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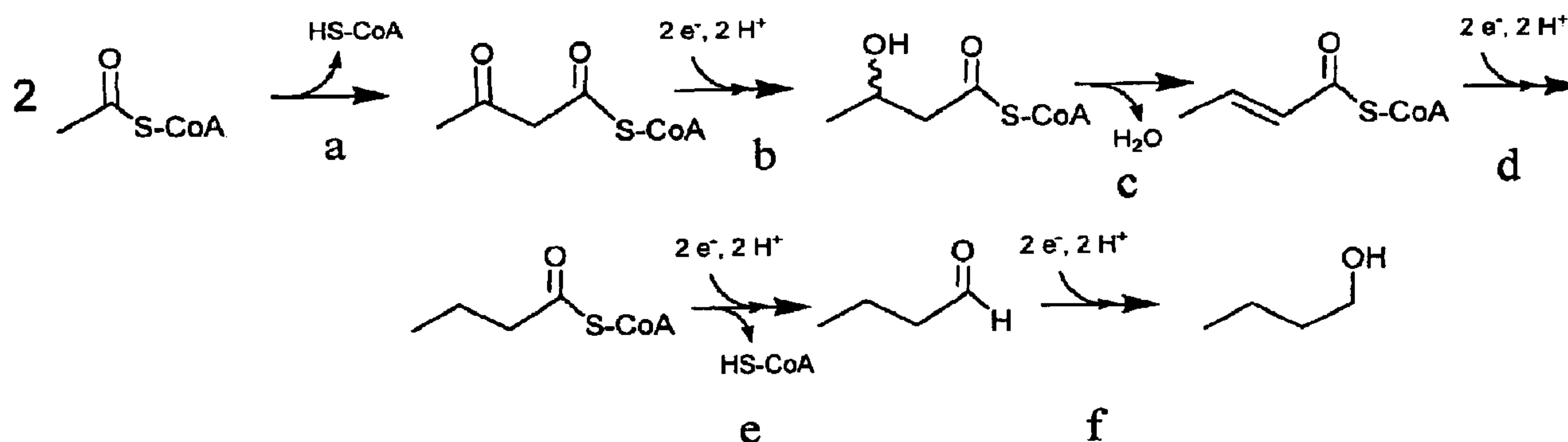
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(54) Title: METHOD FOR THE PRODUCTION OF 1-BUTANOL

Figure 1



(57) Abrégé/Abstract:

A method for the production of 1-butanol by fermentation using a microbial production host is disclosed. The method employs a reduction in temperature during the fermentation process that results in a more robust tolerance of the production host to the butanol product.

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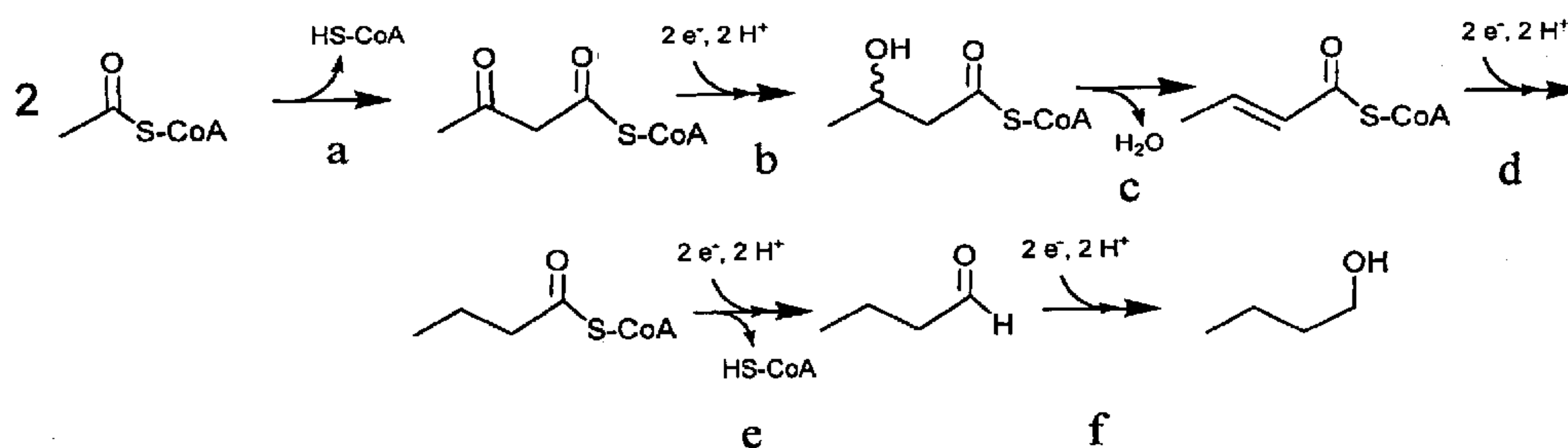
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(54) Title: METHOD FOR THE PRODUCTION OF 1-BUTANOL

Figure 1



(57) Abstract: A method for the production of 1-butanol by fermentation using a microbial production host is disclosed. The method employs a reduction in temperature during the fermentation process that results in a more robust tolerance of the production host to the butanol product.

TITLE

METHOD FOR THE PRODUCTION OF 1-BUTANOL

FIELD OF THE INVENTION

5 The invention relates to a method for the production of 1-butanol by fermentation using a recombinant microbial host. Specifically, the method employs a decrease in temperature during fermentation that results in more robust tolerance of the production host to the 1-butanol product.

BACKGROUND OF THE INVENTION

10 Butanol is an important industrial chemical, useful as a fuel additive, as a feedstock chemical in the plastics industry, and as a foodgrade extractant in the food and flavor industry. Each year 10 to 12 billion pounds of butanol are produced by petrochemical means and the need for this commodity chemical will likely increase.

15 Methods for the chemical synthesis of 1-butanol are known, such as the Oxo Process, the Reppe Process, and the hydrogenation of crotonaldehyde (*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
20 petrochemicals and are generally expensive and are not environmentally friendly. The production of 1-butanol from plant-derived raw materials would minimize greenhouse gas emissions and would represent an advance in the art.

 Methods of producing 1-butanol by fermentation are also known,
25 where the most popular process produces a mixture of acetone, 1-butanol and ethanol and is referred to as the ABE process (Blaschek et al., U.S. Patent No. 6,358,717). Acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fermentations, and the pathways and genes responsible for the production
30 of these solvents have been reported (Girbal et al., *Trends in Biotechnology* 16:11-16 (1998)). Additionally, recombinant microbial production hosts expressing a 1-butanol biosynthetic pathway have been described (Donaldson et al., copending and commonly owned U.S. Patent Application No. 11/527995). However, biological production of 1-butanol

is believed to be limited by butanol toxicity to the host microorganism used in the fermentation.

Some microbial strains that are tolerant to 1-butanol are known in the art (see for example, Jain et al. U.S. Patent No. 5,192,673; Blaschek et al. U.S. Patent No. 6,358,717; Papoutsakis et al. U.S. Patent No. 6,960,465; and Bramucci et al., copending and commonly owned U.S. Patent Application Nos. 11/743220, 11/761497, and 11/949793). However, biological methods of producing 1-butanol to higher levels are required for cost effective commercial production.

There have been reports describing the effect of temperature on the tolerance of some microbial strains to ethanol. For example, Amartei et al. (*Biotechnol. Lett.* 13(9):627-632 (1991)) disclose that *Bacillus stearothermophilus* is less tolerant to ethanol at 70 °C than at 60 °C. Herrero et al. (*Appl. Environ. Microbiol.* 40(3):571-577 (1980)) report that the optimum growth temperature of a wild-type strain of *Clostridium thermocellum* decreases as the concentration of ethanol challenge increases, whereas the optimum growth temperature of an ethanol-tolerant mutant remains constant. Brown et al. (*Biotechnol. Lett.* 4(4):269-274 (1982)) disclose that the yeast *Saccharomyces uvarum* is more resistant to growth inhibition by ethanol at temperatures 5 °C and 10 °C below its growth optimum of 35 °C. However, fermentation became more resistant to ethanol inhibition with increasing temperature. Additionally, Van Uden (*CRC Crit. Rev. Biotechnol.* 1(3):263-273 (1984)) report that ethanol and other alkanols depress the maximum and the optimum growth temperature for growth of *Saccharomyces cerevisiae* while thermal death is enhanced. Moreover, Lewis et al. (U.S. patent Application Publication No. 2004/0234649) describe methods for producing high levels of ethanol during fermentation of plant material comprising decreasing the temperature during saccharifying, fermenting, or simultaneously saccharifying and fermenting.

Much less is known about the effect of temperature on the tolerance of microbial strains to 1-butanol. Harada (*Hakko Kyokaishi* 20:155-156 (1962)) discloses that the yield of 1-butanol in the ABE process is increased from 18.4%-18.7% to 19.1%-21.2% by lowering the

temperature from 30 °C to 28 °C when the growth of the bacteria reaches a maximum. Jones et al. (*Microbiol. Rev.* 50(4):484-524 (1986)) review the role of temperature in ABE fermentation. They report that the solvent yields of three different solvent producing strains remains fairly constant at 5 31% at 30 °C and 33 °C, but decreases to 23 to 25% at 37 °C. Similar results were reported for *Clostridium acetobutylicum* for which solvent yields decreased from 29% at 25 °C to 24% at 40 °C. In the latter case, the decrease in solvent yield was attributed to a decrease in acetone production while the yield of 1-butanol was unaffected. However, 10 Carnarius (U.S. Patent No. 2,198,104) reports that an increase in the butanol ratio is obtained in the ABE process by decreasing the temperature of the fermentation from 30 °C to 24 °C after 16 hours. However, the effect of temperature on the production of 1-butanol by recombinant microbial hosts is not known in the art.

15 There is a need, therefore, for a cost-effective process for the production of 1-butanol by fermentation that provides higher yields than processes known in the art. The present invention addresses this need through the discovery of a method for producing 1-butanol by fermentation using a recombinant microbial host, which employs a decrease in 20 temperature during fermentation, resulting in more robust tolerance of the production host to the 1-butanol product.

SUMMARY OF THE INVENTION

The invention provides a method for the production of 1-butanol by fermentation using a recombinant microbial host, which employs a 25 decrease in temperature during fermentation that results in more robust tolerance of the production host to the 1-butanol product.

Accordingly, the invention provides a method for the production of 1-butanol comprising:

- 30 a) providing a recombinant microbial production host which produces 1-butanol;
- b) seeding the production host of (a) into a fermentation medium comprising a fermentable carbon substrate to create a fermentation culture;

- c) growing the production host in the fermentation culture at a first temperature for a first period of time;
- d) lowering the temperature of the fermentation culture to a second temperature; and
- 5 e) incubating the production host at the second temperature of step (d) for a second period of time;
- whereby 1-butanol is produced.

BRIEF DESCRIPTION OF THE FIGURES AND SEQUENCE DESCRIPTIONS

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

Figure 1 shows the 1-butanol biosynthetic pathway. The steps labeled “a”, “b”, “c”, “d”, “e”, and “f” represent the substrate to product
15 conversions described below.

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

25

Table 1Summary of Gene and Protein SEQ ID Numbers

Description	SEQ ID NO Nucleic acid	SEQ ID NO Peptide
Acetyl-CoA acetyltransferase thIA from <i>Clostridium acetobutylicum</i> ATCC 824	1	2

Acetyl-CoA acetyltransferase thlB from <i>Clostridium acetobutylicum</i> ATCC 824	3	4
Acetyl-CoA acetyltransferase from <i>Escherichia coli</i>	128	129
Acetyl-CoA acetyltransferase from <i>Bacillus subtilis</i>	130	131
Acetyl-CoA acetyltransferase from <i>Saccharomyces cerevisiae</i>	132	133
3-Hydroxybutyryl-CoA dehydrogenase from <i>Clostridium acetobutylicum</i> ATCC 824	5	6
3-Hydroxybutyryl-CoA dehydrogenase from <i>Bacillus subtilis</i>	134	135
3-Hydroxybutyryl-CoA dehydrogenase from <i>Ralstonia eutropha</i>	136	137
3-Hydroxybutyryl-CoA dehydrogenase from <i>Alcaligenes eutrophus</i>	138	139
Crotonase from <i>Clostridium</i> <i>acetobutylicum</i> ATCC 824	7	8
Crotonase from <i>Escherichia coli</i>	140	141
Crotonase from <i>Bacillus subtilis</i>	142	143
Crotonase from <i>Aeromonas</i> <i>caviae</i>	144	145
Putative trans-enoyl CoA reductase from <i>Clostridium</i> <i>acetobutylicum</i> ATCC 824	9	10
Butyryl-CoA dehydrogenase from <i>Euglena gracilis</i>	146	147

Butyryl-CoA dehydrogenase from <i>Streptomyces collinus</i>	148	149
Butyryl-CoA dehydrogenase from <i>Streptomyces coelicolor</i>	150	151
Butyraldehyde dehydrogenase from <i>Clostridium beijerinckii</i> NRRL B594	11	12
Butyraldehyde dehydrogenase from <i>Clostridium acetobutylicum</i>	152	153
Butanol dehydrogenase bdhB from <i>Clostridium acetobutylicum</i> ATCC 824	13	14
Butanol dehydrogenase bdhA from <i>Clostridium</i> <i>acetobutylicum</i> ATCC 824	15	16
Butanol dehydrogenase from <i>Clostridium acetobutylicum</i>	152	153
Butanol dehydrogenase from <i>Escherichia coli</i>	154	155

SEQ ID NOs:17-44 are the nucleotide sequences of oligonucleotide primers used to amplify the genes of the 1-butanol biosynthetic pathway.

5 SEQ ID NOs:45-72 are the nucleotide sequences of oligonucleotide primers used for sequencing.

SEQ ID NOs:73-75 are the nucleotide sequences of oligonucleotide primers used to construct the transformation vectors described in Example 13.

10 SEQ ID NO:76 is the nucleotide sequence of the codon-optimized CAC0462 gene, referred to herein as CaTER.

SEQ ID NO:77 is the nucleotide sequence of the codon-optimized EgTER gene, referred to herein as EgTER(opt).

SEQ ID NO:78 is the nucleotide sequence of the codon-optimized *ald* gene, referred to herein as ald (opt).

SEQ ID NO:79 is the nucleotide sequence of the plasmid pFP988.

SEQ ID NO:'s 80-127, 160-185, and 190-207 are the nucleic acid sequences of cloning, sequencing, or PCR screening primers used for the cloning, sequencing, or screening of the genes of the 1-butanol

5 biosynthetic pathway described herein, and are more fully described in Tables 4 and 5.

SEQ ID NO:156 is the nucleotide sequence of the *cscBKA* gene cluster.

10 SEQ ID NO:157 is the amino acid sequence of sucrose hydrolase (CscA).

SEQ ID NO:158 is the amino acid sequence of D-fructokinase (CscK).

SEQ ID NO:159 is the amino acid sequence of sucrose permease (CscB).

15 SEQ ID NO:186 is the nucleotide sequence of the codon optimized *tery* gene described in Example 21.

SEQ ID NO:187 is the amino acid sequence of the butyl-CoA dehydrogenase (*ter*) encoded by the codon optimized *tery* gene (SEQ ID NO:186).

20 SEQ ID NO:188 is the nucleotide sequence of the codon optimized *aldy* gene described in Example 21.

SEQ ID NO:189 is the amino acid sequence of the butyraldehyde dehydrogenase (*ald*) encoded by the codon optimized *aldy* gene (SEQ ID NO:188).

25 SEQ ID NO:208 is the nucleotide sequence of the template DNA used in Example 18.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for the production of 1-butanol using recombinant microorganisms that employs a decrease in
30 temperature during fermentation, resulting in more robust tolerance of the production host to the 1-butanol product and therefore a higher titer of 1-butanol. The present invention meets a number of commercial and industrial needs. 1-Butanol is an important industrial commodity chemical with a variety of applications, where its potential as a fuel or fuel additive is

particularly significant. 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
5 combustion engine. Additionally 1-butanol is less corrosive than ethanol, the most preferred fuel additive to date.

In addition to its utility as a biofuel or fuel additive, 1-butanol has the potential of impacting hydrogen distribution problems in the emerging fuel cell industry. Fuel cells today are plagued by safety concerns associated
10 with hydrogen transport and distribution. 1-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 vehicles.

Finally the present invention produces 1-butanol from plant derived carbon sources, avoiding the negative environmental impact associated
15 with standard petrochemical processes for butanol production.

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

As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having,” “contains” or “containing,” or any other
20 variation thereof, are intended to cover a non-exclusive inclusion. For example, a composition, a mixture, process, method, article, or apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent to such composition, mixture, process, method, article, or apparatus.
25 Further, unless expressly stated to the contrary, “or” refers to an inclusive or and not to an exclusive or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

30 Also, the indefinite articles “a” and “an” preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances (i.e. occurrences) of the element or component. Therefore “a” or “an” should be read to include one or at least one, and

the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

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.

As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or use solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or carry out the methods; and the like. The term “about” also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities. In one embodiment, the term “about” means within 10% of the reported numerical value, preferably within 5% of the reported numerical value.

“ABE” is the abbreviation for the Acetone-Butanol-Ethanol fermentation process.

The term “1-butanol biosynthetic pathway” means the enzyme pathway to produce 1-butanol from acetyl-coenzyme A (acetyl-CoA).

The term “acetyl-CoA acetyltransferase” refers to an enzyme that catalyzes the conversion of two molecules of acetyl-CoA to acetoacetyl-CoA and coenzyme A (CoA). Preferred acetyl-CoA acetyltransferases are acetyl-CoA acetyltransferases with substrate preferences (reaction in the forward direction) for a short chain acyl-CoA and acetyl-CoA and are classified as E.C. 2.3.1.9 [*Enzyme Nomenclature* 1992, Academic Press, San Diego]; although, enzymes with a broader substrate range (E.C. 2.3.1.16) will be functional as well. Acetyl-CoA acetyltransferases are available from a number of sources, for example, *Escherichia coli* (GenBank Nos: NP_416728 (SEQ ID NO:129), NC_000913 (SEQ ID NO:128); NCBI (National Center for Biotechnology

Information) amino acid sequence, NCBI nucleotide sequence),
Clostridium acetobutylicum (GenBank Nos: NP_349476.1 (SEQ ID NO:2),
 NC_003030 (SEQ ID NO:1); NP_149242 (SEQ ID NO:4), NC_001988
 (SEQ ID NO:3), *Bacillus subtilis* (GenBank Nos: NP_390297 (SEQ ID
 5 NO:131), NC_000964 (SEQ ID NO:130)), and *Saccharomyces cerevisiae*
 (GenBank Nos: NP_015297 (SEQ ID NO:133), NC_001148 (SEQ ID
 NO:132)).

The term “3-hydroxybutyryl-CoA dehydrogenase” refers to an
 enzyme that catalyzes the conversion of acetoacetyl-CoA to 3-
 10 hydroxybutyryl-CoA. 3-Hydroxybutyryl-CoA dehydrogenases may be
 reduced nicotinamide adenine dinucleotide (NADH)-dependent, with a
 substrate preference for (S)-3-hydroxybutyryl-CoA or (R)-3-hydroxybutyryl-
 CoA and are classified as E.C. 1.1.1.35 and E.C. 1.1.1.30, respectively.
 Additionally, 3-hydroxybutyryl-CoA dehydrogenases may be reduced
 15 nicotinamide adenine dinucleotide phosphate (NADPH)-dependent, with a
 substrate preference for (S)-3-hydroxybutyryl-CoA or (R)-3-hydroxybutyryl-
 CoA and are classified as E.C. 1.1.1.157 and E.C. 1.1.1.36, respectively.
 3-Hydroxybutyryl-CoA dehydrogenases are available from a number of
 sources, for example, *C. acetobutylicum* (GenBank NOs: NP_349314
 20 (SEQ ID NO:6), NC_003030 (SEQ ID NO:5)), *B. subtilis* (GenBank NOs:
 AAB09614 (SEQ ID NO:135), U29084 (SEQ ID NO:134)), *Ralstonia*
eutropha (GenBank NOs: YP_294481 (SEQ ID NO:137), NC_007347
 (SEQ ID NO:136)), and *Alcaligenes eutrophus* (GenBank NOs: AAA21973
 (SEQ ID NO:139), J04987 (SEQ ID NO:138)).

25 The term “crotonase” refers to an enzyme that catalyzes the
 conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA and H₂O. Crotonases
 may have a substrate preference for (S)-3-hydroxybutyryl-CoA or (R)-3-
 hydroxybutyryl-CoA and are classified as E.C. 4.2.1.17 and E.C. 4.2.1.55,
 respectively. Crotonases are available from a number of sources, for
 30 example, *E. coli* (GenBank NOs: NP_415911 (SEQ ID NO:141),
 NC_000913 (SEQ ID NO:140)), *C. acetobutylicum* (GenBank NOs:
 NP_349318 (SEQ ID NO:8), NC_003030 (SEQ ID NO:6)), *B. subtilis*
 (GenBank NOs: CAB13705 (SEQ ID NO:143), Z99113 (SEQ ID NO:142)),

and *Aeromonas caviae* (GenBank NOs: BAA21816 (SEQ ID NO:145), D88825 (SEQ ID NO:144)).

The term “butyryl-CoA dehydrogenase” refers to an enzyme that catalyzes the conversion of crotonyl-CoA to butyryl-CoA. Butyryl-CoA dehydrogenases may be either NADH-dependent or NADPH-dependent and are classified as E.C. 1.3.1.44 and E.C. 1.3.1.38, respectively.

Butyryl-CoA dehydrogenases are available from a number of sources, for example, *C. acetobutylicum* (GenBank NOs: NP_347102 (SEQ ID NO:10), NC_003030 (SEQ ID NO:9))), *Euglena gracilis* (GenBank NOs: Q5EU90 SEQ ID NO:147), AY741582 SEQ ID NO:146)), *Streptomyces collinus* (GenBank NOs: AAA92890 (SEQ ID NO:149), U37135 (SEQ ID NO:148)), and *Streptomyces coelicolor* (GenBank NOs: CAA22721 (SEQ ID NO:151), AL939127 (SEQ ID NO:150)).

The term “butyraldehyde dehydrogenase” refers to an enzyme that catalyzes the conversion of butyryl-CoA to butyraldehyde, using NADH or NADPH as cofactor. Butyraldehyde dehydrogenases with a preference for NADH are known as E.C. 1.2.1.57 and are available from, for example, *Clostridium beijerinckii* (GenBank NOs: AAD31841 (SEQ ID NO:12), AF157306 (SEQ ID NO:11)) and *C. acetobutylicum* (GenBank NOs: NP_149325 (SEQ ID NO:153), NC_001988 (SEQ ID NO:152)).

The term “butanol dehydrogenase” refers to an enzyme that catalyzes the conversion of butyraldehyde to 1-butanol, using either NADH or NADPH as cofactor. Butanol dehydrogenases are available from, for example, *C. acetobutylicum* (GenBank NOs: NP_149325 (SEQ ID NO:153), NC_001988 SEQ ID NO:152; note: this enzyme possesses both aldehyde and alcohol dehydrogenase activity); NP_349891 (SEQ ID NO:14), NC_003030 (SEQ ID NO:13); and NP_349892 (SEQ ID NO:16), NC_003030 (SEQ ID NO:15)) and *E. coli* (GenBank NOs: NP_417484 (SEQ ID NO:155), NC_000913 (SEQ ID NO:154)).

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

The term “carbon substrate” or “fermentable carbon substrate” refers to a carbon source capable of being metabolized by host organisms disclosed herein and particularly carbon sources selected from the group

consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof.

The term “gene” refers to a nucleic acid fragment that is capable of being expressed as a specific protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. “Native gene” refers to a gene as found in nature with its own regulatory sequences. “Chimeric gene” refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. “Endogenous gene” refers to a native gene in its natural location in the genome of an organism. A “foreign” 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-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, 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

5 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

10 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.

20 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

25 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

30 greater than 100 nucleotides in length, equations for calculating T_m have 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 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
5 about 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as 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
10 polypeptide or the nucleotide sequence of a gene to putatively identify that polypeptide or gene, either by manual evaluation of the sequence by one 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
15 contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising
20 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). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid
25 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
30 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 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.

5 The terms “homology” and “homologous” are used interchangeably herein. They refer to nucleic acid fragments wherein changes in one or 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
10 invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid 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.

15 Moreover, the skilled artisan recognizes that homologous nucleic acid sequences encompassed by this invention are also defined by their 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
20 equivalent to any of the nucleic acid sequences disclosed herein.

 “Codon degeneracy” refers to the nature in the genetic code 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
25 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
30 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”

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 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).

10 Preferred methods to determine identity are designed to give the 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
15 computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the 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);
20 Higgins, D.G. et al., *Comput. Appl. Biosci.*, 8:189-191 (1992)) and found in 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
25 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
30 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:

5 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
10 software when first initialized.

As used herein the term “coding sequence” refers to a DNA sequence that codes for a specific amino acid sequence. “Suitable regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding
15 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.

20 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
25 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
30 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 affecting 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 disclosed herein. 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 molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. “Transformation vector” refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host cell.

As used herein the term “codon degeneracy” refers to the nature in the genetic code permitting variation of the nucleotide sequence without 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 “codon-optimized” as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA.

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

The 1-Butanol Biosynthetic Pathway

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. Subsequently, pyruvate is transformed to acetyl-coenzyme A (acetyl-CoA) via a variety of means, including reaction with the pyruvate dehydrogenase complex, pyruvate-formate lyase, and pyruvate-ferredoxin oxidoreductase. Acetyl-CoA serves as a key intermediate, for example, in generating fatty acids, amino acids and secondary metabolites. The combined reactions of sugar conversion to acetyl-CoA 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. O₂, NO₃⁻ and SO₄²⁻), the reducing equivalents may be used to augment the energy pool; alternatively, a reduced carbon by-product may be formed. The production of ethanol and 1-butanol resulting from the fermentation of carbohydrate are examples of the latter. As described by Donaldson,
5 *supra*, 1-butanol can be produced from carbohydrate sources by recombinant microorganisms comprising a complete 1-butanol biosynthetic pathway from acetyl-CoA to 1-butanol, as shown in Figure 1."

This biosynthetic pathway, generally lacking in the microbial
10 community due to the absence of genes or the lack of appropriate gene regulation, comprises the following substrate to product conversions:

- a) acetyl-CoA to acetoacetyl-CoA, as catalyzed for example by acetyl-CoA acetyltransferase;
- b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA, as catalyzed for
15 example by 3-hydroxybutyryl-CoA dehydrogenase;
- c) 3-hydroxybutyryl-CoA to crotonyl-CoA, as catalyzed for example by crotonase;
- d) crotonyl-CoA to butyryl-CoA, as catalyzed for example by butyryl-CoA dehydrogenase;
- e) butyryl-CoA to butyraldehyde, as catalyzed for example by
20 butyraldehyde dehydrogenase; and
- f) butyraldehyde to 1-butanol, as catalyzed for example by butanol dehydrogenase.

The pathway requires no ATP and generates NAD⁺ and/or NADP⁺,
25 thus, balances with the central metabolic routes that generate acetyl-CoA. The ability of natural organisms to produce 1-butanol by fermentation is rare and exemplified most prominently by *Clostridium beijerinckii* and *Clostridium acetobutylicum*. The gene organization and gene regulation for *Clostridium acetobutylicum* has been described (L. Girbal and P.
30 Soucaille, *Trends in Biotechnology* 216:11-16 (1998)). However, many of these enzyme activities are associated also with alternate pathways, for example, hydrocarbon utilization, fatty acid oxidation, and poly-hydroxyalkanoate metabolism. Thus, in providing a recombinant pathway from acetyl-CoA to 1-butanol, there exist a number of choices to fulfill the

- individual reaction steps, and the person of skill in the art will be able to utilize publicly available sequences to construct the relevant pathways. A listing of a representative number of genes known in the art and useful in the construction of the 1-butanol biosynthetic pathway are listed below in
- 5 Table 2 and in Donaldson et al., copending and commonly owned U.S. Patent Application No. 11/527995, incorporated herein by reference.

Table 2Sources of 1-Butanol Pathway Genes

Gene	GenBank Citation
acetyl-CoA acetyltransferase	NC_000913 <i>Escherichia coli</i> K12, complete genome gi 49175990 ref NC_000913.2 [49175990]
	NC_001988 <i>Clostridium acetobutylicum</i> ATCC 824 plasmid pSOL1, complete sequence gi 15004705 ref NC_001988.2 [15004705]
	NC_000964 <i>Bacillus subtilis</i> subsp. subtilis str. 168, complete genome gi 50812173 ref NC_000964.2 [50812173]
	NC_001148 <i>Saccharomyces cerevisiae</i> chromosome XVI, complete chromosome sequence gi 50593503 ref NC_001148.3 [50593503]
	CP000017 <i>Streptococcus pyogenes</i> MGAS5005, complete genome gi 71852596 gb CP000017.1 [71852596]
	NC_005773 <i>Pseudomonas syringae</i> pv. phaseolicola 1448A, complete genome gi 71733195 ref NC_005773.3 [71733195]
	CR931997 <i>Corynebacterium jeikeium</i> K411 complete genome gi 68262661 emb CR931997.1 [68262661]
3-hydroxybutyryl- CoA dehydrogenase	NC_003030 <i>Clostridium acetobutylicum</i> ATCC 824, complete genome

	gi 15893298 ref NC_003030.1 [15893298]
	U29084 <i>Bacillus subtilis</i> (mmgA), (mmgB), (mmgC), and citrate synthase III (mmgD) genes, complete cds, and (mmgE) gene, partial cds gi 881603 gb U29084.1 BSU29084[881603]
	NC_007347 <i>Ralstonia eutropha</i> JMP134 Raeut01_1, whole genome shotgun sequence gi 45517296 ref NZ_AADY01000001.1 [45517296]
	J04987 <i>A. eutrophus</i> beta-ketothiolase (phbA) and acetoacetyl-CoA reductase (phbB) genes, complete cds gi 141953 gb J04987.1 AFAKTLAACA[141953]
	NC_004129 <i>Pseudomonas fluorescens</i> Pf-5, complete genome gi 70728250 ref NC_004129.6 [70728250]
	NC_000913 <i>Escherichia coli</i> K12, complete genome gi 49175990 ref NC_000913.2 [49175990]
	NC_004557 <i>Clostridium tetani</i> E88, complete genome gi 28209834 ref NC_004557.1 [28209834]
	NC_006350 <i>Burkholderia pseudomallei</i> K96243 chromosome 1, complete sequence gi 53717639 ref NC_006350.1 [53717639]
	NC_002947 <i>Pseudomonas putida</i> KT2440, complete genome gi 26986745 ref NC_002947.3 [26986745]
crotonase	NC_000913 <i>Escherichia coli</i> K12, complete genome gi 49175990 ref NC_000913.2 [49175990]
	NC_003030 <i>Clostridium acetobutylicum</i> ATCC 824, complete genome gi 15893298 ref NC_003030.1 [15893298]

	Z99113 <i>Bacillus subtilis</i> complete genome (section 10 of 21): from 1807106 to 2014934 gi 32468758 emb Z99113.2 BSUB0010[32468758]
	D88825 <i>Aeromonas caviae</i> phaC gene for PHA synthase, complete cds gi 2335048 dbj D88825.1 [2335048]
	NC_006274 <i>Bacillus cereus</i> ZK, complete genome gi 52140164 ref NC_006274.1 [52140164]
	NC_004557 <i>Clostridium tetani</i> E88, complete genome gi 28209834 ref NC_004557.1 [28209834]
butyryl-CoA dehydrogenase	NC_003030 <i>Clostridium acetobutylicum</i> ATCC 824, complete genome gi 15893298 ref NC_003030.1 [15893298]
	AY741582 <i>Euglena gracilis</i> trans-2-enoyl-CoA reductase mRNA, complete cds gi 58201539 gb AY741582.1 [58201539]
	U37135 <i>Streptomyces collinus</i> crotonyl-CoA reductase (ccr) gene, complete cds gi 1046370 gb U37135.1 SCU37135[1046370]
	AL939127 <i>Streptomyces coelicolor</i> A3(2) complete genome; segment 24/29 gi 24429552 emb AL939127.1 SCO939127[24429552]
	AP006716 <i>Staphylococcus haemolyticus</i> JCSC1435, complete genome gi 68445725 dbj AP006716.1 [68445725]
	NC_006274 <i>Bacillus cereus</i> ZK, complete genome gi 52140164 ref NC_006274.1 [52140164]
	NC_004557 <i>Clostridium tetani</i> E88, complete genome gi 28209834 ref NC_004557.1 [28209834]
butyraldehyde	AF157306 <i>Clostridium beijerinckii</i> strain NRRL

dehydrogenase	B593 hypothetical protein, coenzyme A acylating aldehyde dehydrogenase (ald), acetoacetate:butyrate/acetate coenzyme A transferase (ctfA), acetoacetate:butyrate/acetate coenzyme A transferase (ctfB), and acetoacetate decarboxylase (adc) genes, complete cds gi 47422980 gb AF157306.2 [47422980]
	NC_001988 <i>Clostridium acetobutylicum</i> ATCC 824 plasmid pSOL1, complete sequence gi 15004705 ref NC_001988.2 [15004705]
	AY251646 <i>Clostridium saccharoperbutylacetonicum</i> sol operon, complete sequence gi 31075382 gb AY251646.1 [31075382]
butanol dehydrogenase	NC_001988 <i>Clostridium acetobutylicum</i> ATCC 824 plasmid pSOL1, complete sequence gi 15004705 ref NC_001988.2 [15004705]
	NC_003030 <i>Clostridium acetobutylicum</i> ATCC 824, complete genome gi 15893298 ref NC_003030.1 [15893298]
	NC_000913 <i>Escherichia coli</i> K12, complete genome gi 49175990 ref NC_000913.2 [49175990]
	NC_003198 <i>Salmonella enterica</i> subsp. enterica serovar Typhi str. CT18, complete genome gi 16758993 ref NC_003198.1 [16758993]
	BX571966 <i>Burkholderia pseudomallei</i> strain K96243, chromosome 2, complete sequence gi 52211453 emb BX571966.1 [52211453]
	Z99120 <i>Bacillus subtilis</i> complete genome (section 17 of 21): from 3213330 to 3414388 gi 32468813 emb Z99120.2 BSUB0017[32468813]
	NC_003366 <i>Clostridium perfringens</i> str. 13,

	complete genome gi 18308982 ref NC_003366.1 [18308982
	NC_004431 <i>Escherichia coli</i> CFT073, complete genome gi 26245917 ref NC_004431.1 [26245917

Pathway Steps:

a) Acetyl-CoA to acetoacetyl-CoA, is catalyzed by acetyl-CoA acetyltransferase. The skilled person will appreciate that polypeptides having by acetyl-CoA acetyltransferase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. Examples of suitable by acetyl-CoA acetyltransferase enzymes are available from a number of sources, for example, for example, *Escherichia coli* (GenBank Nos: NP_416728 (SEQ ID NO:129), NC_000913 (SEQ ID NO:128); NCBI (National Center for Biotechnology Information) amino acid sequence, NCBI nucleotide sequence), *Clostridium acetobutylicum* (GenBank Nos: NP_349476.1 (SEQ ID NO:2), NC_003030 (SEQ ID NO:1); NP_149242 (SEQ ID NO:4), NC_001988 (SEQ ID NO:3), *Bacillus subtilis* (GenBank Nos: NP_390297 (SEQ ID NO:131), NC_000964 (SEQ ID NO:130)), and *Saccharomyces cerevisiae* (GenBank Nos: NP_015297 (SEQ ID NO:133), NC_001148 (SEQ ID NO:132)). Preferred by acetyl-CoA acetyltransferase enzymes are those that have at least 80% – 85% identity to SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:129, SEQ ID NO:131, or SEQ ID NO:133, 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) Acetoacetyl-CoA to 3-hydroxybutyryl-CoA is catalyzed by 3-hydroxybutyryl-CoA dehydrogenase. The skilled person will appreciate that polypeptides having 3-hydroxybutyryl-CoA dehydrogenase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. Example of suitable

3-hydroxybutyryl-CoA dehydrogenase enzymes are available from a number of sources, for example, *C. acetobutylicum* (GenBank NOs: NP_349314 (SEQ ID NO:6), NC_003030 (SEQ ID NO:5)), *B. subtilis* (GenBank NOs: AAB09614 (SEQ ID NO:135), U29084 (SEQ ID NO:134)),

5 *Ralstonia eutropha* (GenBank NOs: YP_294481 (SEQ ID NO:137), NC_007347 (SEQ ID NO:136)), and *Alcaligenes eutrophus* (GenBank NOs: AAA21973 (SEQ ID NO:139), J04987 (SEQ ID NO:138)). Preferred 3-hydroxybutyryl-CoA dehydrogenase enzymes are those that have at least 80% – 85% identity to SEQ ID NO:6, SEQ ID NO:135, SEQ ID

10 NO:137, or SEQ ID NO:139 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.

15 c) 3-hydroxybutyryl-CoA to crotonyl-CoA is catalyzed by crotonase. The skilled person will appreciate that polypeptides having crotonase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. Examples of suitable crotonase enzymes are available from a number of sources, for example,

20 *E. coli* (GenBank NOs: NP_415911 (SEQ ID NO:141), NC_000913 (SEQ ID NO:140)), *C. acetobutylicum* (GenBank NOs: NP_349318 (SEQ ID NO:8), NC_003030 (SEQ ID NO:6)), *B. subtilis* (GenBank NOs: CAB13705 (SEQ ID NO:143), Z99113 (SEQ ID NO:142)), and *Aeromonas caviae* (GenBank NOs: BAA21816 (SEQ ID NO:145), D88825 (SEQ ID

25 NO:144)). Preferred crotonase enzymes are those that have at least 80% – 85% identity to SEQ ID NO:8, SEQ ID NO:141, SEQ ID NO:143, and SEQ ID NO:145 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

30 PENALTY=0.1, and Gonnet 250 series of protein weight matrix, is most preferred.

d) Crotonyl-CoA to butyryl-CoA, is catalyzed by butyryl-CoA dehydrogenase. The skilled person will appreciate that polypeptides having butyryl-CoA dehydrogenase activity isolated from a variety of

sources will be useful in the present invention independent of sequence homology. Examples of suitable butyryl-CoA dehydrogenase enzymes are available from a number of sources, for example, *C. acetobutylicum* (GenBank NOs: NP_347102 (SEQ ID NO:10), NC_003030 (SEQ ID NO:9))), *Euglena gracilis* (GenBank NOs: Q5EU90 SEQ ID NO:147), AY741582 SEQ ID NO:146)), *Streptomyces collinus* (GenBank NOs: AAA92890 (SEQ ID NO:149), U37135 (SEQ ID NO:148)), and *Streptomyces coelicolor* (GenBank NOs: CAA22721 (SEQ ID NO:151), AL939127 (SEQ ID NO:150)). Preferred butyryl-CoA dehydrogenase enzymes are those that have at least 80% – 85% identity to SEQ ID NO:10, SEQ ID NO:147, SEQ ID NO:149, SEQ ID NO:151, and SEQ ID NO:187 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.

e) Butyryl-CoA to butyraldehyde, is catalyzed by butyraldehyde dehydrogenase. The skilled person will appreciate that polypeptides having butyraldehyde dehydrogenase activity isolated from a variety of sources will be useful in the present invention independent of sequence homology. Examples of suitable butyraldehyde dehydrogenase enzymes are available from a number of sources, for example, *Clostridium beijerinckii* (GenBank NOs: AAD31841 (SEQ ID NO:12), AF157306 (SEQ ID NO:11)) and *C. acetobutylicum* (GenBank NOs: NP_149325 (SEQ ID NO:153), NC_001988 (SEQ ID NO:152)). Preferred butyraldehyde dehydrogenase enzymes are those that have at least 80% – 85% identity to SEQ ID NO:12, SEQ ID NO:153, and SEQ ID NO:189 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.

f) Butyraldehyde to 1-butanol, is catalyzed by butanol dehydrogenase. 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, *C. acetobutylicum* (GenBank NOs: NP_149325 (SEQ ID NO:153), NC_001988 SEQ ID NO:152; note: this enzyme possesses both aldehyde and alcohol dehydrogenase activity);

5 NP_349891 (SEQ ID NO:14), NC_003030 (SEQ ID NO:13); and NP_349892 (SEQ ID NO:16), NC_003030 (SEQ ID NO:15)) and *E. coli* (GenBank NOs: NP_417484 (SEQ ID NO:155), NC_000913 (SEQ ID NO:154)). Preferred butanol dehydrogenase enzymes are those that have at least 80% – 85% identity to SEQ ID NO:14, SEQ ID NO:16, SEQ

10 ID NO:153, SEQ ID NO:155, and SEQ ID NO:157 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.

15 Microbial Hosts for 1-Butanol Production

Microbial hosts for 1-butanol production may be selected from bacteria, cyanobacteria, filamentous fungi and yeasts. The microbial host used for 1-butanol production is preferably tolerant to 1-butanol so that the yield is not limited by butanol toxicity. Microbes that are metabolically

20 active at high titer levels of 1-butanol are not well known in the art. Although 1-butanol-tolerant mutants have been isolated from solventogenic *Clostridia*, little information is available concerning the 1-butanol tolerance of other potentially useful bacterial strains. Most of the studies on the comparison of alcohol tolerance in bacteria suggest that 1-

25 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 fermentation in *Clostridium acetobutylicum* may be limited by toxicity. The primary effect of 1-butanol on *Clostridium*

30 *acetobutylicum* is disruption of membrane functions (Hermann et al., *Appl. Environ. Microbiol.* 50:1238-1243 (1985)).

The microbial hosts selected for the production of 1-butanol are preferably tolerant to 1-butanol and are able to convert carbohydrates to 1-butanol. The criteria for selection of suitable microbial hosts include the

following: intrinsic tolerance to 1-butanol, high rate of glucose utilization, availability of genetic tools for gene manipulation, and the ability to generate stable chromosomal alterations.

Suitable host strains with a tolerance for 1-butanol may be identified
5 by screening based on the intrinsic tolerance of the strain. The intrinsic tolerance of microbes to 1-butanol may be measured by determining the concentration of 1-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
10 microbes of interest may be grown in the presence of various amounts of 1-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 1-butanol that produces 50% inhibition of
15 growth may be determined from a graph of the percent inhibition of growth versus the 1-butanol concentration. Preferably, the host strain should have an IC50 for 1-butanol of greater than about 0.5% weight/volume.

The microbial host for 1-butanol production should also utilize glucose at a high rate. Most microbes are capable of utilizing
20 carbohydrates. However, certain environmental microbes cannot utilize carbohydrates to high efficiency, and therefore would not be suitable hosts.

The ability to genetically modify the host is essential for the production of any recombinant microorganism. The mode of gene transfer
25 technology may be by electroporation, conjugation, transduction or natural transformation. A broad range of host conjugative plasmids and drug resistant markers are available. The cloning vectors are tailored to the host organisms based on the nature of antibiotic resistance markers that can function in that host.

30 The microbial host also has to be manipulated in order to inactivate competing pathways for carbon flow by deleting various genes. This requires the availability of either transposons to direct inactivation or chromosomal integration vectors. Additionally, the production host should

be amenable to chemical mutagenesis so that mutations to improve intrinsic 1-butanol tolerance may be obtained.

Based on the criteria described above, suitable microbial hosts for the production of 1-butanol include, but are not limited to, members of the
5 genera *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Rhodococcus*,
Pseudomonas, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Alcaligenes*,
Klebsiella, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*,
Pichia, *Candida*, *Hansenula* and *Saccharomyces*. Preferred hosts
include: *Escherichia coli*, *Alcaligenes eutrophus*, *Bacillus licheniformis*,
10 *Paenibacillus macerans*, *Rhodococcus erythropolis*, *Pseudomonas putida*,
Lactobacillus plantarum, *Enterococcus faecium*, *Enterococcus gallinarum*,
Enterococcus faecalis, *Bacillus subtilis* and *Saccharomyces cerevisiae*.

Construction of Production Host

Recombinant organisms containing the necessary genes that will
15 encode the enzymatic pathway for the conversion of a fermentable carbon
substrate to 1-butanol may be constructed using techniques well known in
the art. Genes encoding the enzymes of the 1-butanol biosynthetic
pathway, i.e., acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA
dehydrogenase, crotonase, butyryl-CoA dehydrogenase, butyraldehyde
20 dehydrogenase, and 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, suitable genomic libraries may be
25 created by restriction endonuclease digestion and may be screened with
probes complementary 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 (Mullis,
U.S. Patent No. 4,683,202) to obtain amounts of DNA suitable for
30 transformation using appropriate vectors. 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 content of some exemplary microbial hosts is given
Table 3.

Table 3
GC Content 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

5

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 or cassettes 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 or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. Both control regions may be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions may also be derived from genes that are not native to the specific species chosen as a production host.

10

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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, *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*); and *lac*, *ara*, *tet*, *trp*, *IP_L*, *IP_R*, *T7*, *tac*, and *trc* (useful for expression in *Escherichia coli*, *Alcaligenes*, and *Pseudomonas*); the *amy*, *apr*, *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 the 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 create 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 to create random mutations in a variety of genomes from commercial sources such as EPICENTRE®.

5 The expression of the 1-butanol biosynthetic pathway in various preferred microbial hosts is described in more detail below.

Expression of the 1-butanol biosynthetic pathway in *E. coli*

Vectors or cassettes useful for the transformation of *E. coli* are common and commercially available from the companies listed above. For example, the genes of the 1-butanol biosynthetic pathway may be
10 isolated from various strains of *Clostridium*, cloned into a modified pUC19 vector and transformed into *E. coli* NM522, as described in Example 15. The expression of the 1-butanol biosynthetic pathway in several other strains of *E. coli* is described in Example 17.

15 Expression of the 1-butanol 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)). Additionally, a series of promoters are available for heterologous gene
20 expression in *R. erythropolis* (see for example Nakashima et al., *Appl. Envir. Microbiol.* 70:5557-5568 (2004), and Tao et al., *Appl. Microbiol. Biotechnol.* 2005, DOI 10.1007/s00253-005-0064). Targeted gene disruption of chromosomal genes in *R. erythropolis* may be created using the method described by Tao et al., *supra*, and Brans et al. (*Appl. Envir. Microbiol.* 66: 2029-2036 (2000)).
25

The heterologous genes required for the production of 1-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. erythropolis* by electroporation, as described by Kostichka et al., *supra*.
30 The recombinants may be grown in synthetic medium containing glucose and the production of 1-butanol can be followed using methods known in the art.

Expression of the 1-butanol biosynthetic pathway in *Bacillus Subtilis*

Methods for gene expression and creation of mutations in *B. Subtilis* are also well known in the art. For example, the genes of the 1-butanol biosynthetic pathway may be isolated from various strains of

5 *Clostridium*, cloned into a modified pUC19 vector and transformed into *Bacillus subtilis* BE1010, as described in Example 16. Additionally, the six genes of the 1-biosynthetic pathway can be split into two operons for expression, as described in Example 18. The first three genes of the pathway (*thl*, *hbd*, and *crt*) were integrated into the chromosome of

10 *Bacillus subtilis* BE1010 (Payne and Jackson, *J. Bacteriol.* 173:2278-2282 (1991)). The last three genes (*EgTER*, *ald*, and *bdhB*) were cloned into expression plasmids and transformed into the *Bacillus* strain carrying the integrated 1-butanol genes

15 Expression of the 1-butanol biosynthetic pathway in *Bacillus licheniformis*

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. For example, the genes required for the production of 1-butanol may be cloned in plasmids pBE20 or pBE60

20 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 also be transformed into *B. licheniformis* to produce a recombinant microbial host that produces 1-

25 butanol.

Expression of the 1-butanol 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

30 transformation to produce a recombinant microbial host that produces 1-butanol.

Expression of the 1-butanol biosynthetic pathway in *Alcaligenes*
(Ralstonia) eutrophus

Methods for gene expression and creation of mutations in *Ralstonia eutrophus* are known in the art (see for example Taghavi et al., *Appl. Environ. Microbiol.*, 60(10):3585-3591 (1994)). The genes for the 1-butanol biosynthetic pathway may be cloned in any of the broad host range vectors described above, and electroporated to generate recombinants that produce 1-butanol. The polyhydroxy butyrate pathway in *Ralstonia* has been described in detail and a variety of genetic techniques to modify the *Ralstonia eutrophus* genome is known, and those tools can be applied for engineering the 1-butanol biosynthetic pathway.

Expression of the 1-butanol biosynthetic pathway in *Pseudomonas putida*

Methods for gene expression in *Pseudomonas putida* are known in the art (see for example Ben-Bassat et al., U.S. Patent No. 6,586,229, which is incorporated herein by reference). For example, the 1-butanol pathway genes 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 1-butanol.

Expression of the 1-butanol biosynthetic pathway in
Saccharomyces cerevisiae

Methods for gene expression in *Saccharomyces cerevisiae* are known in the art (see for example *Methods in Enzymology*, Volume 194, *Guide to Yeast Genetics and Molecular and Cell Biology* (Part A, 2004, Christine Guthrie and Gerald R. Fink (Eds.), Elsevier Academic Press, San Diego, CA). Expression of genes in yeast typically requires a promoter, followed by the gene of interest, and a transcriptional terminator. A number of yeast promoters can be used in constructing expression cassettes for genes encoding the 1-butanol biosynthetic pathway, including, but not limited to constitutive promoters FBA, GPD, and GPM, and the inducible promoters GAL1, GAL10, and CUP1. Suitable transcriptional terminators include, but are not limited to FBA_t, GPD_t, GPM_t, ERG10_t, and GAL1_t. Suitable promoters, transcriptional

terminators, and the genes of the 1-butanol biosynthetic pathway may be cloned into yeast 2 micron (2 μ) plasmids, as described in Example 21.

Expression of the 1-butanol biosynthetic pathway in *Lactobacillus plantarum*

5 The *Lactobacillus* genus belongs to the *Lactobacillales* family and many plasmids and vectors used in the transformation of *Bacillus subtilis* and *Streptococcus* may be used for *lactobacillus*. Non-limiting examples of suitable vectors include pAM β 1 and derivatives thereof (Renault et al., *Gene* 183:175-182 (1996); and O'Sullivan et al., *Gene* 137:227-231
10 (1993)); pMBB1 and pHW800, a derivative of pMBB1 (Wyckoff et al. *Appl. Environ. Microbiol.* 62:1481-1486 (1996)); pMG1, a conjugative plasmid (Tanimoto et al., *J. Bacteriol.* 184:5800-5804 (2002)); pNZ9520 (Kleerebezem et al., *Appl. Environ. Microbiol.* 63:4581-4584 (1997)); pAM401 (Fujimoto et al., *Appl. Environ. Microbiol.* 67:1262-1267 (2001));
15 and pAT392 (Arthur et al., *Antimicrob. Agents Chemother.* 38:1899-1903 (1994)). Several plasmids from *Lactobacillus plantarum* have also been reported (e.g., van Kranenburg R, Golic N, Bongers R, Leer RJ, de Vos WM, Siezen RJ, Kleerebezem M. *Appl. Environ. Microbiol.* 2005 Mar; 71(3): 1223-1230). For example, expression of the 1-butanol biosynthetic
20 pathway in *Lactobacillus plantarum* is described in Example 22.

Expression of the 1-butanol biosynthetic pathway in *Enterococcus faecium*, *Enterococcus gallinarum*, and *Enterococcus faecalis*

 The *Enterococcus* genus belongs to the *Lactobacillales* family and many plasmids and vectors used in the transformation of *Lactobacillus*,
25 *Bacillus subtilis*, and *Streptococcus* may be used for *Enterococcus*. 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
30 conjugative plasmid (Tanimoto et al., *J. Bacteriol.* 184:5800-5804 (2002)); pNZ9520 (Kleerebezem et al., *Appl. Environ. Microbiol.* 63:4581-4584 (1997)); pAM401 (Fujimoto et al., *Appl. Environ. Microbiol.* 67:1262-1267 (2001)); and pAT392 (Arthur et al., *Antimicrob. Agents Chemother.*

38:1899-1903 (1994)). 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)). For example, expression of the 1-butanol biosynthetic pathway in *Enterococcus faecalis* is described in Example 23.

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 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 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 yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion et al., *Microb. Growth C1 Compd.*, [Int. Symp.], 7th (1993), 415-32. Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter et al., *Arch. Microbiol.* 153:485-489 (1990)). Hence it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon containing substrates and will only be limited by the choice of organism.

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. Sucrose may be derived from renewable sugar sources such as sugar cane, sugar beets, cassava, sweet sorghum, and mixtures thereof. Glucose and

dextrose may be derived from renewable grain sources through saccharification of starch based feedstocks including grains such as corn, wheat, rye, barley, oats, and mixtures thereof. In addition, fermentable sugars may be derived from renewable cellulosic or lignocellulosic biomass through processes of pretreatment and saccharification, as described, for example, in co-owned and co-pending U.S. Patent Application Publication No. 2007/0031918A1, 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 may comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for 1-butanol production.

Culture Conditions with Temperature Lowering

In the present method, the recombinant microbial production host which produces 1-butanol is seeded into a fermentation medium comprising a fermentable carbon substrate to create a fermentation culture. The production host is grown in the fermentation culture at a first

temperature for a first period of time. The first temperature is typically from about 25 °C to about 40 °C.

Suitable fermentation media in the present invention include common commercially prepared media such as Luria Bertani (LB) broth, 5 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., 10 cyclic adenosine 2':3'-monophosphate, may also be incorporated into the fermentation medium.

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

Fermentations may be performed under aerobic or anaerobic 15 conditions, where anaerobic or microaerobic conditions are preferred.

The first period of time to grow the production host at the first temperature may be determined in a variety of ways. For example, during this period of growth a metabolic parameter of the fermentation culture may be monitored. The metabolic parameter that is monitored may be 20 any parameter known in the art, including, but not limited to the optical density, pH, respiratory quotient, fermentable carbon substrate utilization, CO₂ production, and 1-butanol production. During this period of growth, additional fermentable carbon substrate may be added, the pH may be adjusted, oxygen may be added for aerobic cells, or other culture 25 parameters may be adjusted to support the metabolic activity of the culture. Though nutrients and culture conditions are supportive of growth, after a period of time the metabolic activity of the fermentation culture decreases as determined by the monitored parameter described above. For example, a decrease in metabolic activity may be indicated by a 30 decrease in one or more of the following parameters: rate of optical density change, rate of pH change, rate of change in respiratory quotient (if the host cells are aerobic), rate of fermentable carbon substrate utilization, rate of 1-butanol production, rate of change in CO₂ production, or rate of another metabolic parameter. The decrease in metabolic activity

is related to the sensitivity of the host cells to the production of 1-butanol and/or the presence of 1-butanol in the culture. When decreased metabolic activity is detected, the temperature of the fermentation culture is lowered to reduce the sensitivity of the host cells to 1-butanol and
5 thereby allow further production of 1-butanol. In one embodiment, the lowering of the temperature coincides with a change in the metabolic parameter that is monitored.

In one embodiment, the change in metabolic activity is a decrease in the rate of 1-butanol production. 1-Butanol production may be
10 monitored by analyzing the amount of 1-butanol present in the fermentation culture medium as a function of time using methods well known in the art, such as using high performance liquid chromatography (HPLC) or gas chromatography (GC), which are described in the Examples herein. GC is preferred due to the short assay time.

15 Alternatively, the lowering of the temperature of the fermentation culture may occur at a predetermined time. The first period of time may be predetermined by establishing a correlation between a metabolic parameter of the fermentation culture and time in a series of test fermentations runs. A correlation between a metabolic parameter, as
20 described above, and time of culture growth may be established for any 1-butanol producing host by one skilled in the art. The specific correlation may vary depending on conditions used including, but not limited to, carbon substrate, fermentation conditions, and the specific recombinant 1-butanol producing microbial production host. The correlation is most
25 suitably made between 1-butanol production or specific glucose consumption rate and time of culture growth. Once the predetermined time has been established from the correlation, the temperature of the fermentation culture in subsequent fermentation runs is lowered at the predetermined time. For example, if it is determined by monitoring a
30 metabolic parameter in the test fermentation runs that the rate of production of 1-butanol decreases after 12 hours, the temperature in subsequent fermentations runs is lowered after 12 hours without the need to monitor 1-butanol production in the subsequent runs.

After the first period of time, the temperature of the fermentation culture is lowered to a second temperature. Typically, the second temperature is about 3 °C to about 25 °C lower than the first temperature. Reduction in temperature to enhance tolerance of the host cells to 1-butanol is balanced with maintaining the temperature at a level where the cells continue to be metabolically active for 1-butanol production. For example, a fermentation culture that has been grown at about 35 °C may be reduced in temperature to about 28 °C; or a culture grown at about 30 °C may be reduced in temperature to about 25 °C. The change in temperature may be done gradually over time or may be made as a step change. The production host is incubated at the second temperature for a second period of time, so that 1-butanol production continues. The second period of time may be determined in the same manner as the first period of time described above, e.g., by monitoring a metabolic parameter or by using a predetermined time.

Additionally, the temperature lowering and incubation steps may be repeated one or more times to more finely balance metabolic activity for 1-butanol production and 1-butanol sensitivity. For example, a culture that has been grown at about 35 °C may be reduced in temperature to about 32 °C, followed by an incubation period. During this period a metabolic parameter of the fermentation culture may be monitored as described above, or a predetermined time may be used. It is particularly suitable to monitor the production of 1-butanol during this incubation period. When monitoring indicates a decrease in metabolic activity or at a predetermined time, the temperature may be reduced a second time. For example, the temperature may be reduced from about 32 °C to about 28 °C. The temperature lowering and incubation steps may be repeated a third time where the temperature is reduced, for example, to about 20 °C. The production host is incubated at the lowered temperature so that 1-butanol production continues. The steps may be repeated further as necessary to obtain the desired 1-butanol titer.

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 fermentation the medium is inoculated with the desired organism or organisms, and fermentation is permitted to occur without adding anything
5 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 systems the metabolite and biomass compositions of the system change constantly up to the time the fermentation is stopped. Within batch
10 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 in log phase generally are responsible for the bulk of production of end product or intermediate.

15 A variation on the standard batch system is the Fed-Batch system. Fed-Batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit
20 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
25 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.

30 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 where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or
5 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
10 by media turbidity, 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
15 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
20 cells may be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for 1-butanol production.

Methods for 1-Butanol Isolation from the Fermentation Medium

The bioproducted 1-butanol may be isolated from the fermentation medium using methods known in the art. For example, solids may be
25 removed from the fermentation medium by centrifugation, filtration, decantation, or the like. Then, the 1-butanol may be isolated from the fermentation medium, which has been treated to remove solids as described above, using methods such as distillation, liquid-liquid extraction, or membrane-based separation. Because 1-butanol forms a
30 low boiling point, azeotropic mixture with water, distillation can only be used to separate the mixture up to its azeotropic composition. Distillation may be used in combination with another separation method to obtain separation around the azeotrope. Methods that may be used in combination with distillation to isolate and purify 1-butanol include, but are

not limited to, decantation, liquid-liquid extraction, adsorption, and membrane-based techniques. Additionally, 1-butanol may be isolated using azeotropic distillation using an entrainer (see for example Doherty and Malone, *Conceptual Design of Distillation Systems*, McGraw Hill, New York, 2001).

The 1-butanol-water mixture forms a heterogeneous azeotrope so that distillation may be used in combination with decantation to isolate and purify the 1-butanol. In this method, the 1-butanol containing fermentation broth is distilled to near the azeotropic composition. Then, the azeotropic mixture is condensed, and the 1-butanol is separated from the fermentation medium by decantation. The decanted aqueous phase may be returned to the first distillation column as reflux. The 1-butanol-rich decanted organic phase may be further purified by distillation in a second distillation column.

The 1-butanol may also be isolated from the fermentation medium using liquid-liquid extraction in combination with distillation. In this method, the 1-butanol is extracted from the fermentation broth using liquid-liquid extraction with a suitable solvent. The 1-butanol-containing organic phase is then distilled to separate the 1-butanol from the solvent.

Distillation in combination with adsorption may also be used to isolate 1-butanol from the fermentation medium. In this method, the fermentation broth containing the 1-butanol is distilled to near the azeotropic composition and then the remaining water is removed by use of an adsorbent, such as molecular sieves (Aden et al. *Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover*, Report NREL/TP-510-32438, National Renewable Energy Laboratory, June 2002).

Additionally, distillation in combination with pervaporation may be used to isolate and purify the 1-butanol from the fermentation medium. In this method, the fermentation broth containing the 1-butanol is distilled to near the azeotropic composition, and then the remaining water is removed by pervaporation through a hydrophilic membrane (Guo et al., *J. Membr. Sci.* 245, 199-210 (2004)).

EXAMPLES

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

10 GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, (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 used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), BD Diagnostic Systems (Sparks, MD), Life Technologies (Rockville, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified. Microbial samples were obtained from The American Type Culture Collection (ATCC; Manassas, VA) unless otherwise noted.

The oligonucleotide primers used for cloning in the following Examples are given in Table 4. The primers used to sequence or screen the cloned genes are given in Table 5. All the oligonucleotide primers were synthesized by Sigma-Genosys (Woodlands, TX).

5

Table 4
Oligonucleotide Cloning Primers

Gene	Primer Name	Sequence	SEQ ID NO:	Description
<i>crt</i>	N3	CACCATGGAACATAACAAT GTCATCCTTG	17	<i>crt</i> forward
<i>crt</i>	N4	CCTCCTATCTATTTTTGAA GCCTTC	18	<i>crt</i> reverse
<i>hbd</i>	N5	CACCATGAAAAAGGTATGT GTTATAGGT	19	<i>hbd</i> forward
<i>hbd</i>	N6	CATTTGATAATGGGGATTCTTGT	20	<i>hbd</i> reverse
<i>thlA</i>	N7	CACCATGAAAGAAGTTGTA ATAGCTAGTGC	21	<i>thlA</i> forward
<i>thlA</i>	N8	CTAGCACTTTTCTAGCAAT ATTGCTG	22	<i>thlA</i> reverse
<i>bdhA</i>	N9	CACCATGCTAAGTTTTGAT TATTCAATAC	23	<i>bdhA</i> forward
<i>bdhA</i>	N10	TTAATAAGATTTTTTAAATA TCTCA	24	<i>bdhA</i> reverse
<i>bdhB</i>	N11	CACCATGGTTGATTTTCGAA TATTCAATACC	25	<i>bdhB</i> forward
<i>bdhB</i>	N12	TTACACAGATTTTTTTGAATA TTTGT	26	<i>bdhB</i> reverse
<i>thlB</i>	N15	CACCATGAGAGATGTAGTA ATAGTAAGTGCTG	27	<i>thlB</i> forward
<i>thlB</i>	N16	CCGCAATTGTATCCATATT GAACC	28	<i>thlB</i> reverse
CAC0462	N17	CACCATGATAGTAAAAGCA AAGTTTG	29	CAC0462 forward
CAC0462	N21	GCTTAAAGCTTAAACCGC TTCTGGCG	30	CAC0462 reverse
<i>ald</i>	N27F1	CACCATGAATAAAGACACA CTAATACC	31	<i>ald</i> forward

<i>ald</i>	N28R1	GCCAGACCATCTTTGAAAA TGCGC	32	<i>ald</i> reverse
<i>thlA</i>	N44	CATGCATGCAAAGGAGGTT AGTAGAATGAAAGAAG	33	<i>thlA</i> forward
<i>thlA</i>	N45	GTCCTGCAGGGCGCGCCC AATACTTTCTAGCACTTTTC	34	<i>thlA</i> reverse
<i>hbd</i>	N42	CATGTCGACAAAGGAGGT CTGTTTAATGAAAAAGGTA TG	35	<i>hbd</i> forward
<i>hbd</i>	N43	GTCGCATGCCTTGTAACCT TATTTTGAA	36	<i>hbd</i> reverse
CAC0462	N68	CATAGATCTGGATCCAAAG GAGGGTGAGGAAATGATA GTAAAAG	37	CAC0462 forward
CAC0462	N69	CATGTCGACGTGCAGCCTT TTTAAGGTTCT	38	CAC0462 reverse
<i>crt</i>	N38	CATGAATTCACGCGTAAAG GAGGTATTAGTCATGGAAC	39	<i>crt</i> forward
<i>crt</i>	N39	GTCGGATCCCTTACCTCCT ATCTATTTTTG	40	<i>crt</i> reverse
<i>ald</i>	N58	CATGCCCGGGGGTCACCA AAGGAGGAATAGTTCATGA ATAAA	41	<i>ald</i> forward
<i>ald</i>	N59	CATGGTTAACAAGAAGTTA GCCGGCAAGTACA	42	<i>ald</i> reverse
<i>bdhB</i>	N64	CATGGTTAACAAGGAGG GGTTAAAATGGTTGATTTC GAAT	43	<i>bdhB</i> forward
<i>bdhB</i>	N65	CATGGCATGCGTTTAAACG TAGGTTTACACAGATTTT	44	<i>bdhB</i> reverse
--	BenF	ACTTTCTTTCGCCTGTTTC AC	73	--
--	BenMA R	CATGAAGCTTGGCGCGCC GGGACGCGTTTTTGAAAAT AATGAAAAT	74	--
--	BenBPR	CATGAAGCTTGTTTAACT CGGTGACCTTGAAAATAAT GAAAATTATATTGTTTTGA AAATAATGAAAATTATATT	75	--

		G		
EgTER(opt)	N85	CATAGATCTGGATCCAAAG GAGGGTGAGGAAATGGCG ATGTTTACG	80	Egter forward
EgTER(opt)	N86	GTCGACTTACTGCTGGGC GG	81	Egter reverse
Ptrc- ald(opt)	T- Ptrc(Bsp EI)	TTCCGTA CTTC CGGACGAC TGCACGGTGCACCAATGC TTCTG	87	Ptrc forward
Ptrc- ald(opt)	B- aldopt(S cal)	CGGATCTTAAGTACTTTAA C CCGCCAGCACACAGCGGC GCTGG	88	ald reverse
ald	AF BamHI	CATTGGATCCATGAATAAA GACACACTAATACCTACAA C	93	ald forward
ald	AR Aat2	CATGACGTCACTAGTGTTA ACAAGAAGTTAGCCGGCA AG	94	ald reverse
EgTER	Forward 1 (E)	CATGTTAACAAAGGAGGAA AGATCTATGGCGATGTTTA CGACCACCGCAA	95	EgTER SOE forward
EgTER	Bottom Reverse 1 (E)	CCCCTCCTTTGGCGCGCC T TACTGCTGGGCGGCGCT CGGCAGA	96	EgTER SOE reverse
bdh	Top Forward 2 (B)	GCCCAGCAGTAAGGCGCG CCAAAGGAGGGGT TAAAAT GGTTGATTTCGAAT	97	bdh SOE forward
bdh	Reverse 2 (B)	GTCGACGTCATACTAGTTT ACACAGATTTTTTTGAATATT TGT	98	bdh SOE reverse
---	Pamy/la cO F	CATTGTACAGAATTCGAGC TCTCGAGGCCCGCACAT ACGAAAAGAC	99	Pamy forward
---	Pamy/la cO R	CATTGTACAGTTTAAACAT AGGTCACCCTCATTTTCGT AGGAATTGTTATCC	100	Pamy reverse
---	Spac F	CATCTCGAGTAATTCTACA CAGCCCAGTCC	101	Pspac forward

---	Spac R	CATGTTTAAACGGTGACCC AAGCTGGGGATCCGCGG	102	Pspac reverse
thl	Top TF	CATTGGTCACCATTCCCGG GCATGCAAAGGAGGTTAG TAGAATG	103	thl SOE Forward
thl	Bot TR	CCTTTACGCGACCGGTACT AGTCAAGTCGACAGGGCG CGCCCAATACTTTC	104	thl SOE reverse
crt	Top CF	CGCGCCCTGTCGACTTGA CTAGTACCGGTCGCGTAAA GGAGGTATTAGTCATGGAA C	105	crt SOE forward
crt	Bot CR	CATCGTTTAACTTGGATC CAGATCCCTTACCTCCTAT	106	crt SOE reverse
ERG10- ERG10t	OT731	AAAGCTGGAGCTCCACCG CGGTGGCGGCCGCTCTAG AAGTTTTCAAAGCAGAGTT TCGTTTGAATATTTTACCA	164	ERG10- ERG10t forward
ERG10- ERG10t	OT732	TTCAATATGCATGCCTCAG AACGTTTACATTGTATCGA CTGCCAGAACCC	165	ERG10- ERG10t reverse
GAL1- GAL10	OT733	GCAGTCGATACAATGTAAA CGTTCTGAGGCATGCATAT TGAATTTTCAAAAATTCTTA CTTTTTTTTTGGATGGACG CA	166	GAL1- GAL10 forward
GAL1- GAL10	OT734	ACCTGCACCTATAACACAT ACCTTTTCCATGGTAGTTT TTTCTCCTTGACGTAAAG TATAGAGGTATATTA	167	GAL1- GAL10 reverse
hbd	OT735	AAAAACTACCATGGAAAAG GTATGTGTTATAGGTGCAG GTACTATGGGTTTCAGGAAT TGC	168	hbd forward
hbd	OT736	GTAAAAAAAAGAAGGCCGT ATAGGCCTTATTTTGAATA ATCGTAGAAACCTTTTCCT GATTTTCTTCCAAG	169	hbd reverse
GAL1t	OT737	ACGATTATTCAAATAAGG CCTATACGGCCTTCTTTTT	170	GAL1t forward

		TTTACTTTGTTCAGAACAA CTTCTCATTCTTTCTACTC ATAA		
GAL1t	OT738	GAATTGGGTACCGGGCCC CCCCTCGAGGTCGACCGA TGCCTCATAAACTTCGGTA GTTATATTACTCTGAGAT	171	GAL1t reverse
thIA	OT797	AAAGTAAGAATTTTGA ATTCAATATGCATGCAAGA AGTTGTAATAGCTAGTGCA GTAAGAAC	172	thIA forward
thIA	OT798	GAAAAAGATCATGAGAAAA TCGCAGAACGTAAGGCGC GCCTCAGCACTTTTCTAGC AATATTGCTGTTCTTG	173	thIA reverse
CUP1	OT806	CTCGAAAATAGGGCGCGC CCCCATTACCGACATTTGG GCGC	174	CUP1 forward
CUP1	OT807	ACTGCACTAGCTATTACAA CTTCTTGCATGCGTGATGA TTGATTGATTGATTGTA	175	CUP1 reverse
GPD promoter	OT808	TCGGTAATGGGGGCGCGC CCTATTTTCGAGGACCTTG TCACCTTGA	176	GPD promoter forward
GPD promoter	OT809	TTTCGAATAAACACACATA AACAAACACCCCATGGAAA AGGTATGTGTTATAGGTGC AGG	177	GPD promoter reverse
FBA1 promoter	OT799	TACCGGGCCCCCCTCGA GGTCGACGGCGCGCCACT GGTAGAGAGCGACTTTGTA TGCCCCA	178	FBA1 promoter forward
FBA1 promoter	OT761	CTTGGCCTTCACTAGCATG CTGAATATGTATTACTTGG TTATGGTTATATATGACAAA AG	179	FBA1 promoter reverse
GPM1 promoter	OT803	CCCTCACTAAAGGGAACAA AAGCTGGAGCTCGATATC GGCGCGCCACATGCAGT GATGCACGCGCGA	180	GPM1 promoter forward

GPM1 promoter	OT804	AAGGATGACATTGTTTAGT TCCATGGTTGTAATATGTG TGTTTGTTTGG	181	GPM1 promoter reverse
crt	OT785	CACACATATTACAACCATG GAACTAAACAATGTCATCC TTGAAAAGGAAGG	182	Crt forward
crt	OT786	ATCATTCAATTGGCCATTCA GGCCTTATCTATTTTTGAA GCCTTCAATTTTTCTTTTCT CTATG	183	Crt reverse
GPM1t terminator	OT787	CAAAAATAGATAAGGCCTG AATGGCCAATGAATGATTT GATGATTTCTTTTTCCCTC CATTTTTTC	184	GPM1t terminator forward
GPM1t terminator	OT805	GAATTGGGTACCGGGCCC CCCCTCGAGGTCGACTTAT AGTATTATATTTTCTGATTT GGTTATAGCAAGCAGCGTT T	185	GPM1t terminator reverse
GPD promoter	OT800	GGGAACAAAAGCTGGAGC TCCACCGCGGTGGGGCGC GCCCTATTTTCGAGGACCT TGTCACCTTGAGCC	190	GPD promoter forward
GPD promoter	OT758	TTAAGGTATCTTTATCCAT GGTGTTTGTTTATGTGTGT TTATTCGAAACT	191	GPD promoter reverse
GPD terminator	OT754	TTGGGTACCGGGCCCCC CTCGAGGTCGACTGGCCA TTAATCTTTCCCATAT	192	GPD terminator forward
GPD terminator	OT755	TGTGTCCTAGCAGGTTAGG GCCTGCAGGGCCGTGAAT TTACTTTAAATCTTG	193	GPD terminator reverse
FBA1 promoter	OT760	CGAAAATAGGGCGCGCCA CTGGTAGAGAGCGACTTT GTATGCCCCAATTG	194	FBA1 promoter forward
FBA1 promoter	OT792	CCCTTGACGAACTTGGCCT TCACTAGCATGCTGAATAT GTATTACTTGGTTATGGTT ATATATGACAAAAG	195	FBA1 promoter reverse

FBA1 terminator	OT791	CCCTTGACGAACTTGGCCT TCACTAGCATGCTGAATAT GTATTACTTGGTTATGGTT ATATATGACAAAAG	196	FBA1 terminator forward
FBA1 terminator	OT765	GGAACAAAAGCTGGAGCT CCACCGCGGTGGTTTAAC GTATAGACTTCTAATATATT TCTCCATACTTGGTATT	197	FBA1 terminator reverse
ldhL	LDH EcoRV F	GACGTCATGACCACCCGC CGATCCCTTTT	198	ldhL forward
ldhL	LDH AatIIR	GATATCCAACACCAGCGAC CGACGTATTAC	199	ldhL reverse
Cm	Cm F	ATTTAAATCTCGAGTAGAG GATCCCAACAAACGAAAAT TGGATAAAG	200	Cm forward
Cm	Cm R	ACGCGTTATTATAAAAGCC AGTCATTAGG	201	Cm reverse
P11	P11 F	TCGAGAGCGCTATAGTTGT TGACAGAATGGACATACTA TGATATATTGTTGCTATAG CGCCC	202	P11 promoter forward
P11	P11 R	GGGCGCTATAGCAACAATA TATCATAGTATGTCCATTCT GTCAACAACCTATAGCGCTC	203	P11 promoter reverse
PldhL	PldhL F	GAGCTCGTCGACAAACCA ACATTATGACGTGTCTGGG C	204	ldhL promoter forward
PldhL	PldhL R	GGATCCTACCATGTTTGTG CAAATAAGTG	205	ldhL promoter reverse
PnisA	F-PnisA (EcoRV)	TTCAGTGATATCGACATAC TTGAATGACCTAGTC	206	PnisA forward
PnisA	R- PnisA(P mel BamHI)	TTGATTAGTTTAAACTGTA GGATCCTTTGAGTGCCTCC TTATAATTTA	207	PnisA reverse

Table 5
Sequencing and PCR Screening Primers

Name	Sequence	Gene-specific	SEQ ID NO:
M13 Forward	GTAAAACGACGGCCAGT	TOPO vector	45
M13 Reverse	AACAGCTATGACCATG	TOPO vector	46
N7SeqF1	GCAGGAGATGCTGACGTAATAA	<i>thlA</i>	47
N7SeqR1	CCAACCTGCTTTTTCAATAGCTGC	<i>thlA</i>	48
N15SeqF1	CAGAGATGGGGTCAAAGAATG	<i>thlB</i>	49
N16SeqR1	GTGGTTTTATTCCGAGAGCG	<i>thlB</i>	50
N5SeqF2	GGTCTATACTTAGAATCTCC	<i>hbd</i>	51
N6SeqR2	CGGAACAGTTGACCTTAATATGGC	<i>hbd</i>	52
N22SeqF1	GCCTCATCTGGGTTTGGTCTTG	CAC0426	53
N22SeqF2	CGCCTAGGAGAAAGGACTATAAAA CTGG	CAC0426	54
N22SeqF3	CAGAGTTATAGGTGGTAGAGCC	CAC0426	55
N23SeqR1	CCATCCCGCTGTTCTTCTTCT	CAC0426	56
N23SeqR2	CCAATCCTCTCCACCCATTACC	CAC0426	57
N23SeqR3	CGTCCATCCTTAATCTTCCC	CAC0426	58
N31SeqF2	CCAACCTATGGAATCCCTAGATGC	<i>ald</i>	59
N31SeqF3	GCATAGTCTGCGAAGTAAATGC	<i>ald</i>	60
N31SeqF4	GGATCTACTGGTGAAGGCATAACC	<i>ald</i>	61
N32SeqR1	GTTAGCCGGCAAGTACACATC	<i>ald</i>	72
N32SeqR2	GGCATCATGAGTTCTGTCATGAC	<i>ald</i>	62
N32SeqR3	GCCTTCAATGATACTCTTACCAGCC	<i>ald</i>	63
N32SeqR4	GCATTTCCAGCAGCTATCATGC	<i>ald</i>	64
N32SeqR5	CCTTCCCATATGTGTTTCTTCC	<i>ald</i>	65
N11SeqF1	GTTGAAGTAGTACTAGCTATAG	<i>bdhB</i>	66
N11 SeqF2	GACATAACACACGGCGTAGGGC	<i>bdhB</i>	67
N12SeqR1	TAAGTGTACACTCCAATTAGTG	<i>bdhB</i>	68
N12SeqR2	GCCATCTAACACAATATCCCATGG	<i>bdhB</i>	69
N9SeqF1	GCGATACATGGGACATGGTTAAAG	<i>bdhA</i>	70
N10SeqR1	TGCACTTAACTCGTGTTCCATA	<i>bdhA</i>	71
T7Primer	TAATACGACTCACTATAGGG	pET23 vector	82

Trc99aF	TTGACAATTAATCATCCGGC	p Trc99a vector	83
N5SeqF4	GGTCAACTGTTCCGGAAATTC	hbd	84
T-ald(BamHI)	TGATCTGGATCCAAGAAGGAGCCC TTCACCATGAATAAAGACACAC	ald	85
B-ald(EgTER)	CATCGCCATTTCTCACCCTCCTTT TTAGCCGGCAAGTACACATCTTCTT TGTC	ald	86
N3SeqF1	CCATCATACCATACTGACCC	crt	107
N3SeqF2	GCTACTGGAGCATTGCTCAC	crt	108
N3SeqF3	CCATTAACAGCTGCTATTACAGGC	crt	109
N4SeqR3	GGTCTCGGAATAACACCTGG	crt	110
N5SeqF3	CAAGCTTCATAACAGGAGCTGG	hbd	111
N7SeqR2	ATCCCACAATCCGTCAGTGATC	thIA	112
N31SeqF1	CTGAGATAAGAAAGGCCGCA	ald	113
N62SeqF2	CAACCCTGGGCGTGTTTCTG	EgTER	114
N62SeqF3	GTGGCGAAGATTGGGAAGT	EgTER	115
N62SeqF4	GGGAAATGGCAGAAGATGTTTCAGC	EgTER	116
N63SeqR1	CGGTCTGATAACCTGCAAAATCGC	EgTER	117
N63SeqR2	CACCAGCGCTTTGGCAACAAC	EgTER	118
N63SeqR3	GAACGTGCATACAGACCTGCTTC	EgTER	119
N63SeqR4	CGGCTGAATAACTTTTGCGG	EgTER	120
Pamy SeqF2	GCCTTTGATGACTGATGATTTGGC	pFP988 vector	121
Pamy SeqF	TCTCCGGTAAACATTACGGCAAAC	pFP988 vector	122
Pamy SeqR	CGGTCAGATGCAATTCGACATGTG	pFP988 vector	123
SpacF Seq	GAAGTGGTCAAGACCTCACT	Pspac promoter	124
sacB Up	CGGGTTTGTTACTGATAAAGCAGG	sacB	125
sacB Dn	CGGTTAGCCATTTGCCTGCTTTTA	sacB	126
HT R	ACAAAGATCTCCATGGACGCGT	pHT01 vector	127
Scr1	CCTTTCTTTGTGAATCGG	csc	160
Scr2	AGAAACAGGGTGTGATCC	csc	161
Scr3	AGTGATCATCACCTGTTGCC	csc	162
Scr4	AGCACGGCGAGAGTCGACGG	csc	163

Methods for Determining 1-Butanol Concentration in Culture Media

The concentration of 1-butanol 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

5 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 H_2SO_4 as the mobile phase with a flow rate of 0.5 mL/min and a column temperature of 50 °C. 1-Butanol had a retention time of 52.8 min under

10 the conditions used. 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

15 head pressure; injector split was 1:25 at 200 °C; oven temperature was 45 °C for 1 min, 45 to 220 °C at 10 °C/min, and 220 °C for 5 min; and FID detection was employed at 240 °C with 26 mL/min helium makeup gas. The retention time of 1-butanol was 5.4 min. A similar GC method using a

20 Varian CP-WAX 58(FFAP) CB column (25 m x 0.25 mm id X 0.2 μm film thickness, Varian, Inc., Palo Alto, CA) was also used.

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), “ μL ” means microliter(s), “mL” means milliliter(s), “L” means liter(s), “mm” means millimeter(s), “nm”

25 means nanometers, “mM” means millimolar, “M” means molar, “mmol” means millimole(s), “ μmole ” means micromole(s), “g” means gram(s), “ μg ” means microgram(s) and “ng” means nanogram(s), “PCR” means polymerase chain reaction, “OD” means optical density, “OD₆₀₀” means the optical density measured at a wavelength of 600 nm, OD₅₅₀” means

30 the optical density measured at a wavelength of 550 nm, “kDa” means kilodaltons, “g” means the gravitation constant, “rpm” means revolutions per minute, “bp” means base pair(s), “kbp” means kilobase pair(s), “% w/v” means weight/volume percent, % v/v” means volume/volume percent, “nt”

means not tested, "HPLC" means high performance liquid chromatography, and "GC" means gas chromatography.

EXAMPLE 1

Increased tolerance of *Lactobacillus plantarum* PN0512 to 1-butanol, iso-butanol and 2-butanol at decreased growth temperatures

Tolerance levels of bacterial strain *Lactobacillus plantarum* PN0512 (ATCC # PTA-7727) were determined at 25 °C, 30 °C and 37 °C as follows. The strain was cultured in S30L medium (i.e., 10 mM ammonium sulfate, 5 mM potassium phosphate buffer, pH 7.0, 50 mM MOPS, pH 7.0, 2 mM MgCl₂, 0.7 mM CaCl₂, 50 µM MnCl₂, 1 µM FeCl₃, 1 µM ZnCl₂, 1.72 µM CuCl₂, 2.53 µM CoCl₂, 2.42 µM Na₂MoO₄, 2 µM thiamine hydrochloride, 10 mM glucose, and 0.2% yeast extract). An overnight culture in the absence of any test compound was started in 15 mL of the S30L medium in a 150 mL flask, with incubation at 37 °C in a shaking water bath. The next morning, the overnight culture was diluted into three 500 mL flasks containing 150 mL of fresh medium to an initial OD₆₀₀ of about 0.08. Each flask was incubated in a shaking water bath, one each at 25 °C, 30 °C and 37 °C. Each large culture was allowed to acclimate at the test temperature for at least 0.5 h. After the acclimation period, each large culture was split into flasks in the absence (control) and in the presence of various amounts of 1-butanol, isobutanol or 2-butanol, as listed in Tables 6, 7, and 8, respectively. Growth was followed by measuring OD₆₀₀ for six hours after addition of the compounds. The results are summarized in Tables 6, 7, and 8 below.

Table 6. Growth of *L. plantarum* PN0512 in the presence of 1-butanol at different temperatures

Concentration 1-butanol (% w/v)	37 °C	30 °C	25 °C
0.0	+	+	+
1.0	+	nt	nt

1.2	+	nt	nt
1.4	+	nt	nt
1.5	+	+	+
1.6	+	nt	nt
1.8	+	nt	nt
2.0	+	+	+
2.1	+	nt	nt
2.2	+	nt	nt
2.3	+	nt	nt
2.4	-	+	+
2.5	-	nt	nt
2.7	-	+	nt
2.9	-	-	+
3.1	-	-	+
3.2	nt	-	-
3.3	nt	nt	-
3.4	nt	-	-

Table 7. Growth of *L. plantarum* PN0512 in the presence of isobutanol at different temperatures

Concentration isobutanol (% w/v)	37 °C	30 °C	25 °C
0.0	+	+	+
0.5	+	nt	nt
1.0	+	nt	nt
1.5	+	+	+
1.6	+	nt	nt
1.8	+	nt	nt
2.0	+	+	+
2.1	+	nt	nt

2.3	+	nt	nt
2.4	+	+	+
2.5	+	nt	nt
2.7	+	+	+
2.9	+	+	+
3.1	+	+	+
3.3	nt	-	+
3.4	-	nt	nt
3.5	nt	nt	+
3.6	nt	nt	-
3.8	-	nt	nt
4.3	-	nt	nt

Table 8. Growth of *L. plantarum* PN0512 in the presence of 2-butanol at different temperatures

Concentration 2-butanol (% w/v)	37 °C	30 °C	25 °C
0.0	+	+	+
1.8	+	nt	nt
2.1	+	nt	nt
2.5	+	nt	nt
2.9	+	+	+
3.1	+	nt	nt
3.5	+	nt	nt
3.6	+	nt	nt
3.8	+	+	+
4.0	nt	+	nt
4.3	+	+	+
4.5	-	+	nt
4.7	-	+	+
4.9	nt	-	+

5.2	-	nt	+
5.6	-	nt	-
6.0	-	nt	nt
6.4	-	nt	nt
7.3	-	nt	nt

“+” = growth observed as an increase in OD₆₀₀.

“-” = no growth observed, i.e. no change in OD₆₀₀.

5 All three butanols showed a similar effect of temperature on growth inhibition of *L. plantarum* PN0512. The concentration that resulted in full growth inhibition was greater at 25 °C than at 37 °C. In the case of 1-butanol, growth was observed at 37 °C in 2.3% 1-butanol, but not 2.4%. However, at 30 °C growth was observed in 2.7%, but not 2.9%, and at 25
10 °C growth was observed even in 3.1% 1-butanol. Thus, the concentration of 1-butanol that completely inhibited growth increased as growth temperature decreased. Likewise, in the case of isobutanol, growth was observed in 3.5% at 25 °C while growth was observed in 3.1% at 30 °C and 37 °C, but not in 3.3% or 3.4%. Similarly, in the case of 2-butanol
15 growth was observed at 37 °C in 4.3%, but not in 4.5%; at 30 °C in 4.7%, but not in 4.9%; and at 25 °C in 5.2 %. Thus the tolerance of *L. plantarum* PN0512 to butanols increased with decreased growth temperature.

EXAMPLE 2

Increased tolerance of *Escherichia coli* to 1-butanol at decreased 20 exposure temperature

The effect of growth and exposure temperature on survival of *Escherichia coli* in the presence of 1-butanol was tested using stationary phase cultures in a rich medium and log phase cultures in a defined medium. For the stationary phase studies, *E. coli* strain MG1655 (ATCC #
25 700926) was grown overnight in LB medium (Teknova, Half Moon Bay, CA) with shaking at 250 rpm at 42 °C, 29 °C or 28 °C. Survival of 1-butanol shock was tested at exposure temperatures of 0 °C, 28 °C or 42 °C. The 1-butanol exposure at 28 °C or 42 °C was started immediately after removing the overnight cultures from the growth incubators. The 1-

butanol exposure at 0 °C was done after allowing the overnight cultures to cool on ice for about 15 min. A series of solutions of 1-butanol at different concentrations in LB medium was made and 90 µL aliquots were put in microfuge tubes. To these were added 10 µL of the overnight cultures and the tubes were immediately placed in shaking incubators at 42 °C or 28 °C or left on ice for 30 min. To stop the effect of 1-butanol on the cultures, a 10⁻² dilution was done by placing 2 µL of the treated culture into 198 µL of LB medium in wells of a microplate. Then, 5 µL of the undiluted treated cultures were spotted on LB agar plates. Subsequent 10-fold serial dilutions of 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ of the exposed cultures were done by serial pipetting of 20 µL, starting with the 10⁻² dilution cultures, into 180 µL of LB medium in the microplate, using a multi-channel pipette. Prior to each transfer, the cultures were mixed by pipetting up and down six times. Each dilution (5 µL) was spotted onto an LB plate using a multi-channel pipette and allowed to soak into the plate. The plates were inverted and incubated overnight at 37 °C. The number of colonies for each dilution was counted and the % growth inhibition was calculated by comparison with a control culture that had not been exposed to 1-butanol. Survival of 0% was recorded when no colonies in the spots of the undiluted or any of the serial dilutions were observed. The results are shown in Table 9.

Table 9. Survival of stationary phase *E. coli* in 1-butanol at 42 °C, 28 °C, or 0 °C

	Grown at 42 °C	Grown at 29 °C	Grown at 42 °C	Grown at 28 °C	Grown at 42 °C	Grown at 29 °C
1- Butanol % (w/v)	% survival after 30 min exposure at 42 °C		% survival after 30 min exposure at 28 °C		% survival after 30 min exposure at 0 °C	
1.0	100	100	100	100	100	100
1.5	0.1	0.1	100	100	100	100
2.0	0	0.1	100	100	100	100
2.5	0	0	100	100	100	100

3.0	0	0	100	100	100	100
3.5	0	0	3	10	100	100
4.0	0	0	0.0004	0.0003	100	100
5.0	nt	nt	nt	nt	1	1
6.0	nt	nt	nt	nt	0	0.001
7.0	nt	nt	nt	nt	0	0

A similar study was done with log-phase cultures of *E. coli* grown in a defined medium. *E. coli* strain MG1655 was allowed to grow overnight in MOPS 0.2% glucose medium (Teknova, Half Moon Bay, CA) at 42 °C or 28 °C. The following day, the cultures were diluted into fresh medium and allowed to grow at the same temperature until in the log phase of growth. The OD₆₀₀ was 0.74 for the 28 °C culture and was 0.72 for the 42 °C culture. Both of these log phase cultures were exposed to 1-butanol at 42 °C, 28 °C and 0 °C as follows. A series of solutions of 1-butanol at different concentrations in MOPS 0.2% glucose medium was made and 90 μL aliquots were put in microfuge tubes. To these were added 10 μL of the log phase cultures and the tubes were immediately placed in shaking incubators at 42 °C or 28 °C or left on ice for 30 min. To stop the effect of 1-butanol on the cultures, a 10⁻² dilution was done by placing 2 μL of the treated culture into 198 μL of LB medium in wells of a microplate. Then 5 μL of the undiluted treated cultures were spotted on LB agar plates. Subsequent 10-fold serial dilutions of 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ of the exposed cultures were done by serial pipetting of 20 μL, starting with the 10⁻² dilution cultures, into 180 μL of LB medium in the microplate, using a multi-channel pipette. Prior to each transfer, the cultures were mixed by pipetting up and down six times. Each dilution (5 μL) was spotted onto an LB plate using a multi-channel pipette and allowed to soak into the plate. The plates were inverted and incubated overnight at 37 °C. The number of colonies for each dilution was counted and the % growth inhibition was calculated by comparison with a control culture that had not been exposed to 1-butanol. Survival of 0% was recorded when no colonies in the spots

of the undiluted or any of the serial dilutions were observed. The results are shown in Table 10.

Table 10. Survival of log-phase *E. coli* in 1-butanol at 42 °C, 28 °C, or 0

5 °C

	Grown at 42 °C	Grown at 28 °C	Grown at 42 °C	Grown at 28 °C	Grown at 42 °C	Grown at 29 °C
1- Butanol % (w/v)	% survival after 30 min exposure at 42 °C		% survival after 30 min exposure at 28 °C		% survival after 30 min exposure at 0 °C	
1.0	100	100	nt	nt	nt	nt
1.5	0	0	100	100	nt	nt
2.0	0	0	100	100	nt	nt
2.5	0	0	0.1	50	100	100
3.0	0	0	0	0	100	100
3.5	0	0	0.01	0	100	100
4.0	0	0	0.001	0	100	100
4.5	nt	nt	0	0	100	100
5.0	nt	nt	nt	nt	10	50
6.0	nt	nt	nt	nt	1	1

For both the stationary phase and log-phase cultures of *E. coli* MG1655, the growth temperature had very little, if any, effect on the survival of a 1-butanol shock. However, the exposure temperature had a major effect on the survival of *E. coli* to 1-butanol shock. As can be seen from the data in Tables 9 and 10, the tolerance of *E. coli* MG1655 to 1-butanol increased with decreasing exposure temperature.

EXAMPLE 3

15 Increased tolerance of *Escherichia coli* to 2-butanone at decreased exposure temperature

The effect of exposure temperature on survival of *Escherichia coli* in the presence of 2-butanone (also referred to herein as methyl ethyl

ketone or MEK) was tested as follows. *E. coli* strain BW25113 (The Coli Genetic Stock Center (CGSC), Yale University; # 7636) was grown overnight in LB medium (Teknova, Half Moon Bay, CA) with shaking at 250 rpm at 37 °C. Survival of MEK shock was tested at exposure

5 temperatures of 28 °C or 37 °C. A series of solutions of MEK at different concentrations in LB medium was made and 90 µL aliquots were put in microfuge tubes. To these were added 10 µL of the overnight culture and the tubes were immediately placed in shaking incubators at 37 °C or 28 °C for 30 min. To stop the effect of MEK on the cultures, a 10⁻² dilution was

10 done by placing 2 µL of the MEK treated culture into 198 µL of LB medium in wells of a microplate. Then 5 µL of the undiluted treated cultures were spotted on LB agar plates. Subsequent 10-fold serial dilutions of 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ of the exposed cultures were done by serial pipetting of 20 µL, starting with the 10⁻² dilution cultures, into 180 µL of LB medium in

15 the microplate, using a multi-channel pipette. Prior to each transfer, the cultures were mixed by pipetting up and down six times. Each dilution (5 µL) was spotted onto LB plates using a multi-channel pipette and allowed to soak into the plate. The plates were inverted and incubated overnight at 37 °C. The number of colonies for each dilution was counted and the %

20 growth inhibition was calculated by comparison with a control culture that had not been exposed to MEK. Survival of 0% was recorded when no colonies in the spots of the undiluted or any of the serial dilutions were observed. The results, given as the average of duplicate experiments, are shown in Table 11.

25

Table 11. Survival of *E. coli* in MEK at 37 °C and 28 °C

MEK % w/v	% Survival at 37 °C	% Survival at 28 °C
0	100	100
4	100	100
6	0	100
8	0	0.002

Reducing the exposure temperature from 37 °C to 28 °C dramatically improved survival of *E. coli* to MEK treatment. At 37 °C there was full survival at 4% w/v and no survival at 6% w/v, while at 28 °C there was full survival at 6% w/v. Thus, the tolerance of *E. coli* to MEK increased with decreasing exposure temperature.

EXAMPLE 4

Increased tolerance of *E. coli* and *L. plantarum* PN0512 to 1-Butanol at decreased exposure temperature

This Example demonstrates that the toxic effects of 1-butanol and 2-butanol on various microbial cells was reduced at lower temperatures. This was demonstrated by incubating *E. coli* (strain MG1655; ATCC # 700926), and *L. plantarum* (strain PN0512; ATCC # PTA-7727) with either 1-butanol or 2-butanol at different temperatures and then determining the fraction of the cells that survived the treatment at the different temperatures.

Using overnight cultures or cells from plates, 30 mL cultures of the microorganisms to be tested were started in the following culture media:

E. coli - Miller's LB medium (Teknova, Half Moon Bay, CA):

L. plantarum PN0512 - *Lactobacilli* MRS Broth (BD Diagnostic Systems, Sparks, MD).

The *E. coli* and *L. plantarum* cultures were grown at 37 °C aerobically with shaking until the cultures were in log phase and the OD₆₀₀ was between 0.6 and 0.8. A 50 µL aliquot of each culture was removed for a time zero sample. The remainder of the cultures was divided into six 5 mL portions and placed in six small incubation flasks or tubes. Different amounts of 1-butanol or 2-butanol were added to the six flasks to bring the concentration to predetermined values, as listed in the tables below. The flasks or tubes were incubated at a desired temperature, aerobically without shaking for 1 h. After the incubation with one of the butanols, 2 µL from each of the flasks (and in addition 2 µL of the time zero sample of the culture before exposure to one of the butanols) were pipetted into the "head" wells of a 96 well (8 X 12) microtiter plate, each containing 198 µL of LB medium to give a 10⁻² dilution of the culture. Subsequently, 10⁻³,

10^{-4} , 10^{-5} , and 10^{-6} serial dilutions of the cultures were prepared as follows. The 10^{-3} dilution was prepared by pipetting 20 μ L of the sample from the head well into the 180 μ L LB medium in the next well using a multi-channel pipette. This procedure was repeated 3 more times on
 5 successive wells to prepare the 10^{-4} , 10^{-5} , and 10^{-6} dilutions. After each liquid transfer, the solution in the well was mixed by pipetting it up and down 10 times with the multi-channel pipetor. A 5 μ L aliquot of each dilution was spotted onto an LB plate using a multi-channel pipette starting with the 10^{-6} dilution, then the 10^{-5} , and so on working from more to less
 10 dilute without a change of tips. The spots were allowed to soak into the agar by leaving the lid of the plate slightly open for 15 to 30 min in a sterile transfer hood. The plates were covered, inverted, and incubated overnight at 37 °C. The following day, the number of colonies in the spots were counted from the different dilutions. The number of living cells/mL in each
 15 of the original culture solutions from which the 2 μ L was withdrawn was calculated and compared to the number of cells in the control untreated culture to determine the % of the cells surviving.

The results of experiments in which *E. coli* cells were treated with 1-butanol at temperatures of 0, 30, and 37 °C are shown Table 12.

20

Table 12. Percentage of *E. coli* cells surviving in 1-butanol at 0, 30 and 37 °C

1-butanol % v/v	% Survival at 0 °C	% Survival at 30 °C	% Survival at 37 °C
0	100	100	100
1	nt	100	72
1.5	nt	100	20
2	nt	100	0
2.5	100	23	0
3	100	0	0
3.5	100	0	nt
4	100	nt	nt
4.5	100	nt	nt

The concentration at which 1-butanol kills *E. coli* cells was affected by the treatment temperature. At 0 °C, concentrations of 1-butanol as high as 4.5% v/v had no toxic effect on *E. coli* cells during a one hour

5 treatment. At 30 °C, *E. coli* cells were killed when treated with 3% v/v 1-butanol for one hour. At 37 °C, *E. coli* cells were killed when treated with 2% v/v 1-butanol for one hour.

The results of experiments in which *L. plantarum* PN0512 cells were treated with 1-butanol at temperatures of 0, 23, and 37 °C for one
10 hour are shown Table 13.

Table 13. Percentage of *L. plantarum* PN0512 cells surviving in 1-butanol at 0, 23 and 37 °C

1-butanol % v/v	% Survival at 0 °C	% Survival at 23 °C	% Survival at 37 °C
0	100	100	100
1	nt	nt	80
1.5	nt	nt	58
2	nt	100	29
2.5	nt	100	8
3	100	82	0
3.5	100	0	0
4	100	0	nt
4.5	100	0	nt
5	0	nt	nt
5.5	0	nt	nt

15

The concentration at which 1-butanol kills *L. plantarum* PN0512 cells was affected by the treatment temperature. At 0 °C, concentrations of 1-butanol as high as 4.5% v/v had no toxic effect on *L. plantarum* PN0512 cells during a one hour treatment. At 23 °C, *L. plantarum*

20 PN0512 cells were killed when treated with 3.5% v/v 1-butanol for one

hour. At 37 °C, *L. plantarum* PN0512 cells were killed when treated with 2.5 % v/v 1-butanol for one hour.

EXAMPLE 5

Cloning and Expression of Acetyl-CoA Acetyltransferase

5 The purpose of this Example was to express the enzyme acetyl-CoA acetyltransferase, also referred to herein as acetoacetyl-CoA thiolase, in *E. coli*. The acetoacetyl-CoA thiolase gene *thlA* was cloned from *C. acetobutylicum* (ATCC 824) and expressed in *E. coli*. The *thlA* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA
10 using PCR, resulting in a 1.2 kbp product.

 The genomic DNA from *Clostridium acetobutylicum* (ATCC 824) was either purchased from the American Type Culture Collection (ATCC, Manassas, VA) or was isolated from *Clostridium acetobutylicum* (ATCC 824) cultures, as described below.

15 Genomic DNA from *Clostridium acetobutylicum* (ATCC 824) was prepared from anaerobically grown cultures. The *Clostridium* strain was grown in 10 mL of *Clostridial* growth medium (Lopez-Contreras et al., *Appl. Env. Microbiol.* 69(2), 869-877 (2003)) in stoppered and crimped 100 mL Bellco serum bottles (Bellco Glass Inc., Vineland, NJ) in an anaerobic
20 chamber at 30 °C. The inoculum was a single colony from a 2X YTG plate (Kishii, et al., *Antimicrobial Agents & Chemotherapy*, 47(1), 77-81 (2003)) grown in a 2.5 L MGC AnaeroPak™ (Mitsubishi Gas Chemical America Inc, New York, NY) at 37 °C.

 Genomic DNA was prepared using the Gentra Puregene® kit
25 (Gentra Systems, Inc., Minneapolis, MN; catalog no. D-6000A) with modifications to the manufacturer's instruction (Wong et al., *Current Microbiology*, 32, 349-356 (1996)). The *thlA* gene was amplified from *Clostridium acetobutylicum* (ATCC 824) genomic DNA by PCR using primers N7 and N8 (see Table 4), given as SEQ ID NOs:21 and 22,
30 respectively. Other PCR amplification reagents were supplied in manufacturers' kits for example, Kod HiFi DNA Polymerase (Novagen Inc., Madison, WI; catalog no. 71805-3) and used according to the

manufacturer's protocol. Amplification was carried out in a DNA Thermocycler GeneAmp 9700 (PE Applied Biosystems, Foster city, CA).

For expression studies the Gateway cloning technology (Invitrogen Corp., Carlsbad, CA) was used. The entry vector pENTR/SD/D-TOPO
5 allowed 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 (CACG) immediately adjacent to the translational start codon to allow directional cloning into pENTR/SD/D-TOPO (Invitrogen) to generate
10 the plasmid pENTRSDD-TOPOthIA. The pENTR construct was transformed into *E. coli* Top10 (Invitrogen) cells and plated according to manufacturer's recommendations. Transformants were grown overnight and plasmid DNA was prepared using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA; catalog no. 27106) according to manufacturer's
15 recommendations. Clones were submitted for sequencing with M13 Forward and Reverse primers (see Table 5), given as SEQ ID NOs:45 and 46, respectively, to confirm that the genes inserted in the correct orientation and to confirm the sequence. Additional sequencing primers, N7SeqF1 and N7SeqR1 (see Table 5), given as SEQ ID NOs:47 and 48,
20 respectively, were needed to completely sequence the PCR product. The nucleotide sequence of the open reading frame (ORF) for this gene and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:1 and SEQ ID NO:2, respectively.

To create an expression clone, the *thIA* gene was transferred to the
25 pDEST 14 vector by recombination to generate pDEST14thIA. The pDEST14thIA vector was transformed into BL21-AI cells. Transformants were inoculated into LB medium supplemented with 50 µg/mL of ampicillin and grown overnight. An aliquot of the overnight culture was used to inoculate 50 mL of LB supplemented with 50 µg/mL of ampicillin. The
30 culture was incubated at 37 °C with shaking until the OD₆₀₀ reached 0.6-0.8. The culture was split into two 25-mL cultures and arabinose was added to one of the flasks to a final concentration of 0.2% by weight. The negative control flask was not induced with arabinose. The flasks were incubated for 4 h at 37 °C with shaking. Cells were harvested by

centrifugation and the cell pellets were resuspended in 50 mM MOPS, pH 7.0 buffer. The cells were disrupted either by sonication or by passage through a French Pressure Cell. The whole cell lysate was centrifuged yielding the supernatant or cell free extract and the pellet or the insoluble fraction. An aliquot of each fraction (whole cell lysate, cell free extract and insoluble fraction) was resuspended in SDS (MES) loading buffer (Invitrogen), heated to 85 °C for 10 min and subjected to SDS-PAGE analysis (NuPAGE 4-12% Bis-Tris Gel, catalog no. NP0322Box, Invitrogen). A protein of the expected molecular weight of about 41 kDa, as deduced from the nucleic acid sequence, was present in the induced culture but not in the uninduced control.

Acetoacetyl-CoA thiolase activity in the cell free extracts was measured as degradation of a Mg^{2+} -acetoacetyl-CoA complex by monitoring the decrease in absorbance at 303 nm. Standard assay conditions were 100 mM Tris-HCl pH 8.0, 1 mM DTT (dithiothreitol) and 10 mM $MgCl_2$. The cocktail was equilibrated for 5 min at 37 °C; then the cell-free extract was added. The reaction was initiated with the addition of 0.05 mM acetoacetyl-CoA plus 0.2 mM CoA. Protein concentration was measured by either the Bradford method or by the Bicinchoninic Kit (Sigma, catalog no. BCA-1). Bovine serum albumin (Bio-Rad, Hercules, CA) was used as the standard in both cases. In one typical assay, the specific activity of the ThIA protein in the induced culture was determined to be 16.0 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to 0.27 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture.

25

EXAMPLE 6

Cloning and Expression of Acetyl-CoA Acetyltransferase

The purpose of this Example was to express the enzyme acetyl-CoA acetyltransferase, also referred to herein as acetoacetyl-CoA thiolase, in *E. coli*. The acetoacetyl-CoA thiolase gene *thlB* was cloned from *C. acetobutylicum* (ATCC 824) and expressed in *E. coli*. The *thlB* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA using PCR.

The *thlB* gene was cloned and expressed in the same manner as the *thlA* gene described in Example 5. The *C. acetobutylicum* (ATCC 824) genomic DNA was amplified by PCR using primers N15 and N16 (see Table 4), given as SEQ ID NOs:27 and 28, respectively, creating a 1.2 kbp product. The forward primer incorporated four bases (CCAC) immediately adjacent to the translational start codon to allow directional cloning into pENTR/SD/D-TOPO (Invitrogen) to generate the plasmid pENTRSDD-TOPOthlB. Clones were submitted for sequencing with M13 Forward and Reverse primers, given as SEQ ID NOs:45 and 46 respectively, to confirm that the genes inserted in the correct orientation and to confirm the sequence. Additional sequencing primers, N15SeqF1 and N16SeqR1 (see Table 5), given as SEQ ID NOs:49 and 50 respectively, were needed to completely sequence the PCR product. The nucleotide sequence of the open reading frame (ORF) for this gene and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:3 and SEQ ID NO:4, respectively.

To create an expression clone, the *thlB* gene was transferred to the pDEST 14 (Invitrogen) vector by recombination to generate pDEST14thlB. The pDEST14thlB vector was transformed into BL21-AI cells and expression from the T7 promoter was induced by addition of arabinose. A protein of the expected molecular weight of about 42 kDa, as deduced from the nucleic acid sequence, was present in the induced culture, but not in the uninduced control. Enzyme assays were performed as described in Example 5. In one typical assay, the specific activity of the ThlB protein in the induced culture was determined to be $14.9 \mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to $0.28 \mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture.

EXAMPLE 7

Cloning and Expression of 3-Hydroxybutyryl-CoA Dehydrogenase

The purpose of this Example was to clone the *hbd* gene from *C. acetobutylicum* (ATCC 824) and express it in *E. coli*. The *hbd* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA using PCR.

The *hbd* gene was cloned and expressed using the method described in Example 5. The *hbd* gene was amplified from *C.*

acetobutylicum (ATCC 824) genomic DNA by PCR using primers N5 and N6 (see Table 4) given as SEQ ID NOs:19 and 20 respectively, creating a 881 bp product. The forward primer incorporated four bases (CACC) immediately adjacent to the translational start codon to allow directional
5 cloning into pENTR/SD/D-TOPO (Invitrogen) to generate the plasmid pENTRSDD-TOPOhbd. Clones were submitted for sequencing with M13 Forward and Reverse primers, given as SEQ ID NOs:45 and 46 respectively, to confirm that the genes inserted in the correct orientation and to confirm the sequence. Additional sequencing primers, N5SeqF2
10 and N6SeqR2 (see Table 5), given as SEQ ID NOs:51 and 52 respectively, were needed to completely sequence the PCR product. The nucleotide sequence of the open reading frame (ORF) for this gene and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:5 and SEQ ID NO:6, respectively.

15 To create an expression clone, the *hbd* gene was transferred to the pDEST 14 (Invitrogen) vector by recombination to generate pDEST14hbd. The pDEST14hbd vector was transformed into BL21-AI cells and expression from the T7 promoter was induced by addition of arabinose, as described in Example 5. A protein of the expected molecular weight of
20 about 31 kDa, as deduced from the nucleic acid sequence, was present in the induced culture, but was absent in the uninduced control.

Hydroxybutyryl-CoA dehydrogenase activity was determined by measuring the rate of oxidation of NADH as measured by the decrease in absorbance at 340 nm. A standard assay mixture contained 50 mM
25 MOPS, pH 7.0, 1 mM DTT and 0.2 mM NADH. The cocktail was equilibrated for 5 min at 37 °C and then the cell free extract was added. Reactions were initiated by addition of the substrate, 0.1 mM acetoacetyl-CoA. In one typical assay, the specific activity of the BHBD protein in the induced culture was determined to be 57.4 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to
30 0.885 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture.

EXAMPLE 8

Cloning and Expression of Crotonase

The purpose of this Example was to clone the *crt* gene from

C. acetobutylicum (ATCC 824) and express it in *E. coli*. The *crt* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA using PCR.

The *crt* gene was cloned and expressed using the method described in Example 5. The *crt* gene was amplified from *C.*

5 *acetobutylicum* (ATCC 824) genomic DNA by PCR using primers N3 and N4 (see Table 4), given as SEQ ID NOs:17 and 18, respectively, creating a 794 bp product. The forward primer incorporated four bases (CACC) immediately adjacent to the translational start codon to allow directional cloning into pENTR/SD/D-TOPO (Invitrogen) to generate the plasmid
10 pENTRSDD-TOPOcrt. Clones were submitted for sequencing with M13 Forward and Reverse primers, given as SEQ ID NOs:45 and 46 respectively, to confirm that the genes inserted in the correct orientation and to confirm the sequence. The nucleotide sequence of the open reading frame (ORF) for this gene and its predicted amino acid sequence
15 are given as SEQ ID NO:7 and SEQ ID NO:8, respectively.

To create an expression clone, the *crt* gene was transferred to the pDEST 14 (Invitrogen) vector by recombination to generate pDEST14crt. The pDEST14crt vector was transformed into BL21-AI cells and expression from the T7 promoter was induced by addition of arabinose, as
20 described in Example 5. A protein of the expected molecular weight of about 28 kDa, as deduced from the nucleic acid sequence, was present in much greater amounts in the induced culture than in the uninduced control.

Crotonase activity was assayed as described by Stern (*Methods Enzymol.* 1, 559-566, (1954)). In one typical assay, the specific activity of the crotonase protein in the induced culture was determined to be 444 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to 47 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture.

EXAMPLE 9

Cloning and Expression of Butyryl-CoA Dehydrogenase

30 The purpose of this Example was to express the enzyme butyryl-CoA dehydrogenase, also referred to herein as trans-2-Enoyl-CoA reductase, in *E. coli*. The CAC0462 gene, a putative trans-2-enoyl-CoA reductase homolog, was cloned from *C. acetobutylicum* (ATCC 824) and

expressed in *E. coli*. The CAC0462 gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA using PCR.

The CAC0462 gene was cloned and expressed using the method described in Example 5. The CAC0462 gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA by PCR using primers N17 and N21 (see Table 4), given as SEQ ID NOs:29 and 30, respectively, creating a 1.3 kbp product. The forward primer incorporated four bases (CACC) immediately adjacent to the translational start codon to allow directional cloning into pENTR/SD/D-TOPO (Invitrogen) to generate the plasmid pENTRSDD-TOPOCAC0462. Clones were submitted for sequencing with M13 Forward and Reverse primers, given as SEQ ID NO:45 and 46 respectively, to confirm that the genes inserted in the correct orientation and to confirm the sequence. Additional sequencing primers, N22SeqF1 (SEQ ID NO:53), N22SeqF2 (SEQ ID NO:54), N22SeqF3 (SEQ ID NO:55), N23SeqR1 (SEQ ID NO:56), N23SeqR2 (SEQ ID NO:57), and N23SeqR3 (SEQ ID NO:58) (see Table 5) were needed to completely sequence the PCR product. The nucleotide sequence of the open reading frame (ORF) for this gene and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:9 and SEQ ID NO:10, respectively.

To create an expression clone, the CAC0462 gene was transferred to the pDEST 14 (Invitrogen) vector by recombination to generate pDEST14CAC0462. The pDEST14CA0462 vector was transformed into BL21-AI cells and expression from the T7 promoter was induced by addition of arabinose, as described in Example 5. Analysis by SDS-PAGE showed no overexpressed protein of the expected molecular weight in the negative control or in the induced culture. The *C. acetobutylicum* CAC0462 gene used many rare *E. coli* codons. To circumvent problems with codon usage the pRARE plasmid (Novagen) was transformed into BL21-AI cells harboring the pDEST14CAC0462 vector. Expression studies with arabinose induction were repeated with cultures carrying the pRARE vector. A protein of the expected molecular weight of about 46 kDa was present in the induced culture but not in the uninduced control.

Trans-2-enoyl-CoA reductase activity was assayed as described by Hoffmeister et al. (*J. Biol. Chem.* 280, 4329-4338 (2005)). In one typical

assay, the specific activity of the TER CAC0462 protein in the induced culture was determined to be $0.694 \mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to $0.0128 \mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture.

EXAMPLE 10

5 Cloning and Expression of Butyraldehyde Dehydrogenase (Acetylating)

The purpose of this Example was to clone the *ald* gene from *C. beijerinckii* (ATCC 35702) and express it in *E. coli*. The *ald* gene was amplified from *C. beijerinckii* (ATCC 35702) genomic DNA using PCR.

10 The *ald* gene was cloned and expressed using the method described in Example 5. The *ald* gene was amplified from *C. beijerinckii* (ATCC 35702) genomic DNA (prepared from anaerobically grown cultures, as described in Example 5) by PCR using primers N27 F1 and N28 R1 (see Table 4), given as SEQ ID NOs:31 and 32 respectively, creating a 1.6 kbp product. The forward primer incorporated four bases (CACC)
15 immediately adjacent to the translational start codon to allow directional cloning into pENTR/SD/D-TOPO (Invitrogen) to generate the plasmid pENTRSDD-TOPOald. Clones were submitted for sequencing with M13 Forward and Reverse primers, given as SEQ ID NOs:45 and 46 respectively, to confirm that the genes inserted in the correct orientation
20 and to confirm the sequence. Additional sequencing primers, N31SeqF2 (SEQ ID NO:59), N31SeqF3 (SEQ ID NO:60), N31SeqF4 (SEQ ID NO:61), N32SeqR1 (SEQ ID NO:72), N31SeqR2 (SEQ ID NO:62), N31SeqR3 (SEQ ID NO:63), N31SeqR4 (SEQ ID NO:64), and N31SeqR5 (SEQ ID NO:65) (see Table 5) were needed to completely sequence the
25 PCR product. The nucleotide sequence of the open reading frame (ORF) for this gene and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:11 and SEQ ID NO:12, respectively.

30 To create an expression clone, the *ald* gene was transferred to the pDEST 14 (Invitrogen) vector by recombination to generate pDEST14ald. The pDEST14ald vector was transformed into BL21-AI cells and expression from the T7 promoter was induced by addition of arabinose, as described in Example 5. A protein of the expected molecular weight of about 51 kDa, as deduced from the nucleic acid sequence, was present in the induced culture, but not in the uninduced control.

Acylating aldehyde dehydrogenase activity was determined by monitoring the formation of NADH, as measured by the increase in absorbance at 340 nm, as described by Husemann et al. (*Appl. Microbiol. Biotechnol.* 31:435-444 (1989)). In one typical assay, the specific activity
5 of the Ald protein in the induced culture was determined to be 0.106 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to 0.01 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture

EXAMPLE 11

Cloning and Expression of Butanol Dehydrogenase

The purpose of this Example was to clone the *bdhB* gene from *C.*
10 *acetobutylicum* (ATCC 824) and express it in *E. coli*. The *bdhB* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA using PCR.

The *bdhB* gene was cloned and expressed using the method described in Example 5. The *bdhB* gene was amplified from *C.*
acetobutylicum (ATCC 824) genomic DNA by PCR using primers N11 and
15 N12 (see Table 4), given as SEQ ID NOs:25 and 26, respectively, creating a 1.2 kbp product. The forward primer incorporated four bases (CACC) immediately adjacent to the translational start codon to allow directional cloning into pENTR/SD/D-TOPO (Invitrogen) to generate the plasmid pENTRSDD-TOPObdhB. The translational start codon was also
20 changed from "GTG" to "ATG" by the primer sequence. Clones were submitted for sequencing with M13 Forward and Reverse primers, given as SEQ ID NOs:45 and 46 respectively, to confirm that the genes inserted in the correct orientation and to confirm the sequence. Additional sequencing primers, N11SeqF1 (SEQ ID NO:66), N11SeqF2 (SEQ ID
25 NO:67), N12SeqR1 (SEQ ID NO:68), and N12SeqR2 (SEQ ID NO:69), (see Table 5) were needed to completely sequence the PCR product. The nucleotide sequence of the open reading frame (ORF) for this gene and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:13 and SEQ ID NO:14, respectively.

30 To create an expression clone, the *bdhB* gene was transferred to the pDEST 14 (Invitrogen) vector by recombination to generate pDEST14bdhB. The pDEST14bdhB vector was transformed into BL21-AI cells and expression from the T7 promoter was induced by addition of arabinose, as described in Example 5. A protein of the expected molecular

weight of about 43 kDa, as deduced from the nucleic acid sequence, was present in the induced culture, but not in the uninduced control.

Butanol dehydrogenase activity was determined from the rate of oxidation of NADH as measured by the decrease in absorbance at 340 nm as described by Husemann and Papoutsakis, *supra*. In one typical assay, the specific activity of the BdhB protein in the induced culture was determined to be $0.169 \mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to $0.022 \mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture.

EXAMPLE 12

Cloning and Expression of Butanol Dehydrogenase

The purpose of this Example was to clone the *bdhA* gene from *C. acetobutylicum* 824 and express it in *E. coli*. The *bdhA* gene was amplified from *C. acetobutylicum* 824 genomic DNA using PCR.

The *bdhA* gene was cloned and expressed using the method described in Example 5. The *bdhA* gene was amplified from *C. acetobutylicum* 824 genomic DNA by PCR using primers N9 and N10 (see Table 4), given as SEQ ID NOs:23 and 24, respectively, creating a 1.2 kbp product. The forward primer incorporated four bases (CACC) immediately adjacent to the translational start codon to allow directional cloning into pENTR/SD/D-TOPO (Invitrogen) to generate the plasmid pENTRSDD-TOPObdhA. Clones, given as SEQ ID NOs:45 and 46 respectively, to confirm that the genes inserted in the correct orientation and to confirm the sequence. Additional sequencing primers, N9SeqF1 (SEQ ID NO:70) and N10SeqR1 (SEQ ID NO:71), (see Table 5) were needed to completely sequence the PCR product. The nucleotide sequence of the open reading frame (ORF) for this gene and the predicted amino acid sequence of the enzyme are given as SEQ ID NO:15 and SEQ ID NO:16, respectively.

To create an expression clone, the *bdhA* gene was transferred to the pDEST 14 (Invitrogen) vector by recombination to generate pDEST14bdhA. The pDEST14bdhA vector was transformed into BL21-AI cells and expression from the T7 promoter was induced by addition of arabinose, as described in Example 5. A protein of the expected molecular weight of about 43 kDa, as deduced from the nucleic acid

sequence, was present in the induced culture, but not in the uninduced control.

Butanol dehydrogenase activity was determined from the rate of oxidation of NADH as measured by the decrease in absorbance at 340 nm, as described by Husemann and Papoutsakis, *supra*. In one typical assay, the specific activity of the BdhA protein in the induced culture was determined to be $0.102 \mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to $0.028 \mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture

EXAMPLE 13

10 Construction of a Transformation Vector for the Genes in the 1-Butanol Biosynthetic Pathway - Lower Pathway

To construct a transformation vector comprising the genes encoding the six steps in the 1-butanol biosynthetic pathway, the genes encoding the 6 steps in the pathway were divided into two operons. The upper pathway comprises the first four steps catalyzed by acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase. The lower pathway comprises the last two steps, catalyzed by butyraldehyde dehydrogenase and butanol dehydrogenase.

20 The purpose of this Example was to construct the lower pathway operon. Construction of the upper pathway operon is described in Example 14.

The individual genes were amplified by PCR with primers that incorporated restriction sites for later cloning and the forward primers contained an optimized *E. coli* ribosome binding site (AAAGGAGG). PCR products were TOPO cloned into the pCR 4Blunt-TOPO vector and transformed into *E. coli* Top10 cells (Invitrogen). Plasmid DNA was prepared from the TOPO clones and the sequence of the genes was verified. Restriction enzymes and T4 DNA ligase (New England Biolabs, Beverly, MA) were used according to manufacturer's recommendations. For cloning experiments, restriction fragments were purified by gel electrophoresis using QIAquick Gel Extraction kit (Qiagen).

After confirmation of the sequence, the genes were subcloned into a modified pUC19 vector as a cloning platform. The pUC19 vector was

modified by a HindIII/SapI digest, creating pUC19dHS. The digest removed the lac promoter adjacent to the MCS (multiple cloning site), preventing transcription of the operons in the vector.

The *ald* gene was amplified from *C. beijerinckii* ATCC 35702 genomic DNA by PCR using primers N58 and N59 (see Table 4), given as SEQ ID NOs:41 and 42, respectively, creating a 1.5 kbp product. The forward primer incorporated the restriction sites Aval and BstEII and a RBS (ribosome binding site). The reverse primer incorporated the HpaI restriction site. The PCR product was cloned into pCRBlunt II-TOPO creating pCRBluntII-ald. Plasmid DNA was prepared from the TOPO clones and the sequence of the genes verified with primers M13 Forward (SEQ ID NO:45), M13 Reverse (SEQ ID NO:46), N31SeqF2 (SEQ ID NO:59), N31SeqF3 (SEQ ID NO:60), N31SeqF4 (SEQ ID NO:61), N32SeqR1 (SEQ ID NO:72), N31SeqR2 (SEQ ID NO:62), N31SeqR3 (SEQ ID NO:63), N31SeqR4 (SEQ ID NO:64), and N31SeqR5 (SEQ ID NO:65) (see Table 5).

The *bdhB* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA by PCR using primers N64 and N65 (see Table 4), given as SEQ ID NOs:43 and 44, respectively, creating a 1.2 kbp product. The forward primer incorporated an HpaI restriction site and a RBS. The reverse primer incorporated a PmeI and a SphI restriction site. The PCR product was cloned into pCRBlunt II-TOPO creating pCRBluntII-bdhB. Plasmid DNA was prepared from the TOPO clones and the sequence of the genes verified with primers M13 Forward (SEQ ID NO:45), M13 Reverse (SEQ ID NO:46), N11SeqF1 (SEQ ID NO:66), N11SeqF2 (SEQ ID NO:67), N12SeqR1 (SEQ ID NO:68), and N12SeqR2 (SEQ ID NO:69) (see Table 5).

To construct the lower pathway operon, a 1.2 kbp SphI and HpaI fragment from pCRBluntII-bdhB, a 1.4 kbp HpaI and SphI fragment from pCRBluntII-ald, and the large fragment from a Aval and SphI digest of pUC19dHS were ligated together. The three-way ligation created pUC19dHS-ald-bdhB.

The pUC19dHS-ald-bdhB vector was digested with BstEII and PmeI releasing a 2.6 kbp fragment that was cloned into pBenBP, an *E.*

coli-Bacillus subtilis shuttle vector. Plasmid pBenBP was created by modification of the pBE93 vector, which is described by Nagarajan, WO 93/24631 (Example 4). The *Bacillus amyloliquefaciens* neutral protease promoter (NPR), signal sequence and the *phoA* gene were removed from pBE93 with a NcoI/HindIII digest. The NPR promoter was PCR amplified from pBE93 by primers BenF and BenBPR, given by SEQ ID NOs:73 and 75, respectively. Primer BenBPR incorporated BstEII, PmeI and HindIII sites downstream of the promoter. The PCR product was digested with NcoI and HindIII and the fragment was cloned into the corresponding sites in the vector pBE93 to create pBenBP. The lower operon fragment was subcloned into the BstEII and PmeI sites in pBenBP creating pBen-ald-bdhB.

Assays for butyraldehyde dehydrogenase and butanol dehydrogenase activity were conducted on crude extracts using the methods described above. Both enzyme activities were demonstrated at levels above the control strain that contained an empty vector.

EXAMPLE 14 (Prophetic)

Construction of a Transformation Vector for the Genes in the 1-Butanol Biosynthetic Pathway - Upper Pathway

The purpose of this prophetic Example is to describe how to assemble the upper pathway operon. The general approach is the same as described in Example 13.

The *thlA* gene is amplified from *C. acetobutylicum* (ATCC 824) genomic DNA by PCR using primer pair N44 and N45 (see Table 4), given as SEQ ID NOs:33 and 34, respectively, creating a 1.2 kbp product. The forward primer incorporates a SphI restriction site and a ribosome binding site (RBS). The reverse primer incorporates AscI and PstI restriction sites. The PCR product is cloned into pCR4 Blunt-TOPO creating pCR4 Blunt-TOPO-thlA. Plasmid DNA is prepared from the TOPO clones and the sequence of the genes is verified with primers M13 Forward (SEQ ID NO:45), M13 Reverse (SEQ ID NO:46), N7SeqF1 (SEQ ID NO:47), and N7SeqR1 (SEQ ID NO:48) (see Table 5).

The *hbd* gene is amplified from *C. acetobutylicum* (ATCC 824) genomic DNA by PCR using primer pair N42 and N43 (see Table 4) given

as SEQ ID NOs:35 and 36, respectively, creating a 0.9 kbp product. The forward primer incorporates a Sall restriction site and a RBS. The reverse primer incorporates a SphI restriction site. The PCR product is cloned into pCR4 Blunt-TOPO creating pCR4 Blunt-TOPO-hbd. Plasmid DNA is
 5 prepared from the TOPO clones and the sequence of the genes verified with primers M13 Forward (SEQ ID NO:45), M13 Reverse (SEQ ID NO:46), N5SeqF2 (SEQ ID NO:51), and N6SeqR2 (SEQ ID NO:52) (see Table 5).

The CAC0462 gene is codon optimized for expression in *E. coli* as
 10 primary host and *B. subtilis* as a secondary host. The new gene called CaTER, given as SEQ ID NO:76, is synthesized by Genscript Corp (Piscataway, NJ). The gene CaTER is cloned in the pUC57 vector as a BamHI-Sall fragment and includes a RBS, producing plasmid pUC57-CaTER.

15 The *crt* gene is amplified from *C. acetobutylicum* (ATCC 824) genomic DNA by PCR using primer pair N38 and N39 (see Table 4), given as SEQ ID NOs:39 and 40, respectively, creating a 834 bp product. The forward primer incorporates EcoRI and MluI restriction sites and a RBS. The reverse primer incorporates a BamHI restriction site. The PCR
 20 product is cloned into pCR4 Blunt-TOPO creating pCR4 Blunt-TOPO-crt. Plasmid DNA is prepared from the TOPO clones and the sequence of the genes is verified with primers M13 Forward (SEQ ID NO:45) and M13 Reverse(SEQ ID NO:46) (see Table 5).

After confirmation of the sequence, the genes are subcloned into a
 25 modified pUC19 vector as a cloning platform. The pUC19 vector was modified by a SphI/SapI digest, creating pUC19dSS. The digest removed the lac promoter adjacent to the MCS, preventing transcription of the operons in the vector.

To construct the upper pathway operon pCR4 Blunt-TOPO-crt is
 30 digested with EcoRI and BamHI releasing a 0.8 kbp *crt* fragment. The pUC19dSS vector is also digested with EcoRI and BamHI releasing a 2.0 kbp vector fragment. The *crt* fragment and the vector fragment are ligated together using T4 DNA ligase (New England Biolabs) to form pUC19dSS-crt. The CaTER gene is inserted into pCU19dSS-crt by digesting pUC57-

CaTER with BamHI and Sall, releasing a 1.2 kbp CaTER fragment. The pUC19dSS-crt is digested with BamHI and Sall and the large vector fragment is ligated with the CaTER fragment, creating pUC19dSS-crt-CaTER. To complete the operon a 884 bp Sall and SphI fragment from pCR4 Blunt-TOPO-hbd, a 1.2 kb SphI and PstI *thlA* fragment from pCR4 Blunt-TOPO-*thlA* and the large fragment from a Sall and PstI digest of pUC19dSS-crt-CaTER are ligated. The product of the 3-way ligation is pUC19dSS-crt-CaTER-hbd-*thlA*.

The pUC19dSS-crt-CaTER-hbd-*thlA* vector is digested with MluI and AscI releasing a 4.1 kbp fragment that is cloned into a derivative of pBE93 (Caimi, WO2004/018645, pp. 39-40) an *E. coli*-*B. subtilis* shuttle vector, referred to as pBenMA. Plasmid pBenMA was created by modification of the pBE93 vector. The *Bacillus amyloliquefaciens* neutral protease promoter (NPR), signal sequence and the *phoA* gene are removed from pBE93 with a NcoI/HindIII digest. The NPR promoter is PCR amplified from pBE93 by primers BenF and BenMAR, given as SEQ ID NOS:73 and 74, respectively. Primer BenMAR incorporates MluI, AscI, and HindIII sites downstream of the promoter. The PCR product was digested with NcoI and HindIII and the fragment is cloned into the corresponding sites in the vector pBE93, creating pBenMA. The upper operon fragment is subcloned into the MluI and AscI sites in pBenMA creating pBen-crt-hbd-CaTER-*thlA*.

EXAMPLE 15 (Prophetic)

Expression of the 1-Butanol Biosynthetic Pathway in *E. coli*

The purpose of this prophetic Example is to describe how to express the 1-butanol biosynthetic pathway in *E. coli*.

The plasmids pBen-crt-hbd-CaTER-*thlA* and pBen-ald-bdhB, constructed as described in Examples 14 and 13, respectively, are transformed into *E. coli* NM522 (ATCC 47000) and expression of the genes in each operon is monitored by SDS-PAGE analysis, enzyme assay and Western analysis. For Westerns, antibodies are raised to synthetic peptides by Sigma-Genosys (The Woodlands, TX). After confirmation of expression of all the genes, pBen-ald-bdhB is digested with EcoRI and PmeI to release the NPR promoter-ald-bdhB fragment. The EcoRI digest

of the fragment is blunt ended using the Klenow fragment of DNA polymerase (New England Biolabs, catalog no. M0210S). The plasmid pBen-crt-hbd-CaTER-thlA is digested with PvuII to create a linearized blunt ended vector fragment. The vector and NPR-ald-bdhB fragment are
 5 ligated, creating p1B1 O.1 and p1B1 O.2, containing the complete 1-butanol biosynthetic pathway with the NPR promoter-ald-bdhB fragment in opposite orientations. The plasmids p1B1 O.1 and p1B1 O.2 are transformed into *E. coli* NM522 and expression of the genes are monitored as previously described.

10 *E. coli* strain NM522/ p1B1 O.1 or NM522/ p1B1 O.1 is inoculated into a 250 mL shake flask containing 50 mL of medium and shaken at 250 rpm and 35 °C. The 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,
 15 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; 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 to 24 h, 1-butanol is detected by HPLC or GC analysis,
 20 as described in the General Methods section.

EXAMPLE 16 (Prophetic)

Expression of the 1-Butanol Biosynthetic Pathway in *Bacillus subtilis*

The purpose of this prophetic Example is to describe how to express the 1-butanol biosynthetic pathway in *Bacillus subtilis*. The same
 25 approach as described in Example 15 is used.

The upper and lower operons constructed as described in Examples 14 and 13, respectively, are used. The plasmids p1B1 O.1 and p1B1 O.2 are transformed into *Bacillus subtilis* BE1010 (*J. Bacteriol.* 173:2278-2282 (1991)) and expression of the genes in each operon is
 30 monitored as previously described .

B. subtilis strain BE1010/p1B1 O.1 or BE1010/ p1B1 O.2 is inoculated into a 250 mL shake flask containing 50 mL of medium and shaken at 250 rpm and 35 °C for 18 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 15), 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 titrated to pH 7.0 with KOH. After 18 to 24 h,
 5 1-butanol is detected by HPLC or GC analysis, as described in the General Methods section.

EXAMPLE 17

Production of 1-Butanol from Glucose using Recombinant *E. coli*.

This Example describes the production of 1-butanol in *E. coli*.

10 Expression of the genes encoding the 6 steps of the 1-butanol biosynthetic pathway was divided into three operons. The upper pathway comprised the first four steps encoded by *thlA*, *hbd*, *crt* and EgTER in one operon. The next step, encoded by *ald*, was provided by a second operon. The last step in the pathway, encoded by *yqhD*, was provided in a third operon.
 15 1-Butanol production was demonstrated in *E. coli* strains comprising the three operons.

Unless otherwise indicated in the text, cloning primers described in this Example are referenced by their SEQ ID NO in Table 4, and sequencing and PCR screening primers are referenced by their SEQ ID
 20 NO in Table 5.

Acetyl-CoA acetyltransferase. The *thlA* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA by PCR using primer pair N44 and N45 (see Table 4), given as SEQ ID NOs:33 and 34, respectively, creating a 1.2 kbp product. The forward primer incorporated
 25 a SphI restriction site and a ribosome binding site (RBS). The reverse primer incorporated Ascl and PstI restriction sites. The PCR product was cloned into pCR4Blunt-TOPO (Invitrogen Corp., Carlsbad, CA) creating pCR4Blunt-TOPO-*thlA*. Plasmid DNA was prepared from the TOPO clones and the sequence of the genes was verified with primers M13
 30 Forward (SEQ ID NO:45), M13 Reverse (SEQ ID NO:46), N7SeqF1 (SEQ ID NO:47), and N7SeqR1 (SEQ ID NO:48) (see Table 5).

3-Hydroxybutyryl-CoA dehydrogenase. The *hbd* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA by PCR using primer pair N42 and N43 (see Table 4) given as SEQ ID NOs:35 and 36,

respectively, creating a 0.9 kbp product. The forward primer incorporated a Sall restriction site and a RBS. The reverse primer incorporated a SphI restriction site. The PCR product was cloned into pCR4Blunt-TOPO creating pCR4Blunt-TOPO-hbd. Plasmid DNA was prepared from the TOPO clones and the sequence of the genes verified with primers M13 Forward (SEQ ID NO:45), M13 Reverse (SEQ ID NO:46), N5SeqF2 (SEQ ID NO:51), and N6SeqR2 (SEQ ID NO:52) (see Table 5).

Crotonase. The *crt* gene was amplified from *C. acetobutylicum* (ATCC 824) genomic DNA by PCR using primer pair N38 and N39 (see Table 4), given as SEQ ID NOs:39 and 40, respectively, creating a 834 bp product. The forward primer incorporated EcoRI and MluI restriction sites and a RBS. The reverse primer incorporated a BamHI restriction site. The PCR product was cloned into pCR4Blunt-TOPO creating pCR4Blunt-TOPO-crt. Plasmid DNA was prepared from the TOPO clones and the sequence of the genes was verified with primers M13 Forward (SEQ ID NO:45) and M13 Reverse (SEQ ID NO:46) (see Table 5).

Butyryl-CoA Dehydrogenase (trans-2-enoyl-CoA reductase). The CAC0462 gene was synthesized for enhanced codon usage in *E. coli* as primary host and *B. subtilis* as a secondary host. The new gene (CaTER, SEQ ID NO:76) was synthesized and cloned by Genscript Corporation (Piscataway, NJ) in the pUC57 vector as a BamHI-Sall fragment and includes a RBS.

An alternative gene for butyryl-CoA dehydrogenase from *Euglena gracilis* (TER, GenBank No. Q5EU90) was synthesized for enhanced codon usage in *E. coli* and *Bacillus subtilis*. The gene was synthesized and cloned by GenScript Corporation into pUC57 creating pUC57::EgTER. Primers N85 and N86, (SEQ ID NO: 80 and 81 respectively) together with pUC57::EgTER as template DNA, provided a PCR fragment comprising 1224 bp from pUC57::EgTER DNA. The sequence of the 1224 bp is given as SEQ ID NO:77, where bp 1-1218 is the coding sequence (cds) of EgTER(opt). EgTER(opt) is a codon optimized TER gene, lacking the normal mitochondrial presequence so as to be functional in *E. coli* (Hoffmeister et al., *J. Biol. Chem.* 280:4329 (2005)).

EgTER(opt) was cloned into pCR4Blunt-TOPO and its sequence was confirmed with primers M13 Forward (SEQ ID NO:45) and M13 Reverse (SEQ ID NO:46). Additional sequencing primers N62SeqF2 (SEQ ID NO:114), N62SeqF3 (SEQ ID NO:115), N62SeqF4 (SEQ ID NO:116), N63SeqR1 (SEQ ID NO:117), N63SeqR2 (SEQ ID NO:118), N63SeqR3 (SEQ ID NO:119) and N63SeqR4 (SEQ ID NO:120) were needed to completely sequence the PCR product. The 1.2 kbp EgTER(opt) sequence was then excised with *HincII* and *PmeI* and cloned into pET23+ (Novagen) linearized with *HincII*. Orientation of the EgTER(opt) gene to the promoter was confirmed by colony PCR screening with primers T7Primer and N63SeqR2 (SEQ ID NOs:82 and 118 respectively). The resulting plasmid, pET23+::EgTER(opt), was transformed into BL21 (DE3) (Novagen) for expression studies.

Trans-2-enoyl-CoA reductase activity was assayed as described by Hoffmeister et al., *J. Biol. Chem.* 280:4329 (2005). In a typical assay, the specific activity of the EgTER(opt) protein in the induced BL21 (DE3) /pET23+::EgTER(opt) culture was determined to be $1.9 \mu\text{mol mg}^{-1} \text{min}^{-1}$ compared to $0.547 \mu\text{mol mg}^{-1} \text{min}^{-1}$ in the uninduced culture.

The EgTER(opt) gene was then cloned into the pTrc99a vector under the control of the *trc* promoter. The EgTER(opt) gene was isolated as a 1287-bp *BamHI/SalI* fragment from pET23+::EgTER(opt). The 4.2 kbp vector pTrc99a was linearized with *BamHI/SalI*. The vector and fragment were ligated creating the 5.4 kbp pTrc99a-EgTER(opt). Positive clones were confirmed by colony PCR with primers Trc99aF and N63SeqR3 (SEQ ID NOs:83 and 119 respectively) producing a 0.5 kb product.

Construction of plasmid pTrc99a-E-C-H-T comprising genes encoding acetyl-CoA acetyltransferase (*thlA*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*), and butyryl-CoA dehydrogenase (trans-2-enoyl-CoA reductase, EgTER(opt)). To initiate the construction of a four gene operon comprising the upper pathway (EgTER(opt), *crt*, *hbd* and *thlA*), pCR4Blunt-TOPO-*crt* was digested with *EcoRI* and *BamHI* releasing a 0.8 kbp *crt* fragment. The pUC19dSS vector (described in Example 14) was also digested with *EcoRI* and *BamHI* releasing a 2.0 kbp

vector fragment. The *crt* fragment and the vector fragment were ligated together using T4 DNA ligase (New England Biolabs) to form pUC19dSS-*crt*. The CaTER gene was inserted into pUC19dSS-*crt* by digesting pUC57-CaTER with BamHI and Sall, releasing a 1.2 kbp CaTER
 5 fragment. The pUC19dSS-*crt* was digested with BamHI and Sall and the large vector fragment was ligated with the CaTER fragment, creating pUC19dSS-*crt*-CaTER. To complete the operon a 884 bp Sall and SphI fragment from pCR4Blunt-TOPO-*hbd*, a 1.2 kb SphI and PstI *thlA* fragment from pCR4Blunt-TOPO-*thlA* and the large fragment from a Sall and PstI digest of pUC19dSS-*crt*-CaTER were ligated. The product of the
 10 3-way ligation was named pUC19dSS-*crt*-CaTER-*hbd*-*thlA* or pUC19dss::Operon1.

Higher butyryl-CoA dehydrogenase activity was obtained from pTrc99a-EgTER(opt) than from CaTER constructs, thus, an operon
 15 derived from pTrc99a-EgTER(opt) was constructed. The CaTER gene was removed from pUC19dss::Operon1 by digesting with BamHI/Sal I and gel purifying the 5327-bp vector fragment. The vector was treated with Klenow and religated creating pUC19dss::Operon 1 ΔCaTer. The 2934-bp *crt*-*hbd*-*thlA* (C-H-T) fragment was then isolated as a EcoRI/PstI fragment
 20 from pUC19dss::Operon 1 ΔCaTer. The C-H-T fragment was treated with Klenow to blunt the ends. The vector pTrc99a-EgTER(opt) was digested with Sall and the ends treated with Klenow. The blunt-ended vector and the blunt-ended C-H-T fragment were ligated to create pTrc99a-E-C-H-T. Colony PCR reactions were performed with primers N62SeqF4 and
 25 N5SeqF4 (SEQ ID NOs: 116 and 84 respectively) to confirm the orientation of the insert.

Construction of plasmids pBHR T7-ald and pBHR-Ptrc-ald(opt) comprising genes encoding butyraldehyde dehydrogenase (*ald* and *ald(opt)*). The PT7-ald operon was sub-cloned from pDEST14ald
 30 (Example 10) into the broad host range plasmid pBHR1 (MoBitec, Goettingen, Germany) to create pBHR1 PT7-ald. The pBHR1 plasmid is compatible with pUC19 or pBR322 plasmids so pBHR1 PT7-ald can be used in combination with pUC19 or pBR322 derivatives carrying the upper

pathway operon for 1-butanol production in *E. coli*. The pDEST14-ald plasmid was digested with Bgl II and treated with the Klenow fragment of DNA polymerase to make blunt ends. The plasmid was then digested with EcoRI and the 2,245 bp PT7-ald fragment was gel-purified. Plasmid
5 pBHR1 was digested with Scal and EcoRI and the 4,883 bp fragment was gel-purified. The PT7-ald fragment was ligated with the pBHR1 vector, creating pBHR T7-ald. Colony PCR amplification of transformants with primers T-ald(BamHI) and B-ald(EgTER) (SEQ ID NOs:85 and 86 respectively) confirmed the expected 1.4 kb PCR product. Restriction
10 mapping of pBHR T7-ald clones with EcoRI and DrdI confirmed the expected 4,757 and 2,405 bp fragments.

For butyraldehyde dehydrogenase activity assays, the plasmid pBHR T7-ald was transformed into BL21Star™(DE3) cells (Invitrogen) and expression from the T7 promoter was induced by addition of L-arabinose
15 as described in Example 5. Acylating aldehyde dehydrogenase activity was determined by monitoring the formation of NADH, as measured by the increase in absorbance at 340 nm, as described in Example 10.

An alternative DNA sequence for the *ald* gene from *Clostridium beijerinckii* ATCC 35702 was synthesized (optimizing for codon usage in
20 *E. coli* and *Bacillus subtilis*) and cloned into pUC57 by GenScript Corporation (Piscataway, NJ), creating the plasmid pUC57-ald(opt). pUC57-ald(opt) was digested with SacI and Sall to release a 1498 bp fragment comprising the condon optimized gene, *ald*(opt) and a RBS already for *E. coli*. The sequence of the 1498 bp fragment is given as
25 SEQ ID NO:78.

pTrc99a was digested with SacI and Sall giving a 4153 bp vector fragment, which was ligated with the 1498 bp *ald*(opt) fragment to create pTrc-ald(opt). Expression of the synthetic gene, *ald*(opt), is under the control of the IPTG-inducible P_{trc} promoter.

30 The P_{trc}-*ald*(opt) operon was subcloned into the broad host range plasmid pBHR1 (MoBitec) in order to be compatible with the upper pathway plasmid described above. The P_{trc}-*ald*(opt) fragment was PCR-amplified from pTrc99A::ald(opt) with T-P_{trc}(BspEI) and B-aldopt(Scal),

(SEQ ID NOs:87 and 88 respectively) incorporating BspEI and Scal restriction sites within the corresponding primers. The PCR product was digested with BspEI and Scal. The plasmid pBHR1 was digested with Scal and BspEI and the 4,883 bp fragment was gel-purified. The P_{trc}-ald(opt) fragment was ligated with the pBHR1 vector, creating pBHR-PcatP_{trc}-ald(opt). Restriction mapping of the pBHR-PcatP_{trc}-ald(opt) clones with Scal and BspEI confirmed the expected 4,883 and 1,704 bp fragments. To remove the plasmid-born cat promoter (P_{cat}) region, plasmid pBHR-PcatP_{trc}-ald(opt) was digested with BspEI and AatII and the 6,172 bp fragment was gel-purified. T-BspEIAatII and B-BspEIAatII (SEQ ID NOs:89 and 90 respectively) were mixed in a solution containing 50 mM NaCl, 10 mM Tris-HCl, and 10 mM MgCl₂ (pH7.9) to a final concentration of 100 μ M and hybridized by incubating at 75 °C for 5 min and slowly cooling to room temperature. The hybridized oligonucleotides were ligated with the 6,172 bp fragment, creating pBHR-P_{trc}-ald(opt).

Construction of *E. coli* strains expressing butanol dehydrogenase (*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 has 40% identity to the gene *adhB* in *Clostridium*, 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:91) 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 native promoter was replaced by the 1.5GI promoter (WO 2003/089621) (SEQ ID NO:92), creating strain MG1655 1.5GI-yqhD::Cm, thus, replacing the 1.6GI promoter of MG1655 1.6yqhD::Cm with the 1.5GI promoter.

A P1 lysate was prepared from MG1655 1.5GI yqhD::Cm and the cassette moved to expression strains, MG1655 (DE3), prepared from *E. coli* strain MG1655 and a lambda DE3 lysogenization kit (Invitrogen), and BL21 (DE3) (Invitrogen) creating MG1655 (DE3) 1.5GI-yqhD::Cm and BL21 (DE3) 1.5GI-yqhD::Cm, respectively.

Demonstration of 1-butanol production from recombinant *E. coli*. *E. coli* strain MG1655 (DE3) 1.5GI-yqhD::Cm was transformed with plasmids pTrc99a-E-C-H-T and pBHR T7-ald to produce the strain, MG1655 (DE3) 1.5GI-yqhD::Cm/ pTrc99a-E-C-H-T/ pBHR T7-ald. Two independent

5 isolates were initially grown in LB medium containing 50 µg/mL kanamycin and 100 µg/mL carbenicillin. The cells were used to inoculate shake flasks (approximately 175 mL total volume) containing 15, 50 and 150 mL of TM3a/glucose medium (with appropriate antibiotics) to represent high, medium and low oxygen conditions, respectively. TM3a/glucose medium

10 contained (per liter): 10 g glucose, 13.6 g KH₂PO₄, 2.0 g citric acid monohydrate, 3.0 g (NH₄)₂SO₄, 2.0 g MgSO₄·7H₂O, 0.2 g CaCl₂·2H₂O, 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 NH₄OH. The solution of trace elements contained: citric acid·H₂O (4.0 g/L), MnSO₄·

15 H₂O (3.0 g/L), NaCl (1.0 g/L), FeSO₄·7H₂O (0.10 g/L), CoCl₂·6H₂O (0.10 g/L), ZnSO₄·7H₂O (0.10 g/L), CuSO₄·5H₂O (0.010 g/L), H₃BO₃ (0.010 g/L), and Na₂MoO₄·2H₂O (0.010 g/L). The flasks were inoculated at a starting OD₆₀₀ of ≤ 0.01 units and incubated at 34 °C with shaking at 300 rpm. The flasks containing 15 and 50 mL of medium were capped with

20 vented caps; the flasks containing 150 mL, were capped with non-vented caps to minimize air exchange. IPTG was added to a final concentration of 0.04 mM; the OD₆₀₀ of the flasks at the time of addition was ≥ 0.4 units.

Approximately 15 h after induction, an aliquot of the broth was analyzed by HPLC (Shodex Sugar SH1011 column) with refractive index

25 (RI) detection and GC (Varian CP-WAX 58(FFAP) CB column, 25 m x 0.25 mm id X 0.2 µm film thickness) with flame ionization detection (FID) for 1-butanol content, as described in the General Methods section. The results of the 1-butanol determinations are given in Table 14.

Table 14

Production of 1-butanol by *E. coli* strain MG1655 (DE3) 1.5GI-yqhD::Cm/
pTrc99a-E-C-H-T/ pBHR T7-ald.

Strain	O ₂ Level	1-butanol, mM	molar yield, %
MG1655 a	high	0.11	0.2
MG1655 b	high	0.12	0.2
MG1655 a	medium	0.13	0.3
MG1655 b	medium	0.13	0.2
MG1655 a	low	0.15	0.4
MG1655 b	low	0.18	0.5

- Values were determined from HPLC analysis.

5 - Strain suffixes "a" and "b" indicate independent isolates.

The two independent isolates of MG1655 (DE3) 1.5GI-yqhD::Cm/
pTrc99a-E-C-H-T/ pBHR T7-ald were tested for 1-butanol production in an
identical manner except that the medium contained 5 g/L yeast extract.

10 The results are shown in Table 15.

Table 15

Production of 1-butanol by *E. coli* strain MG1655 (DE3) 1.5GI-yqhD::Cm/
pTrc99a-E-C-H-T/ pBHR T7-ald.

Strain	O ₂ Level	1-butanol, mM	molar yield, %
MG1655 a	high	-	-
MG1655 b	high	-	-
MG1655 a	medium	0.08	0.1
MG1655 b	medium	0.06	0.1
MG1655 a	low	0.14	0.3
MG1655 b	low	0.14	0.3

- Quantitative values were determined from HPLC analysis.

15 - " - " = not detected.

- Strain suffixes "a" and "b" indicate independent isolates.

E. coli strain BL21 (DE3) 1.5GI-yqhD::Cm was transformed with
plasmids pTrc99a-E-C-H-T and pBHR T7-ald to produce the strain, BL21
20 (DE3) 1.5GI-yqhD::Cm / pTrc99a-E-C-H-T/ pBHR T7-ald. Two

independent isolates were tested for 1-butanol production exactly as described above. The results are given in Tables 16 and 17.

Table 16

Production of 1-butanol by *E. coli* strain BL21 (DE3) 1.5GI-yqhD::Cm /
pTrc99a-E-C-H-T/ pBHR T7-ald.

5

Strain	O ₂ Level	1-butanol, mM	molar yield, %
DE a	high	+	+
DE b	high	-	-
DE a	medium	0.80	1.4
DE b	medium	0.77	1.4
DE a	low	0.06	0.2
DE b	low	0.07	0.2

- Quantitative values were determined from HPLC analysis.

- " - " indicates not detected.

" + " indicates positive, qualitative identification by GC with a lower detection limit than with HPLC.

10

- Strain suffixes "a" and "b" indicate independent isolates.

Table 17

Production of 1-butanol by *E. coli* strain BL21 (DE3) 1.5GI-yqhD::Cm /
pTrc99a-E-C-H-T/ pBHR T7-ald.

Strain	O ₂ Level	1-butanol, mM	molar yield, %
DE a	high	+	+
DE b	high	+	+
DE a	medium	0.92	1.7
DE b	medium	1.03	1.9
DE a	low	+	+
DE b	low	+	+

15

- Quantitative values were determined from HPLC analysis.

- " - " indicates not detected.

" + " indicates positive, qualitative identification by GC with a lower detection limit than with HPLC.

- Strain suffixes "a" and "b" indicate independent isolates.

20

E. coli strain MG1655 1.5GI-yqhD::Cm was transformed with plasmids pTrc99a-E-C-H-T and pBHR-Ptrc-ald(opt) to produce the strain, MG1655 1.5GI-yqhD::Cm/ pTrc99a-E-C-H-T/ pBHR-Ptrc-ald(opt). Two isolates were initially grown in LB medium containing 50 µg/mL kanamycin and 100 µg/mL carbenicillin. The cells were used to inoculate shake flasks (approximately 175 mL total volume) containing 50 and 150 mL of TM3a/glucose medium (with appropriate antibiotics). The flasks were inoculated at a starting OD₅₅₀ of ≤ 0.04 units and incubated as described above, with and without induction. IPTG was added to a final concentration of 0.4 mM; the OD₅₅₀ of the flasks at the time of addition was between 0.6 and 1.2 units. In this case, induction was not necessary for 1-butanol pathway gene expression because of the leakiness of the IPTG inducible promoters and the constitutive nature of the 1.5GI promoter; however, induction provided a wider range of expression.

Approximately 15 h after induction, an aliquot of the broth was analyzed by GC with flame ionization detection for 1-butanol content, as described above. The results are given in Table 18. For the recombinant *E. coli* strains, 1-butanol was produced in all cases; in separate experiments, wild type *E. coli* strains were shown to produce no detectable 1-butanol (data not shown).

Table 18

Production of 1-butanol by *E. coli* strain MG1655 1.5GI-yqhD::Cm/ pTrc99a-E-C-H-T/ pBHR-Ptrc-ald(opt).

Strain	O ₂ Level	1-butanol, mM	IPTG Induction
MG1655 a	medium	0.14	No
MG 1655 b	medium	0.14	No
MG1655 a	medium	0.03	Yes
MG 1655 b	medium	0.07	Yes
MG1655 a	low	0.04	No
MG 1655 b	low	0.04	No

MG1655 a	low	0.02	Yes
MG 1655 b	low	0.03	Yes

- Strain suffixes "a" and "b" indicate separate isolates.

EXAMPLE 18

Production of 1-Butanol from Glucose using Recombinant *B. subtilis*.

5 This Example describes the production of 1-butanol in *Bacillus subtilis*. The six genes of the 1-biosynthetic pathway, encoding six enzyme activities, were split into two operons for expression. The first three genes of the pathway (*thl*, *hbd*, and *crt*) were integrated into the chromosome of *Bacillus subtilis* BE1010 (Payne and Jackson, *J. Bacteriol.*
10 173:2278-2282 (1991)). The last three genes (*EgTER*, *ald*, and *bdhB*) were cloned into an expression plasmid and transformed into the *Bacillus* strain carrying the integrated 1-butanol genes.

Unless otherwise indicated in the text, cloning primers described in this Example are referenced by their SEQ ID NO in Table 4, and
15 sequencing and PCR screening primers are referenced by their SEQ ID NO in Table 5.

Integration Plasmid. Plasmid pFP988 is a *Bacillus* integration vector that contains an *E. coli* replicon from pBR322, an ampicillin antibiotic marker for selection in *E. coli* and two sections of homology to
20 the *sacB* gene in the *Bacillus* chromosome that directs integration of the vector and intervening sequence by homologous recombination. Between the *sacB* homology regions is the Pamy promoter and signal sequence that can direct the synthesis and secretion of a cloned gene, a His-Tag and erythromycin as a selectable marker for *Bacillus*. The Pamy promoter and signal sequence is from *Bacillus amyloliquefaciens* alpha-amylase.
25 The promoter region also contains the *lacO* sequence for regulation of expression by a *lacI* repressor protein. The sequence of pFP988 (6509 bp) is given as SEQ ID NO:79.

Since the 1-butanol pathway genes were to be expressed in the
30 cytoplasm, the amylase signal sequence was deleted. Plasmid pFP988 was amplified with primers Pamy/lacO F and Pamy/lacO R creating a 317

bp (0.3 kbp) product that contained the Pamy/lacO promoter. The 5' end of the Pamy/lacO F primer incorporated a BsrGI restriction site followed by an EcoRI site. The 5' end of the Pamy/lacO R primer incorporated a BsrGI restriction site followed by a PmeI restriction site. The PCR product was TOPO cloned into pCR4Blunt-TOPO creating pCR4Blunt-TOPO-Pamy/lacO. Plasmid DNA was prepared from overnight cultures and submitted for sequencing with M13 Forward and M13 Reverse primers (SEQ ID NO:45 and SEQ ID NO:46, respectively) to ensure no mutation had been introduced into the promoter. A clone of pCR4Blunt-TOPO-Pamy/lacO was digested with BsrGI and the 0.3 kbp fragment was gel-purified. The vector pFP988 was digested with BsrGI resulting in deletion of 11 bp from the 5' *sacB* homology region and the removal of the Pamy/lacO promoter and signal sequence and His-tag. The 6 kbp BsrGI digested vector was gel-purified and ligated with Pamy/lacO BsrGI insert. The resulting plasmids were screened with primers Pamy SeqF2 and Pamy SeqR to determine orientation of the promoter. The correct clone restored the Pamy/lacO promoter to its original orientation and was named pFP988Dss.

The cassette with genes *thl-crt* was constructed by SOE (splicing by overlap extension). The genes were amplified using as template pUC19dss::Operon1. The *thl* primers were Top TF and Bot TR amplifying a 0.9 kbp product. The *crt* primers were Top CF and Bot CR amplifying a 1.3 kbp product. The two genes were joined by SOE with PCR amplification using primers Top TF and Bot CR generating a 2.1 kbp product that was TOPO cloned into pCR4Blunt-TOPO creating pCR4Blunt-TOPO-T-C. Clones were submitted for sequencing to confirm the sequence. The plasmid pCR4Blunt-TOPO-T-C was digested with BstEII and PmeI releasing a 2.1 kbp fragment that was gel-purified. The insert was treated with Klenow polymerase to blunt the BstEII site. Vector pFP988Dss was digested with PmeI and treated with calf intestinal alkaline phosphatase (New England BioLabs) to prevent self-ligation. The 2.1 kbp *thl-crt* fragment and the digested pFP988Dss were ligated and transformed into *E. coli* Top10 cells. Transformants were screened by

PCR amplification with Pamy SeqF2 and N7SeqR2 for a 0.7 kbp product, the correct product was called pFP988Dss-T-C.

Construction of the *thl-crt* cassette created unique Sall and SpeI sites between the two genes. To add the *hbd* gene to the cassette, the
 5 *hbd* gene was subcloned from pCR4Blunt-TOPO-hbd as a 0.9 kbp Sall/SpeI fragment. Vector pFP988Dss-T-C was digested with Sall and SpeI and the 8 kbp vector fragment was gel-purified. The vector and *hbd* insert were ligated and transformed into *E. coli* Top10 cells.

Transformants were screened by PCR amplification with primers Pamy
 10 SeqF and N3SeqF3 for a 3.0 kbp fragment. The resulting plasmid was named pFP988Dss-T-H-C.

The Pamy promoter subsequently was replaced with the Pspac promoter from plasmid pMUTIN4 (Vagner *et al.*, Microbiol. 144:3097-3104 (1998)). The Pspac promoter was amplified from pMUTIN4 with primers
 15 Spac F and Spac R as a 0.4 kbp product and TOPO cloned into pCR4Blunt-TOPO. Transformants were screened by PCR amplification with M13 Forward and M13 Reverse primers for the presence of a 0.5 kbp insert. Positive clones were submitted for sequencing with the same primers. Plasmid pCR4Blunt-TOPO-Pspac was digested with SmaI and
 20 XhoI and the 0.3 kbp fragment was gel-purified. Vector pFP988Dss-T-H-C was digested with SmaI and XhoI and the 9 kbp vector was isolated by gel purification. The digested vector and Pspac insert were ligated and transformed into *E. coli* Top10 cells. Transformants were screened by PCR amplification with primers SpacF Seq and N7SeqR2. Positive
 25 clones gave a 0.7 kbp product. Plasmid DNA was prepared from positive clones and further screened by PCR amplification with primers SpacF Seq and N3SeqF2. Positive clones gave a 3 kbp PCR product and were named pFP988DssPspac-T-H-C.

Integration into *B. subtilis* BE1010 to form *B. subtilis* Δ sacB::T-H-C::erm #28 comprising exogenous *thl*, *hbd*, and *crt* genes. Competent
 30 cells of *B. subtilis* BE1010 were prepared as described in Doyle *et al.*, *J. Bacteriol.* 144:957-966 (1980). Competent cells were harvested by centrifugation and the cell pellets were resuspended in a small volume of

the cell supernatant. To 1 volume of competent cells, 2 volumes of SP11-EGTA medium (*Methods for General and Molecular Bacteriology*, P. Gerhardt et al., Eds, American Society for Microbiology, Washington, DC (1994)) was added. Aliquots of 0.3 mL of cells were dispensed into test
5 tubes and the plasmid pFP988DssPspac-T-H-C was added to the tubes. Cells were incubated for 30 minutes at 37 °C with shaking, after which 0.1 mL of 10% yeast extract was added to each tube and the cells were further incubated for 60 min. Transformants were plated for selection on LB erythromycin plates using the double agar overlay method (*Methods*
10 *for General and Molecular Bacteriology, supra*). Transformants were initially screened by PCR amplification with primers Pamy SeqF and N5SeqF3. Positive clones that amplified the expected 2 kbp PCR product were further screened by PCR amplification. If insertion of the cassette into the chromosome had occurred via a double crossover event then
15 primer set sacB Up and N7SeqR2 and primer set sacB Dn and N4SeqR3 would amplify a 1.7 kbp and a 2.7 kbp product respectively. A positive clone was identified and named *B. subtilis* Δ sacB::T-H-C::erm #28.

Plasmid Expression of EgTER, *ald*, and *bdhB* genes. The three remaining 1-butanol genes were expressed from plasmid pHT01
20 (MoBitec). Plasmid pHT01 is a *Bacillus-E. coli* shuttle vector that replicates via a theta mechanism. Cloned proteins are expressed from the GroEL promoter fused to a *lacO* sequence. Downstream of the *lacO* is the efficient RBS from the *gsiB* gene followed by a MCS. The *ald* gene was amplified by PCR with primers AF BamHI and AR Aat2 using pUC19dHS-
25 ald-bdhB (described in Example 13) as template, creating a 1.4 kbp product. The product was TOPO cloned into pCR4-TOPO and transformed into *E. coli* Top10 cells. Transformants were screened with M13 Forward and M13 Reverse primers. Positive clones amplified a 1.6 kbp product. Clones were submitted for sequencing with primers M13
30 forward and M13 reverse, N31SeqF2, N31SeqF3, N32SeqR2, N32SeqR3 and N32SeqR4. The plasmid was named pCR4TOPO-B/A-ald.

Vector pHT01 and plasmid pCR4TOPO-B/A-ald were both digested with BamHI and AatII . The 7.9 kbp vector fragment and the 1.4 kbp *ald*

fragment were ligated together to create pHT01-ald. The ligation was transformed into *E. coli* Top10 cells and transformants were screened by PCR amplification with primers N31SeqF1 and HT R for a 1.3 kbp product.

To add the last two steps of the pathway to the pHT01 vector, two
5 cloning schemes were designed. For both schemes, EgTER and *bdhB* were amplified together by SOE. Subsequently, the EgTER-*bdh* fragment was either cloned into pHT01-ald creating pHT01-ald-EB or cloned into pCR4-TOPO-B/A-ald creating pCR4-TOPO-ald-EB. The ald-EgTer-bdhB fragment from the TOPO vector was then cloned into pHT01 creating
10 pHT01-AEB.

An EgTER-*bdhB* fragment was PCR amplified using primers Forward 1 (E) and Reverse 2 (B), using template DNA given as SEQ ID NO:208. The resulting 2.5 kbp PCR product was TOPO cloned into pCR4Blunt-TOPO, creating pCR4Blunt-TOPO-E-B. The TOPO reaction
15 was transformed into *E. coli* Top10 cells. Colonies were screened with M13 Forward and M13 Reverse primers by PCR amplification. Positive clones generated a 2.6 kbp product. Clones of pCR4Blunt-TOPO-E-B were submitted for sequencing with primers M13 Forward and Reverse, N62SeqF2, N62SeqF3, N62SeqF4, N63SeqR1, N63SeqR2, N63SeqR3,
20 N11Seq F1 and N11Seq F2, N12SeqR1 and N12SeqR2.

Plasmid pCR4Blunt-TOPO-E-B was digested with HpaI and AatII to release a 2.4 kbp fragment. The E-B fragment was treated with Klenow polymerase to blunt the end and then was gel-purified. Plasmid pHT01-ald was digested with AatII and treated with Klenow polymerase to blunt
25 the ends. The vector was then treated with calf intestinal alkaline phosphatase and was gel-purified. The E-B fragment was ligated to the linearized vector pHT01-ald, transformed into *E. coli* Top10 cells, and selected on LB plates containing 100 µg/mL ampicillin. Transformants were screened by PCR amplification with primers N3SeqF1 and
30 N63SeqR1 to give a 2.4 kbp product. The resulting plasmid, pHT01-ald-EB, was transformed into JM103 cells, a *recA*⁺ *E. coli* strain. Plasmids prepared from *recA*⁺ strains form more multimers than *recA*⁻ strains. *Bacillus subtilis* transforms more efficiently with plasmid multimers rather

than monomers (*Methods for General and Molecular Bacteriology, supra*). Plasmid DNA was prepared from JM103 and transformed into competent *B. subtilis* Δ sacB::T-H-C::erm #28 forming strain *B. subtilis* Δ sacB::T-H-C::erm #28/pHT01-ald-EB. Competent cells were prepared and
5 transformed as previously described. Transformants were selected on LB plates containing 5 μ g/mL chloramphenicol and screened by colony PCR with the primers N31SeqF1 and N63SeqR4 for a 1.3 kbp product.

In the alternate cloning strategy, pCR4Blunt-TOPO-E-B was digested with HpaI and AatII releasing a 2.4 kbp fragment that was gel-
10 purified. Plasmid pCR4-TOPO-B/A-ald was digested with HpaI and AatII and the 5.4 kbp vector fragment was gel-purified. The vector fragment from pCR4-TOPO-B/A-ald was ligated with the HpaI-AatII E-B fragment creating pCR4-TOPO-ald-EB. The ligation was transformed into *E. coli* Top10 cells and the resulting transformants were screened by PCR
15 amplification with primers N11SeqF2 and N63SeqR4 for a 2.1 kbp product. Plasmid pCR4-TOPO-ald-EB was digested with BamHI and AatII and SphI. The BamHI/AatII digest releases a 3.9 kbp ald-EB fragment that was gel-purified. The purpose of the SphI digest was to cut the remaining vector into smaller fragments so that it would not co-migrate on
20 a gel with the ald-EB insert. Vector pHT01 was digested with BamHI and AatII and the 7.9 kbp vector fragment was gel-purified. The vector and ald-EB insert fragments were ligated to form plasmid pHT01-AEB and transformed into *E. coli* Top10 cells. Colonies were screened by PCR amplification with primers N62SeqF4 and HT R for a 1.5 kbp product.
25 Plasmid was prepared and transformed into JM103. Plasmid DNA was prepared from JM103 and transformed into competent *B. subtilis* Δ sacB::T-H-C::erm #28 forming strain *B. subtilis* Δ sacB::T-H-C::erm #28/pHT01-AEB. Competent BE1010 cells were prepared and transformed as previously described. *Bacillus* transformants were screened by PCR
30 amplification with primers N31SeqF1 and N63SeqR4 for a 1.3 kbp product.

Demonstration of 1-butanol production from recombinant *B. subtilis*.

Three independent isolates of each strain of *B. subtilis* Δ sacB::T-H-C::erm #28/pHT01-ald-EB and *B. subtilis* Δ sacB::T-H-C::erm #28/ pHT01-AEB were inoculated into shake flasks (approximately 175 mL total volume) containing 15 mL of medium. A *B. subtilis* BE1010 strain lacking the exogenous 1-butanol, six gene pathway was also included as a negative control. The medium contained (per liter): 10 mL of 1 M (NH₄)₂SO₄; 5 mL of 1 M potassium phosphate buffer, pH 7.0; 100 mL of 1 M MOPS/KOH buffer, pH 7.0; 20 mL of 1 M L-glutamic acid, potassium salt; 10 g glucose; 10 mL of 5 g/L each of L-methionine, L-tryptophan, and L-lysine; 0.1 g each of yeast extract and casamino acids; 20 mL of metal mix; and appropriate antibiotics (5 mg chloramphenicol and erythromycin for the recombinant strains). The metal mix contained 200 mM MgCl₂, 70 mM CaCl₂, 5 mM MnCl₂, 0.1 mM FeCl₃, 0.1 mM ZnCl₂, 0.2 mM thiamine hydrochloride, 172 μ M CuSO₄, 253 μ M CoCl₂, and 242 μ M Na₂MoO₄. The flasks were inoculated at a starting OD₆₀₀ of ≤ 0.1 units, sealed with non-vented caps, and incubated at 37 °C with shaking at approximately 200 rpm.

Approximately 24 h after inoculation, an aliquot of the broth was analyzed by HPLC (Shodex Sugar SH1011 column) with refractive index (RI) detection and GC (Varian CP-WAX 58(FFAP) CB column, 0.25 mm X 0.2 μ m X 25 m) with flame ionization detection (FID) for 1-butanol content, as described in the General Methods section. The results of the 1-butanol determinations are given in Table 19.

Table 19

Production of 1-butanol by strains *B. subtilis* Δ sacB::T-H-C::erm #28/pHT01-ald-EB and *B. subtilis* Δ sacB::T-H-C::erm #28/ pHT01-AEB

Strain	1-butanol, HPLC RI peak area	1-butanol, mM*
BE1010 control	Not detected	Not detected
pHT01-ald-EB a	4629	0.19
pHT01-ald-EB b	3969	Not determined
pHT01-ald-EB c	4306	Not determined

pHT01-AEB a	4926	0.16
pHT01-AEB b	3984	Not determined
pHT01-AEB c	3970	Not determined

* Concentration determined by GC.

-Strain suffixes "a", "b", and "c" indicate separate isolates.

EXAMPLE 19

5 Production of 1-Butanol from Glucose or Sucrose by Recombinant *E. coli*.

To endow *E. coli* MG1655 with the ability to use sucrose as the carbon and energy source for 1-butanol production, a sucrose utilization gene cluster (*cscBKA*) from plasmid pScr1 (described below) was subcloned into pBHR-Ptrc-ald(opt) (described in Example 17) in this
10 organism. The sucrose utilization genes (*cscA*, *cscK*, and *cscB*) encode a sucrose hydrolase (*CscA*), given as SEQ ID NO:157, D-fructokinase (*CscK*), given as SEQ ID NO:158, and sucrose permease (*CscB*), given as SEQ ID NO:159. To allow constitutive expression of the three genes from their natural promoter, the sucrose-specific repressor gene, *cscR*,
15 that regulates the gene cluster is not present in the construct.

Cloning and expression of the sucrose utilization gene cluster *cscBKA* in plasmid pBHR-Ptrc-ald(opt). The sucrose utilization gene cluster *cscBKA*, given as SEQ ID NO:156, was isolated from genomic DNA of a sucrose-utilizing *E. coli* strain derived from *E. coli* strain ATCC
20 13281. The genomic DNA was digested to completion with BamHI and EcoRI. Fragments having an average size of about 4 kbp were isolated from an agarose gel, ligated to plasmid pLitmus28 (New England Biolabs, Beverly, MA), which was then digested with BamHI and EcoRI. The resulting DNA was transformed into ultracompetent *E. coli* TOP10F'
25 (Invitrogen, Carlsbad, CA). The transformants were plated on MacConkey agar plates containing 1% sucrose and 100 µg/mL ampicillin and screened for purple colonies. Plasmid DNA was isolated from the purple transformants and sequenced using primers M13 Forward (SEQ ID NO:45), M13 Reverse (SEQ ID NO:46), scr1 (SEQ ID NO:160), scr2 (SEQ
30 ID NO:161), scr3 (SEQ ID NO:162), and scr4 (SEQ ID NO:163). The plasmid containing *cscB*, *cscK*, and *cscA* (*cscBKA*) genes was designated pScr1.

Plasmid pScrl was digested with XhoI and treated with the Klenow fragment of DNA polymerase to make blunt ends. The plasmid was then digested with AgeI, and the 4,179 bp *cscBKA* gene cluster fragment was gel-purified. Plasmid pBHR-Ptrc-ald(opt) was prepared as described in
 5 Example 17 and was digested with AgeI and NaeI. The resulting 6,003 bp pBHR-Ptrc-ald(opt) fragment was gel-purified. The *cscBKA* fragment was ligated with the pBHR-Ptrc-ald(opt), yielding pBHR-Ptrc-ald(opt)-*cscAKB*. Plasmid pBHR-Ptrc-ald(opt)-*cscAKB* was transformed into *E. coli* NovaXG electrocompetent cells (Novagen, Madison, WI) and sucrose utilization
 10 was confirmed by plating the transformants on McConkey agar plates containing 2% sucrose and 25 µg/mL kanamycin. In the pBHR-Ptrc-ald(opt)-*cscAKB* construct, the sucrose utilization genes were cloned downstream of Ptrc-ald(opt) as a separate fragment in the order *cscA*, *cscK*, and *cscB*.

15 Alternatively, the sucrose utilization genes were cloned in the opposite direction in pBHR-Ptrc-ald(opt). Plasmid pBHR-Ptrc-ald(opt) was digested with Scal and AgeI, and the 5,971 bp pBHR-Ptrc-ald(opt) fragment was gel-purified. The 4,179 bp *cscBKA* fragment, prepared as described above, was ligated with the pBHR-Ptrc-ald(opt) fragment,
 20 yielding pBHR-Ptrc-ald(opt)-*cscBKA*. Plasmid pBHR-Ptrc-ald(opt)-*cscBKA* was transformed into *E. coli* NovaXG electrocompetent cells (Novagen, Madison, WI) and sucrose utilization was confirmed by plating the transformants on McConkey agar plates containing 2% sucrose and 25 µg/mL kanamycin. In the pBHR-Ptrc-ald(opt)-*cscBKA* construct, the
 25 sucrose utilization genes were cloned as a separate fragment downstream of Ptrc-ald(opt) in the order *cscB*, *cscK*, and *cscA*.

Demonstration of 1-butanol production from glucose or sucrose using recombinant *E. coli*. *E. coli* strain MG1655 1.5GI-yqhD::Cm (described in Example 17) was transformed with plasmids
 30 pTrc99a-E-C-H-T (prepared as described in Example 17) and pBHR-Ptrc-ald(opt)-*cscAKB* or pBHR-Ptrc-ald(opt)-*cscBKA* to produce two strains, MG1655 1.5GI-yqhD::Cm/pTrc99a-E-C-H-T/pBHR-Ptrc-ald(opt)-*cscAKB* #9 and MG1655 1.5GI-yqhD::Cm/pTrc99a-E-C-H-T/pBHR-Ptrc-ald(opt)-

cscBKA #1. Starter cultures of the two strains were prepared by growing the cells in LB medium containing 25 µg/mL of kanamycin and 100 µg/mL of carbenicillin. These cells were then used to inoculate shake flasks (approximately 175 mL total volume) containing 50, 70 and 150 mL of
5 TM3a/glucose medium (with appropriate antibiotics) to represent high, medium and low oxygen conditions, respectively, as described in Example 17. A third strain, *E. coli* MG1655/pScr1, grown in TM3a/glucose medium containing 100 µg/mL carbenicillin, was used as a negative control. For each of the strains, an identical set of flasks was prepared with
10 TM3a/sucrose medium (with appropriate antibiotics). TM3a/sucrose medium is identical to TM3a/glucose medium except that sucrose (10 g/L) replaces glucose. The flasks were inoculated at a starting OD₅₅₀ of ≤ 0.03 units and incubated as described in Example 17. With the exception of the negative control flasks, IPTG was added to the flasks (final
15 concentration of 0.04 mM) when the cultures reached an OD₅₅₀ between 0.2 and 1.8 units. The cells were harvested when the OD₅₅₀ of the cultures increased at least 3-fold.

Approximately 24 h after inoculation, an aliquot of the broth was analyzed by HPLC (Shodex Sugar SH1011 column) with refractive index
20 (RI) detection and GC (HP-INNOWax column, 30 m x 0.53 mm id, 1 µm film thickness) with flame ionization detection (FID) for 1-butanol content, as described in the General Methods section.

The concentrations of 1-butanol in cultures following growth in the glucose and sucrose-containing media are given in Table 20 and Table
25 21, respectively. Both recombinant *E. coli* strains containing the 1-butanol biosynthetic pathway produced 1-butanol from glucose and sucrose under all oxygen conditions, while the negative control strain produced no detectable 1-butanol.

Table 20Production of 1-butanol from glucose by recombinant *E. coli* strainsMG1655 1.5GI-yqhD::Cm/pTrc99a-E-C-H-T/pBHR-Ptrc-ald(opt)-cscAKB#9 and MG1655 1.5GI-yqhD::Cm/pTrc99a-E-C-H-T/pBHR-Ptrc-ald(opt)-

5

cscBKA #1

Strain	O ₂ Level	1-butanol, mM	molar yield, %
cscBKA #1	high	0.01	0.03
cscBKA #1	medium	0.20	0.43
cscBKA #1	low	0.07	0.21
cscAKB #9	high	0.01	0.02
cscAKB #9	medium	0.17	0.35
cscAKB #9	low	0.04	0.12
pScr1	high	Not detected	Not detected
pScr1	medium	Not detected	Not detected
pScr1	low	Not detected	Not detected

Table 21Production of 1-butanol from sucrose by recombinant *E. coli* strains.

Strain	O ₂ Level	1-butanol, mM	molar yield, %
cscBKA #1	high	0.02	0.10
cscBKA #1	medium	0.02	0.11
cscBKA #1	low	0.01	0.09
cscAKB #9	high	0.03	0.11
cscAKB #9	medium	0.03	0.15
cscAKB #9	low	0.02	0.10
pScr1	high	Not detected	Not detected

pScr1	medium	Not detected	Not detected
pScr1	low	Not detected	Not detected

EXAMPLE 20

Production of 1-Butanol from Sucrose using Recombinant *B. subtilis*.

This example describes the production of 1-butanol from sucrose using recombinant *Bacillus subtilis*. Two independent isolates of *B. subtilis* strain Δ sacB::T-H-C::erm #28/pHT01-ald-EB (Example 18) were examined for 1-butanol production essentially as described in Example 18. The strains were inoculated into shake flasks (approximately 175 mL total volume) containing either 20 mL or 100 mL of medium to simulate high and low oxygen conditions, respectively. Medium A was exactly as described in Example 18, except that glucose was replaced with 5 g/L of sucrose. Medium B was identical to the TM3a/glucose medium described in Example 17, except that glucose was replaced with 10 g/L sucrose and the medium was supplemented with (per L) 10 mL of a 5 g/L solution of each of L-methionine, L-tryptophan, and L-lysine. The flasks were inoculated at a starting OD₅₅₀ of ≤ 0.1 units, capped with vented caps, and incubated at 34 °C with shaking at 300 rpm.

Approximately 24 h after inoculation, an aliquot of the broth was analyzed by GC (HP-INNOWax column, 30 m x 0.53 mm id, 1.0 μ m film thickness) with FID detection for 1-butanol content, as described in the General Methods section. The results of the 1-butanol determinations are given in Table 22. The recombinant *Bacillus* strain containing the 1-butanol biosynthetic pathway produced detectable levels of 1-butanol under high and low oxygen conditions in both media.

Table 22

Production of 1-butanol from sucrose by *B. subtilis* strain Δ sacB::T-H-C::erm #28/pHT01-ald-EB

Strain	Medium	O ₂ Level	1-BuOH, mM ^{1, 2}
none	A	Not applicable	Not detected

pHT01-ald-EB a	A	high	+
pHT01-ald-EB b	A	high	+
pHT01-ald-EB a	A	low	0.01
pHT01-ald-EB b	A	low	0.01
none	B	Not applicable	Not detected
pHT01-ald-EB a	B	high	+
pHT01-ald-EB b	B	high	+
pHT01-ald-EB a	B	low	0.04
pHT01-ald-EB b	B	low	0.03

¹ Concentration determined by GC.

² "+" indicates qualitative presence of 1-butanol.

Strain suffixes "a" and "b" indicate separate isolates.

5

EXAMPLE 21

Production of 1-Butanol from Glucose and Sucrose Using Recombinant *Saccharomyces cerevisiae*

This Example describes the production of 1-butanol in the yeast *Saccharomyces cerevisiae*. Of the six genes encoding enzymes catalyzing the steps in the 1-butanol biosynthetic pathway, five were cloned into three compatible yeast 2 micron (2 μ) plasmids and co-expressed in *Saccharomyces cerevisiae*. The "upper pathway" is defined as the first three enzymatic steps, catalyzed by acetyl-CoA acetyltransferase (*thIA*, *thiolase*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), and crotonase (*crt*). The lower pathway is defined as the fourth (butyl-CoA dehydrogenase, *ter*) and the fifth (butylaldehyde dehydrogenase, *ald*) enzymatic steps of the pathway. The last enzymatic step of the 1-butanol pathway is catalyzed by alcohol dehydrogenase, which may be encoded by endogenous yeast genes, e.g., *adhI* and *adhII*.

Expression of genes in yeast typically requires a promoter, followed by the gene of interest, and a transcriptional terminator. A number of constitutive yeast promoters were used in constructing expression cassettes for genes encoding the 1-butanol biosynthetic pathway, including FBA, GPD, and GPM promoters. Some inducible promoters,

e.g. GAL1, GAL10, CUP1 were also used in intermediate plasmid construction, but not in the final demonstration strain. Several transcriptional terminators were used, including FBA_t, GPD_t, GPM_t, ERG10_t, and GAL1_t. Genes encoding the 1-butanol biosynthetic pathway were first subcloned into a yeast plasmid flanked by a promoter and a terminator, which yielded expression cassettes for each gene. Expression cassettes were optionally combined in a single vector by gap repair cloning, as described below. For example, the three gene cassettes encoding the upper pathway were subcloned into a yeast 2 μ plasmid. The *ter* and *ald* genes were each expressed individually in the 2 μ plasmids. Co-transformation of all three plasmids in a single yeast strain resulted in a functional 1-butanol biosynthetic pathway. Alternatively, several DNA fragments encoding promoters, genes, and terminators were directly combined in a single vector by gap repair cloning.

Methods for constructing plasmids and strains in yeast *Saccharomyces cerevisiae*. Basic yeast molecular biology protocols including transformation, cell growth, gene expression, gap repair recombination, etc. are described in *Methods in Enzymology*, Volume 194, *Guide to Yeast Genetics and Molecular and Cell Biology* (Part A, 2004, Christine Guthrie and Gerald R. Fink (Eds.), Elsevier Academic Press, San Diego, CA).

The plasmids used in this Example were *E. coli*-*S. cerevisiae* shuttle vectors, pRS423, pRS424, pRS425, and pRS426 (American Type Culture Collection, Rockville, MD), which contain an *E. coli* replication origin (e.g., pMB1), a yeast 2 μ origin of replication, and a marker for nutritional selection. The selection markers for these four vectors are His3 (vector pRS423), Trp1 (vector pRS424), Leu2 (vector pRS425) and Ura3 (vector pRS426). These vectors allow strain propagation in both *E. coli* and yeast strains. A yeast haploid strain *BY4741* (*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0*) (Research Genetics, Huntsville, AL, also available from ATCC 201388) and a diploid strain *BY4743* (*MATa/alpha his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 lys2 Δ 0/LYS2 MET15/met15 Δ 0 ura3 Δ 0/ura3 Δ 0*) (Research Genetics, Huntsville, AL, also available from ATCC 201390) were used as

hosts for gene cloning and expression. Construction of expression vectors for genes encoding 1-butanol biosynthetic pathway enzymes were performed by either standard molecular cloning techniques in *E. coli* or by the gap repair recombination method in yeast.

5 The gap repair cloning approach takes advantage of the highly efficient homologous recombination in yeast. Typically, a yeast vector DNA is digested (e.g., in its multiple cloning site) to create a “gap” in its sequence. A number of insert DNAs of interest are generated that contain a ≥ 21 bp sequence at both the 5’ and the 3’ ends that sequentially overlap
10 with each other, and with the 5’ and 3’ terminus of the vector DNA. For example, to construct a yeast expression vector for “Gene X”, a yeast promoter and a yeast terminator are selected for the expression cassette. The promoter and terminator are amplified from the yeast genomic DNA, and Gene X is either PCR amplified from its source organism or obtained
15 from a cloning vector comprising Gene X sequence. There is at least a 21 bp overlapping sequence between the 5’ end of the linearized vector and the promoter sequence, between the promoter and Gene X, between Gene X and the terminator sequence, and between the terminator and the 3’ end of the linearized vector. The “gapped” vector and the insert DNAs
20 are then co-transformed into a yeast strain and plated on the SD minimal dropout medium, and colonies are selected for growth of cultures and mini preps for plasmid DNAs. The presence of correct insert combinations can be confirmed by PCR mapping. The plasmid DNA isolated from yeast (usually low in concentration) can then be transformed into an *E. coli*
25 strain, e.g. *TOP10*, followed by mini preps and restriction mapping to further verify the plasmid construct. Finally the construct can be verified by sequence analysis. Yeast transformants of positive plasmids are grown in SD medium for performing enzyme assays to characterize the activities of the enzymes expressed by the genes of interest.

30 Yeast cultures were grown in YPD complex medium or Synthetic Minimal dropout medium containing glucose (SD medium) and the appropriate compound mixtures that allow complementation of the nutritional selection markers on the plasmids (*Methods in Enzymology*,

Volume 194, *Guide to Yeast Genetics and Molecular and Cell Biology*, 2004, Part A, pp.13-15). The sugar component in the SD drop out medium was 2% glucose. For 1-Butanol production, yeast cultures were also grown in Synthetic Minimal dropout medium with 2% sucrose (SS
5 medium).

For enzyme activity analysis, a single colony of each strain was streaked onto a fresh plate containing SD minimal drop out medium and incubated at 30 °C for 2 days. The cells on this plate were used to inoculate 20 mL of SD drop out medium and in a 125 mL shake flask and
10 grown overnight at 30 °C, with shaking at 250 rpm. The optical density (OD₆₀₀) of the overnight culture was measured, and the culture was diluted to an OD₆₀₀ = 0.1 in 250 mL of the same medium in a 1.0 L shake flask, and grown at 30 °C with shaking at 250 rpm to an OD₆₀₀ of between 0.8 to 1.0. The cells were then harvested by centrifugation at 2000 x g for 10
15 min, and resuspended in 20 mL of 50 mM Tris-HCl buffer, pH 8.5. Enzyme assays were performed as described above.

Construction of plasmid pNY102 for *thlA* and *hbd* co-expression. A number of dual expression vectors were constructed for the co-expression of *thlA* and *hbd* genes. The *Saccharomyces cerevisiae* ERG10 gene is a
20 functional ortholog of the *thlA* gene. Initially, a dual vector of ERG10 and *hbd* was constructed using the yeast GAL1-GAL10 divergent dual promoter, the GAL1 terminator (GAL1t) and the ERG10 terminator (ERG10t). The ERG10 gene-ERG10t DNA fragment was PCR amplified from genomic DNA of *Saccharomyces cerevisiae* strain BY4743, using
25 primers OT731 (SEQ ID NO:164) and OT732 (SEQ ID NO:165). The yeast GAL1-GAL10 divergent promoter was also amplified by PCR from BY4743 genomic DNA using primers OT733 (SEQ ID NO:166) and OT734 (SEQ ID NO:167). The *hbd* gene was amplified from *E. coli* plasmid pTrc99a-E-C-H-T (described in Example 17) using PCR primers OT735
30 (SEQ ID NO:168) and OT736 (SEQ ID NO:169). GAL1t was amplified from BY4743 genomic DNA using primers OT737 (SEQ ID NO:170) and OT738 (SEQ ID NO:171). Four PCR fragments, *erg10*-ERG10t, GAL1-GAL10 promoters, *hbd*, and GAL1t, were thus obtained with approximately 25 bp overlapping sequences between each adjacent PCR fragment.

GAL1t and ERG10-ERG10t fragments each contain approximately 25 bp overlapping sequences with the yeast vector pRS425. To assemble these sequences by gap repair recombination, the DNA fragments were co-transformed into the yeast strain *BY4741* together with vector pRS425 which was digested with BamHI and HindIII enzymes. Colonies were selected from SD-Leu minimal plates, and clones with inserts were identified by PCR amplification. The new plasmid was named pNY6 (pRS425.ERG10t-erg10-GAL10-GAL1-hbd-GAL1t). Further confirmation was performed by restriction mapping.

10 The yeast strain *BY4741* (pNY6), prepared by transforming plasmid pNY6 into *S. cerevisiae* BY4741, showed good Hbd activity but no thiolase activity. Due to the lack of thiolase activity, the ERG10 gene was replaced with the *thIA* gene by gap repair recombination. The *thIA* gene was amplified from *E. coli* vector pTrc99a-E-C-H-T by PCR using primers
15 OT797 (SEQ ID NO:172) which adds a SphI restriction site, and OT798 (SEQ ID NO:173) which adds an AscI restriction site. Plasmid pNY6 was digested with SphI and PstI restriction enzymes, gel-purified, and co-transformed into yeast BY4741 along with the PCR product of *thIA*. Due to the 30 bp overlapping sequences between the PCR product of *thIA* and
20 the digested pNY6, the *thIA* gene was recombined into pNY6 between the GAL10 promoter and the ERG10t terminator. This yielded plasmid pNY7 (pRS425.ERG10t-thIA- GAL10-GAL1-hbd-GAL1t), which was verified by PCR and restriction mapping.

 In a subsequent cloning step based on gap repair recombination,
25 the GAL10 promoter in pNY7 was replaced with the CUP1 promoter, and the GAL1 promoter was replaced with the strong GPD promoter. This plasmid, pNY10 (pRS425. ERG10t-thIA- CUP1- GPD -hbd-GAL1t) allows for the expression of the *thIA* gene under CUP1, a copper inducible promoter, and the expression of the *hbd* gene under the GPD promoter.
30 The CUP1 promoter sequence was PCR amplified from yeast BY4743 genomic DNA using primers OT806 (SEQ ID NO:174), and OT807 (SEQ ID NO:175). The GPD promoter was amplified from BY4743 genomic DNA using primers OT808 (SEQ ID NO:176) and OT809 (SEQ ID NO:177). PCR products of the CUP1 and the GPD promoters were

combined with pNY7 plasmid digested with NcoI and SphI restriction enzymes. From this gap repair cloning step, plasmid pNY10 was constructed, which was verified by PCR and restriction mapping. Yeast *BY4741* strain containing pNY10 had Hbd activity, but no ThIA activity.

- 5 The Hbd activity under GPD promoter was significantly improved compared to the GAL1 promoter controlled Hbd activity (1.8 U/mg vs. 0.40 U/mg). Sequencing analysis revealed that the *thIA* gene in pNY10 had a one base deletion near the 3' end, which resulted in a truncated protein. This explains the lack of thiolase activity in the strain.

- 10 Plasmid pNY12 was constructed with the correct *thIA* gene sequence. The *thIA* gene was cut from the vector pTrc99a-E-C-H-T by digestion with SphI and Ascl. The FBA1 promoter was PCR amplified from BY4743 genomic DNA using primers OT799 (SEQ ID NO:178) and OT761 (SEQ ID NO:179), and digested with Sall and SphI restriction
15 enzymes. The *thIA* gene fragment and FBA1 promoter fragment were ligated into plasmid pNY10 at Ascl and Sall sites, generating plasmid pNY12 (pRS425.ERG10t-thIA-FBA1), which was confirmed by restriction mapping. pNY12 was transformed into yeast strain *BY4741* and the resulting transformant showed a ThIA activity of 1.66 U/mg.

- 20 The FBA1 promoter-*thIA* gene fragment from pNY12 was re-subcloned into pNY10. The pNY10 vector was cut with the Ascl restriction enzyme and ligated with the Ascl digested FBA1 promoter-*thIA* gene fragment isolated from plasmid pNY12. This created a new plasmid with two possible insert orientations. The clones with FBA1 and GPD
25 promoters located adjacent to each other in opposite orientation were chosen and this plasmid was named pNY102. pNY102 (pRS425.ERG10t-thIA- FBA1- GPD -hbd-GAL1t) was verified by restriction mapping. Strain *DPD5206* was made by transforming pNY102 into yeast strain *BY4741*. The ThIA activity of *DPD5206* was 1.24 U/mg and the Hbd
30 activity was 0.76 U/mg.

Construction of plasmid pNY11 for *crt* expression. The *crt* gene expression cassette was constructed by combining the GPM1 promoter, the *crt* gene, and the GPM1t terminator into vector pRS426 using gap repair recombination in yeast. The GPM1 promoter was PCR amplified

from yeast BY4743 genomic DNA using primers OT803 (SEQ ID NO:180) and OT804 (SEQ ID NO:181). The *crt* gene was amplified using PCR primers OT785 (SEQ ID NO:182) and OT786 (SEQ ID NO:183) from *E. coli* plasmid pTrc99a-E-C-H-T. The GPM1t terminator was PCR amplified
5 from yeast BY4743 genomic DNA using OT787 (SEQ ID NO:184) and OT805 (SEQ ID NO:185). Yeast vector pRS426 was digested with BamHI and HindIII and was gel-purified. This DNA was co-transformed with the PCR products of the GPM1 promoter, the *crt* gene and the GPM1 terminator into yeast *BY4741* competent cells. Clones with the correct
10 inserts were verified by PCR and restriction mapping and the resulting yeast strain *BY4741* (pNY11: pRS426-GPM1-crt-GPM1t) had a Crt activity of 85 U/mg.

Construction of plasmid pNY103 for *thlA*, *hbd* and *crt* co-expression. For the co-expression of the upper 1-butanol pathway
15 enzymes, the *crt* gene cassette from pNY11 was subcloned into plasmid pNY102 to create an *hbd*, *thlA*, and *crt* expression vector. A 2,347 bp DNA fragment containing the GPM1 promoter, the *crt* gene, and the GPM1 terminator was cut from plasmid pNY11 with SacI and NotI restriction enzymes and cloned into vector pNY102, which was digested with NotI
20 and partially digested with SacI, producing the expression vector pNY103 (pRS425. ERG10t-thlA- FBA1- GPD -hbd-GAL1t-GPM1t-crt-GPM1). Following confirmation of the presence of all three cassettes in pNY103 by digestion with HindIII, the plasmid was transformed into yeast *BY4743* cells and the transformed yeast strain was named *DPD5200*. When
25 grown under standard conditions, *DPD5200* showed ThlA, Hbd, and Crt enzyme activities of 0.49 U/mg, 0.21 U/mg and 23.0 U/mg, respectively.

Construction of plasmid pNY8 for *ald* expression. A codon optimized gene named *tery* (SEQ ID NO:186), encoding the Ter protein (SEQ ID NO:187), and a codon optimized gene named *aldy* (SEQ ID
30 NO:188), encoding the Ald protein (SEQ ID NO:189) were synthesized using preferred codons of *Saccharomyces cerevisiae*. Plasmid pTERy containing the codon optimized *ter* gene and pALDy containing the codon optimized *ald* gene were made by DNA2.0 (Palo Alto, CA).

To assemble pNY8 (pRS426.GPD-ald-GPDt), three insert fragments including a PCR product of the GPD promoter (synthesized from primers OT800 (SEQ ID NO:190) and OT758, (SEQ ID NO:191), and BY4743 genomic DNA), an *aldy* gene fragment excised from pALDy by
5 digestion with NcoI and SfiI (SEQ ID NO:188), and a PCR product of the GPD terminator (synthesized from primers OT754 (SEQ ID NO:192) and OT755 (SEQ ID NO:193), and BY4743 genomic DNA) were recombined with the BamHI, HindIII digested pRS426 vector *via* gap repair recombination cloning. Yeast *BY4741* transformation clones were
10 analyzed by PCR mapping. The new plasmid thus constructed, pNY8, was further confirmed by restriction mapping. The yeast *BY4741* transformants containing pNY8 were analyzed for Ald activity and the specific activity towards butyryl-CoA was approximately 0.07 U/mg.

Construction of plasmids pNY9 and pNY13 for *ter* expression. The
15 codon optimized *tery* gene was cloned into vector pRS426 under control of the FBA1 promoter by gap repair cloning. The FBA1 promoter was PCR amplified from yeast BY4743 genomic DNA using primers OT760 (SEQ ID NO:194) and OT792 (SEQ ID NO:195). The *tery* gene was obtained by digestion of plasmid pTERy by SphI and NotI restriction enzymes that
20 resulted in the fragment given as SEQ ID NO:186. The PCR fragment of FBA1 terminator was generated by PCR from yeast BY4743 genomic DNA using primers OT791 (SEQ ID NO:196) and OT765 (SEQ ID NO:197). Three DNA fragments, the FBA1 promoter, the *ter* gene and the FBA1 terminator, were combined with the BamHI, HindIII digested
25 pRS426 vector and transformed into yeast BY4741 by gap repair recombination. The resulting plasmid, pNY9 (pRS426-FBA1-tery-FBA1t) was confirmed by PCR mapping, as well as restriction digestion. The yeast *BY4741* transformant of pNY9 produced a Ter activity of 0.26 U/mg.

To make the final 1-butanol biosynthetic pathway strain, it was
30 necessary to construct a yeast expression strain that contained several plasmids, each with a unique nutritional selection marker. Since the parent vector pRS426 contained a Ura selection marker, the *ter* expression cassette was subcloned into vector pRS423, which contained a His3 marker. A 3.2 kb fragment containing the FBA1-tery-FBA1t

cassette was isolated from plasmid pNY9 by digestion with *SacI* and *XhoI* restriction enzymes, and ligated into vector pRS423 that was cut with these same two enzymes. The new plasmid, pNY13 (pRS423--FBA1-*ter*-FBA1t) was mapped by restriction digestion. pNY13 was transformed into
5 *BY4741* strain and the transformant was cultured in SD-His medium, yielding a strain with a *Ter* activity of 0.19 U/mg.

Construction of a yeast strain containing 1-butanol biosynthetic pathway genes for demonstration of 1-butanol production. As described above, yeast strain *DPD5200* was constructed by transformation of
10 plasmid pNY103 into *S. cerevisiae* strain *BY4743*, which allows co-expression of *thlA*, *hbd* and *crt* genes. Yeast competent cells of *DPD5200* were prepared as described above, and plasmids pNY8 and pNY13 were co-transformed into *DPD5200*, generating strain *DPD5213*. *DPD5213* allows for the simultaneous constitutive expression of five genes in the 1-
15 butanol biosynthetic pathway, *thlA*, *hbd*, *crt*, *ter* and *ald*. Strain *DPD5212* (*S. cerevisiae* strain *BY4743* transformed with empty plasmids, pRS425 and pRS426) was used as a negative control. Four independent isolates of strain *DPD5213* were grown on SD-Ura,-Leu,-His dropout minimal medium in the presence of either 2% glucose or 2% sucrose to allow the
20 growth complementation of all three plasmids. A single isolate of *DPD5212* was similarly grown in appropriate medium.

To demonstrate 1-butanol production by aerobic cultures, a single colony of each strain was streaked onto a fresh agar plate containing SD minimal drop out growth medium (containing 2% glucose) or SS minimal
25 drop out growth medium (containing 2% sucrose) and incubated at 30 °C for 2 days. Cells from these plates were used to inoculate 20 mL of the minimal drop out medium (either SD or SS) in 125 mL plastic shake flasks and were grown overnight at 30 °C with shaking at 250 rpm. The optical density (OD₆₀₀) of the overnight culture was measured, the culture was
30 diluted to OD₆₀₀ of 0.1 in 25 mL of the same medium in a 125 mL shake flask, and grown at 30 °C with shaking at 250 rpm.

Aliquots of the culture were removed at 24 h and 48 h for GC analysis of 1-butanol production (HP-INNOWax column, 30 m x 0.53 mm

id, 1 μ m film thickness) with FID detection, as described in the General Methods section. The results of the GC analysis are given in Table 23.

Table 23

Production of 1-butanol from glucose and sucrose by

S. cerevisiae strain DPD5213

5

Strain ¹	Sugar	1-butanol at 24 h, mg/L ²	1-butanol at 48 h, mg/L ²
DPD5212	Glucose	Not detected	Not detected
DPD5213 a	Glucose	0.4	0.5
DPD5213 b	Glucose	0.9	0.2
DPD5213 c	Glucose	1.0	0.6
DPD5213 d	Glucose	0.8	0.3
DPD5212	Sucrose	Not detected	Not detected
DPD5213 a	Sucrose	Not detected	1.7
DPD5213 b	Sucrose	Not detected	1.3
DPD5213 c	Sucrose	0.2	1.5
DPD5213 d	Sucrose	0.6	0.9

¹ Independent isolates are indicated by a-d.

² Concentration determined by GC.

10

EXAMPLE 22 (Prophetic)

Expression of the 1-Butanol Biosynthetic Pathway in *Lactobacillus plantarum*

The purpose of this prophetic Example is to describe how to
 15 express the 1-butanol biosynthetic pathway in *Lactobacillus plantarum*.
 The six genes of the 1-butanol pathway, encoding six enzyme activities,
 are divided into two operons for expression. The first three genes of the
 pathway (*thl*, *hbd*, and *crt*, encoding the enzymes acetyl-CoA
 acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, and crotonase,

respectively) are integrated into the chromosome of *Lactobacillus plantarum* by homologous recombination using the method described by Hols et al. (*Appl. Environ. Microbiol.* 60:1401–1413 (1994)). The last three genes (EgTER, *ald*, and *bdhB*, encoding the enzymes butyryl-CoA
5 dehydrogenase, butyraldehyde dehydrogenase and butanol dehydrogenase, respectively) are cloned into an expression plasmid and transformed into the *Lactobacillus* strain carrying the integrated upper pathway 1-butanol genes. *Lactobacillus* is grown in MRS medium (Difco Laboratories, Detroit, MI) at 37 °C. Chromosomal DNA is isolated from
10 *Lactobacillus plantarum* as described by Moreira et al. (*BMC Microbiol.* 5:15 (2005)).

Integration. The *thl-hbd-crt* cassette under the control of the synthetic P11 promoter (Rud et al., *Microbiology* 152:1011-1019 (2006)) is integrated into the chromosome of *Lactobacillus plantarum* ATCC BAA-
15 793 (NCIMB 8826) at the *ldhL1* locus by homologous recombination. To build the *ldhL* integration targeting vector, a DNA fragment from *Lactobacillus plantarum* (Genbank NC_004567) with homology to *ldhL* is PCR amplified with primers LDH EcoRV F (SEQ ID NO:198) and LDH AatIIR (SEQ ID NO:199). The 1986 bp PCR fragment is cloned into
20 pCR4Blunt-TOPO and sequenced. The pCR4Blunt-TOPO-*ldhL1* clone is digested with EcoRV and AatII releasing a 1982 bp *ldhL1* fragment that is gel-purified. The integration vector pFP988, described in Example 18, is digested with HindIII and treated with Klenow DNA polymerase to blunt the ends. The linearized plasmid is then digested with AatII and the 2931 bp
25 vector fragment is gel-purified. The EcoRV/AatII *ldhL1* fragment is ligated with the pFP988 vector fragment and transformed into *E. coli* Top10 cells. Transformants are selected on LB agar plates containing ampicillin (100 µg/mL) and are screened by colony PCR to confirm construction of pFP988-*ldhL*.

30 To add a selectable marker to the integrating DNA, the *Cm* gene with its promoter is PCR amplified from pC194 (Genbank NC_002013) with primers *Cm* F (SEQ ID NO:200) and *Cm* R (SEQ ID NO:201), amplifying a 836 bp PCR product. The amplicon is cloned into pCR4Blunt-

TOPO and transformed into *E. coli* Top10 cells, creating pCR4Blunt-TOPO-Cm. After sequencing to confirm that no errors are introduced by PCR, the Cm cassette is digested from pCR4Blunt-TOPO-Cm as an 828 bp MluI/SwaI fragment and is gel-purified. The *ldhL*-homology containing
 5 integration vector pFP988-*ldhL* is digested with MluI and SwaI and the 4740 bp vector fragment is gel-purified. The Cm cassette fragment is ligated with the pFP988-*ldhL* vector creating pFP988-DldhL::Cm.

Finally the *thl-hbd-crt* cassette from pFP988Dss-T-H-C, described in Example 18, is modified to replace the amylase promoter with the
 10 synthetic P11 promoter. Then, the whole operon is moved into pFP988-DldhL::Cm. The P11 promoter is built by oligonucleotide annealing with primer P11 F (SEQ ID NO:202) and P11 R (SEQ ID NO:203). The annealed oligonucleotide is gel-purified on a 6% Ultra PAGE gel (Embi Tec, San Diego, CA). The plasmid pFP988Dss-T-H-C is digested with
 15 XhoI and SmaI and the 9 kbp vector fragment is gel-purified. The isolated P11 fragment is ligated with the digested pFP988Dss-T-H-C to create pFP988-P11-T-H-C. Plasmid pFP988-P11-T-H-C is digested with XhoI and BamHI and the 3034 bp P11-T-H-C fragment is gel-purified. pFP988-DldhL::Cm is digested with XhoI and BamHI and the 5558 bp vector
 20 fragment isolated. The upper pathway operon is ligated with the integration vector to create pFP988-DldhL-P11-THC::Cm.

Integration of pFP988-DldhL-P11-THC::Cm into *L. plantarum* BAA-793 to form *L. plantarum* Δ *ldhL1*::T-H-C::Cm comprising exogenous *thl*, *hbd*, and *crt* genes. Electrocompetent cells of *L. plantarum* are prepared
 25 as described by Aukrust, T.W., et al. (In: *Electroporation Protocols for Microorganisms*; Nickoloff, J.A., Ed.; *Methods in Molecular Biology*, Vol. 47; Humana Press, Inc., Totowa, NJ, 1995, pp 201-208). After electroporation, cells are outgrown in MRSSM medium (MRS medium supplemented with 0.5 M sucrose and 0.1 M MgCl₂) as described by
 30 Aukrust et al. *supra* for 2 h at 37 °C without shaking. Electroporated cells are plated for selection on MRS plates containing chloramphenicol (10 µg/mL) and incubated at 37 °C. Transformants are initially screened by colony PCR amplification to confirm integration, and initial positive clones

are then more rigorously screened by PCR amplification with a battery of primers.

Plasmid Expression of EgTER, *ald*, and *bdhB* genes. The three remaining 1-butanol genes are expressed from plasmid pTRKH3 (O'Sullivan DJ and Klaenhammer TR, *Gene* 137:227-231 (1993)) under the control of the *L. plantarum* *ldhL* promoter (Ferain et al., *J. Bacteriol.* 176:596-601 (1994)). The *ldhL* promoter is PCR amplified from the genome of *L. plantarum* ATCC BAA-793 with primers PldhL F (SEQ ID NO:204) and PldhL R (SEQ ID NO:205). The 369 bp PCR product is cloned into pCR4Blunt-TOPO and sequenced. The resulting plasmid, pCR4Blunt-TOPO-PldhL is digested with *SacI* and *BamHI* releasing the 359 bp PldhL fragment.

pHT01-ald-EB, described in Example 18, is digested with *SacI* and *BamHI* and the 10503 bp vector fragment is recovered by gel purification. The PldhL fragment and vector are ligated creating pHT01-PldhL-ald-EB.

To subclone the *ldhL* promoter-ald-EgTER-*bdh* cassette, pHT01-PldhL-ald-EB is digested with *MluI* and the ends are treated with Klenow DNA polymerase. The linearized vector is digested with *Sall* and the 4270 bp fragment containing the PldhL-AEB fragment is gel-purified. Plasmid pTRKH3 is digested with *Sall* and *EcoRV* and the gel-purified vector fragment is ligated with the PldhL-AEB fragment. The ligation mixture is transformed into *E. coli* Top 10 cells and transformants are plated on Brain Heart Infusion (BHI, Difco Laboratories, Detroit, MI) plates containing erythromycin (150 mg/L). Transformants are screened by PCR to confirm construction of pTRKH3-ald-E-B. The expression plasmid, pTRKH3-ald-E-B is transformed into *L. plantarum* Δ *ldhL*1::T-H-C::Cm by electroporation, as described above.

L. plantarum Δ *ldhL*1::T-H-C::Cm containing pTRKH3-ald-E-B is inoculated into a 250 mL shake flask containing 50 mL of MRS medium plus erythromycin (10 μ g/mL) and grown at 37 °C for 18 to 24 h without shaking. After 18 h to 24, 1-butanol is detected by HPLC or GC analysis, as described in the General Methods section.

EXAMPLE 23 (Prophetic)Expression of the 1-Butanol Biosynthetic Pathway in *Enterococcus faecalis*

The purpose of this prophetic Example is to describe how to
5 express the 1-butanol biosynthetic pathway in *Enterococcus faecalis*. The complete genome sequence of *Enterococcus faecalis* strain V583, which is used as the host strain for the expression of the 1-butanol biosynthetic pathway in this Example, has been published (Paulsen et al., *Science* 299:2071-2074 (2003)). Plasmid pTRKH3 (O'Sullivan DJ and
10 Klaenhammer TR, *Gene* 137:227-231 (1993)), an *E. coli*/Gram-positive shuttle vector, is used for expression of the six genes (*thlA*, *hbd*, *crt*, *EgTER*, *ald*, *bdhB*) of the 1-butanol pathway in one operon. pTRKH3 contains an *E. coli* plasmid p15A replication origin and the pAM β 1 replicon, and two antibiotic resistance selection markers, tetracycline
15 resistance and erythromycin resistance. Tetracycline resistance is only expressed in *E. coli*, and erythromycin resistance is expressed in both *E. coli* and Gram-positive bacteria. Plasmid pAM β 1 derivatives can replicate in *E. faecalis* (Poyart et al., *FEMS Microbiol. Lett.* 156:193-198 (1997)). The inducible *nisA* promoter (P_{nisA}), which has been used for efficient
20 control of gene expression by nisin in a variety of Gram-positive bacteria including *Enterococcus faecalis* (Eichenbaum et al., *Appl. Environ. Microbiol.* 64:2763-2769 (1998)), is used to control expression of the six desired genes encoding the enzymes of the 1-butanol biosynthetic pathway.

25 The linear DNA fragment (215 bp) containing the *nisA* promoter (Chandrapati et al., *Mol. Microbiol.* 46(2):467-477 (2002)) is PCR-amplified from *Lactococcus lactis* genomic DNA with primers F-P_{nisA}(EcoRV) (SEQ ID NO:206) and R-P_{nisA}(P_{meI} BamHI) (SEQ ID NO:207). The 215 bp PCR fragment is digested with EcoRV and BamHI, and the resulting P_{nisA}
30 fragment is gel-purified. Plasmid pTRKH3 is digested with EcoRV and BamHI and the vector fragment is gel-purified. The linearised pTRKH3 is ligated with the P_{nisA} fragment. The ligation mixture is transformed into *E. coli* Top10 cells by electroporation and transformants are selected

following overnight growth at 37 °C on LB agar plates containing erythromycin (25 µg/mL). The transformants are then screened by colony PCR with primers F-PnisA(EcoRV) and R-PnisA(BamHI) to confirm the correct clone of pTRKH3-PnisA.

5 Plasmid pTRKH3-PnisA is digested with PmeI and BamHI, and the vector is gel-purified. Plasmid pHTO1-*ald-EgTER-bdhB* is constructed as described in Example 18 and is digested with SmaI and BamHI, and the 2,973 bp *ald-EgTER-bdhB* fragment is gel-purified. The 2,973 bp *ald-EgTER-bdhB* fragment is ligated into the pTRKH3-PnisA vector at the
10 PmeI and BamHI sites. The ligation mixture is transformed into *E. coli* Top10 cells by electroporation and transformants are selected following incubation at 37 °C overnight on LB agar plates containing erythromycin (25 µg/mL). The transformants are then screened by colony PCR with primers *ald* forward primer N27F1 (SEQ ID NO: 31) and *bdhB* reverse
15 primer N65 (SEQ ID NO: 44). The resulting plasmid is named pTRKH3-PnisA-*ald-EgTER-bdhB* (= pTRKH3-A-E-B).

Plasmid pTRKH3-A-E-B is purified from the transformant and used for further cloning of the remaining genes (*thlA*, *hbd*, *crt*) into the BamHI site located downstream of the *bdhB* gene. Plasmid pTRKH3-A-E-B is
20 digested with BamHI and treated with the Klenow fragment of DNA polymerase to make blunt ends. Plasmid pFP988Dss-*thlA-hbd-crt* (=pFP988Dss-T-H-C) is constructed as described in Example 18 and is digested with SmaI and BamHI. The resulting 2,973 bp *thlA-hbd-crt* fragment is treated with the Klenow fragment of DNA polymerase to make
25 blunt ends and is gel-purified. The 2,973 bp *thlA-hbd-crt* fragment is ligated with the linearised pTRKH3-A-E-B. The ligation mixture is transformed into *E. coli* Top10 cells by electroporation and transformants are selected following overnight growth at 37 °C on LB agar plates containing erythromycin (25 µg/mL). The transformants are then screened
30 by colony PCR with primers *thlA* forward primer N7 (SEQ ID NO: 21) and *crt* reverse primer N4 (SEQ ID NO: 18). The resulting plasmid is named pTRKH3-PnisA-*ald-EgTER-bdhB-thlA-hbd-crt* (= pTRKH3-A-E-B-T-H-C). Plasmid pTRKH3-A-E-B-T-H-C is prepared from the *E. coli* transformants

and transformed into electro-competent *E. faecalis* V583 cells by electroporation using methods known in the art (Aukrust, T.W., et al. In: *Electroporation Protocols for Microorganisms*; Nickoloff, J.A., Ed.; *Methods in Molecular Biology*, Vol. 47; Humana Press, Inc., Totowa, NJ., 1995, pp 217-226), resulting in *E. faecalis* V583/pTRKH3-A-E-B-T-H-C.

The second plasmid containing *nisA* regulatory genes, *nisR* and *nisK*, the *add9* spectinomycin resistance gene, and the pSH71 origin of replication is transformed into *E. faecalis* V583/pTRKH3-A-E-B-T-H-C by electroporation. The plasmid containing pSH71 origin of replication is compatible with pAM β 1 derivatives in *E. faecalis* (Eichenbaum et al., *supra*). Double drug resistant transformants are selected on LB agar plates containing erythromycin (25 μ g/mL) and spectinomycin (100 μ g/mL).

The resulting *E. faecalis* strain V583B harboring two plasmids, i.e., an expression plasmid (pTRKH3-A-E-B-T-H-C) and a regulatory plasmid (pSH71-nisRK), is inoculated into a 250 mL shake flask containing 50 mL of Todd-Hewitt broth supplemented with yeast extract (0.2%) (Fischetti et al., *J. Exp. Med.* 161:1384-1401 (1985)), nisin (20 μ g/mL) (Eichenbaum et al., *supra*), erythromycin (25 μ g/mL), and spectinomycin (100 μ g/mL). The flask is incubated without shaking at 37 °C for 18 to 24 h, after which time, 1-butanol production is measured by HPLC or GC analysis, as described in the General Methods section.

EXAMPLE 24

Increased tolerance of *Saccharomyces cerevisiae* to 1-butanol at decreased growth temperatures

Tolerance levels were determined for yeast strain *Saccharomyces cerevisiae* BY4741 (described in Example 21) at 25 °C and 30 °C as follows. The strain was cultured in YPD medium. Overnight cultures in the absence of any test compound were started in 25 mL of YPD medium in 150 mL flasks with incubation at 30 °C or at 25 °C in shaking water baths. The next morning, each overnight culture was diluted into a 500 mL flask-containing 300 mL of fresh medium to an initial OD₆₀₀ of about 0.1. The

flasks were incubated in shaking water baths at 30 °C or 25 °C, using the same temperature as used for each overnight culture. The large cultures were incubated for 3 hours and then were split into flasks in the absence (control) and in the presence of 1% or 2% of 1-butanol. Growth was

5 followed by measuring OD₆₀₀ for six hours after addition of the 1-butanol. The ΔOD_{600} was calculated by subtracting the initial OD₆₀₀ from the final OD₆₀₀ at 6 hours. The percent growth inhibition relative to the control culture was calculated as follows: % Growth Inhibition = 100 – [100(Sample ΔOD_{600} /Control ΔOD_{600})]. The results are summarized in

10 Table 24 below and indicate that growth of strain BY4741 was less inhibited by 1% 1-butanol at 25 °C than by 1% 1-butanol at 30 °C.

Table 24. Growth of *Saccharomyces cerevisiae* Strain BY4741 at 25 °C and 30 °C with 1-Butanol.

% 1-Butanol	Temperature °C	% Growth Inhibition
1	30	64
1	25	43
2	30	No growth
2	25	No growth

CLAIMS

What is claimed is:

5

1. A method for the production of 1-butanol comprising:
 - a) providing a recombinant microbial production host which produces 1-butanol;
 - b) seeding the production host of (a) into a fermentation medium comprising a fermentable carbon substrate to create a fermentation culture;
 - c) growing the production host in the fermentation culture at a first temperature for a first period of time;
 - d) lowering the temperature of the fermentation culture to a second temperature; and
 - e) incubating the production host at the second temperature of step (d) for a second period of time; whereby 1-butanol is produced.

10

15

20

2. A method according to Claim 1 wherein the fermentable carbon substrate is derived from a grain or sugar source selected from the group consisting of wheat, corn, barley, oats, rye, sugar cane, sugar beets, cassava, sweet sorghum, and mixtures thereof.

25

3. A method according to Claim 1 wherein the fermentable carbon substrate is derived from cellulosic or lignocellulosic biomass selected from the group consisting of corn cobs, crop residues, corn husks, corn stover, grasses, wheat straw, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

30

4. A method according to Claim 1 wherein the fermentable carbon substrate is selected from the group consisting of monosaccharides, oligosaccharides, and polysaccharides.
5. A method according to Claim 1 wherein the fermentation culture is maintained under conditions selected from the group consisting of anaerobic conditions and microaerobic conditions.
6. A method according to Claim 1 wherein while growing the production host of (c) at a first temperature over a first period of time, a metabolic parameter of the fermentation culture is monitored.
7. A method according to Claim 6 wherein the metabolic parameter that is monitored is selected from the group consisting of optical density, pH, respiratory quotient, fermentable carbon substrate utilization, CO₂ production, and 1-butanol production.
8. A method according to Claim 1 wherein lowering the temperature of the fermentation culture of step (d) occurs at a predetermined time.
9. A method according to Claim 1 wherein the lowering of the temperature of the fermentation culture of step (d) coincides with a change in a metabolic parameter.
10. A method according to Claim 9 wherein the change in metabolic parameter is a decrease in the rate of 1-butanol production.
11. A method according to Claim 1 wherein the first temperature is from about 25 °C to about 40 °C.
12. A method according to Claim 1 wherein the second temperature is from about 3 °C to about 25 °C lower than the first temperature.

13. A method according to Claim 1 wherein steps (d) and (e) are repeated one or more times.

5 14. A method according to Claim 1 wherein the recombinant microbial production host is selected from the group consisting of *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*,
10 *Saccharomyces*, and *Pichia*.

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

- a) acetyl-CoA to acetoacetyl-CoA;
- b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA;
- c) 3-hydroxybutyryl-CoA to crotonyl-CoA;
- d) crotonyl-CoA to butyryl-CoA;
- 20 e) butyryl-CoA to butyraldehyde; and
- f) butyraldehyde to 1-butanol;

wherein the at least one DNA molecule is heterologous to said microbial production host cell.

25 16. A method according to Claim 15 wherein the polypeptide that catalyzes a substrate to product conversion of acetyl-CoA to acetoacetyl-CoA is acetyl-CoA acetyltransferase.

30 17. A method according to Claim 15 wherein the polypeptide that catalyzes a substrate to product conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA is 3-hydroxybutyryl-CoA dehydrogenase.

18. A method according to Claim 15 wherein the polypeptide that catalyzes a substrate to product conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA is crotonase.

5 19. A method according to Claim 15 wherein the polypeptide that catalyzes a substrate to product conversion of crotonyl-CoA to butyryl-CoA is butyryl-CoA dehydrogenase.

10 20. A method according to Claim 15 wherein the polypeptide that catalyzes a substrate to product conversion of butyryl-CoA to butyraldehyde is butyraldehyde dehydrogenase.

15 21. A method according to Claim 15 wherein the polypeptide that catalyzes a substrate to product conversion of butyraldehyde to 1-butanol is butanol dehydrogenase.

22. A method according to Claim 16 wherein the acetyl-CoA acetyltransferase has an amino acid sequence having at least 95% identity to an amino acid sequence selected from the group consisting of
20 SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:129, SEQ ID NO:131, and SEQ ID NO:133 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.

25 23. A method according to Claim 17 wherein the 3-hydroxybutyryl-CoA 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:6, SEQ ID NO:135, SEQ ID NO:137, and SEQ ID NO:139 based on the Clustal W method of alignment using the default
30 parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

24. A method according to Claim 18 wherein the crotonase has an amino acid sequence having at least 95% identity to an amino acid

sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:141, SEQ ID NO:143, and SEQ ID NO:145 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
5 matrix.

25. A method according to Claim 19 wherein the butyryl-CoA dehydrogenase has an amino acid sequence having at least 95% identity to an amino acid sequence selected from the group consisting of SEQ ID
10 NO:10, SEQ ID NO:147, SEQ ID NO:149, SEQ ID NO:151, and SEQ ID NO:187 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 26. A method according to Claim 20 wherein the butyraldehyde 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:12, SEQ ID NO:153, and SEQ ID NO:189 based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10,
20 GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

27. A method according to Claim 21 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
25 NO:14, SEQ ID NO:16, SEQ ID NO:153, SEQ ID NO:155, and SEQ ID NO:157 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.

Figure 1

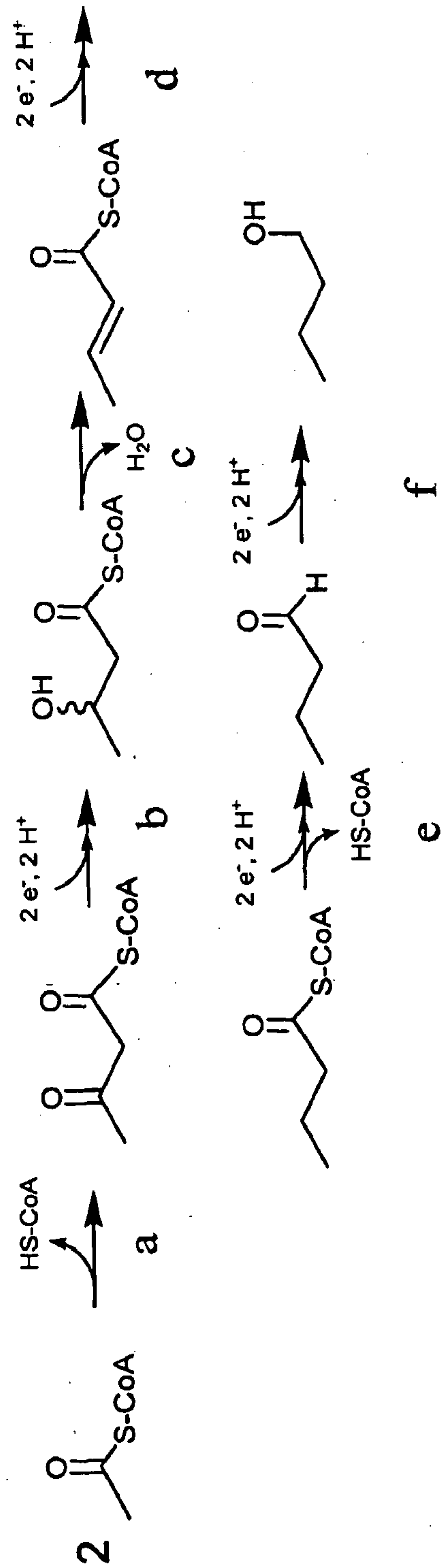


Figure 1

