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(71) Demandeur/Applicant:
E. I. DU PONT DE NEMOURS AND COMPANY, US
(72) Inventeurs/Inventors:
DONALDSON, GAIL K., US;
ELIOT, ANDREW C., US;
HUANG, LISA L., US;
NAGARAJAN, VASANTHA, US;
NAKAMURA, CHARLES E., US
(74) Agent: TORYS LLP

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(57) **Abrégé/Abstract:**

Methods for the fermentive production of four carbon alcohols are provided. Specifically, butanol, preferably 2-butanol is produced by the fermentive growth of a recombinant bacteria expressing a 2- butanol biosynthetic pathway. The recombinant microorganisms and methods of the invention can also be adapted to produce 2-butanone, an intermediate in the 2-butanol biosynthetic pathways disclosed herein.

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(71) Applicant (for all designated States except US): **E. I. DU PONT DE NEMOURS AND COMPANY** [US/US]; 1007 Market Street, Wilmington, Delaware 19898 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DONALDSON, Gail, K.** [US/US]; 3 Great Circle Road, Newark, Delaware 19711 (US). **ELIOT, Andrew, C.** [US/US]; 1407 Stapler Place, Wilmington, Delaware 19806 (US). **HUANG, Lisa, L.** [US/US]; 1423 Old Wilmington Road, Hockessin, Delaware 19707 (US). **NAGARAJAN, Vasantha** [US/US]; 702 Hopeton Road, Wilmington, Delaware 19807 (US). **NAKAMURA, Charles, E.** [US/US]; 2 Mount Vernon Drive, Claymont, Delaware 19703 (US).

(74) Agent: **FELTHAM, S., Neil**; E. I. du Pont de Nemours and Company, Legal Patent Records Center, 4417 Lancaster Pike, Wilmington, Delaware 19805 (US).

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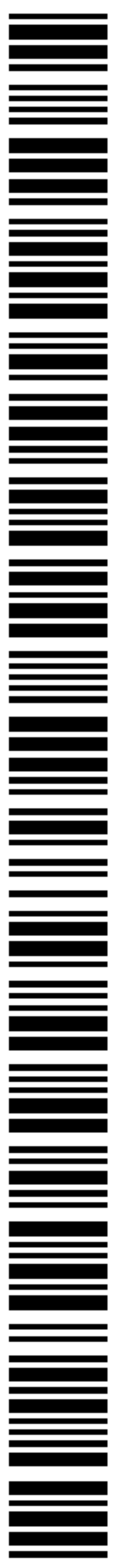
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(54) Title: FERMENTIVE PRODUCTION OF FOUR CARBON ALCOHOLS

(57) Abstract: Methods for the fermentive production of four carbon alcohols are provided. Specifically, butanol, preferably 2-butanol is produced by the fermentive growth of a recombinant bacteria expressing a 2- butanol biosynthetic pathway. The recombinant microorganisms and methods of the invention can also be adapted to produce 2-butanone, an intermediate in the 2-butanol biosynthetic pathways disclosed herein.



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FERMENTIVE PRODUCTION OF FOUR CARBON ALCOHOLS
CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority under 35 U.S.C. §119 from U.S.
5 Provisional Application Serial No. 60/796816, filed May 2, 2006 and U.S.
Provisional Application Serial No. 60/871156, filed December 21, 2006.

FIELD OF THE INVENTION

The invention relates to the field of industrial microbiology and the
production of alcohols. More specifically, 2-butanol is produced via
10 industrial fermentation of a recombinant microorganism. The recombinant
microorganisms and methods of the invention can also be adapted to
produce 2-butanone, an intermediate in the 2-butanol biosynthetic
pathways disclosed herein.

BACKGROUND OF THE INVENTION

15 Butanol is an important industrial chemical, useful as a fuel additive,
as a feedstock chemical in the plastics industry, and as a food-grade
extractant in the food and flavor industry. Each year 10 to 12 billion
pounds of butanol are produced by petrochemical means and the need for
this commodity chemical will likely increase. 2-Butanone, also referred to
20 as methyl ethyl ketone (MEK), is a widely used solvent and is the most
important commercially produced ketone, after acetone. It is used as a
solvent for paints, resins, and adhesives, as well as a selective extractant
and activator of oxidative reactions.

Methods for the chemical synthesis of 2-butanone are known, such
25 as by dehydrogenation of 2-butanol or in a process where liquid butane is
catalytically oxidized giving 2-butanone and acetic acid (*Ullmann's
Encyclopedia of Industrial Chemistry*, 6th edition, 2003, Wiley-VCH Verlag
GmbH and Co., Weinheim, Germany, Vol. 5, pp.727-732). 2-Butanone
may also be converted chemically to 2-butanol by hydrogenation (Breen et
30 al., *J. of Catalysis* 236: 270-281 (2005)). Methods for the chemical
synthesis of 2-butanol are known, such as n-butene hydration (*Ullmann's
Encyclopedia of Industrial Chemistry*, 6th edition, 2003, Wiley-VCH Verlag
GmbH and Co., Weinheim, Germany, Vol. 5, pp. 716-719). These

processes use starting materials derived from petrochemicals and are generally expensive, and are not environmentally friendly. The production of 2-butanone and 2-butanol from plant-derived raw materials would minimize greenhouse gas emissions and would represent an advance in the art.

Methods for producing 2-butanol by biotransformation of other organic chemicals are also known. For example, Stampfer et al. (WO 03/078615) describe the production of secondary alcohols, such as 2-butanol, by the reduction of ketones which is catalyzed by an alcohol dehydrogenase enzyme obtained from *Rhodococcus ruber*. Similarly, Kojima et al. (EP 0645453) describe a method for preparing secondary alcohols, such as 2-butanol, by reduction of ketones which is catalyzed by a secondary alcohol dehydrogenase enzyme obtained from *Candida parapsilosis*. Additionally, Kuehnle et al. (EP 1149918) describe a process that produces both 1-butanol and 2-butanol by the oxidation of hydrocarbons by various strains of *Rhodococcus ruber*. The process favored 1-butanol production with a selectivity of 93.8%.

The production of 2-butanol by certain strains of *Lactobacilli* is also known (Speranza et. al. *J. Agric. Food Chem.* (1997) 45:3476-3480). The 2-butanol is produced by the transformation of *meso*-2,3-butanediol. The production of 2-butanol from acetolactate and acetoin by these *Lactobacilli* strains was also demonstrated. However, there have been no reports of a recombinant microorganism designed to produce 2-butanol.

There is a need, therefore, for environmentally responsible, cost-effective processes for the production of 2-butanol and 2-butanone. The present invention addresses this need through the discovery of recombinant microbial production hosts expressing 2-butanol and 2-butanone biosynthetic pathways.

SUMMARY OF THE INVENTION

The invention provides a recombinant microorganism having an engineered 2-butanol biosynthetic pathway. Also provided is a recombinant microorganism having an engineered 2-butanone biosynthetic pathway, which is the same as the 2-butanol biosynthetic

pathway with omission of the last step. The engineered microorganisms may be used for the commercial production of 2-butanol or 2-butanone.

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

- i) pyruvate to alpha-acetolactate;
- ii) alpha-acetolactate to acetoin;
- iii) acetoin to 3-amino-2-butanol ;
- 10 iv) 3-amino-2-butanol to 3-amino-2-butanol phosphate;
- v) 3-amino-2-butanol phosphate to 2-butanone; and
- vi) 2-butanone to 2-butanol;

wherein the at least one DNA molecule is heterologous to said microbial host cell and wherein said microbial host cell produces 2-butanol.

Similarly the invention provides a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

- 20 i) pyruvate to alpha-acetolactate ;
- ii) alpha-acetolactate to acetoin;
- iii) acetoin to 3-amino-2-butanol;
- iv) 3-amino-2-butanol to 3-amino-2-butanol phosphate; and
- v) 3-amino-2-butanol phosphate to 2-butanone;

25 wherein the at least one DNA molecule is heterologous to said microbial host cell and wherein said microbial host cell produces 2-butanone.

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

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

- i) pyruvate to alpha-acetolactate ;

- ii) alpha-acetolactate to acetoin;
- iii) acetoin to 3-amino-2-butanol;
- iv) 3-amino-2-butanol to 3-amino-2-butanol phosphate;
- v) 3-amino-2-butanol phosphate to 2-butanone; and
- vi) 2-butanone to 2-butanol;

5

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

- 2) contacting the host cell of (1) with a fermentable carbon substrate in a fermentation medium under conditions whereby 2-butanol is produced.

10

Similarly the invention provides a method for the production of 2-butanone comprising:

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

15

- i) pyruvate to alpha-acetolactate;
- ii) alpha-acetolactate to acetoin;
- iii) acetoin to 3-amino-2-butanol;
- iv) 3-amino-2-butanol to 3-amino-2-butanol phosphate; and
- v) 3-amino-2-butanol phosphate to 2-butanone;

20

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

- 2) contacting the host cell of (1) with a fermentable carbon substrate in a fermentation medium under conditions whereby 2-butanone is produced.

25

In another embodiment the invention provides a 2-butanol or 2-butanone containing fermentation product medium produced by the methods of the invention.

BRIEF DESCRIPTION OF THE FIGURES AND SEQUENCE DESCRIPTIONS

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

Figure 1 shows four different pathways for biosynthesis of 2-butanone and 2-butanol.

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

Table 1

Summary of Nucleic Acid and Protein SEQ ID Numbers

Description	SEQ ID Nucleic acid	SEQ ID Protein
<i>budA</i> , acetolactate decarboxylase from <i>Klebsiella pneumoniae</i> ATCC 25955	1	2
<i>alsD</i> , acetolactate decarboxylase from <i>Bacillus subtilis</i>	80	81
<i>budA</i> , acetolactate decarboxylase from <i>Klebsiella terrigena</i>	82	83
<i>budB</i> , acetolactate synthase from <i>Klebsiella pneumoniae</i> ATCC 25955	3	4
<i>alsS</i> , acetolactate synthase from <i>Bacillus subtilis</i>	76	77
<i>budB</i> , acetolactate synthase from <i>Klebsiella terrigena</i>	78	79
<i>budC</i> butanediol dehydrogenase from <i>Klebsiella pneumoniae</i> IAM1063	5	6

butanediol dehydrogenase from <i>Bacillus cereus</i>	84	85
butanediol dehydrogenase from <i>Bacillus cereus</i>	86	87
<i>butB</i> , butanediol dehydrogenase from <i>Lactococcus lactis</i>	88	89
<i>pddA</i> , butanediol dehydratase alpha subunit from <i>Klebsiella oxytoca</i> ATCC 8724	7	8
<i>pddB</i> , butanediol dehydratase beta subunit from <i>Klebsiella oxytoca</i> ATCC 8724	9	10
<i>pddC</i> , butanediol dehydratase gamma subunit from <i>Klebsiella oxytoca</i> ATCC 8724	11	12
<i>pduC</i> , B12 dependent diol dehydratase large subunit from <i>Salmonella typhimurium</i>	92	93
<i>pduD</i> , B12 dependent diol dehydratase medium subunit from <i>Salmonella typhimurium</i>	94	95
<i>pduE</i> , B12 dependent diol dehydratase small subunit from <i>Salmonella typhimurium</i>	96	97
<i>pduC</i> , B12 dependent diol dehydratase large subunit from <i>Lactobacillus collinoides</i>	98	99
<i>pduD</i> , B12 dependent diol dehydratase medium subunit from <i>Lactobacillus collinoides</i>	100	101
<i>pduE</i> , B12 dependent diol dehydratase small subunit from <i>Lactobacillus collinoides</i>	102	103
<i>pddC</i> , adenosylcobalamin-dependent diol dehydratase alpha subunit from <i>Klebsiella pneumoniae</i>	104	105
<i>pddD</i> , adenosylcobalamin-dependent diol dehydratase beta subunit from <i>Klebsiella pneumoniae</i>	106	107
<i>pddD</i> , adenosylcobalamin-dependent diol dehydratase gamma subunit from <i>Klebsiella pneumoniae</i>	108	109
<i>ddrA</i> , diol dehydratase reactivating factor large subunit from <i>Klebsiella oxytoca</i>	110	111
<i>ddrB</i> , diol dehydratase reactivating factor small subunit from <i>Klebsiella oxytoca</i>	112	113

<i>pduG</i> , diol dehydratase reactivating factor large subunit from <i>Salmonella typhimurium</i>	114	115
<i>pduH</i> , diol dehydratase reactivating factor small subunit from <i>Salmonella typhimurium</i>	116	117
<i>pduG</i> , diol dehydratase reactivating factor large subunit from <i>Lactobacillus collinoides</i>	118	119
<i>pduH</i> , diol dehydratase reactivating factor small subunit from <i>Lactobacillus collinoides</i>	120	121
<i>sadH</i> , butanol dehydrogenase from <i>Rhodococcus ruber</i> 219	13	14
<i>adhA</i> , butanol dehydrogenase from <i>Pyrococcus furiosus</i>	90	91
<i>chnA</i> , cyclohexanol dehydrogenase from <i>Acintebacter</i> sp.	71	72
<i>yqhD</i> , butanol dehydrogenase from <i>Escherichia coli</i>	74	75
amine:pyruvate transaminase from <i>Vibrio fluvialis</i> (an acetoin aminase)	144 codon opt.	122
Aminobutanol kinase from <i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	123	124
amino alcohol O-phosphate lyase from <i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	125	126
<i>budC</i> , acetoin reductase (butanediol dehydrogenase) from <i>Klebsiella terrigena</i> (now <i>Raoultella terrigena</i>)	133	134
glycerol dehydratase alpha subunit from <i>Klebsiella pneumoniae</i>	145	146
glycerol dehydratase beta subunit from <i>Klebsiella pneumoniae</i>	147	148
glycerol dehydratase gamma subunit from <i>Klebsiella pneumoniae</i>	149	150
glycerol dehydratase reactivase large subunit from <i>Klebsiella pneumoniae</i>	151	152
glycerol dehydratase reactivase small subunit from <i>Klebsiella pneumoniae</i>	153	154

SEQ ID NOs:15 - 65 are the nucleotide sequences of oligonucleotide PCR, cloning, screening, and sequencing primers used in the Examples.

5 SEQ ID NO:66 is nucleotide sequence of the deleted region of the *yqhD* gene in *E. coli* strain MG1655 $\Delta yqhCD$, described in Example 11.

SEQ ID NO:67 is the nucleotide sequence of a variant of the glucose isomerase promoter 1.6GI.

SEQ ID NO:68 is the nucleotide sequence of the 1.5GI promoter.

10 SEQ ID NO:69 is the nucleotide sequence of the diol dehydratase operon from *Klebsiella oxytoca*.

SEQ ID NO:70 is the nucleotide sequence of the diol dehydratase reactivating factor operon from *Klebsiella oxytoca*.

15 SEQ ID NO:73 is the nucleotide sequence of pDCQ2, which is described in Example 9.

SEQ ID NOs:127 - 132 are the nucleotide sequences of additional oligonucleotide PCR and cloning primers used in the Examples.

SEQ ID NO:155 is a codon optimized coding region for the amino alcohol kinase of *Erwinia carotovora* subsp. *atroseptica*.

20 SEQ ID NO:156 is a codon optimized coding region for the amino alcohol O-phosphate lyase of *Erwinia carotovora* subsp. *atroseptica*.

SEQ ID NOs:157-163 are the nucleotide sequences of additional oligonucleotide PCR and cloning primers used in the Examples.

25 SEQ ID NO:164 is the nucleotide sequence of an operon from *Erwinia carotovora* subsp. *atroseptica*.

Table 2.

Additional glycerol and diol dehydratase large, medium and small subunits

<u>^aDescription</u>	<u>^bsubunit</u>	<u>protein</u> <u>SEQ ID</u>
Corresponding subunits from same organism ^c		
Glycerol dehydratase alpha subunit from <i>Clostridium pasteurianum</i>	L	135
Glycerol dehydratase beta subunit from <i>Clostridium</i>	M	136

<i>pasteurianum</i>		
Glycerol dehydratase gamma subunit from <i>Clostridium</i> <i>pasteurianum</i>	S	137
Glycerol dehydratase alpha subunit from <i>Escherichia</i> <i>blattae</i>	L	138
Glycerol dehydratase beta subunit from <i>Escherichia</i> <i>blattae</i>	M	139
Glycerol dehydratase gamma subunit from <i>Escherichia</i> <i>blattae</i>	S	140
Glycerol dehydratase alpha subunit from <i>Citrobacter</i> <i>freundii</i>	L	141
Glycerol dehydratase beta subunit from <i>Citrobacter</i> <i>freundii</i>	M	142
Glycerol dehydratase gamma subunit from <i>Citrobacter</i> <i>freundii</i>	S	143

^aDescription: from the Genbank annotation of the sequence and may not be correct including the glycerol or diol designation, or may not include subunit information.

^bSubunit: identified by sequence homology to the large, medium, or small subunit of the *Klebsiella oxytoca* enzyme.

5 ^c Subunits are listed together that are from the same organism and have annotations as the same enzyme, or have Genbank numbers close together indicating proximity in the genome.

DETAILED DESCRIPTION OF THE INVENTION

10 The present invention relates to methods for the production of 2-butanol using recombinant microorganisms. The present invention meets a number of commercial and industrial needs. Butanol is an important industrial commodity chemical with a variety of applications, where its potential as a fuel or fuel additive is particularly significant.

15 Although only a four-carbon alcohol, butanol has an energy content similar to that of gasoline and can be blended with any fossil fuel. Butanol is favored as a fuel or fuel additive as it yields only CO₂ and little or no SO_x or NO_x when burned in the standard internal combustion engine. Additionally butanol is less corrosive than ethanol, the most preferred fuel

20 additive to date.

In addition to its utility as a biofuel or fuel additive, butanol has the potential of impacting hydrogen distribution problems in the emerging fuel

cell industry. Fuel cells today are plagued by safety concerns associated with hydrogen transport and distribution. Butanol can be easily reformed for its hydrogen content and can be distributed through existing gas stations in the purity required for either fuel cells or combustion engines in vehicles.

Finally the present invention produces 2-butanol from plant derived carbon sources, avoiding the negative environmental impact associated with standard petrochemical processes for butanol production.

The present invention also provides recombinant microorganisms and methods for producing 2-butanone, an intermediate in the 2-butanol biosynthetic pathways disclosed herein. 2-Butanone, also known as methyl ethyl ketone (MEK), is useful as a solvent in paints and other coatings. It is also used in the synthetic rubber industry and in the production of paraffin wax.

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

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

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

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

The term "acetolactate synthase", also known as "acetohydroxy acid synthase", refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of two molecules of pyruvic acid to one molecule of alpha-acetolactate. Acetolactate synthase, known as EC 2.2.1.6 [formerly 4.1.3.18] (*Enzyme Nomenclature 1992*, Academic Press, San Diego) may be dependent on the cofactor thiamin pyrophosphate for its activity. Suitable acetolactate synthase enzymes are available from a number of sources, for example, *Bacillus subtilis* [GenBank Nos: AAA22222 NCBI (National Center for Biotechnology Information) amino

acid sequence (SEQ ID NO:77), L04470 NCBI nucleotide sequence (SEQ ID NO:76)], *Klebsiella terrigena* [GenBank Nos: AAA25055 (SEQ ID NO:79), L04507 (SEQ ID NO:78)], and *Klebsiella pneumoniae* [GenBank Nos: AAA25079 (SEQ ID NO:4), M73842 (SEQ ID NO:3)].

5 The term "acetolactate decarboxylase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of alpha-acetolactate to acetoin. Acetolactate decarboxylases are known as EC 4.1.1.5 and are available, for example, from *Bacillus subtilis* [GenBank Nos: AAA22223 (SEQ ID NO:81), L04470 (SEQ ID NO:80)], *Klebsiella*
10 *terrigena* [GenBank Nos: AAA25054 (SEQ ID NO:83), L04507 (SEQ ID NO:82)] and *Klebsiella pneumoniae* [GenBank Nos: AAU43774 (SEQ ID NO:2), AY722056 (SEQ ID NO:1)].

 The term "acetoin aminase" or "acetoin transaminase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the
15 conversion of acetoin to 3-amino-2-butanol. Acetoin aminase may utilize the cofactor pyridoxal 5'-phosphate or NADH (reduced nicotinamide adenine dinucleotide) or NADPH (reduced nicotinamide adenine dinucleotide phosphate). The resulting product may have (*R*) or (*S*) stereochemistry at the 3-position. The pyridoxal phosphate-dependent
20 enzyme may use an amino acid such as alanine or glutamate as the amino donor. The NADH- and NADPH-dependent enzymes may use ammonia as a second substrate. A suitable example of an NADH-dependent acetoin aminase, also known as amino alcohol dehydrogenase, is described by Ito et al. (U.S. Patent No. 6,432,688). An
25 example of a pyridoxal-dependent acetoin aminase is the amine:pyruvate aminotransferase (also called amine:pyruvate transaminase) described by Shin and Kim (*J. Org. Chem.* 67:2848-2853 (2002)).

 The term "butanol dehydrogenase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the interconversion
30 of 2-butanone and 2-butanol. Butanol dehydrogenases are a subset of a broad family of alcohol dehydrogenases. Butanol dehydrogenase may be NAD- or NADP-dependent. The NAD-dependent enzymes are known as EC 1.1.1.1 and are available, for example, from *Rhodococcus ruber*

[GenBank Nos: CAD36475 (SEQ ID NO:14), AJ491307 (SEQ ID NO:13)]. The NADP-dependent enzymes are known as EC 1.1.1.2 and are available, for example, from *Pyrococcus furiosus* [GenBank Nos: AAC25556 (SEQ ID NO:91), AF013169 (SEQ ID NO:90)]. Additionally, a butanol dehydrogenase is available from *Escherichia coli* [GenBank Nos:NP_417484 (SEQ ID NO:75), NC_000913 (SEQ ID NO:74)] and a cyclohexanol dehydrogenase is available from *Acinetobacter* sp. [GenBank Nos: AAG10026 (SEQ ID NO:72), AF282240 (SEQ ID NO:71)].

The term "acetoin kinase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of acetoin to phosphoacetoin. Acetoin kinase may utilize ATP (adenosine triphosphate) or phosphoenolpyruvate as the phosphate donor in the reaction. Although there are no reports of enzymes catalyzing this reaction on acetoin, there are enzymes that catalyze the analogous reaction on the similar substrate dihydroxyacetone, for example, enzymes known as EC 2.7.1.29 (Garcia-Alles et al. (2004) *Biochemistry* 43:13037-13046).

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

The term "aminobutanol phosphate phospho-lyase", also called "amino alcohol O-phosphate lyase", refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of 3-amino-2-butanol O-phosphate to 2-butanone. Aminobutanol phosphate phospho-lyase may utilize the cofactor pyridoxal 5'-phosphate. There are

no previous reports of enzymes catalyzing this reaction on aminobutanol phosphate, though there are reports of enzymes that catalyze the analogous reaction on the similar substrate 1-amino-2-propanol phosphate (Jones et al. (1973) *Biochem J.* 134:167-182). The present invention
5 describes a newly identified aminobutanol phosphate phospho-lyase (SEQ ID NO: 126) from the organism *Erwinia carotovora*, with the activity demonstrated in Example 15 herein.

The term "aminobutanol kinase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of
10 3-amino-2-butanol to 3-amino-2-butanol O-phosphate. Aminobutanol kinase may utilize ATP as the phosphate donor. Although there are no reports of enzymes catalyzing this reaction on 3-amino-2-butanol, there are reports of enzymes that catalyze the analogous reaction on the similar substrates ethanolamine and 1-amino-2-propanol (Jones et al., *supra*).
15 The present invention describes, in Example 14, an amino alcohol kinase of *Erwinia carotovora* subsp. *atroseptica* (SEQ ID NO:124). The term "butanediol dehydrogenase" also known as "acetoin reductase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of acetoin to 2,3-butanediol. Butanediol dehydrogenases are a
20 subset of the broad family of alcohol dehydrogenases. Butanediol dehydrogenase enzymes may have specificity for production of (R) - or (S)-stereochemistry in the alcohol product. (S)-specific butanediol dehydrogenases are known as EC 1.1.1.76 and are available, for example, from *Klebsiella pneumoniae* (GenBank Nos: BBA13085 (SEQ ID
25 NO:6), D86412 (SEQ ID NO:5)). (R)-specific butanediol dehydrogenases are known as EC 1.1.1.4 and are available, for example, from *Bacillus cereus* [GenBank Nos. NP_830481 (SEQ ID NO:85), NC_004722 (SEQ ID NO:84); AAP07682 (SEQ ID NO:87), AE017000 (SEQ ID NO:86)], and *Lactococcus lactis* [GenBank Nos. AAK04995 (SEQ ID NO:89), AE006323
30 (SEQ ID NO:88)].

The term "butanediol dehydratase", also known as "diol dehydratase" or "propanediol dehydratase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of

2,3-butanediol to 2-butanone. Butanediol dehydratase may utilize the cofactor adenosyl cobalamin (vitamin B12). Adenosyl cobalamin-dependent enzymes are known as EC 4.2.1.28 and are available, for example, from *Klebsiella oxytoca* [GenBank Nos: BAA08099 (alpha subunit) (SEQ ID NO:8), D45071 (SEQ ID NO:7); BAA08100 (beta subunit) (SEQ ID NO:10), D45071 (SEQ ID NO:9); and BBA08101 (gamma subunit) (SEQ ID NO:12), D45071 (SEQ ID NO:11) (Note all three subunits are required for activity)], and *Klebsiella pneumoniae* [GenBank Nos: AAC98384 (alpha subunit) (SEQ ID NO:105), AF102064 (SEQ ID NO:104); GenBank Nos: AAC98385 (beta subunit) (SEQ ID NO:107), AF102064 (SEQ ID NO:106), GenBank Nos: AAC98386 (gamma subunit) (SEQ ID NO:109), AF102064 (SEQ ID NO:108)]. Other suitable diol dehydratases include, but are not limited to, B12-dependent diol dehydratases available from *Salmonella typhimurium* [GenBank Nos: AAB84102 (large subunit) (SEQ ID NO:93), AF026270 (SEQ ID NO:92); GenBank Nos: AAB84103 (medium subunit) (SEQ ID NO:95), AF026270 (SEQ ID NO:94); GenBank Nos: AAB84104 (small subunit) (SEQ ID NO:97), AF026270 (SEQ ID NO:96)]; and *Lactobacillus collinoides* [GenBank Nos: CAC82541 (large subunit) (SEQ ID NO:99), AJ297723 (SEQ ID NO:98); GenBank Nos: CAC82542 (medium subunit) (SEQ ID NO:101), AJ297723 (SEQ ID NO:100); GenBank Nos: CAD01091 (small subunit) (SEQ ID NO:103), AJ297723 (SEQ ID NO:102)]; and enzymes from *Lactobacillus brevis* (particularly strains CNRZ 734 and CNRZ 735, Speranza et al., *supra*), and nucleotide sequences that encode the corresponding enzymes. Methods of diol dehydratase gene isolation are well known in the art (e.g., U.S. Patent No. 5,686,276).

The term "glycerol dehydratase" refers to a polypeptide (or polypeptides) having an enzyme activity that catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde. Adenosyl cobalamin-dependent glycerol dehydratases are known as EC 4.2.1.30. The glycerol dehydratases of EC 4.2.1.30 are similar to the diol dehydratases in sequence and in having three subunits. The glycerol dehydratases can also be used to convert 2,3-butanediol to 2-butanone. Some examples of

glycerol dehydratases of EC 4.2.1.30 include those from *Klebsiella pneumoniae* (alpha subunit, SEQ ID NO:145, coding region and SEQ ID NO:146, protein; beta subunit, SEQ ID NO:147, coding region and SEQ ID NO:148, protein; and gamma subunit SEQ ID NO:149, coding region and
 5 SEQ ID NO:150, protein); from *Clostridium pasteurianum* [GenBank Nos: 3360389 (alpha subunit, SEQ ID NO:135), 3360390 (beta subunit, SEQ ID NO:136), and 3360391 (gamma subunit, SEQ ID NO:137)]; from *Escherichia blattae* [GenBank Nos: 60099613 (alpha subunit, SEQ ID NO:138), 57340191 (beta subunit, SEQ ID NO:139), and 57340192
 10 (gamma subunit, SEQ ID NO:140)]; and from *Citrobacter freundii* [GenBank Nos: 1169287 (alpha subunit, SEQ ID NO:141), 1229154 (beta subunit, SEQ ID NO:142), and 1229155 (gamma subunit, SEQ ID NO:143)]. Note that all three subunits are required for activity. Additional glycerol dehydratases are listed in Table 2.

15 Diol and glycerol dehydratases may undergo suicide inactivation during catalysis. A reactivating factor protein, also referred to herein as "reactivase", can be used to reactivate the inactive enzymes (Mori et al., *J. Biol. Chem.* 272:32034 (1997)). Preferably, the reactivating factor is obtained from the same source as the diol or glycerol dehydratase used.
 20 For example, suitable diol dehydratase reactivating factors are available from *Klebsiella oxytoca* [GenBank Nos: AAC15871 (large subunit) (SEQ ID NO:111), AF017781 (SEQ ID NO:110); GenBank Nos: AAC15872 (small subunit) (SEQ ID NO:113), AF017781 (SEQ ID NO:112)]; *Salmonella typhimurium* [GenBank Nos: AAB84105 (large subunit) (SEQ
 25 ID NO:115), AF026270 (SEQ ID NO:114), GenBank Nos: AAD39008 (small subunit) (SEQ ID NO:117), AF026270 (SEQ ID NO:116)]; and *Lactobacillus collinoides* [GenBank Nos: CAD01092 (large subunit) (SEQ ID NO:119), AJ297723 (SEQ ID NO:118); GenBank Nos: CAD01093 (small subunit) (SEQ ID NO:121), AJ297723 (SEQ ID NO:120)]. Both the
 30 large and small subunits are required for activity. For example, suitable glycerol dehydratase reactivating factors are available from *Klebsiella pneumoniae* (large subunit, SEQ ID NO:151, coding region and SEQ ID

NO:152, protein;, and small subunit, SEQ ID NO:153, coding region and SEQ ID NO:154, protein).

The term "a facultative anaerobe" refers to a microorganism that can grow in both aerobic and anaerobic environments.

5 The term "carbon substrate" or "fermentable carbon substrate" refers to a carbon source capable of being metabolized by host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof.

10 The term "gene" refers to a nucleic acid fragment that is capable of being expressed as a specific protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene"

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

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

25 native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

As used herein, an "isolated nucleic acid fragment" or "isolated nucleic acid molecule" or "genetic construct" will be used interchangeably and will mean a polymer of RNA or DNA that is single- or double-stranded,

30 optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

A nucleic acid fragment is "hybridizable" to another nucleic acid fragment, such as a cDNA, genomic DNA, or RNA molecule, when a single-stranded form of the nucleic acid fragment can anneal to the other nucleic acid fragment under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989), particularly Chapter 11 and Table 11.1 therein (entirely incorporated herein by reference). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar fragments (such as homologous sequences from distantly related organisms), to highly similar fragments (such as genes that duplicate functional enzymes from closely related organisms). Post-hybridization washes determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45 °C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50 °C for 30 min. A more preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60 °C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65 °C. An additional set of stringent conditions include hybridization at 0.1X SSC, 0.1% SDS, 65 °C and washes with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS, for example.

Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of

nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have
5 been derived (see Sambrook et al., *supra*, 9.50-9.51). For hybridizations with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., *supra*, 11.7-11.8). In one embodiment the length for a hybridizable nucleic acid
10 is at least about 10 nucleotides. Preferably a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least about 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as
15 necessary according to factors such as length of the probe.

A "substantial portion" of an amino acid or nucleotide sequence is that portion comprising enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to putatively identify that polypeptide or gene, either by manual evaluation of the sequence by one
20 skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Altschul, S. F., et al., *J. Mol. Biol.*, 215:403-410 (1993)). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as
25 homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques).
30 In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to specifically

identify and/or isolate a nucleic acid fragment comprising the sequence. The instant specification teaches the complete amino acid and nucleotide sequence encoding particular fungal proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a
5 substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

The term "complementary" is used to describe the relationship
10 between nucleotide bases that are capable of hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine.

The terms "homology" and "homologous" are used interchangeably herein. They refer to nucleic acid fragments wherein changes in one or
15 more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid
20 fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences.

Moreover, the skilled artisan recognizes that homologous nucleic acid sequences encompassed by this invention are also defined by their
25 ability to hybridize, under moderately stringent conditions (e.g., 0.5 X SSC, 0.1% SDS, 60 °C) with the sequences exemplified herein, or to any portion of the nucleotide sequences disclosed herein and which are functionally equivalent to any of the nucleic acid sequences disclosed herein.

"Codon degeneracy" refers to the nature in the genetic code
30 permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when

synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

The term "percent identity", as known in the art, is a relationship
5 between two or more polypeptide sequences or two or more
polynucleotide sequences, as determined by comparing the sequences.
In the art, "identity" also means the degree of sequence relatedness
between polypeptide or polynucleotide sequences, as the case may be, as
determined by the match between strings of such sequences. "Identity"
10 and "similarity" can be readily calculated by known methods, including but
not limited to those described in: 1.) Computational Molecular Biology
(Lesk, A. M., Ed.) Oxford University: NY (1988); 2.) Biocomputing:
Informatics and Genome Projects (Smith, D. W., Ed.) Academic: NY
(1993); 3.) Computer Analysis of Sequence Data, Part I (Griffin, A. M., and
15 Griffin, H. G., Eds.) Humana: NJ (1994); 4.) Sequence Analysis in
Molecular Biology (von Heinje, G., Ed.) Academic (1987); and
5.) Sequence Analysis Primer (Gribskov, M. and Devereux, J., Eds.)
Stockton: NY (1991).

Preferred methods to determine identity are designed to give the
20 best match between the sequences tested. Methods to determine identity
and similarity are codified in publicly available computer programs.
Sequence alignments and percent identity calculations may be performed
using the MegAlign™ program of the LASERGENE bioinformatics
computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the
25 sequences is performed using the "Clustal method of alignment" which
encompasses several varieties of the algorithm including the "Clustal V
method of alignment" corresponding to the alignment method labeled
Clustal V (described by Higgins and Sharp, *CABIOS*. 5:151-153 (1989);
Higgins, D.G. et al., *Comput. Appl. Biosci.*, 8:189-191 (1992)) and found in
30 the MegAlign™ program of the LASERGENE bioinformatics computing
suite (DNASTAR Inc.). For multiple alignments, the default values
correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10.
Default parameters for pairwise alignments and calculation of percent

identity of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program. Additionally the "Clustal W method of alignment" is available and corresponds to the alignment method labeled Clustal W (described by Higgins and Sharp, *CABIOS*. 5:151-153 (1989); Higgins, D.G. et al., *Comput. Appl. Biosci.* 8:189-191(1992)) and found in the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). Default parameters for multiple alignment (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs(%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB). After alignment of the sequences using the Clustal W program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying polypeptides, from other species, wherein such polypeptides have the same or similar function or activity. Useful examples of percent identities include, but are not limited to: 24%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any integer percentage from 24% to 100% may be useful in describing the present invention, such as 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. Suitable nucleic acid fragments not only have the above homologies but typically encode a polypeptide having at least 50 amino acids, preferably at least 100 amino acids, more

preferably at least 150 amino acids, still more preferably at least 200 amino acids, and most preferably at least 250 amino acids.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include, but is not limited to: 1.) the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI); 2.) BLASTP, BLASTN, BLASTX (Altschul et al., *J. Mol. Biol.*, 215:403-410 (1990)); 3.) DNASTAR (DNASTAR, Inc. Madison, WI); 4.) Sequencher (Gene Codes Corporation, Ann Arbor, MI); and 5.) the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.*, [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Plenum: New York, NY). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

As used herein the term "coding sequence" or "CDS" refers to a DNA sequence that codes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector binding site and stem-loop structure.

The term "promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be

composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of effecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

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

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

The terms "plasmid" and "vector" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or

recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation vector" refers to a specific vector containing a foreign gene and having
5 elements in addition to the foreign gene that facilitates transformation of a particular host cell.

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

15 The term "codon-optimized" as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA.

20 The term "fermentation product medium" refers to a medium in which fermentation has occurred such that product is present in the medium.

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

The 2-Butanol and 2-Butanone Biosynthetic Pathways

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

The invention enables the production of 2-butanone or 2-butanol from carbohydrate sources with recombinant microorganisms by providing a complete biosynthetic pathway from pyruvate to 2-butanone or 2-butanol. Three additional pathways are described. Although 2-butanol is not known to be the major product of any bacterial fermentation, there are a number of possible pathways for the production of 2-butanol via known biochemical reaction types. These pathways are shown in Figure 1. The letters and roman numerals cited below correspond to the letters and roman numerals in Figure 1, which are used to depict the conversion steps and products, respectively. As described below, 2-butanone is an intermediate in all of these 2-butanol biosynthetic pathways.

All of the pathways begin with the initial reaction of two pyruvate molecules to yield alpha-acetolactate (I), shown as the substrate to product conversion (a) in Figure 1. From alpha-acetolactate, there are 4 possible pathways to 2-butanone (V), referred to herein as 2-butanone biosynthetic pathways:

Pathway 1) I--->II--->III--->IV--->V (substrate to product conversions b,c,d,e); This is the pathway of the present invention.

2) I--->II--->VII--->IV--->V (substrate to product conversions b,g,h,e)

5 3) I--->II--->VIII--->V (substrate to product conversions b,i,j):

4) I--->IX--->X--->V (substrate to product conversions k,l,m)

The 2-butanol biosynthetic pathways conclude with the conversion of 2-butanone (V) to 2-butanol (VI). A detailed discussion of the substrate to product conversions in each pathway is given below.

Pathway 1:

(a) pyruvate to alpha-acetolactate:

The initial step in pathway 1 is the conversion of two molecules of pyruvate to one molecule of alpha-acetolactate (compound I in Figure 1) and one molecule of carbon dioxide catalyzed by a thiamin pyrophosphate-dependent enzyme. Enzymes catalyzing this substrate to product conversion (generally called either acetolactate synthase or acetohydroxy acid synthase; EC 2.2.1.6 [switched from 4.1.3.18 in 2002]) are well-known, and they participate in the biosynthetic pathway for the proteinogenic amino acids leucine and valine, as well as in the pathway for fermentative production of 2,3-butanediol and acetoin of a number of organisms.

The skilled person will appreciate that polypeptides having acetolactate synthase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. Some example of suitable acetolactate synthase enzymes are available from a number of sources, for example, *Bacillus subtilis* [GenBank Nos: AAA22222 NCBI (National Center for Biotechnology Information) amino acid sequence (SEQ ID NO:77), L04470 NCBI nucleotide sequence (SEQ ID NO:76)], *Klebsiella terrigena* [GenBank Nos: AAA25055 (SEQ ID NO:79), L04507 (SEQ ID NO:78)], and *Klebsiella pneumoniae* [GenBank Nos: AAA25079 (SEQ ID NO:4), M73842 (SEQ ID NO:3)]. Preferred acetolactate synthase enzymes are those that have at least 80% – 85%

identity to SEQ ID NO's 4, 77, and 79, where at least 85% - 90% identity is more preferred and where at least 95% identity based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix, is most preferred.

(b) alpha-acetolactate to acetoin:

Alpha-acetolactate (I) is converted to acetoin (II) by the action of an enzyme such as acetolactate decarboxylase (EC 4.1.1.5). Like acetolactate synthase, this enzyme is thiamin pyrophosphate-dependent and is also involved in the production of 2,3-butanediol and acetoin by a number of organisms. The enzymes from different sources vary quite widely in size (25-50 kilodaltons), oligomerization (dimer-hexamer), localization (intracellular or extracellular), and allosteric regulation (for example, activation by branched-chain amino acids). For the purpose of the present invention, an intracellular location is preferable to extracellular, but other variations are generally acceptable.

The skilled person will appreciate that polypeptides having acetolactate decarboxylase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. Some example of suitable acetolactate decarboxylase enzymes are available from a number of sources, for example, *Bacillus subtilis* [GenBank Nos: AAA22223 (SEQ ID NO:81), L04470 (SEQ ID NO:80)], *Klebsiella terrigena* [GenBank Nos: AAA25054 (SEQ ID NO:83), L04507 (SEQ ID NO:82)] and *Klebsiella pneumoniae* [GenBank Nos: AAU43774 (SEQ ID NO:2), AY722056 (SEQ ID NO:1)].

Preferred acetolactate decarboxylase enzymes are those that have at least 80% – 85% identity to SEQ ID NO's 2, 81 and 83, where at least 85% - 90% identity is more preferred and where at least 95% identity based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix, is most preferred.

(c) acetoin to 3-amino-2-butanol:

There are two known types of biochemical reactions that could effect the substrate to product conversion of acetoin (II) to 3-amino-2-butanol (III), specifically, pyridoxal phosphate-dependent transamination utilizing an accessory amino donor and direct reductive amination with ammonia. In the latter case, the reducing equivalents are supplied in the form of a reduced nicotinamide cofactor (either NADH or NADPH). An example of an NADH-dependent enzyme catalyzing this reaction with acetoin as a substrate is reported by Ito et al. (U.S. Patent No. 6,432,688). Any stereospecificity of this enzyme has not been assessed. An example of a pyridoxal phosphate-dependent transaminase that catalyzes the conversion of acetoin to 3-amino-2-butanol has been reported by Shin and Kim (*supra*). This enzyme was shown in Example 13 herein to convert both the (*R*) isomer of acetoin to the (2*R*,3*S*) isomer of 3-amino-2-butanol and the (*S*) isomer of acetoin to the (2*S*,3*S*) isomer of 3-amino-2-butanol. Either type of enzyme (i.e., transaminase or reductive aminase) is considered to be an acetoin aminase and may be utilized in the production of 2-butanol. Other enzymes in this group may have different stereospecificities.

The skilled person will appreciate that polypeptides having acetoin aminase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. One example of this activity has is described herein and is identified as SEQ ID NO:122. Accordingly preferred acetoin aminase enzymes are those that have at least 80% – 85% identity to SEQ ID NO:122, where at least 85% - 90% identity is more preferred and where at least 95% identity based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix, is most preferred.

(d) 3-amino-2-butanol to 3-amino-2-butanol O-phosphate:

There are no enzymes known in the art that catalyze the substrate to product conversion of 3-amino-2-butanol (III) to 3-amino-2-butanol phosphate (IV). However, a few *Pseudomonas* and *Erwinia* species have been shown to express an ATP-dependent ethanolamine kinase (EC

2.7.1.82) which allows them to utilize ethanolamine or 1-amino-2-propanol as a nitrogen source (Jones et al. (1973) *Biochem. J.* 134:167-182). It is likely that this enzyme also has activity towards 3-amino-2-butanol or could be engineered to do so, thereby providing an aminobutanol kinase.

5 The present invention describes in Example 14, a gene of *Erwinia carotovora* subsp. *atroseptica* (SEQ ID NO:123) that encodes a protein (SEQ ID NO:24). This protein has been identified as an amino alcohol kinase. This enzyme may be used to convert 3-amino-2-butanol to 3-amino-2-butanol O-phosphate.

10 The skilled person will appreciate that polypeptides having aminobutanol kinase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. One example of this activity has is described herein and is identified as SEQ ID NO:124. Accordingly preferred aminobutanol kinase enzymes are those
15 that have at least 80% – 85% identity to SEQ ID NO:124, where at least 85% - 90% identity is more preferred and where at least 95% identity based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix, is most preferred.

20 (e) 3-amino-2-butanol phosphate to 2-butanone:

Although there are no enzymes reported to catalyze the substrate to product conversion of 3-amino-2-butanol phosphate (IV) to 2-butanone (V), the substrate is very similar to those utilized by the pyridoxal phosphate-dependent phosphoethanolamine phospho-lyase enzyme,
25 which has been found in a small number of *Pseudomonas* and *Erwinia* species. These enzymes have activity towards phosphoethanolamine and both enantiomers of 2-phospho-1-aminopropane (Jones et al. (1973) *Biochem. J.* 134:167-182), and may also have activity towards 3-amino-2-butanol O-phosphate. Identified herein is a gene of *Erwinia carotovora*
30 subsp. *atroseptica* (SEQ ID NO:125) that encodes a protein (SEQ ID NO:126) with homology to class III aminotransferases. Example 15 demonstrates that this enzyme is active on both aminopropanol phosphate and aminobutanol phosphate substrates. The newly identified and

characterized enzyme was able to catalyze the conversion of a mixture of (*R*)-3-amino-(*S*)-2-butanol and (*S*)-3-amino-(*R*)-2-butanol O-phosphate, and a mixture of (*R*)-3-amino-(*R*)-2-butanol and (*S*)-3-amino-(*S*)-2-butanol O-phosphate to 2-butanone. The newly identified and
5 characterized enzyme was also able to catalyze the conversion of both (*R*) and (*S*)-2-amino-1-propanol phosphate to propanone, with a preference for (*S*)-2-amino-1-propanol phosphate. The highest activity was observed with the proposed natural substrate DL-1-amino-2-propanol phosphate, which was converted to propionaldehyde.

10 The skilled person will appreciate that polypeptides having aminobutanol phosphate phospho-lyase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. One example of a suitable aminobutanol phosphate phospho-lyase enzyme is described herein as SEQ ID NO: 126. Accordingly
15 preferred aminobutanol phosphate phospho-lyase enzymes are those that have at least 80% – 85% identity to SEQ ID NO's 126, where at least 85% - 90% identity is more preferred and where at least 95% identity based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250
20 series of protein weight matrix, is most preferred.

(f) 2-butanone to 2-butanol:

The final step in all pathways to produce 2- butanol from pyruvic acid is the reduction of 2-butanone (V) to 2-butanol (VI). This substrate to product conversion is catalyzed by some members of the broad class of
25 alcohol dehydrogenases (types utilizing either NADH or NADPH as a source of hydride, depending on the enzyme) that may be called butanol dehydrogenases. Enzymes of each type that catalyze the reduction of 2-butanone are well known, as described above in the definition for butanol dehydrogenase.

30 The skilled person will appreciate that polypeptides having butanol dehydrogenase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. Some example of suitable butanol dehydrogenase enzymes are available from a number

of sources, for example, *Rhodococcus ruber* [GenBank Nos: CAD36475 (SEQ ID NO:14), AJ491307 (SEQ ID NO:13)]. The NADP-dependent enzymes are known as EC 1.1.1.2 and are available, for example, from *Pyrococcus furiosus* [GenBank Nos: AAC25556 (SEQ ID NO:91), AF013169 (SEQ ID NO:90)]. Additionally, a butanol dehydrogenase is available from *Escherichia coli* [GenBank Nos: NP_417484 (SEQ ID NO:75), NC_000913 (SEQ ID NO:74)] and a cyclohexanol dehydrogenase is available from *Acinetobacter* sp. [GenBank Nos: AAG10026 (SEQ ID NO:72), AF282240 (SEQ ID NO:71)]. Preferred butanol dehydrogenase enzymes are those that have at least 80% – 85% identity to SEQ ID NO's 14, 91, 75, and 72, where at least 85% - 90% identity is more preferred and where at least 95% identity based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix, is most preferred.

Pathway 2:

(a) pyruvate to alpha-acetolactate:

This substrate to product conversion is the same as described above for Pathway 1.

(b) alpha-acetolactate to acetoin:

This substrate to product conversion is the same as described above for Pathway 1.

(g) acetoin to phosphoacetoin:

Although enzymes that catalyze the substrate to product conversion of acetoin (II) to phosphoacetoin (VII) have not been described, the structure of the substrate acetoin is very similar to that of dihydroxyacetone, and therefore acetoin may be an acceptable substrate for dihydroxyacetone kinase (EC 2.7.1.29), an enzyme which catalyzes phosphorylation of dihydroxyacetone. Protein engineering techniques for the alteration of substrate specificity of enzymes are well known (Antikainen and Martin (2005) *Bioorg. Med. Chem.* 13:2701-2716) and may be used to generate an enzyme with the required specificity. In this conversion, the phosphate moiety may be supplied by any high energy

biological phosphate donor, with the common substrates being phosphoenolpyruvate (as in the *E. coli* dihydroxyacetone kinase) and ATP (as in the *Citrobacter freundii* dihydroxyacetone kinase) (Garcia-Alles et al. (2004) *Biochemistry* 43:13037-13045).

5 (h) phosphoacetoin to 3-amino-2-butanol O-phosphate:

Although enzymes that catalyze the substrate to product conversion of phosphoacetoin (VII) to 3-amino-2-butanol O-phosphate (IV) have not been described, the structure of the substrate is very similar to that of dihydroxyacetone phosphate a substrate for the proposed serinol
10 phosphate aminotransferase encoded by the 5' portion of the *rtxA* gene in some species of *Bradyrhizobium* (Yasuta et al., *supra*). Thus a serinol phosphate aminotransferase may be functional in this step.

(e) 3-amino-2-butanol O-phosphate to 2-butanone:

This substrate to product conversion is the same as described
15 above for Pathway 1.

(f) 2-butanone to 2-butanol:

This substrate to product conversion is the same as described above for Pathway 1.

Pathway 3:

20 (a) pyruvate to alpha-acetolactate:

This substrate to product conversion is the same as described above for Pathway 1. (b) alpha-acetolactate to acetoin:

This substrate to product conversion is the same as described above for Pathway 1.

25 (i) acetoin to 2,3-butanediol:

The substrate to product conversion of acetoin (II) to 2,3-butanediol (VIII) may be catalyzed by a butanediol dehydrogenase that may either utilize NADH or NADPH as the source of reducing equivalents when carrying out reductions. Enzymes with activity towards acetoin participate
30 in the pathway for production of 2,3-butanediol in organisms that produce that compound. The reported enzymes (e.g., BudC from *Klebsiella pneumoniae* (Ui et al. (2004) *Letters in Applied Microbiology* 39:533-537)

generally utilize NADH. Either cofactor is acceptable for use in the production of 2-butanol by this pathway.

(j) 2,3-butanediol to 2-butanone:

The substrate to product conversion of 2,3-butanediol (VIII) to 2-
5 butanone (V) may be catalyzed by diol dehydratase enzymes (EC 4.2.1.28) and glycerol dehydratase enzymes (EC 4.2.1.30). The best characterized diol dehydratase is the coenzyme B12-dependent *Klebsiella oxytoca* enzyme, but similar enzymes are found in a number of enteric bacteria. The *K. oxytoca* enzyme has been shown to accept *meso*-2,3-
10 butanediol as a substrate (Bachovchin *et al.* (1977) *Biochemistry* 16:1082-1092), producing the desired product 2-butanone. Example 17 demonstrates that the *Klebsiella pneumoniae* glycerol dehydratase was able to convert *meso*-2,3-butanediol to 2-butanone. The three subunits of the *Klebsiella pneumoniae* glycerol dehydratase (alpha: SEQ ID NO:145
15 (coding region) and 146 (protein); beta: SEQ ID NO: 147 (coding region) and 148 (protein); and gamma: SEQ ID NO: 149 (coding region) and 150 (protein)) were expressed in conjunction with the two subunits of the *Klebsiella pneumoniae* glycerol dehydratase reactivase (large subunit, SEQ ID NO: 151 (coding region) and 152 (protein); and small subunit,
20 SEQ ID NO: 153 (coding region) and 154 (protein)) to provide activity.

There are also reports in the literature of a B12-independent diol dehydratase from *Clostridium glycolicum* (Hartmanis *et al.* (1986) *Arch. Biochem. Biophys.* 245:144-152). This enzyme has activity towards 2,3-butanediol, although this activity is less than 1% of the activity towards
25 ethanediol, but the enzyme may be engineered to improve that activity. A better-characterized B12-independent dehydratase is the glycerol dehydratase from *Clostridium butyricum* (O'Brien *et al.* (2004) *Biochemistry* 43:4635-4645), which has high activity towards 1,2-propanediol as well as glycerol. This enzyme uses S-adenosylmethionine
30 as a source of adenosyl radical. There are no reports of activity towards 2,3-butanediol, but such activity, if not already present, may possibly be engineered.

(f) 2-butanone to 2-butanol:

This substrate to product conversion is the same as described above for Pathway 1.

Pathway 4:

5 (a) pyruvate to alpha-acetolactate:

This substrate to product conversion is the same as described above for Pathway 1.

 (k) alpha-acetolactate to 2,3-dihydroxy-2-methylbutanoic acid:

 The substrate to product conversion of acetolactate (I) to 2,3-
10 dihydroxy-2-methylbutanoic acid (IX) is not known in the art. However, the
product of this conversion has been reported as a component of
fermentation broths (Ziadi et al. (1973) *Comptes Rendus des Seances de
l'Academie des Sciences, Serie D: Sciences Naturelles* 276:965-8), but
the mechanism of formation is unknown. The likely mechanism of
15 formation is reduction of acetolactate with NADH or NADPH as the
electron donor. To utilize this pathway for production of 2-butanol, an
enzyme catalyzing this reaction needs to be identified or engineered.
However, the precedent for enzymatic reduction of ketones to alcohols is
well established.

20 (l) 2,3-dihydroxy-2-methylbutanoic acid to 2-hydroxy-2-methyl-3-
phosphobutanoic acid:

 There are no enzymes known that catalyze the substrate to product
conversion of 2,3-dihydroxy-2-methylbutanoic acid (IX) to 2-hydroxy-2-
methyl-3-phosphobutanoic acid (X). However, there are a large number of
25 kinases in Nature that possess varying specificity. It is therefore likely that
an enzyme could be isolated or engineered with this activity.

 (m) 2-hydroxy-2-methyl-3-phosphobutanoic acid to 2-butanone:

 There are no known enzymes that catalyze the substrate to product
conversion of 2-hydroxy-2-methyl-3-phosphobutanoic acid (X) to 2-
30 butanone (V). The combination of this reaction with the previous one is
very similar to the multi-step reaction catalyzed by mevalonate-5-
pyrophosphate (M5PP) decarboxylase, which consists of initial
phosphorylation of M5PP to 3-phosphomevalonate-5-PP, followed by

decarboxylation-dependent elimination of phosphate (Alvear *et al.* (1982) *Biochemistry* 21:4646-4650).

(f) 2-butanone to 2-butanol:

5 This substrate to product conversion is the same as described above for Pathway 1.

Thus, in providing multiple recombinant pathways from pyruvate to 2-butanol, there exists a number of choices to fulfill the individual conversion steps, and the person of skill in the art will be able to utilize publicly available sequences and sequences disclosed herein to construct
10 the relevant pathways. A listing of a representative number of genes known in the art and useful in the construction of 2-butanol biosynthetic pathways is given above in Tables 1 and 2.

Microbial Hosts for 2-Butanol and 2-Butanone Production

Microbial hosts for 2-butanol or 2-butanone production may be
15 selected from bacteria, cyanobacteria, filamentous fungi and yeasts. The microbial host used for 2-butanol or 2-butanone production should be tolerant to the product produced, so that the yield is not limited by toxicity of the product to the host. The selection of a microbial host for 2-butanol production is described in detail below. The same criteria apply to the
20 selection of a host for 2-butanone production.

Microbes that are metabolically active at high titer levels of 2-butanol are not well known in the art. Although butanol-tolerant mutants have been isolated from solventogenic *Clostridia*, little information is available concerning the butanol tolerance of other potentially useful
25 bacterial strains. Most of the studies on the comparison of alcohol tolerance in bacteria suggest that butanol is more toxic than ethanol (de Cavalho *et al.*, *Microsc. Res. Tech.* 64:215-22 (2004) and Kabelitz *et al.*, *FEMS Microbiol. Lett.* 220:223-227 (2003)). Tomas *et al.* (*J. Bacteriol.* 186:2006-2018 (2004)) report that the yield of 1-butanol during
30 fermentation in *Clostridium acetobutylicum* may be limited by butanol toxicity. The primary effect of 1-butanol on *Clostridium acetobutylicum* is disruption of membrane functions (Hermann *et al.*, *Appl. Environ. Microbiol.* 50:1238-1243 (1985)).

The microbial hosts selected for the production of 2-butanol should be tolerant to 2-butanol and should be able to convert carbohydrates to 2-butanol using the introduced biosynthetic pathway. The criteria for selection of suitable microbial hosts include the following: intrinsic
5 tolerance to 2-butanol, high rate of carbohydrate utilization, availability of genetic tools for gene manipulation, and the ability to generate stable chromosomal alterations.

Suitable host strains with a tolerance for 2-butanol may be identified by screening based on the intrinsic tolerance of the strain. The intrinsic
10 tolerance of microbes to 2-butanol may be measured by determining the concentration of 2-butanol that is responsible for 50% inhibition of the growth rate (IC50) when grown in a minimal medium. The IC50 values may be determined using methods known in the art. For example, the microbes of interest may be grown in the presence of various amounts of
15 2-butanol and the growth rate monitored by measuring the optical density at 600 nanometers. The doubling time may be calculated from the logarithmic part of the growth curve and used as a measure of the growth rate. The concentration of 2-butanol that produces 50% inhibition of growth may be determined from a graph of the percent inhibition of growth
20 versus the 2-butanol concentration. Preferably, the host strain should have an IC50 for 2-butanol of greater than about 0.5%. More suitable is a host strain with an IC50 for 2-butanol that is greater than about 1.5%. Particularly suitable is a host strain with an IC50 for 2-butanol that is greater than about 2.5%.

25 The microbial host for 2-butanol production should also utilize glucose and/or other carbohydrates at a high rate. Most microbes are capable of utilizing carbohydrates. However, certain environmental microbes cannot efficiently use carbohydrates, and therefore would not be suitable hosts.

30 The ability to genetically modify the host is essential for the production of any recombinant microorganism. Modes of gene transfer technology that may be used include by electroporation, conjugation, transduction or natural transformation. A broad range of host conjugative

plasmids and drug resistance markers are available. The cloning vectors used with an organism are tailored to the host organism based on the nature of antibiotic resistance markers that can function in that host.

5 The microbial host also may be manipulated in order to inactivate competing pathways for carbon flow by inactivating various genes. This requires the availability of either transposons or chromosomal integration vectors to direct inactivation. Additionally, production hosts that are amenable to chemical mutagenesis may undergo improvements in intrinsic 2-butanol tolerance through chemical mutagenesis and mutant
10 screening.

Based on the criteria described above, suitable microbial hosts for the production of 2-butanol and 2-butanone include, but are not limited to, members of the genera *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Lactobacillus*,
15 *Enterococcus*, *Pediococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*, *Pichia*, *Candida*, *Hansenula* and *Saccharomyces*. Preferred hosts include: *Escherichia coli*, *Alcaligenes eutrophus*, *Bacillus licheniformis*, *Paenibacillus macerans*, *Rhodococcus erythropolis*, *Pseudomonas putida*, *Lactobacillus plantarum*, *Enterococcus faecium*, *Enterococcus gallinarum*,
20 *Enterococcus faecalis*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Bacillus subtilis* and *Saccharomyces cerevisiae*.

Construction of Production Host

Recombinant organisms containing the necessary genes that
25 encode the enzymatic pathway for the conversion of a fermentable carbon substrate to 2-butanol or 2-butanone may be constructed using techniques well known in the art. In the present invention, genes encoding the enzymes of the 2-butanol biosynthetic Pathway 1: acetolactate synthase, acetolactate decarboxylase, acetoin aminase (or amine:pyruvate
30 transaminase), aminobutanol kinase, aminobutanol O-phosphate lyase and butanol dehydrogenase; or 2-butanone biosynthetic Pathway 1 omitting the butanol dehydrogenase, may be isolated from various sources, as described above.

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

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

In preparing a synthetic DNA fragment containing a coding sequence, this sequence may be optimized for expression in the target host cell. Tools for codon optimization for expression in a heterologous host are readily available. Some tools for codon optimization are available based on the GC content of the host organism. The GC contents of some exemplary microbial hosts are given Table 3.

Table 3
GC Contents of Microbial Hosts

Strain	% GC
<i>B. licheniformis</i>	46
<i>B. subtilis</i>	42
<i>C. acetobutylicum</i>	37

<i>E. coli</i>	50
<i>P. putida</i>	61
<i>A. eutrophus</i>	61
<i>Paenibacillus macerans</i>	51
<i>Rhodococcus erythropolis</i>	62
<i>Brevibacillus</i>	50
<i>Paenibacillus polymyxa</i>	50

Once the relevant pathway genes are identified and isolated they may be transformed into suitable expression hosts by means well known in the art. Vectors useful for the transformation of a variety of host cells are common and commercially available from companies such as EPICENTRE[®] (Madison, WI), Invitrogen Corp. (Carlsbad, CA), Stratagene (La Jolla, CA), and New England Biolabs, Inc. (Beverly, MA). Typically the vector contains a selectable marker and sequences allowing autonomous replication or chromosomal integration in the desired host. In addition, suitable vectors comprise a promoter region which harbors transcriptional initiation controls and a transcriptional termination control region, between which a coding region DNA fragment may be inserted, to provide expression of the inserted coding region. Both control regions may be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions may also be derived from genes that are not native to the specific species chosen as a production host.

Initiation control regions or promoters, which are useful to drive expression of the relevant pathway coding regions in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genetic elements is suitable for the present invention including, but not limited to, promoters derived from the following genes: *CYC1*, *HIS3*, *GAL1*, *GAL10*, *ADH1*, *PGK*, *PHO5*, *GAPDH*, *ADC1*, *TRP1*, *URA3*, *LEU2*, *ENO*, *TPI*, *CUP1*, *FBA*, *GPD*, and *GPM* (useful for expression in *Saccharomyces*); *AOX1* (useful for expression in *Pichia*); as well as the *lac*, *ara*, *tet*, *trp*, *IP_L*, *IP_R*, *T7*, *tac*, and

trc promoters (useful for expression in *Escherichia coli*, *Alcaligenes*, and *Pseudomonas*); the *amy*, *apr*, and *npr* promoters, and various phage promoters useful for expression in *Bacillus subtilis*, *Bacillus licheniformis*, and *Paenibacillus macerans*; *nisA* (useful for expression Gram-positive bacteria, Eichenbaum et al. *Appl. Environ. Microbiol.* 64(8):2763-2769 (1998)); and the synthetic P11 promoter (useful for expression in *Lactobacillus plantarum*, Rud et al., *Microbiology* 152:1011-1019 (2006)).

Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary, however, it is most preferred if included.

Certain vectors are capable of replicating in a broad range of host bacteria and can be transferred by conjugation. The complete and annotated sequence of pRK404 and three related vectors: pRK437, pRK442, and pRK442(H), are available. These derivatives have proven to be valuable tools for genetic manipulation in Gram-negative bacteria (Scott et al., *Plasmid* 50(1):74-79 (2003)). Several plasmid derivatives of broad-host-range Inc P4 plasmid RSF1010 are also available with promoters that can function in a range of Gram-negative bacteria. Plasmid pAYC36 and pAYC37, have active promoters along with multiple cloning sites to allow for heterologous gene expression in Gram-negative bacteria.

Chromosomal gene replacement tools are also widely available. For example, a thermosensitive variant of the broad-host-range replicon pWV101 has been modified to construct a plasmid pVE6002 which can be used to effect gene replacement in a range of Gram-positive bacteria (Maguin et al., *J. Bacteriol.* 174(17):5633-5638 (1992)). Additionally, in vitro transposomes are available from commercial sources such as EPICENTRE[®] to create random mutations in a variety of genomes.

The expression of a 2-butanol biosynthetic pathway in various preferred microbial hosts is described in more detail below. For the expression of a 2-butanone biosynthetic pathway, the same description

applies, but the final substrate to product conversion of 2-butanone to 2-butanol is omitted.

Expression of a 2-butanol or 2-butanone biosynthetic pathway in *E. coli*

5 Vectors useful for the transformation of *E. coli* are common and commercially available from the companies listed above. For example, the genes of a 2-butanol biosynthetic pathway may be isolated from various sources, as described above, cloned onto a modified pUC19 vector and transformed into *E. coli* NM522, as described in Examples 6
10 and 7. Alternatively, the genes encoding a 2-butanol biosynthetic pathway may be divided into multiple operons, cloned onto expression vectors, and transformed into various *E. coli* strains, as described in Examples 9, 10, and 11. The 2-butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

15 Expression of a 2-butanol or 2-butanone biosynthetic pathway in *Rhodococcus erythropolis*

 A series of *E. coli*-*Rhodococcus* shuttle vectors are available for expression in *R. erythropolis*, including, but not limited to pRhBR17 and pDA71 (Kostichka et al., *Appl. Microbiol. Biotechnol.* 62:61-68 (2003)).
20 Additionally, a series of promoters are available for heterologous gene expression in *R. erythropolis* (see for example Nakashima et al., *Appl. Environ. Microbiol.* 70:5557-5568 (2004), and Tao et al., *Appl. Microbiol. Biotechnol.* 2005, DOI 10.1007/s00253-005-0064). Targeted gene
25 disruptions in chromosomal genes of *R. erythropolis* may be created using the methods described by Tao et al., *supra*, and Brans et al. (*Appl. Environ. Microbiol.* 66: 2029-2036 (2000)).

 The heterologous genes required for the production of 2-butanol, as described above, may be cloned initially in pDA71 or pRhBR71 and transformed into *E. coli*. The vectors may then be transformed into *R.*
30 *erythropolis* by electroporation, as described by Kostichka et al., *supra*. The recombinants may be grown in synthetic medium containing glucose and the production of 2-butanol can be followed using fermentation

methods known in the art. The 2-butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

Expression of a 2-butanol or 2-butanone biosynthetic pathway in *B. Subtilis*

5 Methods for gene expression and creation of mutations in *B. subtilis* are also well known in the art. For example, the genes of a 2-butanol biosynthetic pathway may be isolated from various sources, as described above, cloned into a modified *E. coli-Bacillus* shuttle vector and transformed into *Bacillus subtilis* BE1010, as described in Example 8, The
10 desired genes may be cloned into a *Bacillus* expression vector and transformed into a strain to make a production host. Alternatively, the genes may be integrated into the *Bacillus* chromosome using conditional replicons or suicide vectors that are known to one skilled in the art. For example, the *Bacillus* Genetic Stock Center carries numerous integration
15 vectors. The 2-butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

Expression of a 2-butanol or 2-butanone biosynthetic pathway in *B. licheniformis*

20 Most of the plasmids and shuttle vectors that replicate in *B. subtilis* may be used to transform *B. licheniformis* by either protoplast transformation or electroporation. The genes required for the production of 2-butanol may be cloned in plasmids pBE20 or pBE60 derivatives (Nagarajan et al., *Gene* 114:121-126 (1992)). Methods to transform *B. licheniformis* are known in the art (for example see Fleming et al. *Appl. Environ. Microbiol.*, 61(11):3775-3780 (1995)). The plasmids constructed for expression in *B. subtilis* may be transformed into *B. licheniformis* to produce a recombinant microbial host that produces 2-butanol. The 2-butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

30 Expression of a 2-butanol or 2-butanone biosynthetic pathway in *Paenibacillus macerans*

Plasmids may be constructed as described above for expression in *B. subtilis* and used to transform *Paenibacillus macerans* by protoplast

transformation to produce a recombinant microbial host that produces 2-butanol. The 2-butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

5 Expression of a 2-butanol or 2-butanone biosynthetic pathway in
Alcaligenes (Ralstonia) eutrophus

Methods for gene expression and creation of mutations in *Alcaligenes eutrophus* are known in the art (see for example Taghavi et al., *Appl. Environ. Microbiol.*, 60(10):3585-3591 (1994)). The genes for a 2-butanol biosynthetic pathway may be cloned in any of the broad host
10 range vectors described above, and electroporated into *Alcaligenes eutrophus* to generate recombinants that produce 2-butanol. The poly(hydroxybutyrate) pathway in *Alcaligenes* has been described in detail, a variety of genetic techniques to modify the *Alcaligenes eutrophus* genome are known, and those tools can be applied for engineering a 2-
15 butanol biosynthetic pathway. The 2-butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

Expression of a 2-butanol or 2-butanone biosynthetic pathway in
Pseudomonas putida

Methods for gene expression in *Pseudomonas putida* are known in
20 the art (see for example Ben-Bassat et al., U.S. Patent No. 6,586,229, which is incorporated herein by reference). The genes of a 2-butanol biosynthetic pathway may be inserted into pPCU18, and this ligated DNA may be electroporated into electrocompetent *Pseudomonas putida* DOT-T1 C5aAR1 cells to generate recombinants that produce 2-butanol. The 2-
25 butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

Expression of a 2-butanol or 2-butanone biosynthetic pathway in
Lactobacillus plantarum

The *Lactobacillus* genus belongs to the *Lactobacillales* family and
30 many plasmids and vectors used in the transformation of *Bacillus subtilis* and *Streptococcus* may be used for *Lactobacillus*. Non-limiting examples of suitable vectors include pAM β 1 and derivatives thereof (Renault et al.,

Gene 183:175-182 (1996); and O'Sullivan et al., *Gene* 137:227-231 (1993)); pMBB1 and pHW800, a derivative of pMBB1 (Wyckoff et al. *Appl. Environ. Microbiol.* 62:1481-1486 (1996)); pMG1, a conjugative plasmid (Tanimoto et al., *J. Bacteriol.* 184:5800-5804 (2002)); pNZ9520
 5 (Kleerebezem et al., *Appl. Environ. Microbiol.* 63:4581-4584 (1997)); pAM401 (Fujimoto et al., *Appl. Environ. Microbiol.* 67:1262-1267 (2001)); and pAT392 (Arthur et al., *Antimicrob. Agents Chemother.* 38:1899-1903 (1994)). Several plasmids from *Lactobacillus plantarum* have also been reported (van Kranenburg et al., *Appl. Environ. Microbiol.* 71(3):1223-
 10 1230 (2005)).

The various genes for a 2-butanol biosynthetic pathway may be assembled into any suitable vector, such as those described above. The codons can be optimized for expression based on the codon index deduced from the genome sequences of *Lactobacillus plantarum* or
 15 *Lactobacillus arizonensis*. The plasmids may be introduced into the host cell using methods known in the art, such as electroporation (Cruz-Rodz et al. *Molecular Genetics and Genomics* 224:1252-154 (1990), Bringel, et al. *Appl. Microbiol. Biotechnol.* 33: 664-670 (1990), Alegre et al., *FEMS Microbiology letters* 241:73-77 (2004)), and conjugation (Shrago et al.,
 20 *Appl. Environ. Microbiol.* 52:574-576 (1986)). The 2-butanol biosynthetic pathway genes can also be integrated into the chromosome of *Lactobacillus* using integration vectors (Hols et al., *Appl. Environ. Microbiol.* 60:1401-1403 (1990), Jang et al., *Micro. Lett.* 24:191-195 (2003)). The 2-butanone biosynthesis pathway may be similarly
 25 expressed, omitting the butanol dehydrogenase.

Expression of a 2-butanol or 2-butanone biosynthetic pathway in *Enterococcus faecium*, *Enterococcus gallinarum*, and *Enterococcus faecalis*

The *Enterococcus* genus belongs to the *Lactobacillales* family and
 30 many plasmids and vectors used in the transformation of *Lactobacillus*, *Bacillus subtilis*, and *Streptococcus*, described above, may be used for *Enterococcus*. Expression vectors for *E. faecalis* using the *nisA* gene from *Lactococcus* may also be used (Eichenbaum et al., *Appl. Environ.*

Microbiol. 64:2763-2769 (1998). Additionally, vectors for gene replacement in the *E. faecium* chromosome may be used (Nallaapareddy et al., *Appl. Environ. Microbiol.* 72:334-345 (2006)).

5 The various genes for a 2-butanol biosynthetic pathway may be assembled into any suitable vector, such as those described above. The codons can be optimized for expression based on the codon index deduced from the genome sequences of *Enterococcus faecalis* or *Enterococcus faecium*. The plasmids may be introduced into the host cell using methods known in the art, such as electroporation, as described by
10 Cruz-Rodz et al. (*Molecular Genetics and Genomics* 224:1252-154 (1990)) or conjugation, as described by Tanimoto et al. (*J. Bacteriol.* 184:5800-5804 (2002)) and Grohamann et al. (*Microbiol. Mol. Biol. Rev.* 67:277-301 (2003)). The 2-butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

15 Expression of a 2-butanol or 2-butanone biosynthetic pathway in *Pediococcus pentosaceus* and *Pediococcus acidilactici*.

The *Pediococcus* genus belongs to the *Lactobacillales* family and many plasmids and vectors used in the transformation of *Bacillus subtilis* and *Streptococcus*, described above, may be used for *Pediococcus*. A
20 non-limiting example of a suitable vector is pHPS9 (Bukhtiyarova et al. *Appl. Environ. Microbiol.* 60:3405-3408 (1994)). Several plasmids from *Pediococcus* have also been reported (Alegre et al., *FEMS Microbiol. Lett.* 250:151-156 (2005); Shareck et al. *Crit. Rev Biotechnol.* 24:155-208 (2004)).

25 The genes for a 2-butanol biosynthetic pathway may be assembled into any suitable vector, such as those described above. The codons can be optimized for expression based on the codon index deduced from the genome sequence of *Pediococcus pentosaceus*. The plasmids may be introduced into the host cell using methods known in the art, such as
30 electroporation (see for example, Osmanagaoglu et al., *J. Basic Microbiol.* 40:233-241 (2000); Alegre et al., *FEMS Microbiol. Lett.* 250:151-156 (2005)) and conjugation (Gonzalez and Kunka, *Appl. Environ. Microbiol.* 46:81-89 (1983)). The 2-butanol biosynthetic pathway genes can also be

integrated into the chromosome of *Pediococcus* using integration vectors (Davidson et al. *Antonie van Leeuwenhoek* 70:161-183 (1996)). The 2-butanone biosynthesis pathway may be similarly expressed, omitting the butanol dehydrogenase.

5 Fermentation Media

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

Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, preferred carbon substrates are glucose, fructose, and sucrose, as well as
30 mixtures of any of these sugars. Sucrose may be obtained from feedstocks such as sugar cane, sugar beets, cassava, and sweet sorghum. Glucose and dextrose may be obtained through saccharification

of starch based feedstocks including grains such as corn, wheat, rye, barley, and oats.

In addition, fermentable sugars may be obtained from cellulosic and lignocellulosic biomass through processes of pretreatment and saccharification, as described, for example, in co-owned and co-pending US patent application US20070031918A1, which is herein incorporated by reference. Biomass refers to any cellulosic or lignocellulosic material and includes materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides.

Biomass may also comprise additional components, such as protein and/or lipid. Biomass may be derived from a single source, or biomass can comprise a mixture derived from more than one source; for example, biomass could comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers and animal manure.

In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of an enzymatic pathway necessary for 2-butanol or 2-butanone production.

Culture Conditions

Typically cells are grown at a temperature in the range of about 25 °C to about 40 °C in an appropriate medium. Suitable growth media in the present invention are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Medium (YM) broth. Other defined or synthetic growth media may also be used,

and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, e.g., cyclic adenosine 2':3'-monophosphate, may also be
5 incorporated into the fermentation medium.

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

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

10 Industrial Batch and Continuous Fermentations

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

A variation on the standard batch system is the fed-batch system. Fed-batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the
30 substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate

concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO₂. Batch and fed-batch fermentations are common and well known in the art and examples may be found in Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36:227, (1992), herein incorporated by reference.

Although the present invention is performed in batch mode it is contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density.

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

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

subjected to fermentation conditions for 2-butanol or 2-butanone production.

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

5 The bioproducted 2-butanol may be isolated from the fermentation medium using methods known in the art for ABE fermentations (see for example, Durre, *Appl. Microbiol. Biotechnol.* 49:639-648 (1998), Groot et al., *Process Biochem.* 27:61-75 (1992), and references therein). For example, solids may be removed from the fermentation medium by
10 centrifugation, filtration, decantation, or the like. Then, the 2-butanol may be isolated from the fermentation medium using methods such as distillation, azeotropic distillation, liquid-liquid extraction, adsorption, gas stripping, membrane evaporation, or pervaporation. These same methods may be adapted to isolate bioproducted 2-butanone from the fermentation
15 medium.

EXAMPLES

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

25 GENERAL METHODS

 Standard recombinant DNA and molecular cloning techniques described in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring
30 Harbor, NY, (1989) (Maniatis) and by T. J. Silhavy, M. L. Bennis, and L. W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1984) and by Ausubel, F. M.

et al., *Current Protocols in Molecular Biology*, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following Examples may be found as set out in *Manual of Methods for General Bacteriology* (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994)) or by Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989). All reagents, restriction enzymes and materials described for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), BD Diagnostic Systems (Sparks, MD), Life Technologies (Rockville, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified. Bacterial strains are obtained from the American Type Culture Collection (ATCC, Manassas, VA) unless otherwise noted.

Oligonucleotide primers described in the following Examples are given in Table 4. All oligonucleotide primers were synthesized by Sigma-Genosys

(Woodlands, TX). Table 4

Cloning and Screening Primers

Gene	Primer Name	Sequence	SEQ ID NO:	Description
<i>budB</i>	B1	CACCATGGACAAACAGTA TCCGGTACGCC	15	<i>budB</i> forward
<i>budB</i>	B2	CGAAGGGCGATAGCTTTA CCAATCC	16	<i>budB</i> reverse
<i>budA</i>	B3	CACCATGAATCATTCTGC TGAATGCACCTGCG	17	<i>budA</i> forward
<i>budA</i>	B4	GATACTGTTTGTCCATGT GACC	18	<i>budA</i> reverse
<i>budC</i>	B5	CACCATGAAAAAAGTCGC ACTTGTTACC	19	<i>budC</i> forward
<i>budC</i>	B6	TTAGTTAAATACCAT	20	<i>budC</i> reverse
<i>pddA</i>	B7	CACCATGAGATCGA AAAGATTG	21	<i>pddABC</i> forward
<i>pddC</i>	B8	CTTAGAGAAGTTAATCGT CGCC	22	<i>pddABC</i> reverse
<i>sadh</i>	B9	CACCATGAAAGCCCTCCA GTACACC	23	<i>sadh</i> forward
<i>sadh</i>	B10	CGTCGTGTCATGCCCGG G	24	<i>sadh</i> reverse

<i>budA</i>	B11	GATCGAATTCGTTTAAACT TAGTTTTCTACCGCACG	25	<i>budABC</i> forward
<i>budC</i>	B12	GATCGCATGCAAGCTTTC ATATAGTCGGAATTCC	26	<i>budABC</i> reverse
<i>pddA</i>	B13	GATCGAATTCGTTTAAACA AAGGAGGTCTGATTCATG AGATCG	27	<i>pddABC</i> forward
<i>pddC</i>	B14	GATCGGATTCTTAATCGT CGCC	28	<i>pddABC</i> reverse
<i>sadh</i>	B15	GATCGGATCCAAAGGAGG TCGGGCGCATGAAAGCC C	29	<i>sadh</i> forward
<i>sadh</i>	B16	GATCTCTAGAAAGCTTTC AGCCCGGGACGACC	30	<i>sadh</i> reverse
--	BenF	ACTTTCTTTCGCCTGTTTC AC	31	--
--	BenBPR	CATGAAGCTTGTTTAAACT CGGTGACCTTGAAAATAA TGAAAACCTTATATTGTTTT GAAAATAATGAAAACCTTAT ATTG	32	--
<i>budAB</i>	BABC F	GAGCTCGAATTCAAAGGA GGAAGTGTATATGAATCA TTC	33	<i>budAB</i> forward
<i>budAB</i>	BAB R	GGATCCTCTAGAATTAGT TAAATACCATCCCGCCG	34	<i>budAB</i> reverse
<i>budC</i>	BC Spe F	ACTAGTAAAGGAGGAAAG AGTATGAAGAAGGTCGCA CT	40	<i>budC</i> forward
<i>budC</i>	BC Xba R	TCTAGAAAGCAGGGGCAA GCCATGTC	41	<i>budC</i> reverse
<i>pddAB</i> C- ddrAB	DDo For	AAGCTTAAAGGAGGCTGA TTCATGAGATCGAAAAGA TT	44	<i>pddABC-ddrAB</i> forward
<i>pddAB</i> C- ddrAB	DDo Rev	TCTAGATTATTCATCCTGC TGTTCTCC	45	<i>pddABC-ddrAB</i> reverse
<i>chnA</i>	ChnA F	CATCAATTGACTACGTAG TCGTACGTGTAAGGAGGT TTGAAATGGAAAAAATTAT G	54	<i>chnA</i> forward
<i>chnA</i>	ChnA R	CATGCTAGCCCCGGGTAT CTTCTACTCATTTTTTATTT CG	55	<i>chnA</i> reverse
--	Top ter F1	CTAGAAGTCAAAGCCTC CGACCGGAGGCTTTTGA	58	forward
--	Top ter F2	CTGCTCGAGTTGCTAGC AAGTTTAAACAAAAAAA GCCCGCTCATTAGGCGG GCTGAGCT	59	forward
--	Bot ter R1	CAGCCCGCCTAATGAGC GGGCTTTTTTTTGTTTAA AC	60	reverse
--	Bot ter R2	TTGCTAGCAACTCGAGCA GTCAAAGCCTCCGGTC GGAGGCTTTTGACTT	61	reverse
<i>KA-AT</i>	OT872	CTCCGGAATTCATGTCTG ACGGACGACTCACCGCA	127	Aminoalcohol kinase/lyase

				operon forward
<i>KA-AT</i>	OT873	TCCAATGCATTGGCTGC AGTTATCTCTGTGCACGA GTGCCGATGA	128	Aminoalcohol kinase/lyase operon reverse
<i>KA</i>	OT879	AACAGCCAAGCTTGGCT GCAGTCATCGCGCATTCT CCGGG	129	Aminoalcohol kinase reverse
<i>AT</i>	OT880	TCTCCGGAATTCATGACG TCTGAAATGACAGCGACA GAAG	130	Aminoalcohol lyase forward
<i>pBAD. HisB</i>	OT909	GCTAACAGGAGGAAGAA TTCATGGGGGGTTCTC	131	Adds EcoRI site to replace NcoI site
<i>pBAD. HisB</i>	OT910	GAGAACCCCCCATGAATT CTTCCTCCTGTTAGC	132	Adds EcoRI site to replace NcoI site
<i>BudAB</i>	N84seqR3	GGACCTGCTTCGCTTTAT CG	159	reverse
<i>APT</i>	APTfor	GCGCGCCCGGGAAGAAG GAGCTCTTCACCATGAAC AAACCACAGTCTTGG	162	APT forward
<i>APT</i>	APTrev	GCGCGCCCGGGTTCATG CCACCTCTGCG	163	APT reverse

Table 5

Sequencing Primers

Name	Sequence	Gene- specific	SEQ ID NO:
M13 Forward	GTAAAACGACGGCCAGT	--	35
M13 Reverse	AACAGCTATGACCATG	--	36
N83 SeqF2	GCTGGATTACCAGCTCGACC	--	37
N83 SeqF3	CGGACGCATTACCGGCAAAG	--	38
N84 Seq R2	GCATCGAGATTATCGGGATG	--	65
N84 SeqR4	CGAAGCGAGAGAAGTTATCC	--	39
Trc F	TTGACAATTAATCATCCGGC	all	42
Trc R	CTTCTCTCATCCGCCAAAAC	all	43
DDko seq F2	GCATGGCGCGGATTTGACGAAC	pddABC- ddrAB	46
DDko seq F5	CATTAAAGAGACCAAGTACGTG	pddABC- ddrAB	47
DDko seq F7	ATATCCTGGTGGTGTCTCGGCGT	pddABC- ddrAB	48
DDko seq F9	TCTTTGTCACCAACGCCCTGCG	pddABC- ddrAB	49
DDko seq R1	GCCCACCGCGCTCGCCGCCGCG	pddABC- ddrAB	50

DDko seq R3	CCCCCAGGATGGCGGCTTCGGC	pddABC-ddrAB	51
DDko seq R7	GGGCCGACGGCGATAATCACTT	pddABC-ddrAB	52
DDko seq R10	TTCTTCGATCCACTCCTTAACG	pddABC-ddrAB	53
chnSeq F1	CTCAACAGGGTGTAAGTGTAGT	chnA	56
chnSeq R1	CGTTTTGATATAGCCAGGATGT	chnA	57
pCL1925 vec F	CGGTATCATCAACAGGCTTACC	all	62
pCL1925 vec R1	AGGGTTTTCCCAGTCACGACGT	all	63
pCL1925 vec R2	CGCAATAGTTGGCGAAGTAATC	all	64
APTseqRev	GCTAGAGATGATAGC	APT	160
APTseqFor	GGAAGAGACTATCCAGCG	APT	161

Methods for Determining 2-Butanol and 2-Butanone Concentration in Culture Media

The concentration of 2-butanol and 2-butanone in the culture media can be determined by a number of methods known in the art. For example, a specific high performance liquid chromatography (HPLC) method utilized a Shodex SH-1011 column with a Shodex SH-G guard column, both purchased from Waters Corporation (Milford, MA), with refractive index (RI) detection. Chromatographic separation was achieved using 0.01 M H₂SO₄ as the mobile phase with a flow rate of 0.5 mL/min and a column temperature of 50 °C. Under the conditions used, 2-butanone and 2-butanol had retention times of 39.5 and 44.3 min, respectively. Alternatively, gas chromatography (GC) methods are available. For example, a specific GC method utilized an HP-INNOWax column (30 m x 0.53 mm id, 1 μm film thickness, Agilent Technologies, Wilmington, DE), with a flame ionization detector (FID). The carrier gas was helium at a flow rate of 4.5 mL/min, measured at 150 °C with constant head pressure; injector split was 1:25 at 200 °C; oven temperature was 45 °C for 1 min, 45 to 220 °C at 10 °C/min, and 220 °C for 5 min; and FID detection was employed at 240 °C with 26 mL/min helium makeup gas. The retention times of 2-butanone and 2-butanol were 3.61 and 5.03 min, respectively.

2-Butanone can also be detected by derivatization with 3-methyl-2-benzothiazolinone hydrazone (MBTH). An aqueous solution containing 2-butanone is mixed with an equal volume of an aqueous solution of 6 mg/mL MBTH in 375 mM glycine-HCl (pH 2.7) and incubated at 100 °C for 3 min. The resulting MBTH-derivatized samples are analyzed on a 25 cm x 4.6 mm (id) Supelosil LC-18-D5 5 μ m column (Supelco) using a mobile phase of 55% acetonitrile in water at a flow rate of 1 mL/min. The 2-butanone derivative appears as two peaks (*cis* and *trans* isomers) with retention times of approximately 12.3 and 13.3 min and absorbance maxima of 230 and 307 nm.

The meaning of abbreviations is as follows: "s" means second(s), "min" means minute(s), "h" means hour(s), "psi" means pounds per square inch, "nm" means nanometers, "d" means day(s), " μ L" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "mm" means millimeter(s), "nm" means nanometers, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), " μ mol" means micromole(s)", "g" means gram(s), " μ g" means microgram(s) and "ng" means nanogram(s), "PCR" means polymerase chain reaction, "OD" means optical density, "OD₆₀₀" means the optical density measured at a wavelength of 600 nm, "kDa" means kilodaltons, "g" means the gravitation constant, "bp" means base pair(s), "kbp" means kilobase pair(s), "% w/v" means weight/volume percent, % v/v" means volume/volume percent, "wt %" means percent by weight, "HPLC" means high performance liquid chromatography, and "GC" means gas chromatography. The term "molar selectivity" is the number of moles of product produced per mole of sugar substrate consumed and is reported as a percent.

EXAMPLE 1

Cloning and Expression of Acetolactate Synthase

The purpose of this Example was to clone and express in *E. coli* the *budB* gene that encodes the enzyme acetolactate synthase. The *budB* gene was amplified from *Klebsiella pneumoniae* strain ATCC 25955 genomic DNA using PCR.

The *budB* sequence which encodes acetolactate synthase was amplified from *Klebsiella pneumoniae* (ATCC 25955) genomic DNA by PCR using the primer pair B1 (SEQ ID NO:15) and B2 (SEQ ID NO:16). Other PCR amplification reagents (e.g. Kod HiFi DNA Polymerase
5 (Novagen Inc., Madison, WI; catalog no. 71805-3)) were supplied in manufacturers' kits and used according to the manufacturer's protocol. *Klebsiella pneumoniae* genomic DNA was prepared using the Gentra Puregene Puregene kit (Gentra Systems, Inc., Minneapolis, MN; catalog number D-5000A). Amplification was carried out in a DNA Thermocycler
10 GeneAmp 9700 (PE Applied Biosystems, Foster city, CA). The nucleotide sequence of the open reading frame (ORF) and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:3 and SEQ ID NO:4, respectively.

For expression studies the Gateway cloning technology (Invitrogen Corp., Carlsbad, CA) was used. The entry vector pENTR/SD/D-TOPO allows directional cloning and provided a Shine-Dalgarno sequence for the gene of interest. The destination vector pDEST14 used a T7 promoter for expression of the gene with no tag. The forward primer incorporated four bases (CACC) immediately adjacent to the translational start codon to
20 allow directional cloning of the *budB* acetolactate synthase coding region PCR product into pENTR/SD/D-TOPO (Invitrogen), generating the plasmid pENTRSDD-TOPObudB. The pENTR construct was transformed into *E. coli* Top10 (Invitrogen) cells and plated according to the manufacturer's recommendations. Transformants were grown overnight and plasmid
25 DNA was prepared using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA; catalog no. 27106) according to the manufacturer's recommendations. To create an expression clone, the *budB* coding region from pENTRSDD-TOPObudB was transferred to the pDEST 14 vector by *in vitro* recombination using the LR Clonase mix (Invitrogen, Corp., Carlsbad,
30 CA). The resulting vector, pDEST14budB, was transformed into BL-21-AI cells (Invitrogen Corp.). BL-21-AI cells carry a chromosomal copy of the T7 RNA polymerase under control of the arabinose-inducible araBAD promoter.

Transformants are inoculated into LB medium supplemented with 50 $\mu\text{g/mL}$ of ampicillin and grown overnight. An aliquot of the overnight culture is used to inoculate 50 mL of LB medium supplemented with 50 $\mu\text{g/mL}$ of ampicillin. The culture is incubated at 37 °C with shaking until
5 the OD₆₀₀ reaches 0.6-0.8. The culture is split into two 25-mL portions and arabinose is added to one of the flasks to a final concentration of 0.2% w/v. The negative control flask is not induced with arabinose. The flasks are incubated for 4 h at 37 °C with shaking. Cells are harvested by centrifugation and the cell pellets are resuspended in 50 mM MOPS, pH
10 7.0 buffer. The cells are disrupted either by sonication or by passage through a French Pressure Cell. Each cell lysate is centrifuged yielding the supernatant and the pellet or the insoluble fraction. An aliquot of each fraction (whole cell lysate, from induced and control cells, is resuspended in SDS (MES) loading buffer (Invitrogen), heated to 85 °C for 10 min and
15 subjected to SDS-PAGE analysis (NuPAGE 4-12% Bis-Tris Gel, catalog no. NP0322Box, Invitrogen). A protein of the expected molecular weight, as deduced from the nucleic acid sequence, is present in the induced culture but not in the uninduced control.

Acetolactate synthase activity in the cell free extracts is measured
20 using the method described by Bauerle et al. (Bauerle et al. (1964) *Biochim. Biophys. Acta* 92:142-149). Protein concentration is measured by either the Bradford method or by the Bicinchoninic Kit (Sigma, catalog no. BCA-1; St. Louis, MO) using Bovine serum albumin (BSA) (Bio-Rad, Hercules, CA) as the standard.

25

EXAMPLE 2

Cloning and Expression of Acetolactate Decarboxylase

The purpose of this Example was to clone and express in *E. coli* the *budA* gene that encodes the enzyme acetolactate decarboxylase. The *budA* gene was amplified from *Klebsiella pneumoniae* strain ATCC 25955
30 genomic DNA using PCR.

The *budA* sequence which encodes acetolactate decarboxylase, was cloned in the same manner as described for budB in Example 1, except that the primers used for PCR amplification were B3 (SEQ ID

NO:17) and B4 (SEQ ID NO:18). The nucleotide sequence of the open reading frame (ORF) and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:1 and SEQ ID NO:2, respectively. The resulting plasmid was named pENTRSDD-TOPObudA.

- 5 Acetolactate decarboxylase activity in the cell free extracts is measured using the method described by Bauerle et al., *supra*.

EXAMPLE 3 (Prophetic)

Cloning and Expression of Butanediol Dehydrogenase

- 10 The purpose of this prophetic Example is to describe how to clone and express in *E. coli* the *budC* gene that encodes the enzyme butanediol dehydrogenase. The *budC* gene is amplified from *Klebsiella pneumoniae* strain IAM1063 genomic DNA using PCR.

- 15 The *budC* sequence encoding butanediol dehydrogenase is cloned and expressed in the same manner as described for *budA* in Example 1, except that the primers used for PCR amplification are B5 (SEQ ID NO:19) and B6 (SEQ ID NO:20) and the genomic template DNA is from *Klebsiella pneumoniae* IAM1063 (which is obtained from the Institute of Applied Microbiology Culture Collection, Tokyo, Japan). *Klebsiella pneumoniae* IAM1063 genomic DNA is prepared using the Gentra Puregene Puregene
20 kit (Gentra Systems, Inc., Minneapolis, MN; catalog number D-5000A). The nucleotide sequence of the open reading frame (ORF) and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:5 and SEQ ID NO:6, respectively.

- 25 Butanediol dehydrogenase activity in the cell free extracts is measured spectrophotometrically by following NADH consumption at an absorbance of 340 nm.

EXAMPLE 4 (Prophetic)

Cloning and Expression of Butanediol Dehydratase

- 30 The purpose of this prophetic Example is to describe how to clone and express in *E. coli* the *pddA*, *pddB* and *pddC* genes that encode butanediol dehydratase. The *pddA*, *pddB* and *pddC* genes are amplified from *Klebsiella oxytoca* ATCC 8724 genomic DNA using PCR.

The *pddA*, *pddB* and *pddC* sequences which encode butanediol dehydratase are cloned and expressed in the same manner as described for *budA* in Example 1, except that the genomic template DNA is from *Klebsiella oxytoca* ATCC 8724, and the primers are B7 (SEQ ID NO:21) and B8 (SEQ ID NO:22). *Klebsiella oxytoca* genomic DNA is prepared using the Gentra Puregene Puregene kit (Gentra Systems, Inc., Minneapolis, MN; catalog number D-5000A). A single PCR product including all three open reading frames (ORFs) is cloned, so that all three coding regions are expressed as an operon from a single promoter on the expression plasmid. The nucleotide sequences of the open reading frames for the three subunits are given as SEQ ID NOs:7, 9, and 11, respectively, and the predicted amino acid sequences of the three enzyme subunits are given as SEQ ID NOs:8, 10, and 12, respectively.

Butanediol dehydratase activity in the cell free extracts is measured by derivatizing the ketone product with 2,4-dinitrophenylhydrazine (DNPH). Briefly, 100 μ L of reaction mixture, cell extract containing approximately 0.0005 units of enzyme, 40 mM potassium phosphate buffer (pH 8.0), 2 μ g of adenosylcobalamin, 5 μ g of 2,3,-butanediol, and 1 μ g of bovine serum albumin, is quenched by addition of an equal volume of 0.05 wt % DNPH in 1.0 N HCl. After 15 min at room temperature, the color is developed by addition of 100 μ L of 4 N NaOH. The amount of product is determined from the absorbance of the final solution at 550 nm compared to a standard curve prepared with 2-butanone. All reactions are carried out at 37 °C under dim red light.

EXAMPLE 5 (Prophetic)

Cloning and Expression of Butanol Dehydrogenase

The purpose of this prophetic Example is to describe how to clone and express in *E. coli* the *sadh* gene that encodes butanol dehydrogenase. The *sadh* gene is amplified from *Rhodococcus ruber* strain 219 genomic DNA using PCR.

The *sadh* sequence encoding butanol dehydrogenase is cloned and expressed in the same manner as described for *budA* in Example 1, except that the genomic template DNA is from *Rhodococcus ruber* strain

219 (Meens, Institut fuer Mikrobiologie, Universitaet Hannover, Hannover, Germany) and the primers are B9 (SEQ ID NO:23) and B10 (SEQ ID NO:24). *Rhodococcus ruber* genomic DNA is prepared using the Ultra Clean™ Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA), according to the manufacturer's protocol. The nucleotide sequence of the open reading frame (ORF) and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:13 and SEQ ID NO:14, respectively.

Butanol dehydrogenase activity in cell free extracts is measured by following the increase in absorbance at 340 nm resulting from the conversion of NAD to NADH when the enzyme is incubated with NAD and 2-butanol.

EXAMPLE 6 (Prophetic)

Construction of a Transformation Vector for the Genes in a 2-Butanol Biosynthetic Pathway

The purpose of this prophetic Example is to describe the preparation of a transformation vector for the genes in a 2-butanol biosynthetic pathway (i.e., Pathway 3 as described above). Like most organisms, *E. coli* converts glucose initially to pyruvic acid. The enzymes required to convert pyruvic acid to 2-butanol following Pathway 3, i.e., acetolactate synthase, acetolactate decarboxylase, butanediol dehydrogenase, butanediol dehydratase, and butanol dehydrogenase, are encoded by the *budA*, *budB*, *budC*, *pddA*, *pddB*, *pddC* and *sadh* genes. To simplify building the 2-butanol biosynthetic pathway in a recombinant organism, the genes encoding the 5 steps in the pathway are divided into two operons. The upper pathway comprises the first three steps catalyzed by acetolactate synthase, acetolactate decarboxylase, and butanediol dehydrogenase. The lower pathway comprises the last two steps catalyzed by butanediol dehydratase and butanol dehydrogenase.

The coding sequences are amplified by PCR with primers that incorporate restriction sites for later cloning, and the forward primers contain an optimized *E. coli* ribosome binding site (AAAGGAGG). PCR products are TOPO cloned into the pCR4Blunt-TOPO vector and

transformed into Top10 cells (Invitrogen). Plasmid DNA is prepared from the TOPO clones, and the sequence of the cloned PCR fragment is verified. Restriction enzymes and T4 DNA ligase (New England Biolabs, Beverly, MA) are used according to manufacturer's recommendations.

- 5 For cloning experiments, restriction fragments are gel-purified using QIAquick Gel Extraction kit (Qiagen).

After confirmation of the sequence, the coding regions are subcloned into a modified pUC19 vector as a cloning platform. The pUC19 vector is modified by a HindIII/SapI digest, followed by treatment with
10 Klenow DNA polymerase to fill in the ends. The 2.4 kB vector fragment is gel-purified and religated creating pUC19dHS. Alternatively the pUC19 vector is modified by a SphI/SapI digest, followed by treatment with Klenow DNA polymerase to blunt the ends. The 2.4 kB vector fragment is gel-purified and religated creating pUC19dSS. The digests remove the lac
15 promoter adjacent to the MCS (multiple cloning sites), preventing transcription of the operons from the vector.

Upper Pathway:

The *budABC* coding regions are amplified from *Klebsiella pneumoniae* genomic DNA by PCR using primer pair B11 and B12 (Table
20 4), given as SEQ ID NOs:25 and 26, respectively. The forward primer incorporates an EcoRI restriction site and a ribosome binding site (RBS). The reverse primer incorporates an SphI restriction site. The PCR product is cloned into pCR4 Blunt-TOPO creating pCR4 Blunt-TOPO-budABC.

To construct the upper pathway operon pCR4 Blunt-TOPO-budABC
25 is digested with EcoRI and SphI releasing a 3.2 kbp *budABC* fragment. The pUC19dSS vector is also digested with EcoRI and SphI, releasing a 2.0 kbp vector fragment. The *budABC* fragment and the vector fragment are ligated together using T4 DNA ligase (New England Biolabs) to form pUC19dSS-budABC.

30 Lower Pathway:

The *pddABC* coding regions are amplified from *Klebsiella oxytoca* ATCC 8724 genomic DNA by PCR using primers B13 and B14 (Table 4), given as SEQ ID NOs:27 and 28, respectively, creating a 2.9 kbp product.

The forward primer incorporates EcoRI and PmeI restriction sites and a RBS. The reverse primer incorporates the BamHI restriction site. The PCR product is cloned into pCRBlunt II-TOPO creating pCRBluntII-pdd.

The *sadh* gene is amplified from *Rhodococcus ruber* strain 219 genomic DNA by PCR using primers B15 and B16 (Table 4), given as SEQ ID NOs:29 and 30, respectively, creating a 1.0 kbp product. The forward primer incorporates a BamHI restriction site and a RBS. The reverse primer incorporates an XbaI restriction site. The PCR product is cloned into pCRBlunt II-TOPO creating pCRBluntII-sadh.

To construct the lower pathway operon, a 2.9 kbp EcoRI and BamHI fragment from pCRBluntII-pdd, a 1.0 kbp BamHI and XbaI fragment from pCRBluntII-sadh, and the large fragment from an EcoRI and XbaI digest of pUC19dHS are ligated together. The three-way ligation creates pUC19dHS-pdd-sadh.

The pUC19dSS-budABC vector is digested with PmeI and HindIII, releasing a 3.2 kbp fragment that is cloned into pBenBP, an *E.coli*-*B. subtilis* shuttle vector. Plasmid pBenBP is created by modification of the pBE93 vector, which is described by Nagarajan (WO 93/2463, Example 4). To generate pBenBP, the *Bacillus amyloliquefaciens* neutral protease promoter (NPR) signal sequence and the *phoA* gene are removed from pBE93 with an NcoI/HindIII digest. The NPR promoter is PCR amplified from pBE93 by primers BenF and BenBPR, given by SEQ ID NOs:31 and 32, respectively. Primer BenBPR incorporates BstEII, PmeI and HindIII sites downstream of the promoter. The PCR product is digested with NcoI and HindIII, and the fragment is cloned into the corresponding sites in the vector pBE93 to create pBenBP. The upper operon fragment is subcloned into the PmeI and HindIII sites in pBenBP creating pBen-budABC.

The pUC19dHS-pdd-sadh vector is digested with PmeI and HindIII releasing a 3.9 kbp fragment that is cloned into the PmeI and HindIII sites of pBenBP, creating pBen-pdd-sadh.

EXAMPLE 7 (Prophetic)

Expression of a 2-Butanol Biosynthetic Pathway in *E. coli*

The purpose of this prophetic Example is to describe how to express a 2-butanol biosynthetic pathway in *E. coli*.

5 The plasmids pBen-budABC and pBen-pdd-sadh, prepared as described in Example 6, are separately transformed into *E. coli* NM522 (ATCC No. 47000), and expression of the genes in each operon is monitored by SDS-PAGE analysis and enzyme assay. After confirmation of expression of all genes, pBen-budABC is digested with EcoRI and
10 HindIII to release the NPR promoter-budABC fragment. The fragment is blunt ended using the Klenow fragment of DNA polymerase (New England Biolabs, catalog no. M0210S). The plasmid pBen-pdd-sadh is digested with EcoRI and similarly blunted to create a linearized, blunt-ended vector fragment. The vector and NPR-budABC fragments are ligated, creating
15 p2BOH. This plasmid is transformed into *E. coli* NM522 to give *E. coli* NM522/p2BOH, and expression of the genes is monitored as previously described.

E. coli NM522/p2BOH is inoculated into a 250 mL shake flask containing 50 mL of medium and shaken at 250 rpm and 35 °C. The
20 medium is composed of: dextrose, 5 g/L; MOPS, 0.05 M; ammonium sulfate, 0.01 M; potassium phosphate, monobasic, 0.005 M; S10 metal mix, 1% (v/v); yeast extract, 0.1% (w/v); casamino acids, 0.1% (w/v); thiamine, 0.1 mg/L; proline, 0.05 mg/L; and biotin 0.002 mg/L, and is titrated to pH 7.0 with KOH. S10 metal mix contains: MgCl₂, 200 mM;
25 CaCl₂, 70 mM; MnCl₂, 5 mM; FeCl₃, 0.1 mM; ZnCl₂, 0.1 mM; thiamine hydrochloride, 0.2 mM; CuSO₄, 172 µM; CoCl₂, 253 µM; and Na₂MoO₄, 242 µM. After 18 h, 2-butanol is detected by HPLC or GC analysis using methods that are well known in the art, for example, as described in the General Methods section above.

EXAMPLE 8 (Prophetic)

Expression of a 2-Butanol Biosynthetic Pathway in *Bacillus subtilis*

The purpose of this prophetic Example is to describe how to express a 2-butanol biosynthetic pathway in *Bacillus subtilis*.

5 The plasmids pBen-budABC and pBen-pdd-sadh, prepared as described in Example 6, are separately transformed into *Bacillus subtilis* BE1010 (*J. Bacteriol.* 173:2278-2282 (1991)) and expression of the genes in each operon is monitored as described in Example 7. The plasmid pBen-budABC is digested with EcoRI and HindIII to release the NPR
10 promoter-budABC fragment. The fragment is blunt ended using the Klenow fragment of DNA polymerase (New England Biolabs, catalog no. M0210S). The plasmid pBen-pdd-sadh is digested with EcoRI and similarly blunted to create a linearized, blunt-ended vector fragment. The vector and NPR-budABC fragments are ligated, creating p2BOH. This
15 plasmid is transformed into *Bacillus subtilis* BE1010 to give *Bacillus subtilis* BE1010 /p2BOH, and expression of the genes is monitored as previously described.

Bacillus subtilis BE1010 /p2BOH is inoculated into a 250 mL shake flask containing 50 mL of medium and shaken at 250 rpm and 35 °C for 18
20 h. The medium is composed of: dextrose, 5 g/L; MOPS, 0.05 M; glutamic acid, 0.02 M; ammonium sulfate, 0.01 M; potassium phosphate, monobasic buffer, 0.005 M; S10 metal mix (as described in Example 7), 1% (v/v); yeast extract, 0.1% (w/v); casamino acids, 0.1% (w/v); tryptophan, 50 mg/L; methionine, 50 mg/L; and lysine, 50 mg/L, and is
25 titrated to pH 7.0 with KOH. After 18 h, 2-butanol is detected by HPLC or GC analysis using methods that are well known in the art, for example, as described in the General Methods section above.

EXAMPLE 9

Construction of a Transformation Vector for the Genes in a 30 2-Butanol Biosynthetic Pathway

The purpose of this Example was to prepare a recombinant *E. coli* host carrying the genes in a 2-butanol biosynthetic pathway (i.e., Pathway 3 as described above). Like most organisms, *E. coli* converts glucose

initially to pyruvic acid. The enzymes required to convert pyruvic acid to 2-butanone in Pathway 3, i.e., acetolactate synthase, acetolactate decarboxylase, butanediol dehydrogenase, and butanediol dehydratase are encoded by the *budA*, *budB*, *budC*, *pddA*, *pddB*, and *pddC* genes. In the last step of the pathway, a butanol dehydrogenase converts 2-butanone to 2-butanol. Dehydrogenases that carry out this last step are promiscuous and may be found in many organisms. To simplify building the 2-butanol biosynthetic pathway in a recombinant organism, the genes encoding the 5 steps in the pathway were divided into multiple operons. The upper pathway operon comprised the first three steps catalyzed by acetolactate synthase, acetolactate decarboxylase, and butanediol dehydrogenase and were cloned onto an expression vector. The lower pathway comprised the last two steps catalyzed by butanediol dehydratase including the reactivating factor (Mori et al., *J. Biol. Chem.* 272:32034 (1997)) and a butanol dehydrogenase. The diol dehydratase can undergo suicide inactivation during catalysis. The reactivating factor protein encoded by *ddrA* and *ddrB* (GenBank AF017781, SEQ ID NO:70) reactivates the inactive enzyme. The *ddrA* and *ddrB* genes flank the diol dehydratase operon. The operons for the dehydratase/reactivating factor and the butanol dehydrogenase were either cloned onto another expression vector or the dehydratase/reactivating factor operon was cloned singly onto another expression vector and the last step was provided by an endogenous activity in the demonstration host.

Construction of Vector pTrc99a-budABC:

The *budAB* coding regions were amplified from *K. pneumoniae* ATCC 25955 genomic DNA by PCR using primer pair BABC F and BAB R, given as SEQ ID NOs:33 and 34, respectively (see Table 4), creating a 2.5 kbp product. The forward primer incorporated *SacI* and *EcoRI* restriction sites and a ribosome binding site (RBS). The reverse primer incorporated a *SpeI* restriction site. The PCR product was cloned into pCR4 Blunt-TOPO creating pCR4 Blunt-TOPO-budAB. Plasmid DNA was prepared from the TOPO clones and the sequence of the genes was verified with primers M13 Forward (SEQ ID NO:35), M13 Reverse (SEQ

ID NO:36), N83 SeqF2 (SEQ ID NO:37), N83 SeqF3 (SEQ ID NO:38) and N84 SeqR4 (SEQ ID NO:39) (see Table 5).

The *budC* coding region was amplified from *K. pneumoniae* ATCC 25955 genomic DNA by PCR using primer pair BC Spe F and BC Xba R given as SEQ ID NOs:40 and 41, respectively, creating a 0.8 kbp product. The forward primer incorporated a SpeI restriction site, a RBS and modified the CDS by changing the second and third codons from AAA to AAG. The reverse primer incorporated an XbaI restriction site. The PCR product was cloned into pCR4 Blunt-TOPO creating pCR4 Blunt-TOPO-
 5 budC. Plasmid DNA was prepared from the TOPO clones and the sequence of the genes was verified with primers M13 Forward (SEQ ID NO:35) and M13 Reverse (SEQ ID NO:36).

To construct the budABC operon, pCR4 Blunt-TOPO-budC was digested with SnaBI and XbaI releasing a 1.0 kbp *budC* fragment. The
 15 vector pTrc99a (Amann et al., *Gene* 69(2):301-315 (1988)) was digested with SmaI and XbaI creating a 4.2 kbp linearized vector fragment. The vector and the budC fragment were ligated to create pTrc99a-budC and transformed into *E. coli* Top 10 cells (Invitrogen). Transformants were analyzed by PCR amplification with primers Trc F (SEQ ID NO:42) and Trc
 20 R (SEQ ID NO:43) for a 1.2 kbp product to confirm the presence of the *budC* insert. The *budAB* genes were subcloned from pCR4 Blunt-TOPO-budAB as a 2.5 kbp EcoRI/SpeI fragment. Vector pTrc99a-budC was digested with EcoRI and SpeI and the resulting 5.0 kbp vector fragment was gel-purified. The purified vector and budAB insert were ligated and
 25 transformed into *E. coli* Top 10 cells. Transformants were screened by PCR amplification with primers Trc F (SEQ ID NO:42) and N84 Seq R2 (SEQ ID NO:65) to confirm creation of pTrc99a-budABC. In this plasmid, the bud A, B, and C coding regions are adjacent to each other, in this order, and between the Trc promoter and the *rrnB* termination sequence.

30 Results:

Three independent isolates of *E. coli* Top 10/pTrc99a-budABC were examined for the production of butanediol, using *E. coli* Top 10/pCL1925-Kodd-ddr (described below) as a negative control. The strains were grown

in LB medium containing 100 $\mu\text{g/mL}$ carbenicillin. The resulting cells were used to inoculate shake flasks (approximately 175 mL total volume) containing 125 mL of TM3a/glucose medium with 100 $\mu\text{g/mL}$ carbenicillin. In addition, the flasks inoculated with strains carrying pTrc99a-budABC contained 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). TM3a/glucose medium contains (per liter): 10 g glucose, 13.6 g KH_2PO_4 , 2.0 g citric acid monohydrate, 3.0 g $(\text{NH}_4)_2\text{SO}_4$, 2.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.33 g ferric ammonium citrate, 1.0 mg thiamine-HCl, 0.50 g yeast extract, and 10 mL trace elements solution, adjusted to pH 6.8 with NH_4OH . The solution of trace elements contained: citric acid $\cdot \text{H}_2\text{O}$ (4.0 g/L), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (3.0 g/L), NaCl (1.0 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.10 g/L), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.10 g/L), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.10 g/L), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.010 g/L), H_3BO_3 (0.010 g/L), and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.010 g/L). The flasks, capped with vented caps, were inoculated at a starting OD_{600} of approximately 0.03 units and incubated at 34 $^\circ\text{C}$ with shaking at 300 rpm.

Approximately 23 h after induction, an aliquot of the broth was analyzed by HPLC (Shodex Sugar SH1011 column) and GC (HP-INNOWax), using the same methods described in the General Methods section for 2-butanol and 2-butanone. The results of the analysis are given in Table 6. The three *E. coli* clones converted glucose to acetoin and meso-2,3-butanediol, the desired intermediates of the pathway, with a molar selectivity of 14%. This selectivity was approximately 35-fold higher than that observed with the *E. coli* control strain lacking budABC.

25

Table 6

Production of Acetoin and meso-2,3-butanediol
by *E. coli* Top 10/pTrc99a-budABC

Strain	OD_{600}	Acetoin, mM	Meso-2,3-Butanediol, mM	Molar Selectivity ^a , %

Negative control	1.4	0.07	0.03	0.4
Isolate #1	1.5	0.64	1.3	14
Isolate #2	1.4	0.70	1.2	14
Isolate #3	1.4	0.74	1.3	15

^a Molar selectivity is (acetoin + meso-2,3-butanediol)/(glucose consumed).

Construction of vector pCL1925-KoDD-ddr:

5 The diol dehydratase (GenBank D45071, SEQ ID NO:69) and reactivating factor (GenBank AF017781, SEQ ID NO:70) operons were PCR amplified from *Klebsiella oxytoca* ATCC 8724 as a single unit with primers DDo For (SEQ ID NO: 44) and DDo Rev (SEQ ID NO:45). The forward primer incorporated an optimized *E. coli* RBS and a HindIII
10 restriction site. The reverse primer included an XbaI restriction site. The 5318 bp PCR product was cloned into pCR4Blunt-TOPO and clones of the resulting pCR4Blunt-TOPO-Kodd-ddr were sequenced with primers M13 Forward (SEQ ID NO:35), M13 Reverse (SEQ ID NO:36), DDko seq F2 (SEQ ID NO:46), DDko seq F5 (SEQ ID NO:47), DDko seq F7 (SEQ ID
15 NO:48), DDko seq F9 (SEQ ID NO:49), DDko seq R1 (SEQ ID NO:50), DDko seq R3 (SEQ ID NO:51), DDko seq R7 (SEQ ID NO:52), and DDko seq R10 (SEQ ID NO:53). A clone having the insert with the expected sequence was identified.

For expression, the diol dehydratase/reactivating factor genes were
20 subcloned into pCL1925 (U.S. Patent No. 7,074,608), a low copy plasmid carrying the glucose isomerase promoter from *Streptomyces*. pCR4Blunt-TOPO-Kodd-ddr was digested with HindIII and XbaI and the resulting 5.3 kbp Kodd-ddr fragment was gel-purified. Vector pCL1925 was digested with HindIII and XbaI and the resulting 4539 bp vector fragment was gel
25 purified. The vector and Kodd-ddr fragment were ligated and transformed

into *E. coli* Top10. Transformants were screened by PCR with primers DDko Seq F7 (SEQ ID NO:48) and DDko Seq R7 (SEQ ID NO: 52). Amplification of the plasmid (pCL1925-Kodd-ddr) carrying the insert resulted in a product of approximately 797 bp.

5 Activity of diol dehydratase towards *meso*-2,3-butanediol was measured by incubating cell extract (total protein ~ 0.8 mg/mL) with 10 mM butanediol and 12 mM coenzyme B₁₂ in 80 mM HEPES (pH 8.2) for 17 h at room temperature. Formation of the expected product, 2-butanone, was determined by HPLC as described in the General Methods.

10 Construction of vector pCL1925-KoDD-ddr::T5 chnA ter:

To provide a heterologous alcohol dehydrogenase activity, the *chnA* gene encoding cyclohexanol dehydrogenase from *Acinetobacter* sp. (Cheng et al., *J. Bacteriol.* 182:4744-4751 (2000)) was cloned into the pCL1925 vector with the diol dehydratase operon, pCL1925-Kodd-ddr.

15 The *chnA* gene, given as SEQ ID NO:71 (Genbank No: AF282240, SEQ ID NO:73) was amplified from pDCQ2, a cosmid carrying the cyclohexanol gene cluster from *Acinetobacter*, with primers ChnA F (SEQ ID NO:54) and ChnA R (SEQ ID NO:55). The resulting 828 bp PCR product was cloned into pCR4Blunt-TOPO to create pCR4Blunt-TOPO-chnA and
20 transformants were screened by colony PCR with primers M13 Forward (SEQ ID NO:35) and M13 Reverse (SEQ ID NO:36). Correct clones produced a PCR product of about 1 kbp and were sequenced with primers M13 Forward (SEQ ID NO:35) and M13 Reverse (SEQ ID NO:36).

After sequencing pCR4Blunt-TOPO-chnA to confirm the correct
25 sequence, the *chnA* gene was subcloned from the plasmid as an 813 bp MfeI/SmaI fragment. The expression vector pQE30 (Qiagen) was digested with MfeI and SmaI and the resulting 3350 bp vector fragment was gel-purified. The *chnA* fragment and the purified vector were ligated and transformed into *E. coli* Top10 cells. Transformants were colony PCR
30 screened with primers chnSeq F1 (SEQ ID NO:56) and chnseq R1 (SEQ ID NO:57) for a 494 bp PCR product. This cloning placed the *chnA* gene under the control of the T5 promoter in the plasmid, pQE30-chnA.

To prepare the pCL1925 vector to carry two operons, terminators were added to the vector. A *tonB* terminator-mcs-*trpA* terminator fragment was prepared by oligonucleotide annealing with primers Top ter F1 (SEQ ID NO:58), Top ter F2 (SEQ ID NO:59), Bot ter R1 (SEQ ID NO:60) and
5 Bot ter R2 (SEQ ID NO:61). The annealed DNA was gel-purified on a 6% PAGE gel (Embi-tec, San Diego, CA). Vector pCL1925 was digested with *SacI* and *XbaI* and gel-purified. The annealed DNA and vector fragment were ligated to create pCL1925-ter. Transformants were screened by colony PCR amplification with primers pCL1925 vec F (SEQ ID NO:62)
10 and pCL1925 vec R1 (SEQ ID NO:63) for the presence of a PCR product of approximately 400 bp. Positive clones from the PCR screen were sequenced with the same primers.

Vector pCL1925-ter was digested with *XhoI* and *PmeI* and the resulting 4622 bp fragment was gel-purified. pQE30-*chnA* was digested
15 with *NcoI* and the DNA was treated with Klenow DNA polymerase to blunt the ends. pQE30-*chnA* was then digested with *XhoI* and the resulting 1.2 kbp T5 promoter-*chnA* fragment was gel-purified. The pCL1925-ter vector and the *chnA* operon fragment were ligated together to give pCL1925-ter-T5*chnA* and transformed into *E. coli* Top10. Transformants were
20 screened by colony PCR amplification with primers pCL1925 vec F (SEQ ID NO:64) and *chnseq* R1 (SEQ ID NO:59) for a product of approximately 1 kbp.

To finish building the pathway vector, the pCL1925-KoDD-ddr plasmid was digested with *XbaI* and *SacI* and the resulting 9504 bp vector
25 fragment was gel-purified. The *chnA* operon flanked by terminators, with the *trpA* terminator (Koichi et al. (1997) Volume 272, Number 51, pp. 32034-32041) 3' to the *chnA* coding sequence, from pCL1925-ter-T5*chnA* was gel-purified as a 1271 bp *XbaI*/*SacI* fragment. After ligation of the fragments and transformation into *E. coli* Top10, transformants were
30 screened by colony PCR. Primers *chnSeq* F1 (SEQ ID NO:58) and pCL1925 vec R2 (SEQ ID NO:64) amplified the expected 1107 bp PCR product in the resulting plasmid, pCL1925-KoDD-ddr::ter-T5*chnA*.

EXAMPLE 10

Expression of a 2-Butanol Biosynthetic Pathway in *E. coli* with Overexpressed Endogenous Alcohol Dehydrogenase

The purpose of this Example was to express a 2-butanol
5 biosynthetic pathway in several *E. coli* strains.

Construction of *E. coli* strains constitutively expressing *yqhD*:

E. coli contains a native gene (*yqhD*) that was identified as a 1,3-propanediol dehydrogenase (U.S. Patent No. 6,514,733). The *yqhD* gene, given as SEQ ID NO:74, has 40% identity to the gene *adhB* in *Clostridium*,
10 a probable NADH-dependent butanol dehydrogenase. The *yqhD* gene was placed under the constitutive expression of a variant of the glucose isomerase promoter 1.6GI (SEQ ID NO:67) in *E. coli* strain MG1655 1.6yqhD::Cm (WO 2004/033646) using λ Red technology (Datsenko and Wanner, *Proc. Natl. Acad. Sci. U.S.A.* 97:6640 (2000)). Similarly, the
15 native promoter was replaced by the 1.5GI promoter (WO 2003/089621) (SEQ ID NO:68), creating strain MG1655 1.5yqhD::Cm, thus, replacing the 1.6GI promoter of MG1655 1.6yqhD::Cm with the 1.5GI promoter. The 1.5GI and 1.6GI promoters differ by 1 bp in the -35 region, thereby altering the strength of the promoters (WO 2004/033646). While replacing the
20 native *yqhD* promoter with either the 1.5GI or 1.6GI promoter, the *yqhC* gene encoding the putative transcriptional regulator for the *yqh* operon was deleted. Butanol dehydrogenase activity was confirmed by enzyme assay using methods that are well known in the art.

Transformation of *E. coli* strains:

25 Pathway plasmids pCL1925-Kodd-ddr and pTrc99a-budABC, described in Example 9, were co-transformed into *E. coli* strains MG1655, MG1655 1.6yqhD, and MG1655 1.5yqhD. The two latter strains overexpress the 1,3-propanediol dehydrogenase, YqhD, which also has butanol dehydrogenase activity. Strains were examined for the production
30 of 2-butanone and 2-butanol essentially as described above. Cells were inoculated into shake flasks (approximately 175 mL total volume) containing either 50 or 150 mL of TM3a/glucose medium (with 0.1 mg/L vitamin B₁₂, appropriate antibiotics and IPTG) to represent medium and

low oxygen conditions, respectively. Spectinomycin (50 µg/mL) and carbenicillin (100 µg/mL) were used for plasmids pCL1925-Kodd-ddr and pTrc99a-budABC, respectively. The flasks were inoculated at a starting OD₆₀₀ of ≤ 0.04 units and incubated at 34 °C with shaking at 300 rpm. The

5 flasks containing 50 mL of medium were capped with vented caps; the flasks containing 150 mL, were capped with non-vented caps to minimize air exchange. IPTG was present at time zero at a concentration of zero or 0.04 mM. Analytical results for 2-butanone and 2-butanol production are presented in Table 7. All the *E. coli* strains comprising a 2-butanol

10 biosynthetic pathway produced 2-butanone under low and medium oxygen conditions and produced 2-butanol under low oxygen conditions.

Table 7

Production of 2-Butanone and 2-Butanol by *E. coli* MG1655 strains
harboring pathway plasmids pCL1925-Kodd-ddr and pTrc99a-budABC

<u>Strain^{a,b}</u>	<u>IPTG, mM</u>	<u>Volume of Medium, mL</u>	<u>2-Butanone, mM</u>	<u>2-Butanol, mM</u>
MG1655 #1	0	50	0.08	Not detected
MG1655 #2	0	50	0.11	Not detected
MG1655 #1	0.04	50	0.12	Not detected
MG1655 #2	0.04	50	0.11	Not detected
MG1655 #1	0	150	0.15	0.047
MG1655 #2	0	150	0.19	0.041
MG1655 #1	0.04	150	0.10	0.015
MG1655 #2	0.04	150	0.11	0.015
MG1655 1.5yqhD #1	0	50	0.10	Not detected
MG1655 1.5yqhD #2	0	50	0.07	Not detected
MG1655 1.5yqhD #1	0.04	50	0.12	Not detected
MG1655 1.5yqhD #2	0.04	50	0.18	Not detected

MG1655 1.5yqhD #1	0	150	0.16	0.030
MG1655 1.5yqhD #2	0	150	0.18	0.038
MG1655 1.5yqhD #1	0.04	150	0.10	0.021
MG1655 1.5yqhD #2	0.04	150	0.09	0.017
MG1655 1.6yqhD #1	0	50	0.08	Not detected
MG1655 1.6yqhD #2	0	50	0.07	Not detected
MG1655 1.6yqhD #1	0.04	50	0.12	Not detected
MG1655 1.6yqhD #2	0.04	50	0.15	Not detected
MG1655 1.6yqhD #1	0	150	0.17	0.019
MG1655 1.6yqhD #2	0	150	0.18	0.041
MG1655 1.6yqhD #1	0.04	150	0.11	0.026
MG1655 1.6yqhD #2	0.04	150	0.11	0.038
Control (uninoculated medium)			Not detected	Not detected

^a #1 and #2 represent independent isolates.

^b MG1655 is MG1655/pCL1925-Kodd-ddr/pTrc99a-budABC

MG1655 1.6yqhD is MG1655 1.6yqhD/pCL1925-Kodd-ddr/pTrc99a-budABC

5 MG1655 1.6yqhD is MG1655 1.5yqhD/pCL1925-Kodd-ddr/pTrc99a-budABC.

EXAMPLE 11

Expression of a 2-Butanol Biosynthetic Pathway in *E. coli* with Heterologous Alcohol Dehydrogenase

Plasmids pCL1925-KoDD-ddr::ter-T5chnA and pTrc99a-budABC,
5 described in Example 9, were transformed into *E. coli* strains MG1655 and
MG1655 $\Delta yqhCD$ for a demonstration of the production of 2-butanol.

MG1655 $\Delta yqhCD$ carries a *yqhCD* inactivation that was made using
the method of Datsenko and Wanner (*Proc. Natl. Acad. Sci. U.S.A.*
97(12):6640-6645 (2000)). After replacement of the region with the FRT-
10 CmR-FRT cassette of pKD3, the chloramphenicol resistance marker was
removed using the FLP recombinase. The sequence of the deleted region
is given as SEQ ID NO:66.

Strains MG1655/pTrc99a-budABC/pCL1925KoDD-ddr::ter-T5 chnA
and MG1655 $\Delta yqhCD$ /pTrc99a-budABC/pCL1925KoDD-ddr::ter-T5 chnA
15 were examined for the production of 2-butanone and 2-butanol essentially
as described above. Strain MG1655 $\Delta yqhCD$ /pCL1925 was used as a
negative control. Cells were inoculated into shake flasks (approximately
175 mL total volume) containing 50 or 150 mL of TM3a/glucose medium
(with 0.1 mg/L vitamin B₁₂ and appropriate antibiotics) to represent
20 medium and low oxygen conditions, respectively. Spectinomycin (50
 μ g/mL) and ampicillin (100 μ g/mL) were used for selection of pCL1925
based plasmids and pTrc99a-budABC, respectively. Enzyme activity
derived from pTrc99a-budABC was detected by enzyme assay in the
absence of IPTG inducer, thus, IPTG was not added to the medium. The
25 flasks were inoculated at a starting OD₆₀₀ of ≤ 0.01 units and incubated at
34 °C with shaking at 300 rpm for 24 h. The flasks containing 50 mL of
medium were capped with vented caps; the flasks containing 150 mL,
were capped with non-vented caps to minimize air exchange. Analytical
results for 2-butanone and 2-butanol production are presented in Table 8.
30 Both *E. coli* strains comprising a 2-butanol biosynthetic pathway produced
2-butanone under low and medium oxygen conditions and produced 2-
butanol under low oxygen conditions, while the negative control strain did
not produce detectable levels of either 2-butanone or 2-butanol.

Table 8
Production of 2-butanone and 2-butanol by *E. coli* strains

Strain ^a	Volume, mL	2-Butanone, mM	2-Butanol, mM
Negative control, MG1655 $\Delta yqhCD/pCL1925$	50	Not detected	Not detected
MG1655/pTrc99a- budABC/pCL1925KoDD-ddr::T5 chnA ter	50	0.33	Not detected
MG1655 $\Delta yqhCD/pTrc99a$ - budABC/pCL1925KoDD-ddr::T5 chnA ter #1	50	0.23	Not detected
MG1655 $\Delta yqhCD/pTrc99a$ - budABC/pCL1925KoDD-ddr::T5 chnA #2	50	0.19	Not detected
Negative control, MG1655 $\Delta yqhCD/pCL1925$	150	Not detected	Not detected
MG1655/pTrc99a- budABC/pCL1925KoDD-ddr::T5 chnA ter	150	0.41	0.12
MG1655 $\Delta yqhCD/pTrc99a$ - budABC/pCL1925KoDD-ddr::T5 chnA #1	150	0.15	0.46
MG1655 $\Delta yqhCD/pTrc99a$ - budABC/pCL1925KoDD-ddr::T5 chnA #2	150	0.44	0.14
Medium		Not detected	Not detected

5 ^a #1 and #2 represent independent isolates.

EXAMPLE 12

Cloning of Amino:Pyruvate Transaminase (APT)

An amino:pyruvate transaminase (APT) from *Vibrio Fluvialis JS17* was identified by Shin et al. (Appl. Microbiol Biotechnol. (2003) 61:463-
5 471). The amino acid sequence (SEQ ID NO:122) was found to have significant homology with ω -amino acid:pyruvate transaminases (Shin and Kim (J. Org. Chem. 67:2848-2853 (2002)). It was shown that the *Vibrio Fluvialis* APT has transaminase activity towards acetoin.

For expression of the APT enzyme in *E. coli*, a codon optimized
10 APT coding region (SEQ ID NO:144) was designed using the preferred *E. coli* codons with additional considerations such as codon balance and mRNA stability, and synthesized (by DNA2.0; Redwood City, CA). The coding region DNA fragment was subcloned into the pBAD.HisB vector (Invitrogen) between the NcoI and HindIII sites and the resulting plasmid,
15 hereafter referred to as pBAD.APT1, was transformed into TOP10 cells.EXAMPE 13

Characterization of *Vibrio Fluvialis* APT Alanine:Acetoin Aminotransferase Activity

A 5 mL volume of LB broth + 100 μ g/mL ampicillin was inoculated
20 with a fresh colony of TOP10/pBAD:APT1 cells. The culture was incubated at 37 °C for approximately 16 h with shaking (225 rpm). A 300 μ L aliquot of this culture was used to inoculate 300 mL of the same medium, which was incubated at 37 °C with shaking (225 rpm). When the culture reached an OD₆₀₀ of 0.8, L-arabinose was added to a final
25 concentration of 0.2 % (w/v). The culture was incubated for an additional 16 h, then harvested. The cells were washed once with 100 mM potassium phosphate buffer (pH 7.8) and then frozen and stored at -80 °C.

To isolate the enzyme, the cell pellet was thawed and resuspended in 8 mL of 100 mM potassium phosphate buffer (pH 7) containing 0.2 mM
30 ethylenediaminetetraacetate, 1 mM dithiothreitol and 1 tablet of protease inhibitor cocktail (Roche; Indianapolis, IN). The cells were lysed by two passes through a French pressure cell at 900 psi, and the resulting lysate was clarified by centrifugation for 30 min at 17000 $\times$ g. Ammonium sulfate

was added to 35 % saturation, and the solution was stirred for 30 min at room temperature, at which point precipitated solids were removed by centrifugation (30 min, 17000 × g). Additional ammonium sulfate was added to the supernatant to give 55% saturation, and the solution was again stirred for 30 min at room temperature. The precipitated solids were removed by centrifugation (30 min, 17000 × g) and then resuspended in 5 mL of 100 mM potassium phosphate buffer (pH 7) containing 10 μM pyridoxal 5'-phosphate and 1 mM dithiothreitol. This solution was desalted by passage through a PD10 column equilibrated with Buffer A (50 mM bis-tris propane buffer (pH 6) containing 10 μM pyridoxal 5'-phosphate and 1 mM dithiothreitol). The desalted extract was then loaded onto a 20 mL Q-Fast Flow column pre-equilibrated with Buffer A. APT was eluted with a linear gradient of 0-0.1 M NaCl in Buffer A. The enzyme was detected in eluted fractions by the presence of a protein band of size ~ 50 kD when analyzed by SDS-polyacrylamide gel electrophoresis and by the characteristic absorbance at 418 nm. Fractions containing the enzyme eluted at ~ 0.3 M NaCl. These fractions were pooled to yield a total of 6 mL of a 5.45 mg/mL solution of enzyme, which was >90% pure, as judged by SDS-polyacrylamide gel electrophoresis.

The alanine:acetoin aminotransferase activity of APT was assayed using a lactic dehydrogenase coupled assay. Reaction mixtures contained 100 mM bis-tris propane (pH 9.0), 10 μM pyridoxal 5'-phosphate, 0-50 mM acetoin, 0-5 mM L-alanine, 0.14 or 0.28 mg/mL purified enzyme, 200 μM NADH and 20 U/mL lactic dehydrogenase (Sigma; St. Louis, MO). The reaction was followed by measuring the change in absorbance at 340 nm, indicative of the oxidation of NADH. Under these conditions, the k_{cat}/K_m for acetoin was 10 M⁻¹ s⁻¹ and that for L-alanine was 400 M⁻¹ s⁻¹.

The identity of the expected product 3-amino-2-butanol was confirmed by comparison to a synthetic standard. A mixture of (*R,R*)- and (*S,S*)-3-amino-2-butanol was synthesized by the method of Dickey *et al.* [*J Amer Chem Soc* 74:944 (1952)]: 5 g of *trans*-2,3-epoxybutane were slowly stirred into 150 mL of cold (4 °C) NH₄OH. The reaction was slowly

warmed to room temperature, sealed and stirred at room temperature for an additional 10 days. At this time, excess ammonia and water and residual epoxybutane were removed by rotary evaporation under vacuum at 40 °C. The resulting clear oil (2.9 g) was resuspended in water to a concentration of 10% (w/v). Production of the desired product was confirmed by NMR analysis and comparison of the spectrum to that reported by Levy *et al.* [*Org. Magnetic Resonance* 14:214 (1980)]. A mixture of the corresponding (2*R*,3*S*)- and (2*S*,3*R*)- isomers was produced using the identical method with the exception that the starting material was the *cis*- isomer of 2,3-epoxybutane.

An analytical method for detection of 3-amino-2-butanol was developed based on the *o*-phthaldialdehyde derivatization method for amino acid determination reported by Roth [*Anal. Chem.* 43:880 (1971)]. A 200 μ L aliquot of 1 mM 3-amino-2-butanol (mixture of isomers) was mixed with 200 μ L of a 50 mM solution of borate (pH 9.5), to which was added 10 μ L of 5 μ L/mL 2-mercaptoethanol in ethanol and 10 μ L of 10 mg/mL *o*-phthaldialdehyde in ethanol. The solution was incubated at room temperature for 10 min, at which time the derivative was extracted into 200 μ L hexane. The hexane was separated from the aqueous solution by decanting, and 10 μ L were injected onto a Chiracel OD HPLC column (Daicel Chemical Industries; Fort Lee, NJ). The column was run isocratically with a mobile phase of 90:10 hexane:isopropanol at a rate of 1 mL/min. The derivatized isomers of 3-amino-2-butanol were detected by absorbance at 340 nm with retention times of approximately 15.7 and 16.8 min [(2*S*,3*S*) and (2*R*,3*R*)], and 18.4 and 21.9 min [(2*R*,3*S*) and (2*S*,3*R*)]. To differentiate the enantiomers in the first mixture, the pure (2*R*,3*R*) isomer (Bridge Organics; Vicksburg, MI) was also run under the identical conditions and found to be the 16.8 min peak. To differentiate the enantiomers in the second mixture, the mixture was first kinetically resolved using the alanine:acetoin aminotransferase: 0.28 mg of purified enzyme was incubated with 10 mM pyruvate and 10 mM 3-amino-2-butanol [1:1 mixture of (2*R*,3*S*) and (2*S*,3*R*) isomers] in 1 mL of 100 mM bis-tris propane (pH 9.0). After 24 h at room temperature, an aliquot was

removed and analyzed as described above. Analysis revealed that the 18.4 min peak was 95 % depleted, while the 21.9 min peak was >90 % retained. A 100 μ L aliquot of the remaining reaction mixture was mixed with 50 μ L of 20 mM NADH and 10 μ L of extract from the TOP10/pTrc99a-
5 BudC strain described in Example 9. The BudC enzyme is known to reduce (*R*)-acetoin to *meso*-2,3-butanediol and (*S*)-acetoin to (*S,S*)-2,3-butanediol [Ui *et al.* (2004) *Letters in Applied Microbiology* 39:533-537]. After 3 h, samples were taken from the reaction and analyzed as described above for acetoin and butanediol. The analysis indicated that
10 the primary product of the reduction was *meso*-2,3-butanediol, indicating that the product of the aminotransferase reaction was (*R*)-acetoin, and therefore the consumed 3-amino-2-butanol isomer was the (*2R,3S*) isomer. Thus the retention time of 18.4 min can be assigned to this isomer and 21.9 to the (*2S,3R*) isomer.

15 To confirm that the product of the APT-catalyzed alanine:acetoin aminotransferase reaction was 3-amino-2-butanol, 0.28 mg of purified enzyme was incubated with 10 mM acetoin, 10 mM L-alanine, 50 U lactic dehydrogenase and 200 μ M NADH in 1 mL of 100 mM bis-tris propane (pH 9.0). The reaction mixture was incubated at room temperature for 20
20 h, after which a 200 μ L aliquot was removed and derivatized as described above. The retention times of the derivatized products were 15.8 min (major product) and 18.5 min (minor product), matching that of the (*2S,3S*)- and (*2R,3S*)-3-amino-2-butanol standards.

EXAMPLE 14

25 Identification and Cloning of *Erwinia carotovora* subsp. *atroseptica* Amino Alcohol Kinase and Amino Alcohol O-Phosphate Lyase

The purpose of this example is to describe the identification and cloning of sequences encoding an amino alcohol kinase and amino alcohol O-phosphate lyase from the bacterium *Erwinia carotovora*. These
30 two enzymes are part of Pathway 1 for the conversion of 3-amino-2-butanol to 2-butanone *via* the intermediate 3-amino-2-butanol phosphate as shown in Figure 1.

Prediction of the *Erwinia* amino alcohol kinase and the amino alcohol O-phosphate lyase

ATP-dependent amino alcohol kinase and amino alcohol O-phosphate lyase activities have been detected in several *Pseudomonas* and *Erwinia* species, including *Pseudomonas* sp. P6 (NCIB10431),
 5 *Pseudomonas putida* NCIB 10558 (Jones et al. (1973) *Biochem. J.* 134:167-182), *Erwinia carotovora*, *Erwinia amanas*, *Erwinia millettiae*, and *Erwinia atroseptica* (Jones et al. (1973) *Biochem. J.* 134:959-968). In these studies, the extracts of the above species were shown to have
 10 activity for the enzymatic conversion of aminopropanol through aminopropanol O-phosphate to propionaldehyde, and the conversion of ethanolamine through ethanolamine O-phosphate to acetaldehyde.

The genomic sequence of the *Erwinia atroseptica* strain in which these activities were reported to exist (now designated as *Erwinia*
 15 *carotovora* subsp. *atroseptica* strain SCRI1043 (ATCC BAA-672)) has been determined at the Sanger Institute (Bell et al. *Proc. Natl. Acad. Sci. USA* 101(30): 11105-11110). Analysis of the putative kinases in the *Erwinia carotovora* subsp. *atroseptica* genome revealed an operon sequence (SEQ ID NO:164) encoding a putative protein (ECA2059; SEQ
 20 ID NO:124) that is 39% identical to a *Rhizobium loti* homoserine kinase and a putative class-III pyridoxal phosphate (PLP)-dependent aminotransferase (ECA2060; SEQ ID NO:126) that is 58% identical to a putative aminotransferase from *Rhizobium meliloti*. Based on the above it was expected that ECA2059 was an amino alcohol kinase and ECA2060
 25 was an amino alcohol O-phosphate lyase which uses PLP as cofactor.

Cloning of the putative amino alcohol kinase and putative amino alcohol O-phosphate lyase from *Erwinia carotovora* subsp. *atroseptica*

Genomic DNA of *Erwinia carotovora* subsp. *atroseptica* (ATCC #: BAA-672D) was obtained from American Type Culture Collection (ATCC).
 30 The operon encoding the putative amino alcohol kinase (KA) and amino alcohol O-phosphate lyase (AT) was named KA-AT (SEQ ID NO:164). This operon was amplified from the *Erwinia* genomic DNA by Phusion DNA polymerase (Finnzymes; via New England Biolabs; Ipswich, MA)

using primers OT872 (SEQ. ID. No. 127) and OT873 (SEQ. ID. No. 128). A DNA fragment of 2.4 kb was obtained by the PCR reaction, which corresponds to the size of the KA-AT operon. The PCR product was digested with EcoRI and PstI restriction enzymes, and cloned into vector pKK223-3 (Amersham Biosciences; Piscataway, NJ) which was digested with the same restriction enzymes. This produced plasmid pKK223.KA-AT, which contained the putative *Erwinia* amino alcohol kinase-lyase operon under control of the *tac* promoter. Similarly, plasmids pKK223.KA and pKK223.AT were made which placed the putative *Erwinia* kinase and the putative *Erwinia* lyase coding regions in separate vectors, each under the control of the *tac* promoter. For the PCR cloning of the KA coding region (SEQ ID NO:123), primers OT872 (SEQ. ID. No. 127) and OT879 (SEQ. ID. No. 129) were used; and for the PCR cloning of AT coding region (SEQ ID NO:125), primers OT873 (SEQ. ID. No. 128) and OT880 (SEQ. ID. No. 130) were used in the PCR amplifications, which generated PCR products of 1.1 kb and 1.3 kb respectively. The PCR products were each digested with EcoRI and PstI, and ligated into vector pKK223-3 to generate pKK223.KA and pKK223.AT.

In vivo activity of the putative amino alcohol kinase and putative amino alcohol O-phosphate lyase from Erwinia carotovora subsp. atroseptica

Plasmids pKK223.KA-AT, pKK223.KA, pKK223.AT and pKK223-3 were transformed into the *E. coli* MG1655 strain. The transformants were restreaked onto a MOPS minimal media plate containing 1% glucose, 0.5% aminopropanol as a sole nitrogen source, 1 mM IPTG and 100 µg/mL ampicillin. Expression of KA-AT, KA and AT genes were induced by the IPTG. A control plate had no IPTG included. The plates were incubated at 37 °C for 7 days. On the plate with IPTG, only the strain MG1655/pKK223.KA-AT grew, while all the other three strains did not grow. On the plate without added IPTG, the strain MG1655/pKK223.KA-AT grew, but the colonies were significantly smaller than those on the IPTG-containing plate, which corresponds to the lower expression levels of KA and AT in the uninduced cells. None of the other three strains grew on this plate. This indicates that the co-expression of the putative *Erwinia*

KA and AT genes provided sufficient enzyme activities that allowed the *E. coli* strain MG1655/pKK223.KA-AT to utilize aminopropanol as a sole nitrogen source. Expression of each individual enzyme of either KA or AT was not sufficient to provide such enzyme activity *in vivo*.

5

EXAMPLE 15

In vitro Activity of *Erwinia* putative Amino Alcohol Kinase and Amino Alcohol O-Phosphate Lyase

Subcloning of the Erwinia KA-AT operon into the pBAD.HisB vector and induction of protein expression

10 The protein expression levels of *Erwinia* putative KA and AT enzymes expressed in MG1655 cells from the pKK223.KA-AT vector were analyzed by SDS-PAGE analysis. The expression level of the *Erwinia* AT enzyme was relatively low, with a new protein band detected at the correct molecular weight of 46 kD in the soluble fraction of a cell extract, while no
15 new protein band was detected at the size predicted for the KA enzyme.

In an effort to improve the expression of the *Erwinia* putative KA and AT genes, the KA-AT operon was subcloned into the EcoRI and HindIII sites of vector pBAD.HisB-EcoRI. pBAD.HisB-EcoRI was derived from the pBAD.HisB vector (Invitrogen), by replacing the NcoI site in
20 pBAD.HisB with an EcoRI site *via* QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) using primers OT909 (SEQ ID.# 131) & OT910 (SEQ ID.# 132). In the constructed plasmid pBAD.KA-AT, the KA-AT operon was placed directly under control of the *araB* promoter (without His-tag).

25 The pBAD.KA-AT plasmid was transformed into the *E. coli* TOP10 strain. A 50 mL culture of TOP10/pBAD.KA-AT strain was grown to mid log phase ($OD_{600}=0.6$) in LB, 100 μ g/mL ampicillin media at 37 °C with shaking at 250 rpm. The culture was induced by addition of L-arabinose to a final concentration of 0.1% (w/v), and it was further incubated at 37 °C
30 for 5 h before harvesting by centrifugation. The cell pellet was resuspended in ice cold 50 mM Tris-HCl, pH 8.0, and disrupted by sonication on ice with a Fischer Sonic Model 300 Dismembrator (Fischer, Pittsburgh, PA) at 50% power, repeating four cycles of 30 seconds

sonication with 60 seconds rest in-between each cycle. Each sonicated sample was centrifuged (15,000 × g, 4 min, 4 °C). Clarified cell free extracts were analyzed for protein expression level and amino alcohol O-phosphate lyase activity.

5 Chemical synthesis of aminobutanol O-phosphate and aminopropanol O-phosphate

The substrate (*R,R*)-3-amino-2-butanol O-phosphate was synthesized by a method based on that reported by Ferrari and Ferrari (US patent 2730542 [1956]) for phosphoethanolamine: 10 mmol of H₃PO₄ in a 50 % (w/v) aqueous solution was mixed with a 50 % (w/v) solution of 10 3-amino-2-butanol (~20:1 (*R,R*):(*S,S*) isomers; Bridge Organics; Vicksburg, MI) while stirring on ice. After mixing, the solution was slowly warmed to room temperature and then stirred under vacuum and heated to 70 °C. After 1 h at 70 °C, the temperature was slowly increased to 185 15 °C and maintained there for an additional 2 h. At that time, the reaction was cooled to room temperature and the vacuum released. The remaining material was dissolved in water, and analysis by NMR indicated that 80% of the starting material was converted to product with 20% remaining unreacted. No additional products were observed.

20 The additional substrates (*2R,3S*)-3-amino-2-butanol O-phosphate and (*2S,3R*)-3-amino-2-butanol O-phosphate were synthesized by the same procedure using a 1:1 mixture of (*2R,3S*)-3-amino-2-butanol and (*2S,3R*)-3-amino-2-butanol (synthesized as described in Example 13) as the starting material. DL -1-amino-2-propanol O-phosphate, (*S*)-2-amino- 25 1-propanol O-phosphate, and (*R*)-2-amino-1-propanol O-phosphate were synthesized by the same procedure using DL-1-amino-2-propanol, (*R*)-2-amino-1-propanol, or (*S*)-2-amino-1-propanol as the starting material.

Analysis of the aminopropanol O-phosphate lyase activity encoded by the putative *Erwinia* KA-AT operon

30 The aminopropanol O-phosphate lyase assay was performed as described by Jones et al. (1973, *Biochem. J.* 134:167-182) and G. Gori et al. (1995, *Chromatographia* 40:336) The formation of propionaldehyde from aminopropanol O-phosphate was assayed colorimetrically with

MBTH, which allows the detection of aldehyde formation. The reaction was performed as follows. In a 1 mL reaction, 100 µg cell free extract of *E. coli* TOP10/pBAD.KA-AT was added to 10 mM DL-1-amino-2-propanol O-phosphate in 100 mM Tris-HCl, pH 7.8, with 0.1 mM PLP. The reaction was incubated at 37 °C for 10 min and 30 min, with an aliquot of 100 µL reaction mixture removed at each time point and mixed with 100 µL of 6 mg/mL MBTH in 375 mM glycine-HCl, pH 2.7. This mixture was incubated at 100 °C for 3 min, cooled on ice for 15-30 s, and 1 mL of 3.3 mg/mL FeCl₃·6H₂O (in 10 mM HCl) was added, followed by incubation for 30 min at room temperature. The absorbance of the reaction mixture which contains the aldehyde-MBTH adduct, was measured at 670 nm. The results of the assay are listed in Table 9. In the presence of the aminopropanol phosphate substrate, PLP and cell free extract, formation of aldehyde was detected, as indicated by an Abs₆₇₀ that was higher than the control background of up to 0.3. In the absence of either the substrate or the cell free extract, no aldehyde formation was detected. In the absence of added PLP, somewhat less amount aldehyde was detected, presumably due to the presence of PLP in the cell free extract. Cell free extract of the uninduced TOP10/pBAD.KA-AT culture did not produce any detectable aldehyde in the reaction. These results indicated that the putative *Erwinia* amino alcohol O-phosphate lyase does catalyze the conversion of aminopropanol O-phosphate to propionaldehyde.

Table 9.

Aminopropanol O-phosphate lyase assay. Sample 1 was the cell free extract of a non-induced control of *E. coli* TOP10/pBAD.KA-AT. Samples 2-5 contained the cell free extract of the induced culture *E. coli* TOP10/pBAD.KA-AT.

Sample Number	Induction by 0.1% arabinose	Aminopropanol O-phosphate	PLP	Enzyme extract (100µg/mL)	OD ₆₇₀ , 10 min	OD ₆₇₀ , 30 min
1	uninduced	(+)	(+)	(+)	0.262	0.255
2	induced	(+)	(+)	(+)	1.229	2.264
3	induced	(-)	(+)	(+)	0.303	0.223
4	induced	(+)	(-)	(+)	0.855	1.454
5	induced	(+)	(+)	(-)	0.156	0.065

Analysis of the activity of the *Erwinia* amino alcohol O-phosphate lyase towards aminobutanol O-phosphate substrate

The activity of the amino alcohol O-phosphate lyase towards the aminobutanol O-phosphate substrates was studied under the same conditions as described above. The reaction was carried out at 37 °C overnight in a 1 mL reaction that contained 100 µg of cell free extract of *E. coli* TOP10/pBAD.KA-AT, 10 mM aminobutanol O-phosphate (either the mixture of (*R,R*)+(*S,S*) or the mixture of (*R,S*) + (*S,R*) isomers described in Example 15) in 100 mM Tris-HCl, pH 7.8, with 0.1 mM PLP. An aliquot of 100 µL reaction mixture was removed and the 2-butanone product was detected using the MBTH derivatization method described in the General Methods. The two peaks representing the derivatized 2-butanone isomers were observed. Therefore the *Erwinia* amino alcohol O-phosphate lyase is an aminobutanol phosphate phospho-lyase in addition to an aminopropanol phosphate phospho-lyase.

Analysis of the activity of the *Erwinia* amino alcohol O-phosphate lyase towards stereoisomers of aminopropanol O-phosphate and aminobutanol O-phosphate

The activity of the *Erwinia* amino alcohol O-phosphate lyase towards various stereoisomers of aminopropanol O-phosphate and aminobutanol O-phosphate was studied under the same conditions as described above. In the presence of the *Erwinia* amino alcohol O-phosphate lyase, both (*R*) and (*S*)-2-amino-1-propanol O-phosphate were converted to propanone by the enzyme, but the product yield was much higher with the (*S*) isomer. The enzyme also produced butanone from both mixtures of 3-amino-2-butanol O-phosphate isomers, with a higher product yield found in the reaction containing the (*R,S*) and (*S,R*) substrate isomers. Both propanone and butanone products were derivatized by MBTH, and detected by HPLC as described in General Methods.

Optimization of the gene expression level for the *Erwinia* amino alcohol kinase and amino alcohol O-phosphate lyase

In order to improve the expression levels for the *Erwinia* amino alcohol kinase and the amino alcohol O-phosphate lyase in *E. coli*, codon optimized coding regions for both enzymes (named EKA: SEQ ID NO:155 and EAT: SEQ ID NO:156 respectively) were synthesized by DNA2.0 (Redwood City, CA). Each coding region was synthesized with 5' and 3' tails including restriction sites for cloning: EKA has 5' BbsI and 3' EcoRI, HindIII sites; EAT has 5' EcoRI and 3' HindIII sites. The EKA and EAT coding regions were provided from DNA2.0 as plasmids pEKA and pEAT, which were in the pJ51 vector of DNA2.0. The EKA optimized coding region was subcloned by ligating a BbsI and HindIII digested fragment of pEKA into the pBAD.HisB vector between the NcoI and HindIII sites, to generate plasmid pBAD.EKA. In the resulting plasmid the coding region is 5' to the His tag, so a coding region for an N-terminus His₆ tag fused to the *Erwinia* amino alcohol kinase was constructed by performing a QuickChange site-directed mutagenesis reaction using primers SEQ ID NO:157 and SEQ ID NO:158 to generate vector pBAD.His-EKA.

pBAD.His-EKA was transformed into *E. coli* strain BL21-AI (*F^o ompT hsdSB (rB⁻ mB⁻) gal dcm araB::T7RNAP-tetA*; Invitrogen) to produce strain BL21-AI/pBAD.HisA-EKA. A 50 mL culture of BL21-AI/pBAD.HisA-EKA was grown to mid-log stage (OD₆₀₀=0.6), induced with 0.1% arabinose, and further incubated at 30 °C overnight. Cell free extracts were prepared by sonication. The His₆-tagged fusion protein of *Erwinia* amino alcohol kinase was purified using the ProBond™ Purification System (Invitrogen) under non-denaturing purification conditions following the manufacturer's instructions.

Prophetic Result

The kinase activity of the His₆-tagged *Erwinia* amino alcohol kinase is analyzed by the ADP Quest Assay (DiscoverX, Fremont, CA) following the manufacture's instructions. This is a biochemical assay that measures the accumulation of ADP, a product of the amino alcohol kinase reaction using either aminopropanol or aminobutanol as substrate. 10 mM

substrate is mixed with His₆-tagged *Erwinia* amino alcohol kinase, in 100 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 2 mM KCl, 0.1 mM ATP, and incubated at 37 °C for 1 h in a 0.2 mL reaction. ADP reagent A (100 µL) and ADP reagent B (200 µL) are added and the mixture is incubated at room temperature for 30 min. The fluorescence signal indicating activity is measured with excitation wavelength of 530 nm and emission wavelength of 590 nm.

EXAMPLE 16

Expression of Entire Pathway 3

10 Construction of Vector pCLBudAB-ter-T5chnA

The vector pTrc99a::BudABC (described in Example 9) is digested with EcoRI, and the DNA is treated with Klenow DNA polymerase to blunt the ends. The blunted vector is subsequently digested with SpeI to yield a 2.5 kb fragment containing the *budA* and *budB* genes. The vector pCL1925-ter-T5chnA (described in Example 9) is digested with HindIII, and the DNA was treated with Klenow DNA polymerase to blunt the ends. The blunted vector is subsequently digested with XbaI to yield a 4.6 kb fragment which is then ligated to the *budAB* fragment from pTrc99a::BudABC. The resulting plasmid, designated pCLBudAB-ter-T5chnA, is used to transform *E. coli* Top10 cells, and single colonies are screened for proper plasmid structure by PCR using primers pCL1925vecF (SEQ ID NO:62) and N84seqR3 (SEQ ID NO:159). Plasmid is prepared from a single colony which yields a PCR product of the expected size of 1.4 kb.

25 Construction of vector pKK223.KA-AT-APT

The APT gene is amplified from the vector pBAD.APT (described in Example 12) by PCR using primers APTfor (SEQ ID NO:162; 5' includes RBS and SmaI site) and APTrev (SEQ ID NO:163; 3' adds SmaI site). The product of expected size of 1.7 kbp is gel purified and digested with SmaI to yield blunt ends. The vector pKK223.KA-AT (described in Example 14) is digested with PstI, and the DNA is treated with Klenow DNA polymerase to blunt the ends. The resulting DNA fragment is ligated with the SmaI-digested PCR product, and the ligation product is used to

transform *E. coli* Top10 cells. Individual ampicillin resistant colonies are screened by PCR using primers OT872 (SEQ ID NO:127) and APTrev (SEQ ID NO:163). The presence of a PCR product of the expected size of 4.1 kbp indicates that the gene encoding APT is present and oriented in the same direction as the genes encoding KA and AT. The sequence of the insert is verified using the primers APTseqRev (SEQ ID NO:160) and APTseqFor (SEQ ID NO:161). This plasmid is named pKK223.KA-AT-APT. Proper expression of all three genes is verified by growing a 5 mL culture of Top10/pKK223.KA-AT-APT in LB + 100 μ g/mL ampicillin at 37 °C with shaking. When the OD₆₀₀ reaches ~0.8, expression of the genes on the plasmid is induced by addition of IPTG to 0.4 mM. The expression is evaluated by SDS PAGE and activity assays as described above.

Construction of 2-butanol production strain and production of 2-butanone and 2-butanol

E. coli strain MG1655 is transformed with both pKK223.KA-AT-APT and pCLBudAB-ter-T5chnA, and transformants selected for ampicillin and spectinomycin resistance, indicative of the presence of the plasmids. The cells are inoculated into shake flasks (approximately 175 mL total volume) containing 50 or 150 mL of TM3a/glucose medium (with appropriate antibiotics) to represent medium and low oxygen conditions, respectively. IPTG is added to 0.4 mM to induce expression of genes from pKK223.KA-AT-APT. As a negative control, MG1655 cells are grown in the same medium lacking antibiotics. The flasks are inoculated at a starting OD₆₀₀ of ≤ 0.01 and incubated at 34 °C with shaking at 300 rpm for 24 h. The flasks containing 50 mL of medium are capped with vented caps; the flasks containing 150 mL are capped with non-vented caps to minimize air exchange. The MG1655/ pKK223.KA-AT-APT/pCLBudAB-ter-T5chnA strain comprising a 2-butanol biosynthetic pathway produces both 2-butanone and 2-butanol under low and medium oxygen conditions while the negative control strain does not produce detectable levels of either 2-butanone or 2-butanol.

EXAMPLE 17

Characterization of Glycerol Dehydratase Butanediol Dehydratase Activity

Glycerol dehydratase (E.C. 4.2.1.30) and diol dehydratase (E.C. 4.2.1.28), while structurally related, are often distinguished in the art based on various differences that include substrate specificity. This example demonstrates that glycerol dehydratase converts *meso*-2,3-butanediol to 2-butanone. The recombinant *E. coli* strain KLP23/pSYCO12, comprising *Klebsiella pneumoniae* genes encoding the multiple subunits of glycerol dehydratase (alpha: SEQ ID NO:145 (coding region) and 146 (protein); beta: SEQ ID NO: 147 (coding region) and 148 (protein); and gamma: SEQ ID NO: 149 (coding region) and 150 (protein)) and *Klebsiella pneumoniae* genes encoding the multiple subunits of glycerol dehydratase reactivase (large subunit, SEQ ID NO: 151 (coding region) and 152 (protein); and small subunit, SEQ ID NO: 153 (coding region) and 154 (protein)), is described in Emptage *et al.* US 6,514,733 and in WO 2003089621, which are herein incorporated by reference. A crude, cell free extract of KLP23/pSYCO12 was prepared by methods known to one skilled in the art. Enzyme assay was performed in the absence of light in 80 mM HEPES buffer, pH 8.2 at 37 °C with 12 μM coenzyme B₁₂ and 10 mM *meso*-2,3-butanediol. The formation of 2-butanone was monitored by HPLC (Shodex SH-1011 column and SH-G guard column with refractive index detection; 0.01 M H₂SO₄ as the mobile phase at a flow rate of 0.5 mL/min and a column temperature of 50 °C; 2-butanone retention time = 40.2 min). The rate of 2-butanone formation by the glycerol dehydratase preparation was determined to be 0.4 nmol/min/mg of crude protein.

CLAIMS

What is claimed is:

1. A recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

- i) pyruvate to alpha-acetolactate;
- ii) alpha-acetolactate to acetoin;
- iii) acetoin to 3-amino-2-butanol ;
- iv) 3-amino-2-butanol to 3-amino-2-butanol phosphate;
- v) 3-amino-2-butanol phosphate to 2-butanone; and
- vi) 2-butanone to 2-butanol;

wherein the at least one DNA molecule is heterologous to said microbial host cell and wherein said microbial host cell produces 2-butanol.

2 A recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:

- i) pyruvate to alpha-acetolactate ;
- ii) alpha-acetolactate to acetoin;
- iii) acetoin to 3-amino-2-butanol;
- iv) 3-amino-2-butanol to 3-amino-2-butanol phosphate; and
- v) 3-amino-2-butanol phosphate to 2-butanone;

wherein the at least one DNA molecule is heterologous to said microbial host cell and wherein said microbial host cell produces 2-butanone.

3 A host cell according to claims 1 or 2 wherein the polypeptide that catalyzes a substrate to product conversion of pyruvate to alpha-acetolactate is acetolactate synthase.

4 A host cell according to claims 1 or 2 wherein the polypeptide that catalyzes a substrate to product conversion of alpha-acetolactate to acetoin is acetolactate decarboxylase.

5. A host cell according to claims 1 or 2 wherein the polypeptide that catalyzes a substrate to product conversion of acetoin to 3-amino-2-butanol is acetoin aminase.
6. A host cell according to claims 1 or 2 wherein the polypeptide that catalyzes a substrate to product conversion of 3-amino-2-butanol to 3-amino-2-butanol phosphate is aminobutanol kinase.
7. A host cell according to claims 1 or 2 wherein the polypeptide that catalyzes a substrate to product conversion of 3-amino-2-butanol phosphate to 2-butanone is aminobutanol phosphate phospho-lyase.
8. A host cell according to claim 1 wherein the polypeptide that catalyzes a substrate to product conversion of 2-butanone to 2-butanol is butanol dehydrogenase.
9. A host cell according to claims 1 or 2 wherein the cell is selected from the group consisting of: a bacterium, a cyanobacterium, a filamentous fungus and a yeast.
10. A host cell according to claim 8 wherein the cell is a member of a genus selected from the group consisting of *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*, *Pichia*, *Candida*, *Hansenula* and *Saccharomyces*.
11. A host cell according to claim 3 wherein the acetolactate synthase has an amino acid sequence having at least 95% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:77, and SEQ ID NO:79 based on the Clustal W method of

alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

12. A host cell according to claim 4 wherein the acetolactate decarboxylase has an amino acid sequence having at least 95% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO: 81, and SEQ ID NO:83 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

13 A host cell according to claim 5 wherein the acetoin aminase has an amino acid sequence having at least 95% identity to an amino acid sequence as set forth in SEQ ID NO:122 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

14. A host cell according to claim 6 wherein the aminobutanol kinase has an amino acid sequence having at least 95% identity to an amino acid sequence as set forth in SEQ ID NO:124 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

15. A host cell according to claim 7 wherein the aminobutanol phosphate phospho-lyase has an amino acid sequence having at least 95% identity to an amino acid sequence as set forth in SEQ ID NO:126 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

16. A host cell according to claim 8 wherein the butanol dehydrogenase has an amino acid sequence having at least 95% identity to an amino

acid sequence selected from the group consisting of SEQ ID NO:14, SEQ ID NO:72, SEQ ID NO:75, and SEQ ID NO:91 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

17. A method for the production of 2-butanol comprising:
 - 1) providing a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:
 - i) pyruvate to alpha-acetolactate ;
 - ii) alpha-acetolactate to acetoin;
 - iii) acetoin to 3-amino-2-butanol;
 - iv) 3-amino-2-butanol to 3-amino-2-butanol phosphate;
 - v) 3-amino-2-butanol phosphate to 2-butanone; and
 - vi) 2-butanone to 2-butanol;wherein the at least one DNA molecule is heterologous to said microbial host cell; and
 - 2) contacting the host cell of (1) with a fermentable carbon substrate in a fermentation medium under conditions whereby 2-butanol is produced.
18. A method for the production of 2-butanone comprising:
 - 1) providing a recombinant microbial host cell comprising at least one DNA molecule encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of:
 - i) pyruvate to alpha-acetolactate;
 - ii) alpha-acetolactate to acetoin;
 - iii) acetoin to 3-amino-2-butanol;
 - iv) 3-amino-2-butanol to 3-amino-2-butanol phosphate; and
 - v) 3-amino-2-butanol phosphate to 2-butanone;

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

- 2) contacting the host cell of (1) with a fermentable carbon substrate in a fermentation medium under conditions whereby 2-butanone is produced.

19. A method according to claims 17 or 18 wherein the fermentable carbon substrate is selected from the group consisting of monosaccharides, oligosaccharides, and polysaccharides.

20. A method according to claims 17 or 18 wherein the polypeptide that catalyzes a substrate to product conversion of pyruvate to alpha-acetolactate is acetolactate synthase.

21. A method according to claims 17 or 18 wherein the polypeptide that catalyzes a substrate to product conversion of alpha-acetolactate to acetoin is acetolactate decarboxylase.

22. A method according to claims 17 or 18 wherein the polypeptide that catalyzes a substrate to product conversion of acetoin to 3-amino-2-butanol is acetoin aminase.

23. A method according to claims 17 or 18 wherein the polypeptide that catalyzes a substrate to product conversion of 3-amino-2-butanol to 3-amino-2-butanol phosphate is aminobutanol kinase.

24. A method according to claims 17 or 18 wherein the polypeptide that catalyzes a substrate to product conversion of 3-amino-2-butanol phosphate to 2-butanone is aminobutanol phosphate phospho-lyase.

25. A method according to claim 17 wherein the polypeptide that catalyzes a substrate to product conversion of 2-butanone to 2-butanol is butanol dehydrogenase.

26. A method according to claims 17 or 18 wherein the cell is selected from the group consisting of: a bacterium, a cyanobacterium, a filamentous fungus, and a yeast.
27. A method according to claim 26 wherein the cell is a member of a genus selected from the group consisting of *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*, *Pichia*, *Candida*, *Hansenula* and *Saccharomyces*.
28. A method according to claim 20 wherein the acetolactate synthase has an amino acid sequence having at least 95% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:77, and SEQ ID NO:79 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.
29. A method according to claim 21 wherein the acetolactate decarboxylase has an amino acid sequence having at least 95% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO: 81, and SEQ ID NO:83 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.
30. A method according to claim 22 wherein the acetoin aminase has an amino acid sequence having at least 95% identity to an amino acid sequence as set forth in SEQ ID NO:122 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

31. A method according to claim 23 wherein the aminobutanol kinase has an amino acid sequence having at least 95% identity to an amino acid sequence as set forth in SEQ ID NO:124 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.
32. A method according to claim 24 wherein the aminobutanol phosphate phospho-lyase has an amino acid sequence having at least 95% identity to an amino acid sequence as set forth in SEQ ID NO:126 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.
33. A method according to claim 25 wherein the butanol dehydrogenase has an amino acid sequence having at least 95% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:14, SEQ ID NO:72, SEQ ID NO:75, and SEQ ID NO:91 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.
34. A 2-butanol containing fermentation product medium produced by the method of claim 17.
35. A 2-butanone containing fermentation product medium produced by the method of claim 18.

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Figure 1

