymes KpnI-HF and HindIII, with the following recipe:

Reagent	Initial Concentration	Final Concentration	Volume in Reaction
NEB Buffer 4	10X	1X	5μL
pBAD(KpnI)-WpiMetC	140ng/μL	4.1μg	28μL
KpnI-HF	20,000U/mL	10U/reaction	0.5μL
HindIII	20,000U/mL	10U/reaction	0.5μL
Water			16μL

0260 The amplified ADH gene was similarly digested with KpnI-HF and HindIII:

Reagent	Initial Concentration	Final Concentration	Volume in Reaction
NEB Buffer 4	10X	1X	5μL
WT ADH	75ng/μL	1.8μg	25μL
KpnI-HF	20,000U/mL	10U/reaction	0.5μL

HindIII	20,000U/mL	10U/reaction	0.5µL
Water			19µL

0261 Both digestions were carried out at 37°C for 16 hours. The digested products were separated on a 1% agarose gel stained with SYBRsafe (Invitrogen). The bands corresponding to digested vector and insert were excised and the DNA was recovered using a gel clean-up kit (Omega Bio-Tek).

Construction of pBAD(KpnI)-ADH

0262 The purified DNA from the ADH and pBAD digestions were ligated, alongside a control ligation, in the following reaction, which contained a 3x molar excess of insert over vector:

Reagent	Initial concentration	Final Concentration	Volume per reaction mixture	
			Insert + Vector	Vector Only (Control)
Ligation buffer	10x	1x	2µL	2µL
Vector	50ng/µL	100µg/reaction	2µL	2µL
Insert	45ng/µL	76.5µg/reaction	1.7µL	-
T4 DNA ligase	20U/µL	0.5U/µL	0.5µL	0.5µL
Water			11.5µL	14.5µL

0263 The reaction was incubated at 16°C for 2 hours. A 2µL aliquot of the ligation mixture was used to transform a 50µL aliquot of *E. coli* MC1061 cells by electroporation. After an hour recovery at 37°C in SOC, aliquots were spread on LB-ampicillin plates and incubated at 37°C overnight. 6 colonies were picked and screened for the presence of the ADH insert by PCR using the pBAD_for and ADH_HindIII_rev primers. A freezer stock was made of a successful clone, with 700µl culture and 300µl sterile 50% (v/v) glycerol. The sequence of the ADH gene was verified by DNA sequencing.

Site directed mutagenesis of ADH

0264 The protocol for constructing all 5 mutants by overlap extension PCR was the same, except each mutant was constructed with its corresponding mutagenic primers. Hence, the protocol to produce one mutant only has been provided.

Generation of Ser199Asp primary products:

0265 The first step in site directed mutagenesis was the generation of two overlapping primary products. The PCR reactions for these are as follows:

Primary product 1.0:

Reagent	Initial concentration	Final Concentration	Volume per reaction
Phusion Buffer	5x	1x	20µL
dNTPs	2mM	200µM each dNTP	10µL
ADH_TEV_KpnI_for	10mM	0.5µM	5µL
Ser199Asp_rev	10mM	0.5µM	5µL
Phusion Polymerase	2U/µL	1U/reaction	1µL
pMTL85147-ThlA-CtfAB-Adc-Adh(LZ) template DNA	50ng/µL	40ng	0.8µL
Water			58.2µL

Primary product 1.1:

Reagent	Initial concentration	Final Concentration	Volume per reaction
Phusion Buffer	5x	1x	20µL
dNTPs	2mM	200µM each dNTP	10µL
Ser199Asp_For	10mM	0.5µM	5µL
ADH_HindIII_Rev	10mM	0.5µM	5µL
Phusion Polymerase	2U/µL	1U/reaction	1µL
pMTL85147-ThlA-CtfAB-Adc-Adh(LZ) template DNA	50ng/µL	40ng	0.8µL
Water			58.2µL

The cycling conditions were as follows:

Step	Temperature (°C)	Duration	
Initial Denaturation	98	30 sec	
Denaturation	98	10 sec	30 Cycles
Annealing	60	30 sec	
Extension	72	10 sec	
Final extension	72	5 min	

The products were cleaned up with the Omega Cycle Pure kit.

Generation of Ser199Asp secondary products:

0266 The next step in site directed mutagenesis was to use overlap extension PCR (Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, 77, 51-59) with the outside primers, to recombine the two primary products into a full-length secondary product that contained the complete gene with the introduced mutation. The two primary products are mixed so that they are in equal molar concentrations. The following PCR recipe was used:

Reagent	Initial concentration	Final Concentration	Volume per reaction
Phusion Buffer	5x	1x	10μL
dNTPs	2mM	200μM each dNTP	5μL
ADH_TEV_KpnI_for	10mM	0.5μM	2.5μL
ADH_HindIII_Rev	10mM	0.5μM	2.5μL
Phusion Polymerase	2U/μL	1U/reaction	0.5μL
Product 1.0	7.5ng/μL	24ng	3.2μL
Product 1.1	7.1ng/μL	19.8ng	2.8μL
Water			23.5μL

The cycling conditions were the same as for amplification of the wild-type ADH.

Construction of pBAD(KpnI)-ADH(Ser199Asp):

0267 Digestion and ligation protocols for cloning each of the five ADH mutants (i.e. bearing the mutations: Ser199Asp; Ser199Glu; Arg200Gln; Arg200Glu; and Ser199Glu/Arg200Gln) were the same as those used to construct pBAD(KpnI)-ADH. The presence of each mutation was confirmed by DNA sequencing.

Expression and Purification of ADH enzymes

0268 Expression and purification protocols were the same for all the mutants as well as the wild type, except where noted. The protocol for expression and purification of the wild-type ADH is provided below.

Testing for soluble expression:

0269 A 5mL LB-ampicillin overnight culture from freezer stocks was grown at 37°C, and used to inoculate 100mL LB-ampicillin the following morning. The culture was incubated at 37°C until an OD_{600} of 0.8 was reached. At this point, 1mL of 20% arabinose was added and the culture was incubated at 28°C for the remainder of the expression. 500μL samples were taken at t = 0h, 2h, 4h, and 16 h (i.e. overnight). For each sample, cells were pelleted, and supernatant decanted. Pellets

were then resuspended in HEPES buffer (50 mM Na-HEPES and 0.2 mM DTT, pH 8.0), and 0.2μL each of Benzonase (Merck, 25 units/μL) and rLysozyme (Merck, 30 kU/μL) were added. The mixtures were incubated at room temperature for 15min and then frozen (at -80°C) and thawed 3 times. 10μL was taken at this point from each mixture as 'total protein' samples. The remainder of the sample was centrifuged at 13000rpm for 1min, and the supernatant removed and placed on ice. A 10μL aliquot of the supernatant was taken from each mixture as 'soluble protein' samples. To each 10μL total and soluble protein sample, 10μL of SDS buffer was added and all mixtures were heated at 98°C for 5min. 15μL of each aliquot was loaded into an SDS-PAGE gel with 12% resolving gel and 4% stacking gel. The gel was run at 200V for 40min. The gel was stained in overnight using Coomassie blue and then de-stained.

0270 At the end of expression, the remainder of the 100mL culture was pelleted in 50mL tubes and stored frozen at -80°C.

Protein purification:

0271 Since the wild-type and mutant ADH enzymes all carried His_6tagsat their N-termini, they could all be purified using immobilized metal affinity chromatography. The frozen cell pellets described above(from 100mL cultures) were resuspended in 10mL ice-coldlysis buffer (50 mM potassium phosphate, 300 mM NaCl, pH 7.0), with 0.5μl rLysozyme (30kU/μl), 0.5μl Benzonase(25U/μl), and 50μl protease inhibitor cocktail (Sigma). After a 30min incubation on ice, the cells were lysed by sonication, insoluble debris was pelleted, and the supernatant was clarified using a 0.2 micron filter. The clarified supernatant was added to Talon resin (Clontech) which had been thoroughly washed with lysis buffer. A bed volume of 500μL was used for purifying the wild-type ADH, and a bed volume of 200μL was used for purifying each mutant. The protein was allowed to bind to the Talon resin for an hour at 4°C and then washed several times with lysis buffer. The protein was eluted from the column using lysis buffer supplemented with 150mM imidazole, and the eluant was collected in 500μL fractions. Aliquots taken at various stages through the purification process, as well as all elution fractions, were run on an SDS page gel to confirm presence of the protein and determine the success of the purification. Proteins were exchanged into a storage buffer(50 mM potassium phosphate, 150 mM NaCl, 10% v/v glycerol, pH 7.0)using Amicon Ultra-4 Centrifugal Filter Units (Millipore).

0272 The wild-type protein was highly soluble when expressed in *E. coli*, and could be readily purified (Figure 1). The Ser199Asp and Arg200Gln mutant proteins were also highly soluble (Figure 2). The solubility of the other two point mutants (Ser199Glu and Arg200Glu) were more variable

(results not shown). Somewhat surprisingly, the double mutant (Ser199Glu + Arg200Gln) was more soluble than Ser199Glu (results not shown).

0273 The sequence of the Ser199Asp mutant is provided in SEQ ID 37 (nucleic acid) and SEQ ID 38 (amino acid).

Activity assays

0274 All activity assays were carried out using a Cary 100 UV/visspectrophotometer with quartz cuvettes. All chemicals used in the assay were sourced from Sigma-Aldrich.

0275 Unless noted otherwise, assays had a cofactor (NADPH/NADH) concentration of 0.2 mM, a substrate (acetaldehyde, acetyl-CoA, acetone, DL-acetoin, MEK) concentration of 3 mM and an ADH concentration of 30 nM. They were carried out in 50 mM Tris-HCl buffer (pH 7.5), with 1mM DTT. All assays were done in triplicate, with freshly prepared substrates and cofactors.

0276 First, the wild-type enzyme of *C. autoethanogenum* was measured and it was shown that the enzyme is strictly NADPH dependent with barely any detectable activity with NADH (Fig.11) as described for the alcohol dehydrogenase enzyme of *C. beijerinckii* (Ismaiel, Zhu, Colby, & Chen, 1993).

0277 Afterwards, kinetics for the purified wild-type enzyme (Fig. 12) of *C. autoethanogenum* DSM10061 were determined as baseline to evaluate mutated enzymes with substitutions (Fig. 9). Activity could be detected with ketones (acetone, MEK, acetoin) and aldehydes (acetaldehyde) which are important in several fermentation pathways (Fig. 14).

0278 Subsequently, generated mutant alcohol dehydrogenases were assayed and compared with the wild-type enzyme. The Ser199Glu mutant could not be assayed, as there was no soluble protein. Assays of the crude cell lysate showed no ADH activity. While soluble, the Arg200Gln mutant also showed no detectable activity. Unsurprisingly, the mutant that combines these two, Ser199Glu + Arg200Gln, was also inactive. Of the three remaining proteins, the activity of Arg200Glu was only measurable when substrate concentrations were increased 5-fold (to 15 mM) and enzyme concentration was increased 6-fold (to 180 nM).

0279 On the other hand, the Ser199Asp mutant was nearly as active as wild-type with acetone (Figure 3). Most interestingly, the Ser199Asp mutant is more specific for acetone (Figure 4). This

indicates that replacing wild-type ADH with the Ser199Asp mutant may lead to increased isopropanol production. This mutant was the basis for further substitutions as described in Example 2.

Example 2 – Change of substrate and co-factor specificity with multiple amino acid substitutions

0280 Based on the results from Example 1, the inventors generated and studied the activity of a further 8 alcohol dehydrogenase mutants with 1 to 4 amino acid substitutions:

Name	Mutation(s)
Mutant 2	S199E
Mutant 7	G198D, S199V, P201E
Mutant 8	G198E, S199V, P201E
Mutant 9	G198D, S199L, P201E
Mutant 10	G198D, S199V, P201E, Y218F
Mutant 11	G198D, S199V, P201E, Y218A
Mutant 12	G198D, S199D, P201E
Mutant 13	Y218A

Materials

Primers

Table 5: Oligonucleotides used for Quikchange mutagenesis.

Primer name	Primer sequence (5′-3′)	SEQ ID No.
Mutant8_for	GCATTATTGGCGTTGAGGTTCGTGAGGTCTGCG	23
Mutant8_rev	CGCAGACCTCACGAACCTCAACGCCAATAATGC	24
Mutant9_for	GGGTCGCATTATTGGCGTTGATCTTCGTGAGGTCT	25
Mutant9_rev	AGACCTCACGAAGATCAACGCCAATAATGCGACCC	26
Mutant10_for	CCACGGACATCGTCAATTTCAAAAATGGCGACATTGT	27
Mutant10_rev	ACAATGTCGCCATTTTTGAAATTGACGATGTCCGTGG	28
Mutant11_for	GTGCCACGGACATCGTCAATGCCAAAAATGGCGACATTGTTG	29
Mutant11_rev	CAACAATGTCGCCATTTTTGGCATTGACGATGTCCGTGGCAC	30
Mutant12_for	CATTATTGGCGTTGATGATCGTGAGGTCTGCGTCG	31
Mutant12_rev	CGACGCAGACCTCACGATCATCAACGCCAATAATG	32
Mutant13_for	ATGGAGCAACTGATATTGTAAATGCTAAAAATGGTGATATAGTTGAAC	33
Mutant13_rev	GTTCAACTATATCACCATTTTTAGCATTTACAATATCAGTTGCTCCAT	34

Plasmids

0281 The gene sequence for Mutant 7 was synthesized by DNA 2.0. The sequence was codon optimized for expression of the protein in *E. coli*. The synthesized gene was provided in the plasmid pJ201. This sequence included HindIII and KpnI restriction sites for subcloning the Mutant 7 ADH gene into the expression vector pBAD(KpnI)-ADH.

Methods

Construction of the expression plasmid for Mutant 7

0282 The pBAD backbone was prepared by digesting pBAD(KpnI)-ADH with the restriction enzymes KpnI-HF and HindIII-HF.

0283 The Mutant 7 gene was similarly digested from the pJ201 vector with the restriction enzymes Kpn-HF and HindIII-HF.

0284 The digested products were separated on a 1% agarose gel stained with SYBRsafe (Invitrogen). The bands corresponding to the digested vector and insert were excised and the DNA was recovered using a gel clean-up kit (Omega Bio-Tek).

0285 The purified insert and vector DNA were ligated using a 3:1 molar ratio of insert to vector, using T4 DNA ligase (NEB) according to the manufacturer's standard protocol.

0286 The ligation mixture was used to transform *E. coli* MC1061 cells by electroporation. Aliquots of the transformed cells were spread on LB-ampicillin and incubated at 37°C overnight. A single colony was picked. The resulting expression plasmid was purified, and the sequence of the Mutant 7 gene was confirmed by DNA sequencing. A freezer stock was made of the successful clone.

Quikchange mutagenesis of ADH

0287 The template DNA used for the construction of Mutants 8, 9, 10, 11 and 12 was pBAD(KpnI)-Mutant 7 ADH. The template DNA used for the construction of Mutant 13 was pBAD(KpnI)-ADH. Aside from the different template DNA, and the corresponding mutagenic primers, the protocol for constructing Mutants 8-13 was the same. Each mutant was constructed using the Quikchange II Site-Directed Mutagenesis Kit from Stratagene, using their standard recommended protocol. The forward and reverse primers used for the construction of each mutant are listed in Table 5.

0288 The products of the Quikchange mutagenesis reaction were used to transform chemically-competent *E. coli* XL1-Blue cells by heat shock. Single colonies were picked. The resulting expression plasmids were purified, and the sequence of each mutant gene was confirmed by DNA sequencing. A freezer stock was made of each successful clone.

Protein expression and purification

0289 The expression vectors for Mutants 2, 7, 10, 11 were used to transform *E. coli* LMG194 that had previously been transformed with plasmid pGro7 (Takara Bio, Inc.). This plasmid facilitates arabinose-inducible expression of the GroEL/ES chaperone proteins. The expression vector for Mutant 13 was used to transform *E. coli* LMG194.

0290 Expression of each ADH mutant was induced in mid-log phase cultures ($OD_{600} \approx 0.5$), by adding L-arabinose to a final concentration of 0.2% (w/v). The cultures were incubated at 28°C for an additional 5 h. Cells were harvested by centrifugation and the pellets were stored at -80°C. Each pellet was resuspended in 10 mL of lysis buffer (50 mM potassium phosphate, 300 mM NaCl, pH 7.0). Protease inhibitor cocktail (150 µL), Benzonase nuclease (37.5 U) and lysozyme (0.2 $mg.mL^{-1}$, final concentration) were added. After 20 minutes incubation at 4°C, cells were lysed by sonication on ice, and the lysates were clarified by centrifugation (21,000*g*, 4°C, 30 minutes). The clarified lysate was mixed with 500 µL Talon metal affinity resin (50% w/v slurry) and the mixture was gently agitated at 4°C for 1 h to allow the His_6-tagged ADH protein to bind the resin. The resin was washed multiple times with lysis buffer, before being transferred to a gravity flow column. For the purification of Mutants 7, 8, 9, 10 and 12, 5 mM ATP/$MgCl_2$ was included in this wash step to facilitate removal of chaperone proteins. After further washes with 10 bed volumes of lysis buffer containing 5 mM imidazole and 10 mM imidazole, respectively, each purified protein was eluted with 5 bed volumes of elution buffer (50 mM potassium phosphate, 300 mM NaCl, 150 mM imidazole, pH 7.0). Amicon Ultra centrifugal filter units (10 kDa molecular weight cut-off; Merck Millipore, Billerica, MA) were used to exchange the purified protein into storage buffer (50 mM potassium phosphate, 150 mM NaCl, 10% (v/v) glycerol, pH 7.5). Aggregates were removed by filtration through a sterile 0.22 µm filter (Millex-GV; Millipore). Each protein was judged to be >95% pure by SDS-PAGE. ADH concentrations were quantified by measuring A_{280} (using extinction coefficients calculated according to (Pace, Vajdos et al. 1995). Aliquots of the purified protein were stored at −80°C. Activity assays verified that these storage conditions, combined with a freeze/thaw cycle, did not lead to any loss of activity.

0291 As shown in Figure 5, Mutants 2, 7 and 11 were all highly soluble when expressed in *E. coli* with the pGro7 plasmid. The yield soluble protein for Mutant 10 was lower than the other variants.

Mutants 8, 9 and 12 were completely insoluble, even when co-expressed with pGro7. Mutant 13 was highly soluble, and could be produced in high quantities under the same conditions as the wild-type ADH protein.

Activity assays

0292 Wild-type and mutant ADH activities were measured using a spectrophotometric assay, based on a method described previously (Ismaiel, Zhu et al. 1993). Activity was quantified by monitoring the decrease in absorbance at 340 nm associated with the oxidation of NADPH or NADH (ε_{340} = 6,220 $M^{-1}.cm^{-1}$). Steady-state kinetic parameters were measured at 25°C using a Cary 100 UV-Vis spectrophotometer with a Peltier temperature controller. The standard assay mixture contained 50 mM Tris-HCl, 1 mM DTT, pH 7.5 with either cofactor (NADPH or NADH) present at 0.2 mM. Initial reaction rates were measured with a substrate concentration of 5 mM. Measurements were made in triplicate and corrected for background. Co-factors NADH and NADPH and substrates acetone, acetoin, MEK were sourced from Sigma-Aldrich. D-Acetoin was purified as described below as there is no commercial source.

0293 Overall, Mutant 11 has the highest activity using NADH as the cofactor (Figure 6). The cofactor usage for Mutant 11 has completely switched (compared to wild-type ADH); Mutant 11 had no detectable activity with NADPH.

0294 Mutant 11 has four mutations (G198D, S199V, P201E, Y218A). All four mutations are required for the observed switch in cofactor usage. The addition of the Y218A mutation was required for activity with NADH, but this mutation alone (i.e. Mutant 13) had no effect on cofactor preference. This could have important advantages in fermentation pathways to Isopropnaol, 2,3-butanediol, 2-butanol, and ethanol as shown in Fig. 14

0295 As shown in Figure 7 some mutants are also more specific for acetone, over either a larger substrate (acetoin and MEK) or a smaller substrate (acetaldehyde). All mutants (2, 7, 10 and 11) have an increased substrate specificity of acetone over MEK. Mutants 2, 10 and 11 have an increased substrate specificity of acetone over acetaldehyde and over acetoin.

0296 As shown in Figure 8 some mutants are also more specific for MEK, over either a larger substrate (acetoin) or a smaller substrate (acetaldehyde). Mutant 2, 10 and 11 have an increased substrate specificity of MEK over acetaldehyde and over acetoin.

0297 As shown in Figure 9 some mutants are also more specific for acetaldehyde over a larger substrate (acetone, acetoin and MEK). Mutant 7 has an increased substrate specificity of acetaldehyde over acetone and over MEK. Mutants 2, 7 and 10 have an increased substrate specificity of acetaldehyde over acetoin.

0298 As shown in Figure 10 some mutants are also more specific for acetoin over a smaller substrate (acetone, acetoin and MEK). Mutant 7 has an increased substrate specificity of acetoin over acetone and over MEK. Mutants 7 and 11 have an increased substrate specificity of acetoin over acetaldehyde. Mutant 10 lost the activity with acetoin, while still having activity with the other substrates acetone, MEK and acetaldehyde.

0299 In summary, the results, as depicted in Figures 7-10, show that:

- mutant 2 has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and acetoin, 2) MEK over acetaldehyde and acetoin;
- mutant 7 has an increased substrate specificity for 1) acetone over MEK, 2) acetaldehyde over MEK,acetone and acetoin, 3) acetoin over acetoneand MEK;
- mutant 11 has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and acetoin, 2) MEK over acetaldehyde and acetoin, 3) acetoin over acetaldehyde; and,
- mutant 10 has an increased substrate specificity for 1) acetone over MEK, acetaldehyde and acetoin, and 2) MEK over acetaldehyde and acetoin, 3) acetalydehyde over acetoin. This could have important advantages in fermentation pathways to Isopropnaol, 2,3-butanediol, 2-butanol, and ethanol shown in Fig. 14.

Purification of D-acetoin

0300 Enantioselective synthesis of D-acetoin (or (S)-acetoin):: D-Acetoin was synthesized as described via the regioselectively-controlled monooxidation of D-(-)-2,3-butanediol (D'Accoloti et al. 1993 *J. Org. Chem.* 58: 3600-1) The requisite dimethyldioxirane (DMDO)-acetone solution was freshly prepared according to a modification of a previously reported procedure and subsequently titrated by iodometry.

0301 Generation of DMDO-acetone solution: A 1000 mL 3-neck round bottom flask was fitted with a gas adaptor and a double distillation head, to which was attached a vacuum adapted receiver. The final joint was stoppered and intended for reagent additions. A round-bottom flask was fastened to the receiving end and cooled to -78 °C in an acetone-dry ice bath. In line with the vacuum was a Buchi® cold finger condenser cooled to -20 °C using salted-ice, followed by connection to a -196 °C liquid nitrogen cold finger trap.

0302 The reaction vessel was charged with a large oval stirring bar, water (220 mL), acetone (160 mL) and sodium bicarbonate (120.063 g). Vigorous stirring was initiated at 0 °C whilst purging with nitrogen, which was continued during addition of solid oxone (250.047 g) in 5 portions at 3 minute intervals. Strong effervescence was exhibited and the rate of oxone addition was controlled to manage this accordingly. After addition of the second portion of oxone, the colourless solution above the white slurry took on a pink colour. Stirring was continued for 15 minutes upon complete addition of oxone. At the subsidence of effervescence, a slight vacuum (900 mbar) was applied, which was slowly increased to about 450 mbar whilst monitoring effervescence. During progressive application of vacuum, a pale yellow solution collected in the cooled receiver. Once about 100 mL of DMDO-acetone solution was collected the reaction was quenched with saturated $Na_2S_2O_3$ solution and the product brought to room temperature.

0303 Iodometric titration of DMDO: The freshly distilled solution of DMDO in acetone (1.00 mL) was pipetted into an acetic acid-acetone solution (2 mL, 3:2). Saturated aqueous potassium iodide solution (2.0 mL) was then added, together with dry ice to de-aerate the solution. This was allowed to mix whilst stored in the dark at room temperature for 10 minutes. The mixture was diluted with water (5 mL) and titrated against aqueous $Na_2S_2O_3$ (0.00099 mol/L) solution using 1% starch solution as the endpoint indicator, yielding a concentration of 29.9 mM. The flask was then, sealed and stored at -18 °C.

0304 **DMDO oxidation of (*R*,*R*)-2,3-butanediol.** The monooxidation of D-(-)-2,3-butanediol to (*R*)-3-hydroxybutanone was conducted as per method reported in the literature (D'Accoloti et al. 1993 *J. Org. Chem.* 58: 3600-1).

Example 3 – Preferential production of 2,3-butanediol over ethanol in carboxydotrophes using optimized alcohol dehydrogenase

0305 Carboxydotropic organism *C. autoethanogenum* was shown to produce both ethanol and 2,3-butanediol from CO (Köpke et al., 2011) (Fig. 14). As demonstrated in example 1, an alcohol dehydrogenase is present in *C. autoethanogenum* that catalyses the last step in both the ethanol and the 2,3-butanediol pathway, the reactions of acetaldehyde to ethanol respectively acetoin to 2,3-butanediol (Fig. 14). The activity of this wild-type enzyme with acetaldehyde is higher than with acetoin (Fig. 12), which leads to ethanol being the dominant product over 2,3-butanediol in fermentations with *C. autoethanogenum* DSM 10061 (Köpke et al., 2011). In example 2, an alcohol dehydrogenase mutant (mutant 7) was generated with amino acid substitutions G198E, S199V, P201E that has an improved substrate specificity for acetoin over acetaldehyde (Fig. 7). This enzyme that

preferentially uses acetoin over acetaldehyde compared to the wild-type enzyme can be used to increase the 2,3-butanediol production over ethanol production compared to the wild-type strain.

0306 The gene (Seq. ID 41) of a mutant alcohol dehydrogenase is cloned into a pMTL85353 shuttle vector (Heap, Pennington, Cartman, & Minton, 2009) carrying a ferredoxin promoter using sites NdeI and EcoRI. The construct is then methylated and transformed into *C. autoethanogenum* by electroporation as described (US 2012/0252083, WO/2012/115527). Thiamphenicol resistant colonies are picked and grown in 5 mL liquid media. The transformed culture is verified by PCR and a fermentation experiment is carried out. When comparing the metabolic end products to the wild-type of *C. autoethanogenum*, the strain carrying the mutant alcohol dehydrogenase will have an increased 2,3-butanediol:ethanol ratio.

0307 In analogy to this, other acetogenic strains such as *C. ljungdahlii* can be modified with the alcohol dehydrogenase mutant, using the same plasmid. Electroporation has been described for several carboxydotrophic acetogens such as *C. ljungdahlii* (Köpke et al. 2010, *Poc. Nat. Acad. Sci. U.S.A.* 107: 13087-92; (Leang, Ueki, & Lovley, 2011) PCT/NZ2011/000203; WO2012/053905), *Acetobacterium woodii* (Straetz et al., 1994, *Appl. Environ. Microbiol.* 60:1033-37) and *Moorella thermoacetica* (Kita et al., 2012). *C. autoethanogenum, C. ljungdahlii* and other acetogenic strains are able to produce ethanol and 2,3-butanediol (Köpke et al., 2011). By expressing the mutant alcohol dehydrogenase, this ratio can be improved in favour of 2,3-butanediol.

Example 4 – Preferential production of ethanol over 2,3-butanediol in carboxydotrophes using optimized alcohol dehydrogenase

0308 As described in example 3, *C. autoethanogenum* is able to produce both ethanol and 2,3-butanediol from CO (Fig. 14). For some processes it may however be favourable to produce only a single product in order to keep the costs (e.g separation) of the process low. Typically this can be achieved by inactivating an enzyme in the pathway of one of the pathways. However, in case of a multifunctional enzyme that catalyzes reaction in multiple pathways, this strategy can't be applied, which is the case for the alcohol dehydrogenase of *C. autoethanogenum* that catalyzes both the reduction of acetaldehyde to ethanol and acetoin to 2,3-butanediol. The current invention gives an alternative means to achieve, for example, production of ethanol with reduced levels or without 2,3-butanediol. In example 2, an alcohol dehydrogenase mutant (mutant 10) was generated with amino acid substitutions G198D, S199V, P201E, Y218F that lost the ability to reduce acetoin, but still has activity with acetaldehyde (Fig. 7). The gene (Seq. ID 47) of this mutant alcohol dehydrogenase is cloned into a vector along with the flanking regions of the alcohol dehydrogenase to allow a double homologous crossover integration (replacing the wild-type with the mutant alcohol dehydrogense). 1

kb 5' (Seq. ID. 55) and 3' (Seq. ID. 56) homology arms of SecAdh genes are PCR amplified using C. autoethanogenum DSM23693 genomic DNA. Primers Sec5f (attcatcctgcaggACAGTTAAAAAGCATATCTAACAGT (SEQ ID 57)) / Sec5r (gactgcggccgcTAAATATATAAGCAAATGTTGTGCC (SEQ ID 58)) and Sec3f (atatgctagCGTATTTTTAATTGCGAACTTAAGA (SEQ ID 59)) / Sec3r (gactggcgcgcCAGTTAAAGTTAGACATCCGATTAT (SEQ ID 60)) are used to amplify the 5' and 3' homology arms, respectively. The two PCR products are cloned into pMTL85151 plasmids between the SbfI/ NotI and NheI/AscI sites to get pMTL85151-SecAdh-KO. The vector is transformed as described above. Following selection on thiamphenicol plates the transformants are screened for swap out of the wild-type alcohol dehydrogenase with the mutant alcohol dehydrogenase using the primers SecOf (TTGGAATTTTAGCTGTAGATAACAA (SEQ ID 61)) and SecOr (TAAGTGATTTTCAATGGACTTTACT (SEQ ID 62)) that flank the homology arms. After sequencing conformation, a fermentation experiment is carried out, confirming production of ethanol with reduced or no 2,3-butanediol production. In *C. autoethanogenum*, a second enzyme with activity towards butanediol is present which can be knocked-out for complete removal of 2,3-butanediol production under certain conditions.

0309 In analogy to this, other acetogenic 2,3-butanediol producing strains such as *C. ljungdahlii* and *C. ragsdalei* can be modified with the alcohol dehydrogenase mutant, using the same plasmid. Electroporation as well as a method for double homologous crossover has been described for *C. ljungdahlii* (Köpke et al. 2010, *Poc. Nat. Acad. Sci. U.S.A.* 107: 13087-92; (Leang et al., 2011).

Example 5 – Production of isopropanol in carboxydotrophes using optimized alcohol dehydrogenase

0310 *C. autoethanogenum* has been modified for isopropanol production from CO by introducing acetone biosynthesis genes, relying on the wild-type alcohol dehydrogenase (US 2012/0252083, WO/2012/115527). To improve the production, a highly acetone specific mutant alcohol dehydrogenase (mutant 11) with substitutions G198D, S199V, P201E, Y218A generated in example 2 can be introduced. The mutant alcohol dehydrogenase gene (Seq. ID 53) is cloned into acetone biosynthesis plasmid (Seq. ID 40) by SalI/XhoI restriction sites. The plasmid is then transformed in *C. autoethanogenum* as described above. Fermentation experiments with the transformed culture will show an increased isopropanol production in which all acetone is converted to isopropanol. In addition to having an increased specificity towards acetone, the organism will use NADPH and NADH for isopropanol synthesis, allowing to tap into both pools.

0311 In analogy to this, other acetogenic strains such as *C. ljungdahlii* can be modified with the alcohol dehydrogenase mutant, using the same plasmid. Electroporation has been described for several carboxydotrophic acetogens as *C. ljungdahlii* (Köpke et al. 2010, *Poc. Nat. Acad. Sci. U.S.A.* 107: 13087-92; (Leang et al., 2011) PCT/NZ2011/000203; WO2012/053905), *Acetobacterium woodii* (Straetz et al., 1994, *Appl. Environ. Microbiol.* 60:1033-37) or *Moorella thermoacetica* (Kita et al., 2012). As *C. autoethanogenum, C. ljungdahlii* and other acetogenic strains are able to produce ethanol and 2,3-butanediol (Köpke et al., 2011). By expressing the mutant alcohol dehydrogenase, this ratio can be improved in favour of 2,3-butanediol.

Example 6 – Production of isopropanol in ABE organism using optimized alcohol dehydrogenase

0312 It has been shown that *C. acetobutylicum* can be metabolically engineered for isopropanol production using a secondary alcohol dehydrogenase from *C. beijerinckii*, but the reported results show only low isopropanol levels with residual acetone that hasen't been converted through to isopropanol [Lee et al, 2012: Metabolic engineering of *Clostridium acetobutylicum* ATCC824 for isopropanol-butanol-ethanol fermentation, *Appl. Environ. Microbiol.* 78: 1416-1423]. To improve this process an optimized alcohol dehydrogenase is required to overcome this limitation.

0313 In example 2 an optimized mutant alcohol dehydrogenase (mutant 11) was generated with substitutions G198D, S199V, P201E, Y218A that has high specificity towards acetoin and is also able to use NADH, which is more abundant than NADPH in *C. acetobutylicum*. The mutant alcohol dehydrogenase gene (Seq. ID 53) is cloned into into a pMTL85354 shuttle vector (Heap et al., 2009) carrying the strong C. acetobutylicum thiolase promoter using sites NdeI and EcoRI. The plasmid is then *in vivo* methylated using a *Bacillus subtilis* phage methyltrasnferase and transformed in C. acetobutylicum as described (Mermelstein & Papoutsakis, 1993). In a fermentation carried out with the transformed culture all acetoin is converted to isopropanol with high specificity.

Example 7 – Production of isopropanol in *E. coli* using optimized alcohol dehydrogenase

0314 E. coli has been a target for isopropanol production in several studies [Hanai T et al (2007) Engineered synthetic pathway for isopropanol production in Escherichia coli. *Applied and environmental microbiology* 73:7814–8; Inokuma K et al (2010) Improvement of isopropanol production by metabolically engineered Escherichia coli using gas stripping. *Journal of bioscience and bioengineering* 110:696–701; Jojima T et al (2008) Production of isopropanol by metabolically engineered Escherichia coli. *Applied microbiology and biotechnology* 77:1219–24]. However all studies use the very same non-optimized alcohol dehydrogenase from *C. beijerinckii*, limiting isopropanol production due to the enzyme's low specificity and NADPH dependency. This becomes

evident as in all studies acetone accumulates as by-product as it is not effectively enough reduced to isopropanol. In *E. coli*, the pool of NADH pool is several times bigger than the NADPH pool (Bennett & San, 2009).

0315 In example 2 an optimized mutant alcohol dehydrogenase (mutant 11) was generated with substitutions G198D, S199V, P201E, Y218A that has high specificity towards acetoin and is also able to use NADH. *E.coli* can be engineered to include this mutant alcohol dehydrogenase using standard techniques used in the art. The recombinant *E.coli* will have increased isopropanol production compared to a wild type organism.

Example 8 – Production of 2-butanol in yeast *S. cerevisiae* using optimized alcohol dehydrogenase

0316 Some strains of yeast *Saccharomyces cerevisiae* are able to produce high levels of acetoin (Romano, Suzzi, Mortimer, & Polsinelli, 1995) beside ethanol. D-Acetoin (or (S)-Acetoin) can be converted to meso-2,3-butanediol by action of the alcohol dehydrogenase of *C. autoethanogenum* described in example 1. Conversion of *meso*-2,3-Butanediol to MEK has been described with diol dehydratase enzyme of for example *A. aerogenes* (Toraya T, Shirakashi T, Kosuga T, 1976) or *Klebsiella pneumonia* (Bachovchin, Eagar, Moore, & Richards, 1977). MEK can then again be converted to 2-butanol with the alcohol dehydrogenase of *C. autoethanogenum* described in example 1 (Fig. 14). As the alcohol dehydrogenase however also has activity towards ethanol it is desirable to have an enzyme with only low activity with acetaldehyde to ethanol but relatively higher activity with acetoin to 2,3-butanediol and MEK to 2-butanol. Mutant 11 of example 2 has exactly these properties and uses NADH as co-factor which is favourable in yeasts.

0317 Codon optimized gene for Mutant 11 (Seq. ID 50) and codon optimized genes for diol dehydratase from *Klebsiella pneumonia* (YP_002236782; YP_002236783; YP_002236784) are cloned under inducible yeast promoter GAL1/10 into an appropriate vector as described (Steen et al., 2008). Transformation of all *S. cerevisiae* strains is performed using the lithium acetate method as described (Gietz RW: RA Guide to Yeast Genetics and Molecular and Cell Biology. Part B. San Diego, CA: Academic Press Inc; 2002:87-96). After successful transformation and verification a fermentation with *S. cerevisiae* in rich YPD medium at 30°C is carried out with 2-butanol and low levels of ethanol as product.

0318 The invention has been described herein, with reference to certain preferred embodiments, in order to enable the reader to practice the invention without undue experimentation. However, a person having ordinary skill in the art will readily recognise that many of the components and parameters may be varied or modified to a certain extent or substituted for known equivalents without departing from the scope of the invention. It should be appreciated that such modifications and

equivalents are herein incorporated as if individually set forth. Titles, headings, or the like are provided to enhance the reader's comprehension of this document, and should not be read as limiting the scope of the present invention.

0319 The entire disclosures of all applications, patents and publications, cited above and below, if any, are hereby incorporated by reference. However, the reference to any applications, patents and publications in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that they constitute valid prior art or form part of the common general knowledge in any country in the world.

0320 Throughout this specification and any claims which follow, unless the context requires otherwise, the words "comprise", "comprising" and the like, are to be construed in an inclusive sense as opposed to an exclusive sense, that is to say, in the sense of "including, but not limited to".

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Toraya T, Shirakashi T, Kosuga T, F. S. (1976). Substrate Specificity Of Coenzyme B12-Dependent Diol Dehydratase. *Biochemical and biophysical research communications*, *69*(2), 475–480.

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Ismaiel, A. A., C. X. Zhu, et al. (1993). "Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*." J. Bacteriol. 175(16): 5097-5105.

Pace, C. N., F. Vajdos, et al. (1995). How to measure and predict the molar absorption coefficient of a protein. Protein Sci. 4(11): 2411-2423.

CLAIMS

1. In one aspect of the present invention there is provided an alcohol dehydrogenase having increased specificity for at least one first substrate over at least one second substrate wherein the at least one first substrate and the at least one second substrate are selected from the group consisting of:
the first substrate is acetone and the second substrate is MEK;
the first substrate is acetone and the second substrate is acetaldehyde;
the first substrate is acetone and the second substrate is acetoin;
the first substrate is MEK and the second substrate is acetaldehyde;
the first substrate is MEK and the second is acetoin;
the first substrate is acetoin and the second acetone;
the first substrate is acetoin and the second substrate is MEK;
the first substrate is acetoin and the second substrate is acetaldehyde
the first substrate is acetaldehyde and the second acetone;
the first substrate is acetaldehyde and the second acetoin; and,
the first substrate is acetaldehyde and the second substrate is MEK; and,
wherein the alcohol dehydrogenase includes at least one or more mutation compared to the corresponding wild type alcohol dehydrogenase.

2. An alcohol dehydrogenase which uses NADH as a co-factor or has increased specificity for an NADH co-factor over an NADPH co-factor wherein the alcohol dehydrogenase includes at least one or more mutation compared to the corresponding wild type alcohol dehydrogenase.

3. An alcohol dehydrogenase as claimed in claim 1 to 2 wherein the alcohol dehydrogenase includes an amino acid substitution at one or more of the following positions: Gly198, Ser199, Arg200, Pro201, Tyr218.

4. An alcohol dehydrogenase as claimed in claim 3 wherein the amino acid substitution is chosen from one or more of: Gly198Asp, Gly198Ile, Gly198Leu, Gly198Val, Ser199Asp, Ser199Glu, Ser199Leu, Ser199Val, Arg200Glu, Pro201Asp, Pro201Glu, Tyr218Ala, Tyr218Phe, Tyr218Gly, Tyr218Ser and Tyr218Val.

5. An alcohol dehydrogenase as claimed in claim 4 wherein the alcohol dehydrogenase includes:
a Ser199Asp substitution;
a Ser199Glu substitution;
a combination of the substitutions Gly198Asp, Ser199Val, and Pro201Glu;
a combination of the substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ala;
a combination of the substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Phe;
a combination of the substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Val;
a combination of the substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Gly; or
a combination of the substitutions Gly198Asp, Ser199Val, Pro201Glu, and Tyr218Ser.

6. An alcohol dehydrogenase as claimed in claim 5 wherein the alcohol dehydrogenase comprises the sequence of SEQ ID 38, SEQ ID 42, SEQ ID 48, SEQ ID 50, SEQ ID 63, SEQ ID 64 or SEQ ID 65.

7. A nucleic acid encoding an alcohol dehydrogenase of any one of claims 1 to 6.

8. A nucleic acid as claimed in claim 7 wherein the nucleic acid comprises the sequence of SEQ ID 37, SEQ ID 41, SEQ ID 47, SEQ ID 49, SEQ ID 67, SEQ ID 68, SEQ ID 69, or SEQ ID 70.

9. A nucleic acid vector comprising a nucleic acid as claimed in claim 7.

10. A host cell comprising a nucleic acid as claimed in claim 7 or 9.

11. A recombinant microorganism which comprises one or more nucleic acid as claimed in claim 7 or 9 wherein the microorganism is capable of producing one or more products chosen from: Isopropanol; 2,3-Butanediol; ethanol; 2-butanol; and optionally one or more other products by fermentation.

12. A recombinant microorganism which comprises one or more nucleic acid as claimed in claim 7 or 9 wherein the microorganism is capable of producing one or more products chosen from: acetoin; MEK; acetaldehyde; acetone; and optionally one or more other products by fermentation.

13. A recombinant microorganism as claimed in claim 12 wherein the microorganism is chosen from the group comprising bacteria, Archae, and fungi.

14. A recombinant microorganism as claimed in claim 13 wherein the microorganism is a carboxydotrophic acetogen selected from the group comprising *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium scatologenes, Clostridium coskatii, Clostridium aceticum, Clostridium magnum, Clostridium sp.,Butyribacterium limosum, Butyribacterium methylotrophicum, Acetobacterium woodii, Alkalibaculum bacchii, Blautia producta, Eubacterium limosum, Moorella thermoacetica, Moorella thermautotrophica, Oxobacter pfennigii,* and *Thermoanaerobacter kiuvi.*

15. A recombinant microorganism as claimed in claim 13 wherein the microorganism is an ABE fermenting microorganism selected from the group comprising *Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharobutylicum, Clostridium saccharoperbutylacetonicum.*

16. A recombinant microorganism wherein the microorganism is selected from the group comprising *E. coli, Klebsiella pneumonia, Klebsiella oxytoca, Bacillus licheniformis, Bacillus subtilis, Lactobacillus brevis,* and *Saccharomyces cerevisiae.*

17. A method for the production of one or more of isopropanol, 2,3-Butanediol, ethanol, 2-butanol, acetoin, MEK, acetaldehyde, and acetone, and optionally one or more other products, by microbial fermentation of a substrate using a microorganism as claimed in any one of claim 11 to 16.

18. A method as claimed in claim 17 comprising at least the steps of:

(a) providing a substrate to a bioreactor containing a culture of one or more microorganism of the invention; and

(b) fermenting the culture in the bioreactor to produce the one or more products.

19. A method as claimed in claim 17 or 18, wherein the substrate is chosen from a substrate comprising one or more of CO, CO_2, and H_2 and/or a substrate comprising one or more carbohydrate.

20. A method as claimed in claim 19 wherein the substrate comprises a substrate comprising CO and the method comprises the steps of:

(a) capturing CO-containing gas produced as a result of the industrial process, before the gas is released into the atmosphere;

(b) the anaerobic fermentation of the CO-containing gas to produce the one or more products by a culture, wherein the culture comprises one or more carboxydotrophic acetogenic microorganism.

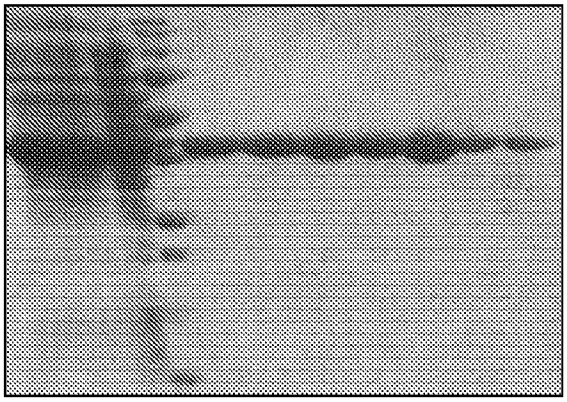

FIG 1

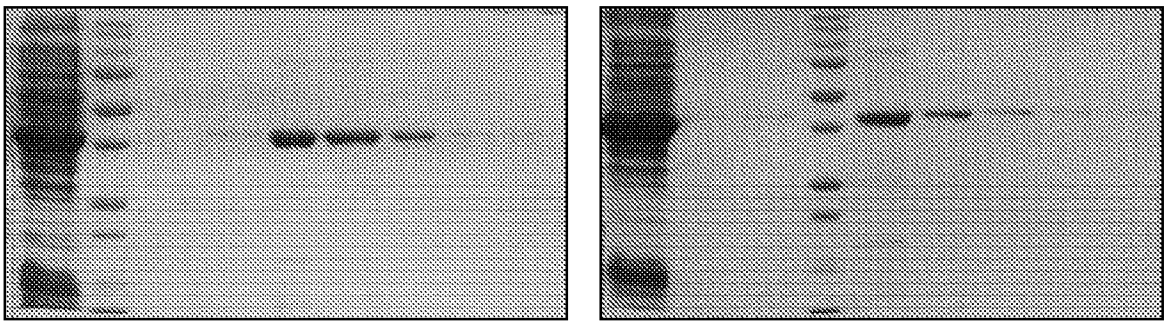

FIG 2

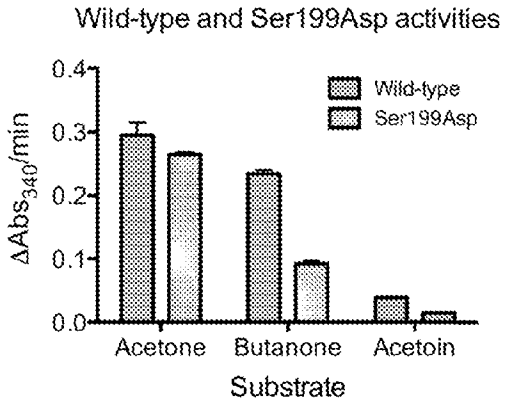
Wild-type and Ser199Asp activities
0.4
0.3
0.2
0.1
0.0
ΔAbs340/min
Wild-type
Ser199Asp
Acetone
Butanone
Acetoin
Substrate

FIG 3

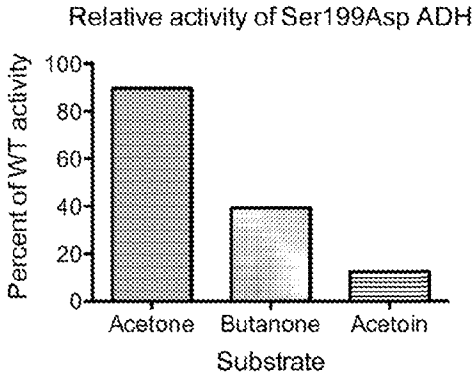
Relative activity of Ser199Asp ADH
100
80
60
40
20
0
Percent of WT activity
Acetone
Butanone
Acetoin
Substrate

FIG 4

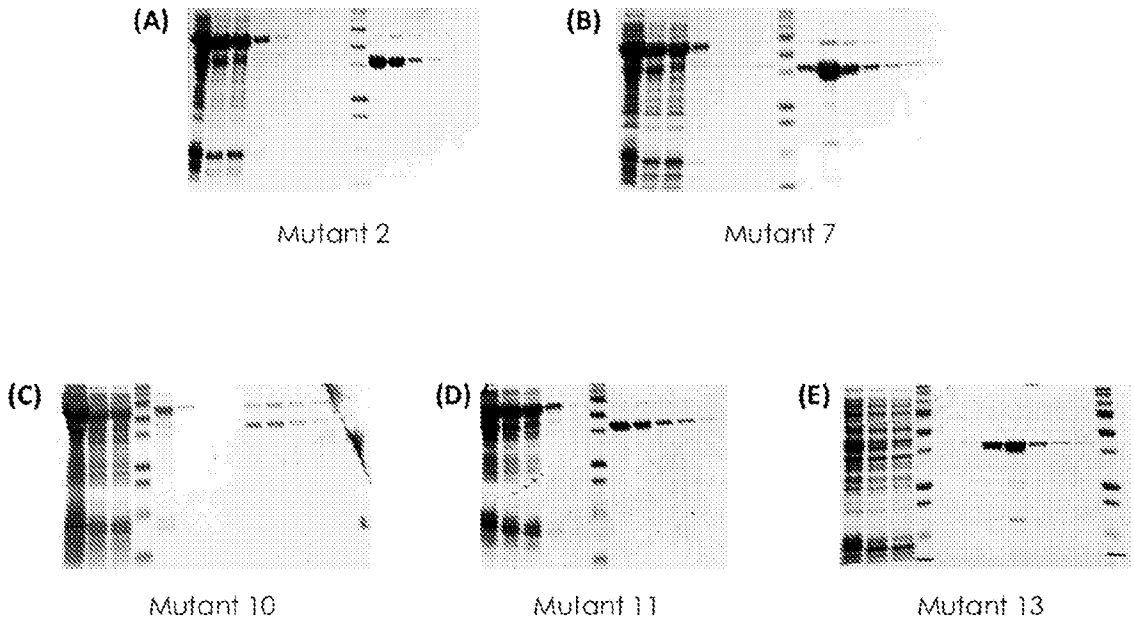

FIG 5

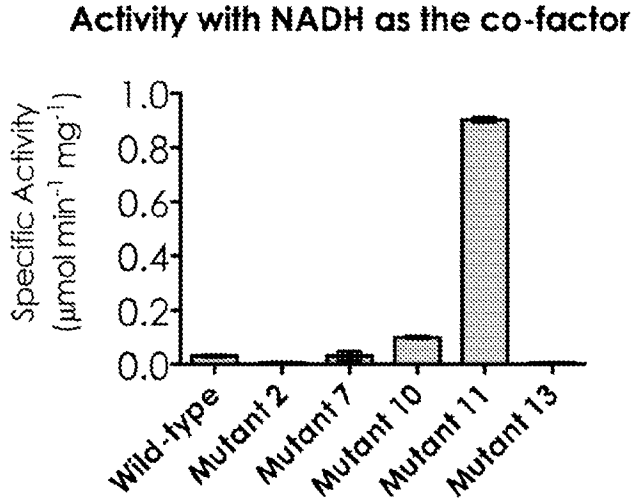

FIG 6

note: 5 mM acetone was the substrate in all assays.

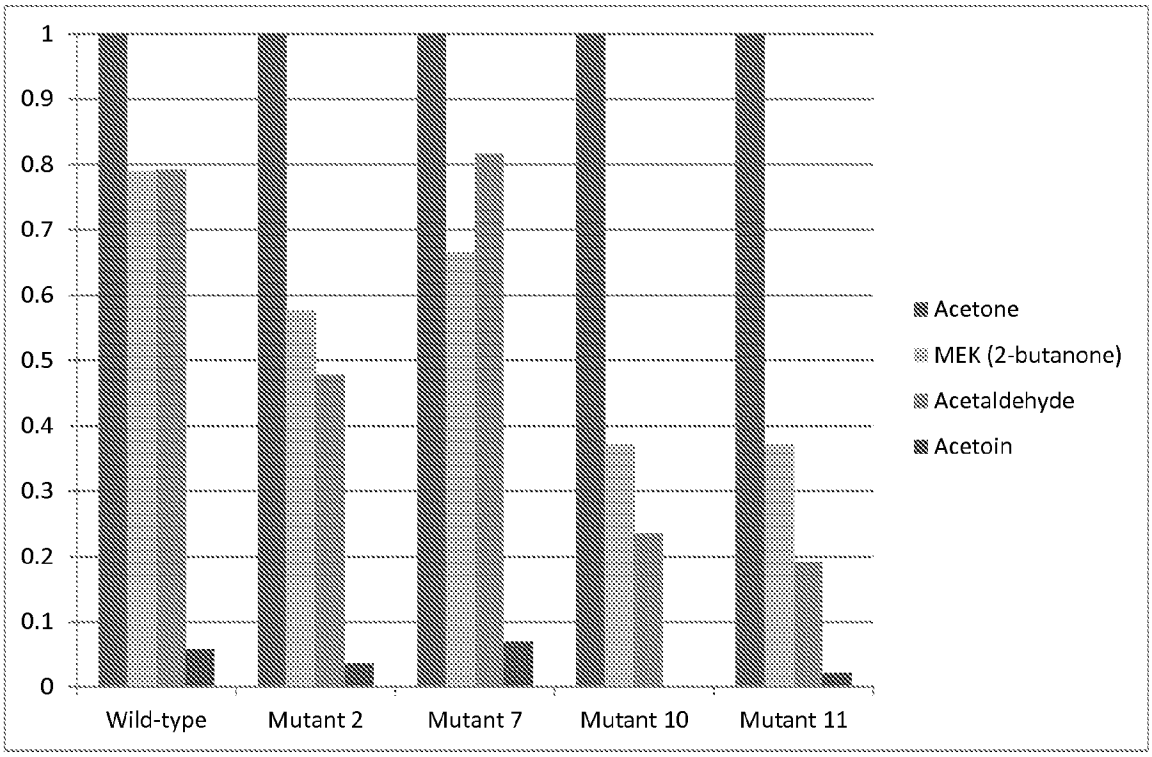
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Wild-type
Mutant 2
Mutant 7
Mutant 10
Mutant 11
Acetone
MEK (2-butanone)
Acetaldehyde
Acetoin

FIG 7

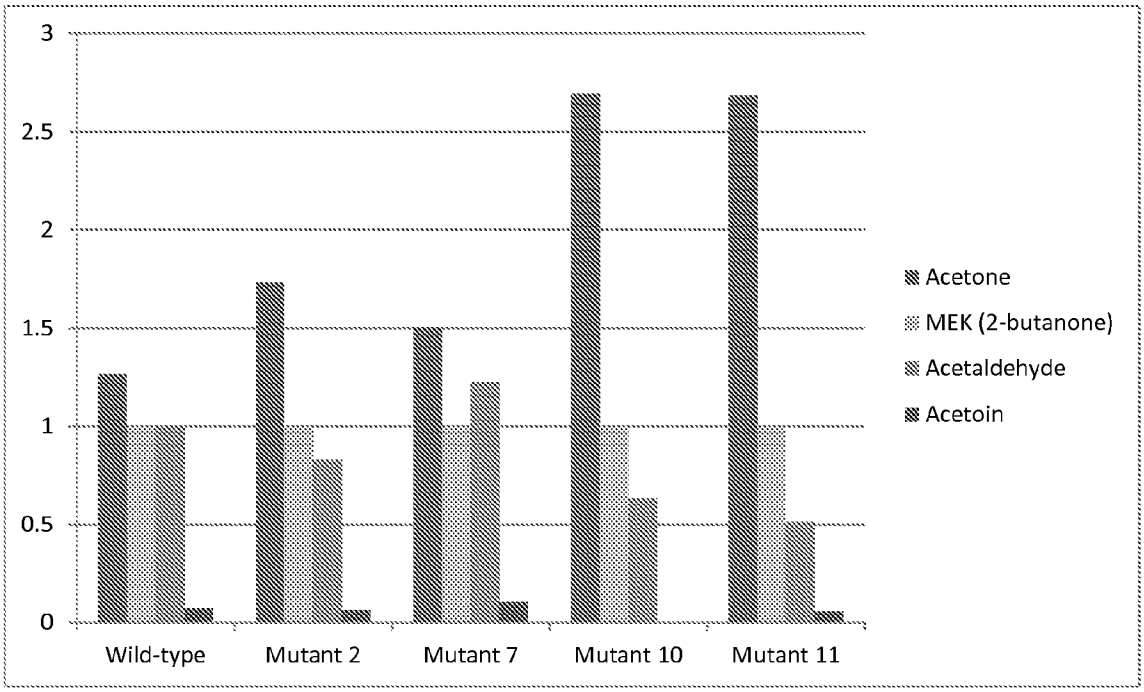
3
2.5
2
1.5
1
0.5
0
Wild-type
Mutant 2
Mutant 7
Mutant 10
Mutant 11
Acetone
MEK (2-butanone)
Acetaldehyde
Acetoin

FIG 8

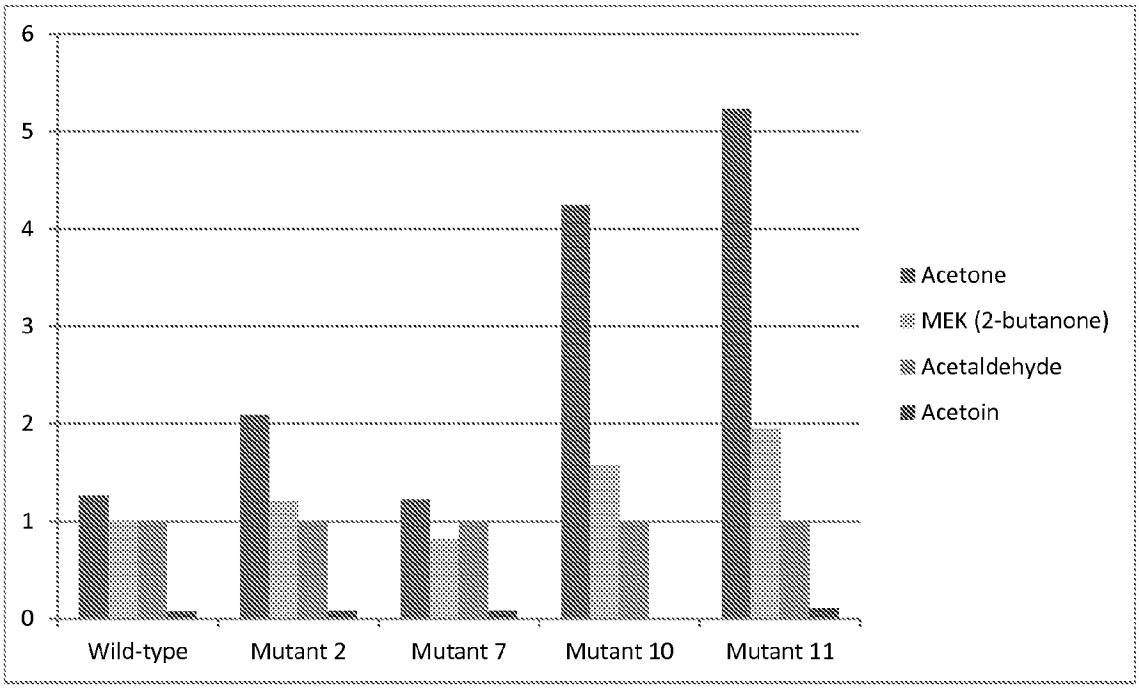
6
5
4
3
2
1
0
Wild-type
Mutant 2
Mutant 7
Mutant 10
Mutant 11
Acetone
MEK (2-butanone)
Acetaldehyde
Acetoin

FIG 9

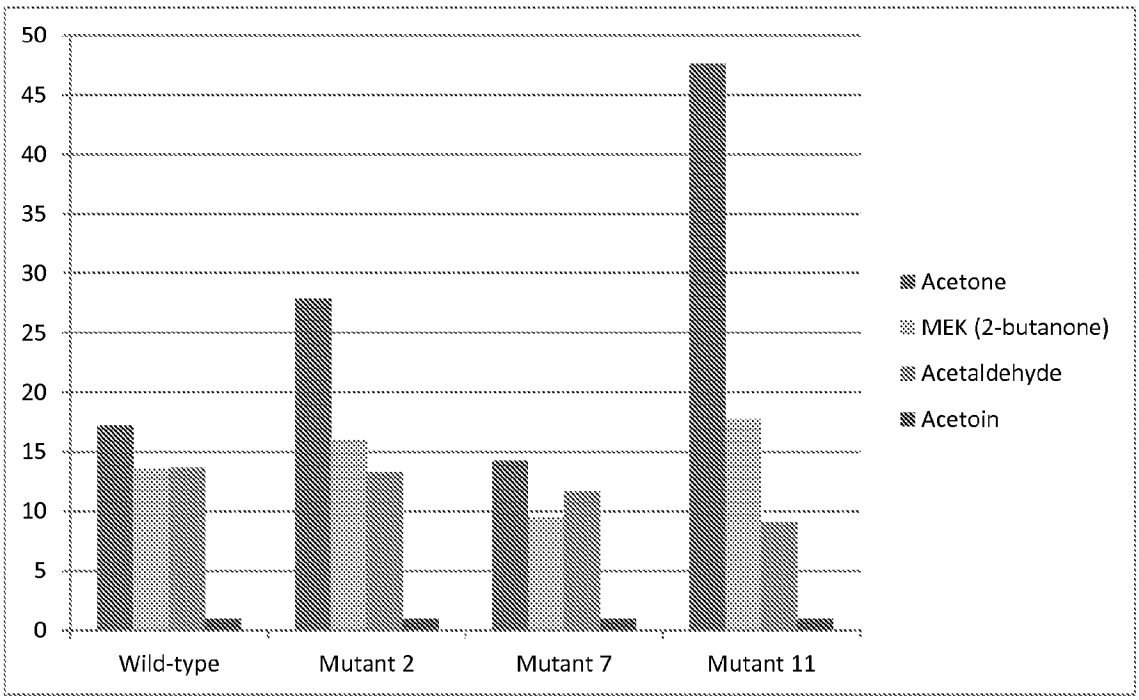
50
45
40
35
30
25
20
15
10
5
0
Wild-type
Mutant 2
Mutant 7
Mutant 11
Acetone
MEK (2-butanone)
Acetaldehyde
Acetoin

FIG 10

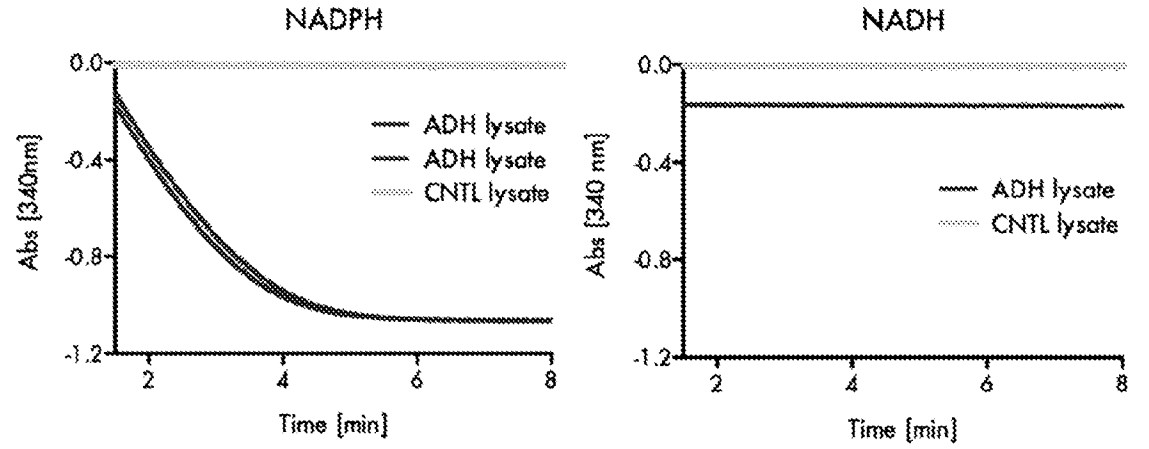

FIG 11

	K_{cat} [sec^{-1}]	K_M [mM]	K_{cat}/K_M [sec^{-1} mM^{-1}]
Primary Adh			
Acetaldehyde to Ethanol	93 ± 6	5.5 ± 1.4	**1.7×10^4**
Secondary Adh			
Acetone to Isopropanol	51.4 ± 0.8	0.60 ± 0.02	**8.6×10^4**
Acetoin to 2,3-Butanediol	41.8 ± 0.7	5.7 ± 1.4	**7.4×10^3**
MEK (2-Butanone) to 2-Butanol	44 ± 2	1.2 ± 0.1	**3.8×10^4**

FIG 12

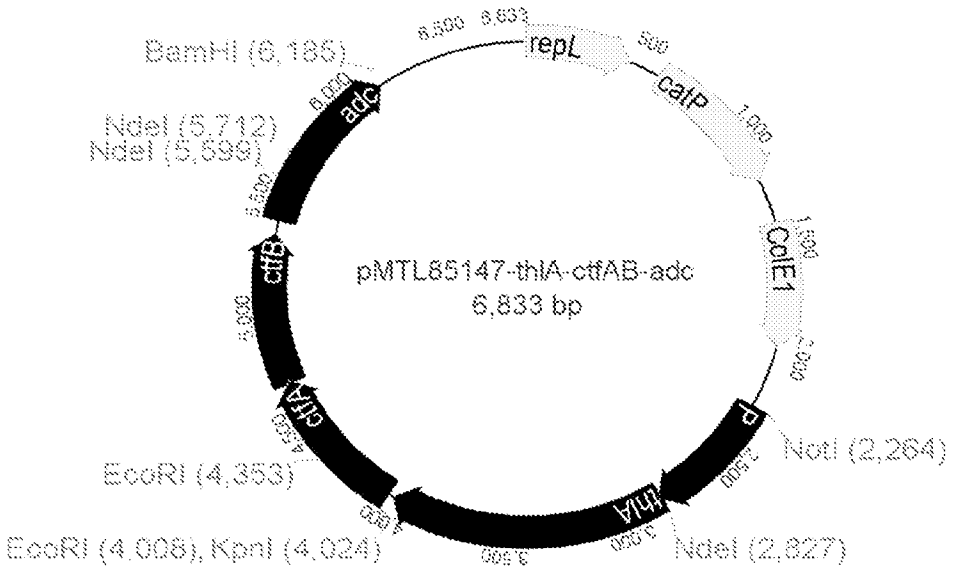
pMTL85147-thlA-ctfAB-adc
6,833 bp
repL
catP
ColE1
P
thlA
ctfB
ctfA
adc
BamHI (6,185)
NdeI (5,712)
NdeI (5,599)
NotI (2,264)
NdeI (2,827)
EcoRI (4,353)
EcoRI (4,008), KpnI (4,024)

FIG 13

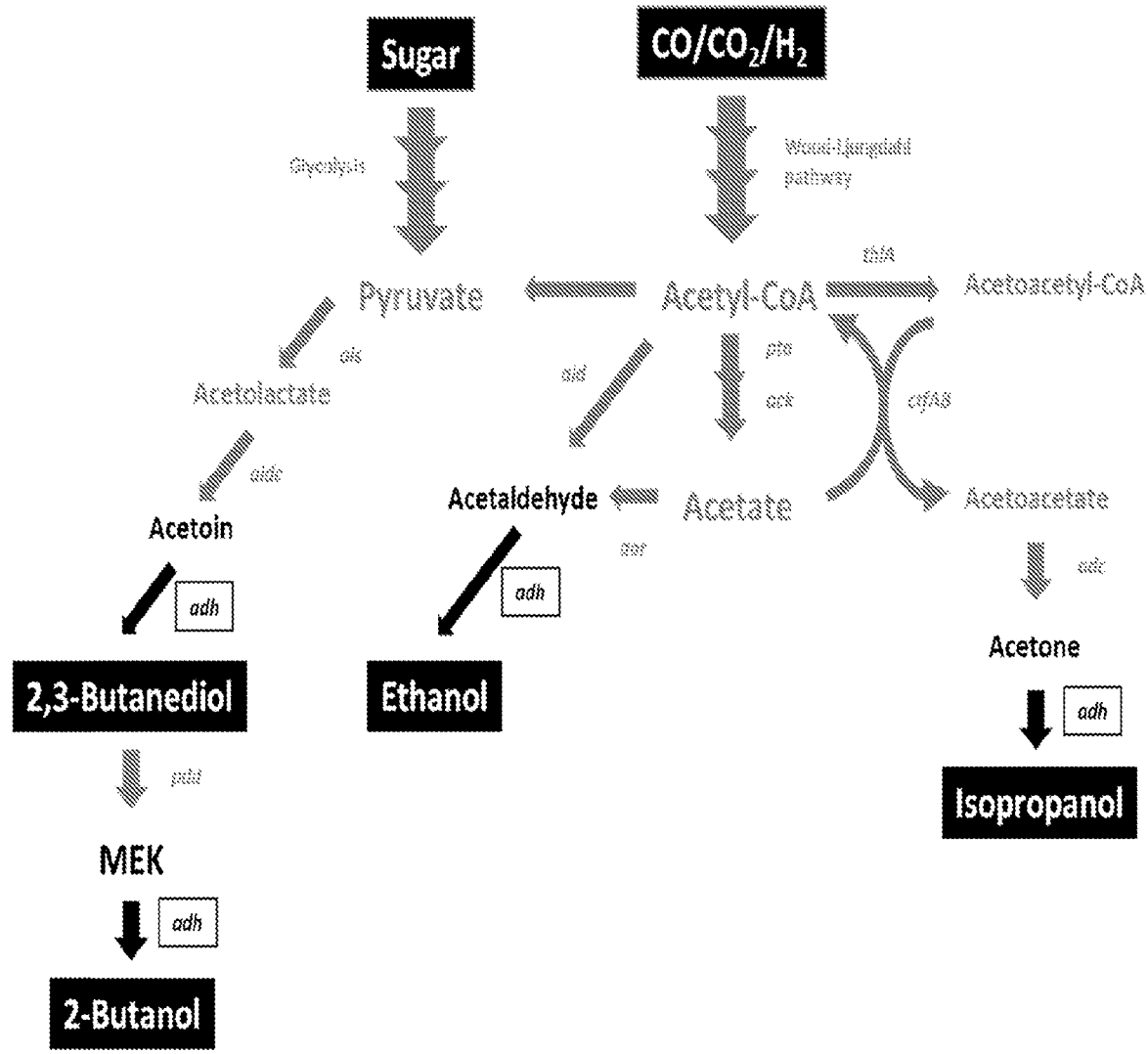
Sugar
$CO/CO_2/H_2$
Glycolysis
Wood-Ljungdahl pathway
Pyruvate
Acetyl-CoA
thlA
Acetoacetyl-CoA
als
Acetolactate
ald
pta
ack
ctfAB
aldc
Acetaldehyde
Acetate
Acetoacetate
Acetoin
adc
adh
adh
2,3-Butanediol
Ethanol
Acetone
adh
Isopropanol
pdu
MEK
adh
2-Butanol

FIG 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/035338

A. CLASSIFICATION OF SUBJECT MATTER

C12N 9/02(2006.01)i, C12N 15/53(2006.01)i, C12N 15/74(2006.01)i, C12P 7/04(2006.01)i

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 9/02; C07H 21/04; C12N 9/04; C12P 7/16; C12P 7/18; C12N 1/21; C12P 7/02; C12N 15/53; C12N 15/74; C12P 7/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: alcohol dehydrogenase, substrate, acetone, MEK, acetaldehyde, acetoin, NADH, recombinant, microorganism

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008-035187 A2 (DOW GLOBAL TECHNOLOGIES INC.) 27 March 2008 See paragraph [0015]; claims 1 and 28.	1
A		2-6,16
X	US 2002-0064847 A1 (YAMAMOTO, HIROAKI et al.) 30 May 2002 See paragraph [0057]; claims 1 and 37.	2,16
A		1,3-6
A	US 2011-0236941 A1 (KOEPKE, MICHAEL et al.) 29 September 2011 See claims 67 and 69.	1-6,16
A	WO 2004-013332 A1 (NEDERLANDS INSTITUUT VOOR ZUIVELONDERZOEK) 12 February 2004 See claims 1-2.	1-6,16
A	US 2011-0177579 A1 (MA, KESEN et al.) 21 July 2011 See claims 1 and 15.	1-6,16

☐ Further documents are listed in the continuation of Box C.

☒ 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 application or patent 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 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
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 2013 (19.08.2013)	**20 August 2013 (20.08.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
HEO Joo Hyung
Telephone No. +82-42-481-8150

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/035338

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 8,9,13-15,18,20
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 8,9,13-15,18 and 20 are unclear since they refer to claims which are not searchable due to not being drafted in accordance with the second and third sentence of Rule 6.4(a).

3. ☒ Claims Nos.: 7,10-12,17,19
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/035338

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008-035187 A2	27/03/2008	WO 2008-035187 A3	10/07/2008
US 2002-0064847 A1	30/05/2002	EP 0875562 A1	04/11/1998
		EP 0875562 A4	17/11/2004
		WO 98-17788 A1	30/04/1998
US 2011-0236941 A1	29/09/2011	AU 2011-318676 A1	02/05/2013
		CA 2813431 A1	26/04/2012
		KR 10-2013-0079599 A	10/07/2013
		WO 2012-053905 A1	26/04/2012
WO 2004-013332 A1	12/02/2004	AU 2003-256152 A1	23/02/2004
US 2011-0177579 A1	21/07/2011	CA 2738574 A1	01/04/2010
		EP 2346992 A1	27/07/2011
		EP 2346992 A4	06/06/2012
		US 8476051 B2	02/07/2013
		WO 2010-034115 A1	01/04/2010