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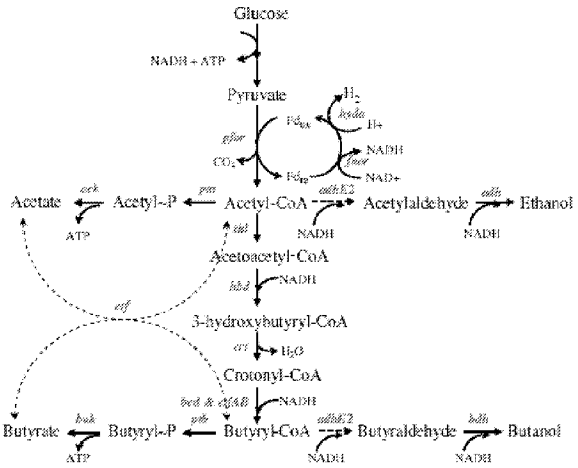

FIG. 1

(57) **Abstract**: This invention relates to compositions, systems, and methods for producing biofuels, such as butanol, and related compounds. More specifically, provided are methods of making recombinant microorganisms having non-naturally occurring metabolic pathways for the production of biofuels, and methods of producing biofuels using such organisms. Also provided are metabolically engineered microorganisms capable of producing butanol from a substrate.

WO 2012/045022 A2

METABOLIC ENGINEERING OF CLOSTRIDIUM TYROBUTYRICUM FOR BUTANOL PRODUCTION

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CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority from US Provisional Patent Application Number 61/389,060, filed October 1, 2010, which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY-SPONSORED R & D

[0002] The present invention was made with government support under a grant awarded by the National Science Foundation. The government has certain rights in the invention under 35 U.S.C. §200 et seq.

TECHNICAL FIELD

[0003] Metabolically engineered microorganisms and methods of producing biofuels, particularly metabolically engineered microorganisms and methods of producing biobutanol from sugars.

BACKGROUND OF THE INVENTION

[0004] With concerns about greenhouse gas emissions and uncertainty about the supply of oil, renewable biofuels have gained increasing attention. Bioethanol, the current major biofuel, is not an ideal replacement of gasoline because of its low energy density, high water solubility and high vapor pressure. Compared to ethanol, butanol is more hydrophobic, has a more similar energy content (27 MJ/L) to that of gasoline (32 MJ/L) and can be transported in the existing pipeline infrastructure. Thus, butanol is considered as a better drop-in biofuel than ethanol.

[0005] Butanol can be produced by anaerobic microorganisms such as *Clostridium acetobutylicum* and *Clostridium beijerinckii* in acetone-butanol-ethanol fermentation (ABE fermentation), which was once the second largest industrial fermentation in the world. In a typical ABE fermentation, butyrate and acetate are produced first, and then the culture undergoes a metabolic shift and solvents (butanol, acetone, and ethanol) are formed. Due to the complicated metabolic pathways involving

acidogenesis and solventogenesis, plus spore-forming life cycle, ABE fermentation is difficult to control or manipulate. Furthermore, ABE fermentation usually suffers from low butanol yield (~20% w/w), titer (<15 g/L) and productivity (<0.5 g/L·h) because of butanol toxicity and production of other byproducts including acetone, ethanol, acetate and butyrate. The low reactor productivity, butanol yield, and final butanol concentration make biobutanol from ABE fermentation uneconomical for the fuel market.

[0006] Since the first oil crisis in the early 1980's, there have been numerous attempts to improve butanol production via metabolic engineering of *C. acetobutylicum* and process engineering to alleviate inhibition caused by butanol and facilitate product recovery. Genetic engineering of *C. acetobutylicum* has failed to result in large increases in butanol yields and product titers to economically favorable levels. This is largely due to the fact that high concentrations of butanol are toxic to the bacteria that produce the solvent. To date, no genetically engineered *C. acetobutylicum* and related strains can meet the requirements for industrial use in butanol production.

[0007] There have been efforts to engineer non-solventogenic microbes, including *E. coli, S. cerevisiae, B. subtilis*, *P. putida* and *L. brevis*, for butanol production because of their potentially higher butanol tolerance and because they are easier to clone than Clostridia. Some microbes, including several strains of *P. putida* can tolerate up to 6% (w/v) butanol, although the butanol toxic threshold is between 1% and 2% (w/v) for most microorganisms. Most *S. cerevisiae* strains are tolerant to 1% (v/v) butanol with 60% relative specific growth rate, and three strains can grow in 2% (v/v) butanol with 10%-20% relative growth rate. For *E. coli,* its growth rate decreased to 20%-40% and 40%-60% at 1% (v/v) butanol at 37℃ and 30℃, respectively, and no growth was observed at 2% (v/v) butanol.

[0008] The Clostridia's solventogenic pathway, which involves multiple genes, has been cloned and expressed in several microorganisms. However, the highest butanol titer obtained so far was only 0.580 g/L in *E. coli*, 0.300 g/L in *L. brevis*, 0.120 g/L in *P. putida*, 0.024 g/L in *B. subtilis,* and 0.0025 g/L in *S. cerevisiae*. The slow and reversible turn-over rate of *Clostridium* butyryl dehydrogenase complex (*bdh*) could limit the butanol production. A keto-acid pathway has also been expressed in *E. coli* for n-butanol production, achieving butanol titer of 800 mg/L. Recently, a higher n-butanol

titer of 4.65 g/L was obtained using a chimeric pathway assembled from three different organisms (*R. eutrophus pha*, *C. acetobutylicum hbd*, *crt*, and *T. denticola ter* replacing *bdh*) and overexpressing native pyruvate dehydrogenase complex (*aceEF.lpd*) in *E. coli*. Furthermore, by increasing the availability of NADH, which is required for butanol biosynthesis, approximately 15 g/L of *n*-butanol was produced from glucose by the recombinant *E. coli* with an approximately 80% theoretical yield. Nevertheless, it has remained desirable to find non-solventogenic hosts that can produce n-butanol at a titer comparable to or higher than that from solventogenic Clostridia, which usually produce 12 g/L to 16 g/L butanol in ABE fermentation.

SUMMARY OF THE INVENTIVE CONCEPT

[0009] Provided herein are metabolically-engineered microorganisms that include recombinant biochemical pathways useful for producing n-butanol from renewable biomass. Also provided herein are methods of producing biofuels using the microorganisms described herein.

[0010] The invention features recombinant microorganisms capable of producing butanol that are constructed by transferring into a host organism which naturally produces butyric acid as a main metabolic product a foreign gene that encodes an enzyme having both alcohol/aldehyde dehydrogenase and butanol dehydrogenase activity so that the recombinant microorganism is capable of converting butyryl-CoA to butanol. Alternatively, the microorganism may be engineered to overexpress aldehyde/alcohol dehydrogenase 2 (*adhE2*), and convert butyryl-CoA to butanol under the control of a native thiolase (*thl*) promoter.

[0011] In certain embodiments the host organism is *Clostridium tyrobutyricum*, and the gene that encodes an enzyme having both alcohol/aldehyde dehydrogenase and butanol dehydrogenase activity comes from *C. acetobutylicum*.

[0012] In certain embodiments the recombinant microorganism is capable of producing butanol from glucose. In other embodiments the recombinant microorganism is capable of producing butanol from other monosachharides, oligosaccharides, and polysaccharides.

[0013] In certain embodiments the recombinant microorganism is constructed by transferring to the host organism a foreign gene for inactivating acetate kinase or for

inactivating phosphotransacetylase or for inactivating phosphotransbutyrylase. Optionally, these foreign genes may be *ack*, *pta*, or *ptb* knockouts, respectively.

[0014] In certain embodiments the recombinant microorganisms may be Ct(pCAAD), Ct(pSAD42), Ct(pMAD72), Ct(pMAD22), AckKO(pMAD72), AckKO(pMAD22), PtaKO(pMAD72), PtaKO(pMAD22), PtbKO(pMAD72), or PtbKO(pMAD22).

[0015] In another aspect, the invention features vectors that enable a host organism to produce increased amounts of butanol. The vectors may be plasmids that contain genes for carrying out the functions of alcohol/aldehyde dehydrogenase and butanol dehydrogenase. In certain embodiments the vectors contain the *adhE2* gene. Depending on the embodiment, the vector may be pSAD42, pMAD72, or pMAD22. The vectors may be transferred into *C. tyrobutyricum* to overexpress *adhE2* for butanol production from glucose or other monosaccharides, oligosaccharides, or polysaccharides, along with *pta, ptb*, or *ack* knockout genes for enhancing butanol production.

[0016] In another aspect, the invention features methods of producing butanol and other biofuels. In certain of these embodiments, the methods include culturing one or more different recombinant microorganisms in a culture medium, and accumulating butanol in the culture medium. In certain embodiments the methods include culturing recombinant microorganisms containing genes for overexpressing *adhE2*. In other embodiments the methods include culturing recombinant microorganisms that also contain genes for inactivating acetate kinase, genes for inactivating phosphotransacetylase, or genes for inactivating phosphotransbutyrylase. In certain embodiments of the methods the host organism is *C. tyrobutyricum*. In certain embodiments of the methods the culture medium includes glucose. In other embodiments of the methods the culture medium includes other carbon sources, such as starch, sucrose, fructose, galactose, mannitol, and xylose.

[0017] In another aspect, the invention features methods of metabolically engineering microorganisms capable of producing butanol from a substrate. The methods include obtaining microbial hosts capable of naturally producing butyric acid, and transferring to the microbial host a foreign gene that encodes an enzyme having

alcohol/aldehyde dehydrogenase and butanol hydrogenase activity. In certain embodiments the methods also include transferring to the microbial host a foreign gene for inactivating acetate kinase, phosphotransbutyrylase, or phosphotransacetylase. In certain embodiments the microbial host is *C. tyrobutyricum*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] **Figure 1** depicts an exemplary metabolic pathway for *Clostridium tyrobutyricum,* where the pathways for butanol and ethanol formation shown in dotted lines are absent in wild-type *C. tyrobutyricum* and are introduced by overexpressing the *adhE2* gene.

[0019] **Figure 2** depicts a map of plasmid pCAAD.

[0020] **Figure 3** depicts a map of plasmid pSAD42.

[0021] **Figure 4** depicts a map of plasmid pMAD72.

[0022] **Figure 5** depicts fermentation kinetics of *C. tyrobutyricum* mutants Ct(pCAAD) (*Panel A)* and Ct(pSAD42) (*Panel B).*

[0023] **Figure 6** depicts a comparison of butyraldehyde dehydrogenase and butanol dehydrogenase activities of *C. tyrobutyricum* mutant Ct(pMAD72) and Ct(pMTL007).

[0024] **Figure 7** depicts fermentation kinetics of *C. tyrobutyricum* mutants with plasmid pMAD72.

[0025] **Figure 8** depicts product distribution and yields from glucose in various *C. tyrobutyricum* mutants.

[0026] **Figure 9** depicts fermentation kinetics of AckKO(pMAD22) with mannitol as the substrate.

[0027] **Figure 10** depicts the effects of butanol on the growth of various *C. tyrobutyricum* mutants.

DETAILED DESCRIPTION

[0028] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent

applications, patents, and other references mentioned herein are incorporated by reference in their entirety for all purposes. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[0029] Any publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[0030] The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way. In addition, the materials, methods, and examples are illustrative only and not intending to be limiting. The use of the singular includes the plural unless specifically stated otherwise. Also, the use of “comprise,” “comprises,” “comprising,” “contain,” “contains,” “containing,” “include,” “includes,” and “including” are not intended to be limiting. It is understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention. The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

[0031] All numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches.

[0032] As utilized in accordance with the embodiments provided herein, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0033] The term "plasmid" refers to a circular nucleic acid vector. Generally, plasmids contain an origin of replication that allows many copies of the plasmid to be produced in a bacterial (or sometimes eukaryotic) cell without integration of the plasmid into the host cell DNA.

[0034] The term "construct" as used herein refers to a recombinant nucleotide sequence, generally a recombinant nucleic acid molecule, that has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences. In general, "construct" is used herein to refer to a recombinant nucleic acid molecule.

[0035] The term "host cell" refers to a cell that is to be transformed using the methods and compositions of the invention. In general, host cell as used herein means a microorganism cell into which a nucleic acid of interest is to be transformed.

[0036] The term "transformation" refers to a permanent or transient genetic change, preferably a permanent genetic change, induced in a cell following incorporation of non-host nucleic acid sequences. Transformation (or transduction, or transfection), can be achieved by any one of a number of means including electroporation, conjugation, microinjection, biolistics (or particle bombardment-mediated delivery), or *agrobacterium* mediated transformation.

[0037] The term "vector" generally refers to a polynucleotide that can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include viruses, bacteriophage, pro-viruses, plasmids, phagemids, transposons, and artificial chromosomes, that are able to replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can be an organism which comprises one or more of the above polynucleotide constructs such as an *agrobacterium*.

[0038] The term "promoter" refers to a minimal nucleic acid sequence sufficient to direct transcription of a nucleic acid sequence to which it is operably linked. The term "promoter" is also meant to encompass those promoter elements sufficient for promoter-

dependent gene expression controllable for cell-type specific expression or inducible by external signals or agents; such elements may be located in the 5′ or 3′ regions of the naturally-occurring gene.

[0039] The term “native” or “wild-type” as used with a protein, enzyme, polynucleotide, gene, or cell, means a protein, enzyme, polynucleotide, gene, or cell that occurs in nature.

[0040] The term “n-butanol” generally refers to a straight chain isomer with the alcohol functional group at the terminal carbon. Recombinant microorganisms provided herein can express a plurality of target enzymes involved in pathways for the production of n-butanol from a suitable carbon substrate.

[0041] Accordingly, metabolically “engineered” or “modified” organisms are produced via the introduction of genetic material into a host or parental microorganism of choice thereby modifying or altering the cellular physiology and biochemistry of the microorganism. Through the introduction of genetic material the parental microorganism acquires new properties, e.g., the ability to produce a new, or greater quantities of, an intracellular metabolite. In an illustrative embodiment, the introduction of genetic material into a parental microorganism acquires new properties, e.g., the ability to produce a new, or greater quantities of, an intracellular metabolite. In an illustrative embodiment, the introduction of genetic material into a parental microorganism results in a new or modified ability to produce n-butanol. The genetic material introduced into the parental microorganism contains gene(s), or parts of genes, coding for one or more of the enzymes involved in a biosynthetic pathway for the production of n-butanol and may also include additional elements for the expression and/or regulation of expression of these genes, e.g. promoter sequences.

[0042] An engineered or modified microorganism can also include in the alternative or in addition to the introduction of a genetic material into a host or parental microorganism, the disruption, deletion, or knocking out of a gene or polynucleotide to alter the cellular physiology and biochemistry of the microorganism. Through the reduction, disruption or knocking out of a gene or polynucleotide the microorganism acquires new or improved properties (e.g., the ability to produce a new or greater

quantities of an intracellular metabolite, improve the flux of a metabolite down a desired pathway, and/or reduce the production of undesirable by-products).

[0043] Microorganisms provided herein are modified to produce metabolites in quantities not available in the parental organism. A "metabolite" refers to any substance produced by metabolism or a substance necessary for taking part in a particular metabolic process. A metabolite can be an organic compound that is a starting material (e.g., glucose), an intermediate (e.g., acetyl-CoA) in, or an end product (e.g., n-butanol) of metabolism.

[0044] The disclosure provides recombinant microorganisms that produce *n*-butanol and include the expression or elevated expression of target enzymes such as aldehyde/alcohol dehydrogenase (*adhE2*) as compared to a parental microorganism. In addition, the modified microorganism may include a disruption, deletion or knockout of expression of phosphotransbutyrylase (*ptb*), phosphotransacetylase (*pta*), or acetate kinase (*ack*), as compared to a parental microorganism.

[0045] EXAMPLES

[0046] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those skilled in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

[0047] As shown in Figure 1, the pathways for butanol and ethanol formation (shown by the dotted lines) are absent in wild-type *C. tyrobutyricum* and are introduced by overexpressing the *adhE2* gene. Key enzymes and genes in the pathway include the

following: hydrogenase (*hydra*); pyruvate: ferredoxin oxidoreductase (*pfor*); ferredoxin NAD^+ oxidoreductase (*fnor*); acetate kinase (*ack*); phosphotransacetylase (*pta*); thiolase (*thl*); beta-hydroxybutyryl-CoA dehydrogenase (*hbd*); crotonase (*crt*); butyryl-CoA dehydrogenase (*bcd*); electron transferring flavoprotein (*etf*); phosphotransbutyrylase (*ptb*); butyrate kinase (*buk*); CoA transferase (*ctf*); alcohol dehydrogenase (*adh*); butanol dehydrogenase (*bdh*); aldehyde-alcohol dehydrogenase (*adhE2*).

[0048] Bacterial strains and growth conditions

[0049] Bacterial strains and plasmids used in the examples are listed in Table 1.

[0050] Table 1: Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Reference or source
Strains		
C. tyrobutyricum	ATCC 25755	ATCC
AckKO	Ack knockout	Liu et al, 2006
PtbKO	Ptb knockout	Zhang, 2009
PtaKO	Pta knockout	Zhu et al, 2005
Ct(pCAAD)	ATCC 25755 with pCAAD	The examples
Ct(pSAD42)	ATCC 25755 with pSAD42	The examples
Ct(pMTL007)	ATCC 25755 with pMTL007	The examples
Ct(pMAD72)	ATCC 25755 with pMAD72	The examples
Ct(pMAD22)	ATCC 25755 with pMAD22	The examples
AckKO(pMAD72)	AckKO with PMAD72	The examples
AckKO(pMAD22)	AckKO with pMAD22	The examples
PtbKO(pMAD72)	PtbKO with pMAD72	The examples
PtbKO(pMAD22)	PtbKO with pMAD22	The examples
PtaKO(pMAD72)	PtaKO with pMAD72	The examples
PtaKO(pMAD22)	PtaKO with pMAD22	The examples
E. coli CA434	*E. coli* HB101 with plasmid R702	Williams et al, 1990

Plasmids		
pCAAD	ColE1 ori; Amp^R; Em^R; pIM13 ori; Paad::*aad*	Nair et al, 1994
pSAD42	ColE1 ori; Amp^R; Em^R; pIM13 ori; Paad::*aad*	The examples
pMTL82151	ColE1 ori; Cm^R; pBP1 ori; TraJ; oriT	Heap et al, 2009
pMTL007	ColE1 ori; Cm^R; pCB102 ori; TraJ; oriT	Heap et al, 2007
pMAD72	From pMTL007; Pthl::*adhE2*	The examples
pMAD22	From pMTL82151; Pthl::*adhE2*	The examples

Ack, acetate kinase; Ptb, phosphotransbutyrylase; Pta, phosphotransacetylase; KO, knock-out; ack, acetate kinase; adhE2, aldehyde-alcohol dehydrogenase; ori, origin; Amp^R, ampicillin resistance; Em^R, erythromycin resistance; Cm^R, chloramphenicol resistance; TraJ and *oriT*: genes for conjugation; ColE1, *E. coli* replicon; Paad, *aad promoter from C. acetobutylicum;* P*thl: thl* promoter from *C. tyrobutyricum;* pIM13, replicon from *B. subtilis;* pCB102, replicon from *C. butyricum*; pBP1, replicon from *C. botulinum.*

[0051] Bacterial strains and culture media

[0052] *Clostridium tyrobutyricum ("C. tyrobutyricum"),* ATCC 25755 was the parental strain of various mutant strains (see Table 1) developed in this work. *C. acetobutylicum* ATCC 824 was used to extract *adhE2* gene from its genome. *E. coli* DH5α (Invitrogen, Carlsbad, CA) was used in the preparation of recombinant plasmids and *E. coli* CA434 as the donor strain in conjugation described later. Unless otherwise noted, *C. tyrobutyricum* and *C. acetobutylicum* were grown anaerobically at 37ºC in Reinforced Clostridial Medium (RCM; Difco, Detroit, MI). Colonies were maintained on RCM (15 g/L agar) plates in the anaerobic chamber. These media were supplemented with 40 µg/mL erythromycin or 30 µg/mL thiamphenicol for transformant selection. *E. coli* was grown aerobically at 37ºC and 250 rpm in Luria-Bertani (LB) media supplemented with chloramphenicol (30 µg/mL), ampicillin (100 µg/mL) or kanamycin (50 µg/mL).

[0053] Plasmid construction

[0054] Several recombinant plasmids were constructed and used to create various butanol-producing *C. tyrobutyricum* mutant strains (See Table 1). Table 2 below provides a list of exemplary oligonucleotide primers, as well as their nucleic acid sequence. It is understood that alternative sequences may also be used to amplify a target nucleic acid. Accordingly, the methods described herein are not limited solely to the primers described below.

[0055] Table 2: Primers used in PCR amplification of *adhE2* gene and *thl* promoter sequence.

Primer	DNA Sequence
Amplification of *adhE2* gene from ATCC 824 genomic DNA – Forward Primer (SEQ ID No: 1)	5′-AT**GGATCC**TTTTATAAAGGAGTGTATATAAATGAAAG-3′
Amplification of *adhE2* gene from ATCC 824 genomic DNA – Reverse Primer (SEQ ID No: 2)	5′-TT**GGCGCC**ATAATGAAGCAAAGACTATTTTACATTC-3′
Amplification of *adhE2* gene from plasmid pSAD42 – Forward Primer (SEQ ID No: 3)	5′-AT***GGATCC***ATAAATATTT**AGGAGG**AATAGTCATGAAAGTT ACAAATCAAAAAGAAC-3′
Amplification of *adhE2* gene from plasmid pSAD42 – Reverse Primer (SEQ ID No: 4)	5′-TCTA***CCGCGG***ATAATGAAGCAAAGACTATTTTACATTC-3′
Amplification of *thl* promoter sequence from ATCC 25755 genomic DNA – Forward Primer (SEQ ID No: 5)	5′-AGCT**AAGCTT**CTGAATATTCAGCGAAAATAG-3′
Amplification of *thl* promoter sequence from ATCC 25755 genomic DNA – Reverse Primer (SEQ ID No: 6)	5′-TCTA***CCGCGG***ACGTC***GGATCC***AAATTTAAATTGATTACAAA CCTTTTTACC-3′

[0056] Plasmid pCAAD, a map of which is depicted in Figure 2, containing *aad* gene and native *aad* promoter from *C. acetobutylicum* ATCC 824, was obtained from Professor G.N. Bennett of Rice University (Nair et al., 1994). Plasmids pSAD42 (map depicted in Figure 3) and pMAD72 (map depicted in Figure 4) were created by cloning *adhE2* into plasmids pSOS94 (Genbank Accession No. AY187685, SEQ ID No: 7) and pMTL007 (Genbank Accession No. EF525477, SEQ. ID No: 8), respectively. The construction of these recombinant plasmids is briefly described below.

[0057] The *adhE*2 gene (Genbank Accession No. AF321779, SEQ. ID No: 9) was PCR-amplified from *C. acetobutylicum* ATCC 824 genomic DNA. The PCR product was purified and digested with *Bam*HI (New England Biolabs, Ipswich, MA) and *Sfo*I (New

England Biolabs, Ipswich, MA), and then ligated into plasmid pSOS94 digested with the same restriction enzymes to generate recombinant pSAD42. To construct recombinant pMAD72, *adhE2* was PCR-amplified from pSAD42, and *thl* promoter (Genbank Accession No. HM989902, SEQ. ID No: 10) was PCR-amplified from *C. tyrobutyricum* ATCC 25755 genomic DNA. The PCR product of *thl* promoter (P*thl*) was ligated into pGEM-T vector (Promega, Madison, WI) to generate pGEM-T-Pthl. Plasmid pGEM-T-Pthl and *adhE2* PCR product were digested with *Bam*HI and *Sac*II (New England Biolabs, Ipswich, MA) and ligated to generate pGEM-T-PthladhE2. PthladhE2, from pGEM-T-PthladhE2 after treating with *Hin*dIII (New England Biolabs, Ipswich, MA) and *Sac*II, was ligated into plasmids pMTL007 and pMTL82151, after *Hin*dIII and *Sac*II digestion, to generate pMAD72 and pMAD22, respectively. All recombinant plasmids were transformed into *E. coli* DH5α (Invitrogen) and purified plasmids were confirmed by DNA sequencing.

[0058] Transformation

[0059] Unless otherwise noted, all transformation procedures were performed in an anaerobic chamber. Plasmids of pCAAD and pSAD42 were transformed into *C. tyrobutyricum* by electroporation following the previously described method. Two mutants, Ct(pCAAD) and Ct(pSAD42), were obtained by transforming ATCC 25755 with pCAAD or pSAD42, respectively.

[0060] *C. tyrobutyricum* was also transformed with pMAD72 via conjugation following the procedures described below. The donor strain, *E. coli* CA434 (*E. coli* HB101 with IncPβ conjugative plasmid R702), was first transformed with the recombinant plasmid to be mobilized by electroporation, and then the transformed cells were collected by centrifugation after culturing in LB medium containing 30 μg/mL chloramphenicol at 37ºC and 250 rpm overnight to reach OD600 1.5~2.0. About 3 mL of the donor *E. coli* cells were then washed once in 1 mL of sterile phosphate-buffered saline (PBS) and resuspended into 0.4 mL of the recipient *C. tyrobutyricum* cells, which were grown overnight to OD600 2.0~3.0 in the RCM medium at 37ºC. The cell mixture was then pipetted onto well-dried RCM agar plate and incubated at 37ºC for 24 hours for mating. One milliliter of the RCM medium was then applied to each conjugation plate to harvest cells, which were then re-plated on RCM plates with 25 μg/mL thiamphenicol

(selection for plasmid uptake) and 200 µg/mL D-cycloserine (counter selection for *E. coli*). Plates were incubated for 48 to 96 hours or until colonies were apparent. Four mutants, Ct(pMAD72), AckKO(pMAD72), PtaKO(pMAD72) and PtbKO(pMAD72), were obtained by transforming pMAD72 into *C. tyrobutyricum* ATCC 25755, AckKO with *ack* knock-out (using a partial *ack* gene having GenBank Accession No. AY706093; SEQ. ID No. 11), PtaKO with *pta* knock-out (using a partial *pta* gene having GenBank Accession No. AY572855; SEQ. ID No. 12) and PtbKO with *ptb* knock-out (using a partial *ptb* fragment identical to the *ptb* sequence from *C. acetobutylicum,* ATCC 824, gene ID1119259, E.C. 2.1.3.19), respectively. Likewise, pMAD22 was also transformed into *C. tyrobutyricum* ATCC 25755, AckKO, PtaKO, and PtbKO to obtain Ct(pMAD22), AckKO(pMAD22), PtaKO(pMAD22), and PtbKO(pMAD22), respectively. In addition, *C. tyrobutyricum* ATCC 25755 was also transformed with pMTL007 (without *adhE2* gene) to obtain the strain Ct(pMTL007), which was used as a control in the fermentation kinetics study described below. AckKO(pMAD72) was deposited with the ATCC in Manassas, Virginia prior to the filing of this application, and was granted ATCC Deposit No. _____.

[0061] Fermentation Kinetics

[0062] Unless otherwise noted, batch fermentations with *C. tyrobutyricum* mutants were carried out in serum bottles containing P2 medium (50 g/L glucose, 3 g/L yeast extract, 1 g/L tryptone, 2 g/L K_2HPO_4, 2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.01 g/L $MnSO_4$, 0.01 g/L $FeSO_4$, 0.01 g/L NaCl) supplemented with 40 µg/mL erythromycin or 25 µg/mL thiamphenicol as required. Each bottle containing 40 mL of the medium was inoculated with 0.4 mL of cells from an overnight culture in RCM. The fermentation was carried out at 37ºC and the medium pH was maintained between 5.0 and 6.5 by adding NaOH solution twice a day. Samples were taken at regular intervals to monitor cell growth, substrate (glucose) consumption and production of butanol, ethanol, acetic acid and butyric acid during the fermentation.

[0063] *AdhE2* Enzyme Activity Assays

[0064] The activities of butyraldehyde dehydrogenase and butanol dehydrogenase of *adhE2* were measured by monitoring NADH consumption at 365 nm according to the method described before with some modifications. Cells were collected

from 100 mL fermentation broth by centrifugation at 10000 g for 10 min using tightly sealed centrifuge tubes purged by nitrogen gas. The cell pellet was washed with Tris-HCl buffer (0.1 M, pH 7.5) once and resuspended in 5 mL of the same Tris-HCl buffer. Lysis was carried out by using a French press with one passage at 77 MPa. Supernatant was collected by centrifugation at 15000 g for 10 minutes and used for enzyme activity assay. Enzyme activity was calculated on the basis of a molar NADH extinction coefficient of 3.4 cm^{-1} mM^{-1}. One unit of enzyme activity was defined as the amount of enzyme which converts 1 µmol NADH per minute under the reaction conditions. Protein concentration in cell extract was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, California) with bovine serum albumin as standard.

[0065] Butanol Tolerance

[0066] To evaluate the butanol tolerance of various *C. tyrobutyricum* mutants and *C. beijerinckii* ATCC 55025, cells were cultured in 50 mL centrifuge tubes, with caps tightly sealed, in an anaerobic chamber. Each tube containing 5 mL RCM medium and butanol at a concentration of up to 2% (v/v) was inoculated with 0.2 mL of cells from an overnight culture in RCM. Cell growth was monitored by measuring the optical density at 600 nm (OD600) of the culture broth. The specific growth rates at various initial butanol concentrations were estimated from OD600 data.

[0067] Analytical Methods

[0068] Cell density was analyzed by measuring the optical density of cell suspension at 600 nm using a spectrophotometer (UV-16-1, Shimadzu, Columbia, MD). The glucose concentration was determined with a YSI 2700 Select Biochemistry Analyzer (Yellow Springs, Ohio). Butanol, ethanol, acetic acid and butyric acid were analyzed with a gas chromatograph (GC) (GC-2014 Shimadzu, Columbia, MD) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (Stabilwax-DA, 0.25 mm film thickness and 0.25 mm ID, Restek, Bellefonte, PA). The GC was operated at an injection temperature of 200ºC with 1 µL of sample injected with an auto injector (AOC-20i, Shimadzu). The column temperature was initially held at 80ºC for 3 min, then increased at a constant rate of 30ºC per min to 150ºC, and held at 150ºC for 3.7 min.

[0069] Butanol Production by Mutants

[0070] The wild-type strain (ATCC 25755) does not produce any detectable butanol from glucose because it does not have aldehyde/alcohol dehydrogenase genes needed to convert butyryl-CoA to butanol. Mutants were selected and tested for alcohol (ethanol and butanol) production in batch fermentations with glucose as the substrate following the procedure outlined under Fermentation Kinetics. Figure 5 is a graphical representation of glucose consumption and product formation from batch fermentations with *C. tyrobutyricum* mutants Ct(pCAAD) and Ct(pSAD42). As shown in Figure 5, both *C. tyrobutyricum* mutants Ct(pCAAD) and Ct(pSAD42) mainly produce butyric acid and acetic acid although butanol and ethanol are also produced. The final butanol titers obtained in these fermentations are 0.067 g/L in Ct(pCAAD) and 0.019 g/L in Ct(pSAD42). Furthermore, these mutants were not stable and could not be maintained in liquid cultures, suggesting poor plasmid stability in the host.

[0071] Plasmid pMTL007 with native *thl* promoter from *C. tyrobutyricum* was used to express *adhE*2 gene in *C. tyrobutyricum* for butanol production. The native *thl* promoter is a strong and constitutive promoter and was thus used to control the expression of *adhE*2 gene. As can be seen in Figure 6, the activities of butyraldehyde dehydrogenase (0.025 U/mg) and butanol dehydrogenase (0.08 U/mg) of *adhE*2 increased about 10-fold in the mutant Ct(pMAD72) as compared to those in the wild-type control. Consequently, mutant Ct(pMAD72) produced 1.1 g/L butanol and 0.14 g/L ethanol in batch fermentation while the control (wild type with pMTL 007 without *adhE2*) produced little or no detectable amounts of butanol and ethanol (see Figure 7). Compared to the control, the mutant Ct(pMAD72) produced less butyric acid (7.8 g/L vs. 9.7 g/L for the control) and more acetic acid (2.8 g/L vs. 2.1 g/L for the control). It is theorized that the decreased butyric acid production can be attributed to the conversion of some butyryl-CoA to butanol, whereas the slightly increased acetic acid production could be due to the need to compensate for the loss of ATP generation from butyric acid biosynthesis in the mutant. Compared to mutants with pCAAD and pSAD42, mutant Ct(pMAD72) with pMAD72 produced much more butanol (1.1 g/L vs. <0.1 g/L).

[0072] Figures 7C and 7D are graphical representations of glucose consumption and product formation from batch fermentations with *ack* and *ptb* knockout mutants of

C. tyrobutyricum expressing *adhE*2. With *ptb* knockout, mutant PtbKO(pMAD72) produced 1.7 g/L butanol, a 55% increase over the 1.1 g/L produced by Ct(pMAD72). The increase in butanol production was expected as less butyryl-CoA was converted to butyric acid because of the *ptb* knockout. However, butyric acid production was still high (9.2 g/L vs. 9.7 g/L by Ct(pMTL007) and 7.8 g/L by Ct(pMAD72)). Compared to Ct(pMAD72), more acetic acid (5.3 g/L) and ethanol (0.2 g/L) were produced in PtbKO(pMAD72), indicating that more carbon substrates were directed towards C2 products.

[0073] A higher butyrate/acetate ratio with higher butyrate titer and yield were obtained with *C. tyrobutyricum* mutant with *ack* knockout. This mutant was used as the host to express *adhE2* for butanol production, and the fermentation results are shown in Figure 7C. Compared to Ct(pMAD72), AckKO(pMAD72) produced significantly more butanol (10.0 g/L vs. 1.1 g/L) and ethanol (0.7 g/L vs. 0.14 g/L) and less butyric acid (5.8 g/L vs. 7.8 g/L) and acetic acid (0.22 g/L vs. 2.8 g/L). The reduced acetic acid production in AckKO(pMAD72) can be attributed to the blocking of acetate biosynthesis pathway by *ack* knockout, which also resulted in increased butanol production because of increased carbon flux toward butyryl-CoA. As expected, AckKO(pMAD72) produced more C4 products (15.8 g/L butanol and butyric acid) and fewer C2 products (0.29 g/L acetic acid and ethanol) than did Ct(pMAD72) (8.9 g/L C4 products and 2.94 g/L C2 products) and PtbKO(pMAD72) (10.9 g/L C4 products and 5.5 g/L C2 products) because of the greater carbon flux toward butyryl-CoA than toward acetyl-CoA in AckKO(pMAD72). Also, AckKO(pMAD72) had a significantly lower specific growth rate than the other mutants studied (see Table 3 below) presumably because reduced acetate and butyrate production resulted in less ATP generation.

[0074] Table 3: Comparison of cell growth and final product concentrations from various mutant strains of *C. tyrobutyricum*.

Strain	Max. OD600	Specific growth rate (h^{-1})	Butanol (g/L)	Butyrate (g/L)	Ethanol (g/L)	Acetate (g/L)	C4/C2 ratio (mol/mol)
Ct-WT	3.4±0.11	0.208±0.003	0	9.26±0.13	0.11±0.02	2.1±0.12	2.82±0.19
Ct(pCAAD)	3.3±0.15	0.189±0.004	0.067±0.004	8.45±0.30	0.12±0.006	3.2±0.21	1.77±0.12

Ct(pSAD42)	3.2±0.10	0.199±0.010	0.019±0.002	8.85±0.32	0.12±0.006	3.0±0.20	1.9±0.13
Ct(pMTL007)	3.4±0.09	0.206±0.004	0	9.7±0.47	0.08±0.02	2.1±0.26	3.0±0.24
Ct(pMAD72)	3.5±0.08	0.180±0.002	1.1±0.1	7.8±0.29	0.14±0.01	2.8±0.30	2.1±0.28
PtbKO(pMAD72)	4.0±0.10	0.195±0.005	1.7±0.25	9.2±0.36	0.2±0.005	5.3±0.22	1.4±0.06
AckKO(pMAD72)	3.5±0.20	0.115±0.011	10.0±0.71	5.8±0.21	0.7±0.035	0.22±0.035	10.6±0.55

Data are mean ± s.d. (n = 3).

[0075] Table 3 summarizes the fermentation results from all mutants. With AckKO(pMAD72), one mol glucose yielded 0.64 mol butanol and 0.31 mol butyric acid, together accounting for 95% glucose fermented. The ratio of its C4 products (butanol and butyric acid) to C2 products is 10.6, much higher than 3.0 in wild-type. With PtbKO(pMAD72) one mol glucose produced 0.11 mol butanol and 0.50 mol butyric acid, and with Ct(pMAD72), one mol glucose produced 0.046 mol butanol and 0.42 mol butyric acid. Figure 8 shows the product distribution and yields from glucose in various mutants (*Panel A:* product distribution, *Panel B:* product yields). The mutant AckKO(pMAD72) produced the highest amount of butanol (10.0 g/L) with 66% theoretical yield or 0.27 g/g glucose fermented.

[0076] Figure 9 shows the fermentation kinetics of AckKO(pMAD22) with mannitol as the substrate in serum bottles. Higher butanol titer (~16 g/L vs. 10 g/L) and yield (~30.6% vs. 27% w/w) were obtained with lower acetate and butyrate production (~1.0 g/L vs. >5 g/L) when the more reduced substrate (mannitol vs. glucose) was used in the fermentation. When the fermentation was carried out in a bioreactor with pH controlled at ~6.0 in a rich medium with mannitol as the substrate, ~20.5 g/L of butanol was produced in ~60 hours with a butanol yield of ~33% (w/w) and productivity of 0.32 g/L/h.

[0077] Reducing hydrogen production by blocking or inhibiting hydrogenase can direct more reducing power to butanol production. Thus, using a hydrogenase inhibitor, such as methyl viologen or neutral red, significantly decreased acids production and enhanced butanol production from glucose, thus increasing the butanol yield and productivity. In batch fermentation in serum bottles, butanol production by AckKO(pMAD22) reached 10.6 g/L within 48 hours and butanol yield increased to 0.28

g/g glucose, whereas acetate and butyrate production decreased to less than 1 g/L. This work illustrated that inhibiting hydrogenase to reduce or block hydrogen production can greatly enhance butanol production and decrease acid production from glucose.

[0078] Besides glucose and mannitol as illustrated in the above examples, other common carbon sources, including starch, sucrose, fructose, galactose, and pentoses such as xylose, can also be readily converted to butanol by the various mutants of *C. tyrobutyricum* expressing the *adhE2* gene.

[0079] Butanol, with a four-carbon chain length, is more toxic to microbes than ethanol. Butanol tolerance is a critical factor limiting a cell's ability to produce butanol. To assess butanol tolerance, the effect of butanol concentration in the culture medium on cell growth was studied, and the results are shown in Figure 10 (*Panel A:* Ct(pMTL007); *Panel B:* Ct(pMAD72); *Panel C:* AckKO(pMAD72); *Panel D:* PtbKO(pMAD72); *Panel E:* Relative Growth Rate. In general, 0.5% (v/v) butanol does not have any apparent effect on cell growth for the strains studied. As the butanol concentration increases, growth inhibition is observed. Cell growth decreases by from about 5% to more than 20% at 1.0% (v/v) butanol. At 1.5% (v/v) butanol, cell growth decreases by from about 30% to about 50%. At 2.0% (v/v) butanol, no significant cell growth was observed for the first 24 hours; however, all strains started to grow after 2 days. These results showed that *C. tyrobutyricum* can tolerate up to 1.5% (v/v) butanol without adaptation and up to about 2.0% (v/v) butanol after adaptation. *C. beijerinckii* ATCC 55025 growth rate was reduced ~20% and ~85% at 1% and 1.5% (v/v) butanol, respectively (Figure 10E), and was completely inhibited by 2% (v/v) butanol as no cell growth was observed even after 10 days incubation. Most solventogenic Clostridia cannot grow at butanol concentrations higher than 1% (v/v) to 1.5% (v/v). Therefore, *C. tyrobutyricum* with high butanol tolerance is a favorable host for butanol production.

[0080] It will be recognized that *Clostridium* strains may be engineered that can not only produce butanol from glucose directly, but also from different polysaccharides and oligosaccharides that may be reduced down to their basic glucose units, and other monosaccharides such as xylose, galactose, and fructose. Lignocellulosic biomass, which is often lower in cost than sugars such as glucose or sucrose, may be used as a substrate for butanol production. Although *C. tyrobutyricum* is used to exemplify the

present invention, other butyric acid producing bacteria including *C. butyricum*, *C. cellulovorans*, *and C. thermobutyricum* can also be used as the host and engineered to produce butanol using the same metabolic engineering technique disclosed in this invention.

[0081] Most butyric acid-producing Clostridia ferment glucose, xylose, lactose, starch and glycerol for cell growth and butyric acid production. Other substrates such as cellulose and CO_2 can also be used by some butyrate-producing Clostridia. For example, *C. carboxidivorans* and *Butyribacterium methylotrophicum* can utilize carbon monoxide and carbon dioxide and hydrogen to produce butyric acid. *C. cellulovorans*, *C. carboxidivorans*, *C. polysaccharolyticum and C. populeti* can use cellulose for butyrate synthesis. *C. kluyveri* can produce butyric acid as a major product from ethanol and acetate as substrates. These bacteria thus can also be used as hosts and engineered to produce butanol from different carbon sources. The preferred strains for commercial uses are those with high butanol tolerance, yield and productivity.

[0082] Other Embodiments

[0083] The foregoing description and Examples detail certain specific embodiments of the invention and describe the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear, the invention can be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

WHAT IS CLAIMED IS:

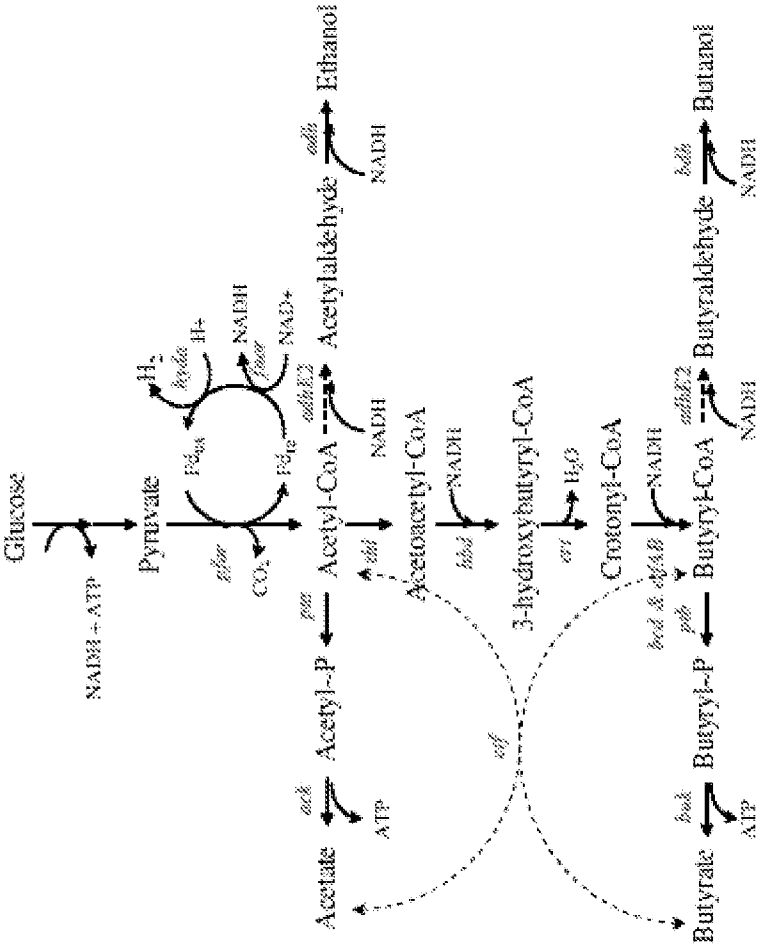
1. A recombinant microorganism capable of producing butanol, which is constructed by transferring a foreign gene that encodes an enzyme having both alcohol/aldehyde dehydrogenase and butanol dehydrogenase activity into a host organism that produces butyric acid as a metabolic product.
2. The recombinant microorganism of claim 1, wherein the foreign gene that encodes an enzyme having both alcohol/aldehyde dehydrogenase and butanol dehydrogenase activity is a DNA comprising the base sequence of SEQ ID. No: 9 or a DNA which hybridizes to a DNA comprising the base sequence of SEQ ID. No: 9 or a complimentary base sequence of SEQ ID. No: 9 under stringent conditions and which encodes a polypeptide having alcohol/aldehyde dehydrogenase and butanol dehydrogenase activity.
3. The recombinant microorganism of claim 1, wherein the host organism is *Clostridium tyrobutyricum*.
4. The recombinant microorganism of claim 1, wherein the host organism is selected from the group consisting of *Clostridium butyricum, Clostridium thermobutyricum, Clostridium cellulovorans, Clostridium carboxidivorans, Butyribacterium methylotrophicum, Clostridium polysaccharolyticum, Clostridium populeti, and Clostridium kluyveri*.
5. The recombinant microorganism of claim 3, wherein the host organism has been transformed with vector pMAD72.
6. The recombinant microorganism of claim 3, wherein the host organism has been transformed with vector pMAD22.
7. The recombinant microorganism of claim 1, wherein the recombinant microorganism is capable of producing butanol from glucose.
8. The recombinant microorganism of claim 7, wherein the recombinant microorganism is capable of producing butanol at a titer greater than 10 g/L and a yield greater than .3 g/g substrate.

9. The recombinant microorganism of claim 1, wherein the recombinant microorganism is capable of producing butanol from a monosaccharide, oligosaccharide, or polysaccharide.
10. The recombinant microorganism of claim 1, which is further constructed by transferring a foreign gene for inactivating acetate kinase to the host organism.
11. The recombinant microorganism of claim 1, which is further constructed by transferring a foreign gene for inactivating phosphotransbutyrylase to the host organism.
12. The recombinant microorganism of claim 1, which is further constructed by transferring a foreign gene for inactivating phosphotransacetylase to the host organism.
13. The recombinant microorganism of claim 3, which is further constructed by transferring a foreign gene for inactivating acetate kinase to the host organism.
14. The recombinant microorganism of claim 3, which is further constructed by transferring a foreign gene for inactivating phosphotransbutyrylase to the host organism.
15. The recombinant microorganism of claim 3, which is further constructed by transferring a foreign gene for inactivating phosphotransacetylase to the host organism.
16. The recombinant microorganism of claim 1, wherein the transfer of the foreign gene that encodes an enzyme having both alcohol/aldehyde dehydrogenase and butanol dehydrogenase activity is accomplished using a vector that includes a thiolase promoter sequence.
17. The recombinant microorganism of claim 5, which is further constructed by transferring a foreign gene for inactivating acetate kinase to the host organism.
18. A method for producing butanol, comprising the steps of culturing a recombinant microorganism of claim 17 in a culture medium containing glucose, and collecting butanol from a culture thereof.
19. A method for producing butanol, comprising the steps of culturing a recombinant microorganism of claim 10 in a culture medium containing glucose, and collecting butanol from a culture thereof.

20. A vector containing the DNA sequence of pMAD72.
21. A vector containing the DNA sequence of pMAD22.
22. A microbial host transformed by the vector of claim 20.
23. A microbial host transformed by the vector of claim 21.
24. The microbial host of claim 22, wherein the host is *C. tyrobutyricum*.
25. The microbial host of claim 23, wherein the host is *C. tyrobutyricum*.
26. The microbial host of claim 24, wherein the host is selected from the group consisting of *Clostridium butyricum, Clostridium thermobutyricum, Clostridium cellulovorans, Clostridium carboxidivorans, Butyribacterium methylotrophicum, Clostridium polysaccharolyticum, Clostridium populeti, and Clostridium kluyveri*.
27. The microbial host of claim 25, wherein the host is selected from the group consisting of *Clostridium butyricum, Clostridium thermobutyricum, Clostridium cellulovorans, Clostridium carboxidivorans, Butyribacterium methylotrophicum, Clostridium polysaccharolyticum, Clostridium populeti, and Clostridium kluyveri*.
28. A method of metabolically engineering a microorganism capable of producing butanol from a substrate, comprising the steps of:

    obtaining a microbial host capable of naturally producing butyric acid, and

    transferring a foreign gene that encodes an enzyme having both alcohol/aldehyde dehydrogenase and butanol hydrogenase activity into the microbial host.
29. The method of Claim 28, further comprising the step of transferring to the microbial host a foreign gene for inactivating acetate kinase.
30. The method of Claim 28, further comprising the step of transferring to the microbial host a foreign gene for inactivating phosphotransbutyrylase.
31. The method of Claim 28, further comprising the step of transferring to the microbial host a foreign gene for inactivating phosphotransacetylase.
32. The method of Claim 28, wherein the microbial host is *C. tyrobutyricum*.
33. The method of Claim 28, wherein the microbial host is selected from the group consisting of *Clostridium butyricum, Clostridium thermobutyricum, Clostridium cellulovorans, Clostridium carboxidivorans, Butyribacterium methylotrophicum, Clostridium polysaccharolyticum, Clostridium populeti, and Clostridium kluyveri*.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Acetyl-P
Acetate
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Acetoacetyl-CoA
NADH
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Crotonyl-CoA
NADH
Butyryl-CoA
Butyryl-P
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Acetylaldehyde
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NADH
NAD+
H+

FIG. 1

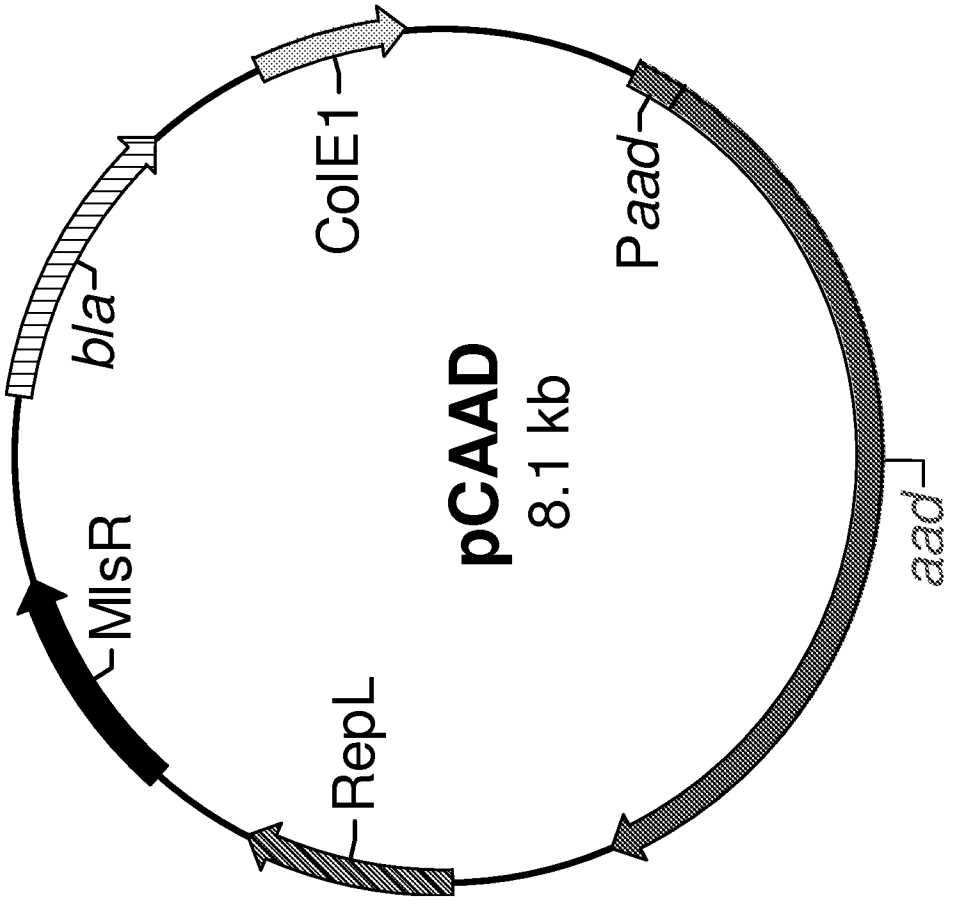

FIG. 2

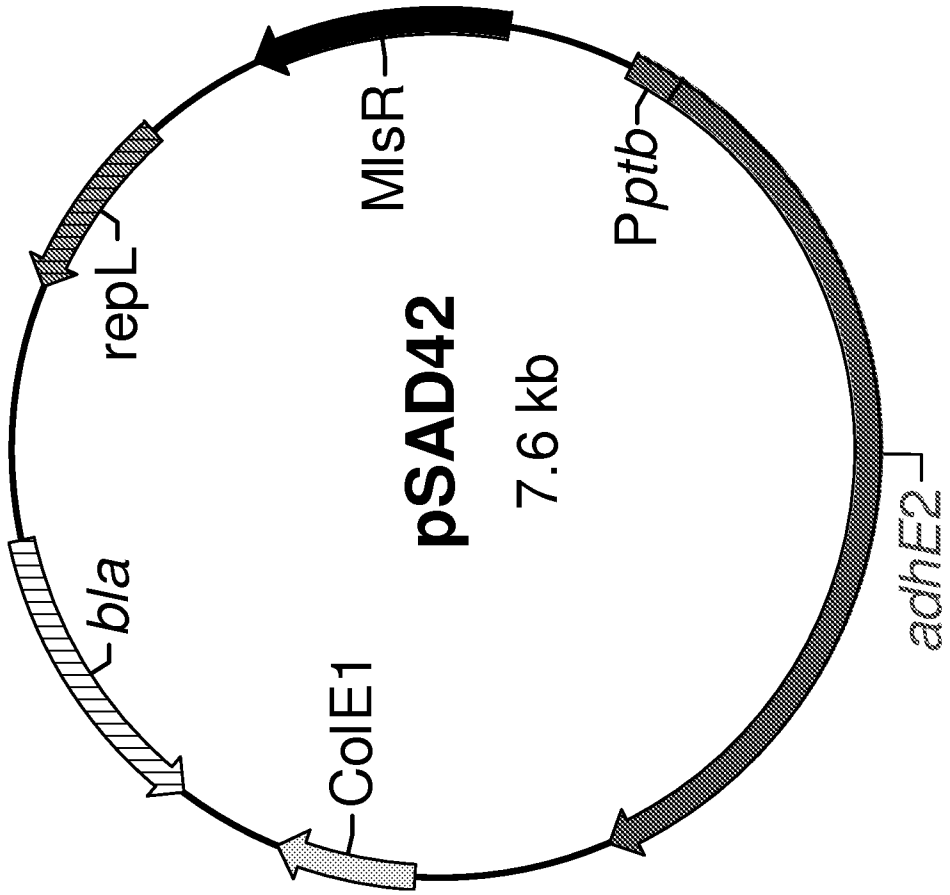

FIG. 3

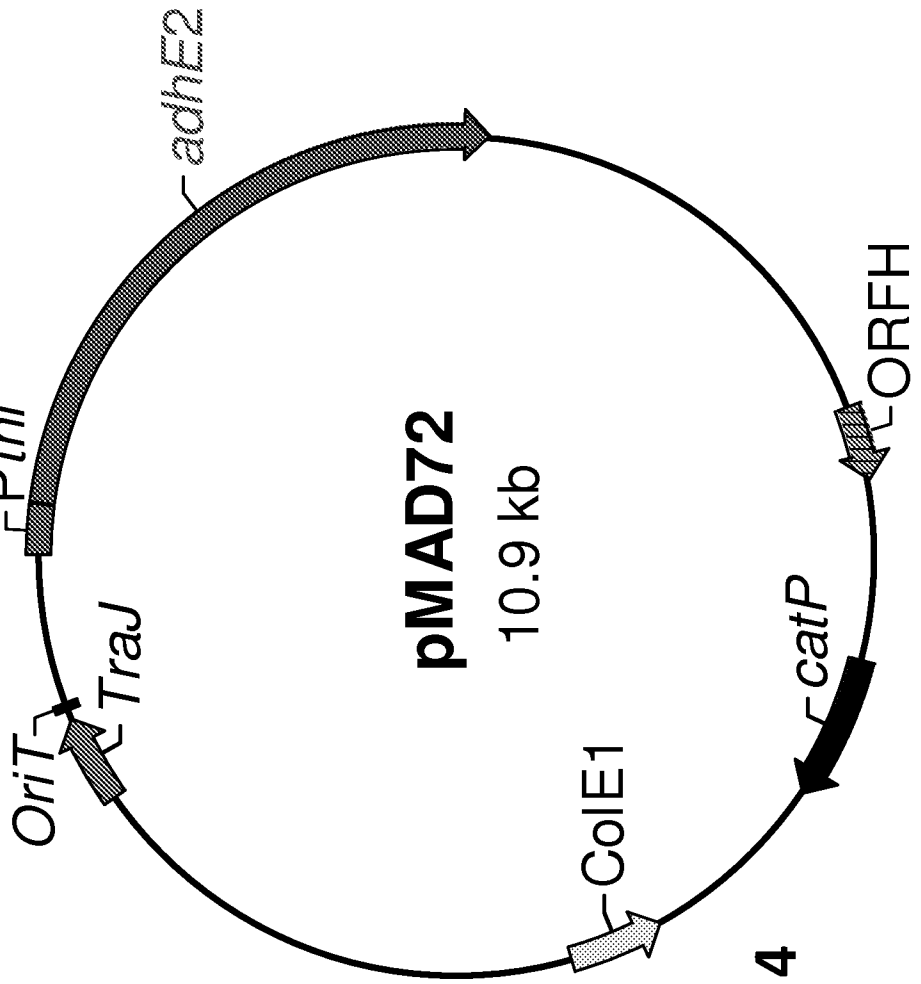

FIG. 4

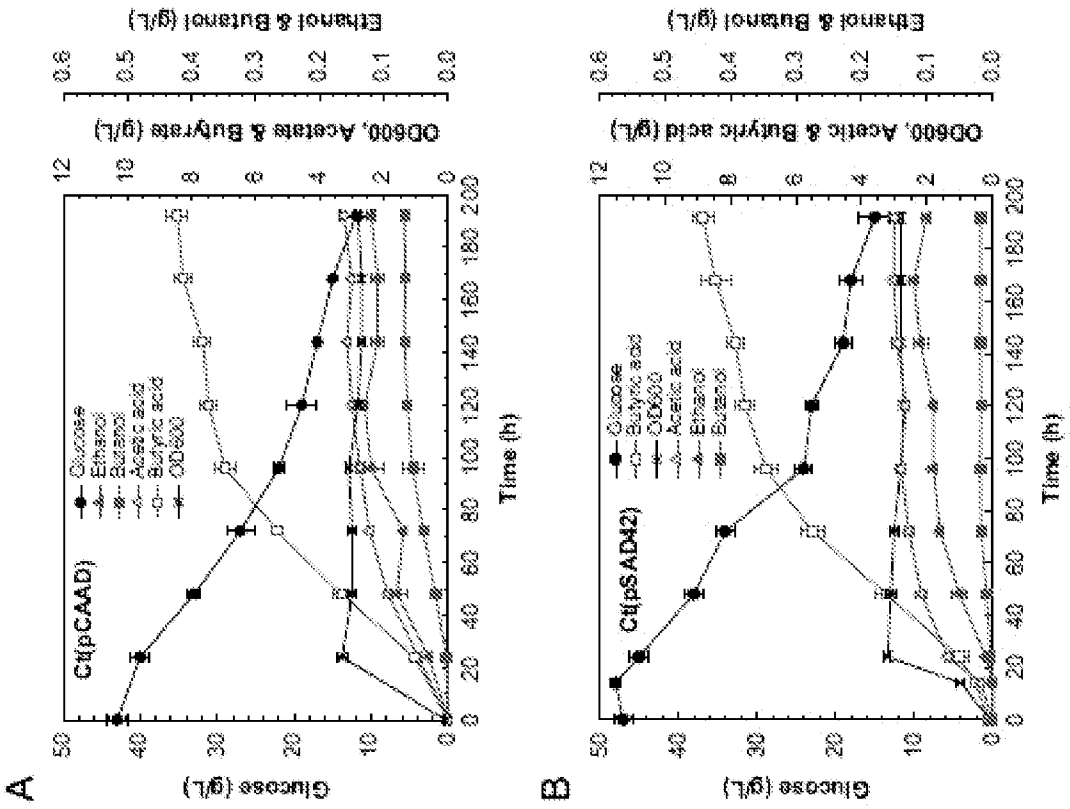
A
Ct(pCAAD)
Glucose
Ethanol
Butanol
Acetic acid
Butyric acid
OD600
Glucose (g/L)
OD600, Acetate & Butyrate (g/L)
Ethanol & Butanol (g/L)
Time (h)
B
Ct(pSAD42)
Glucose
Butyric acid
OD600
Acetic acid
Ethanol
Butanol
Glucose (g/L)
OD600, Acetic & Butyric acid (g/L)
Ethanol & Butanol (g/L)
Time (h)

FIG. 5

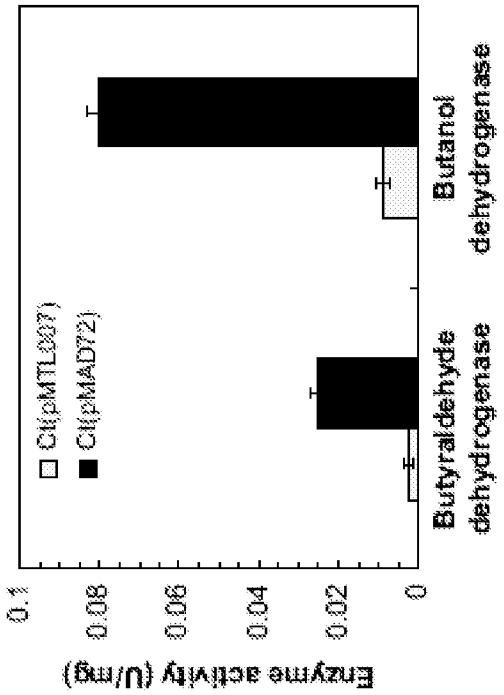
Enzyme activity (U/mg)
0.1
0.08
0.06
0.04
0.02
0
Cl(pMTL007)
Cl(pMAD72)
Butyraldehyde dehydrogenase
Butanol dehydrogenase

FIG. 6

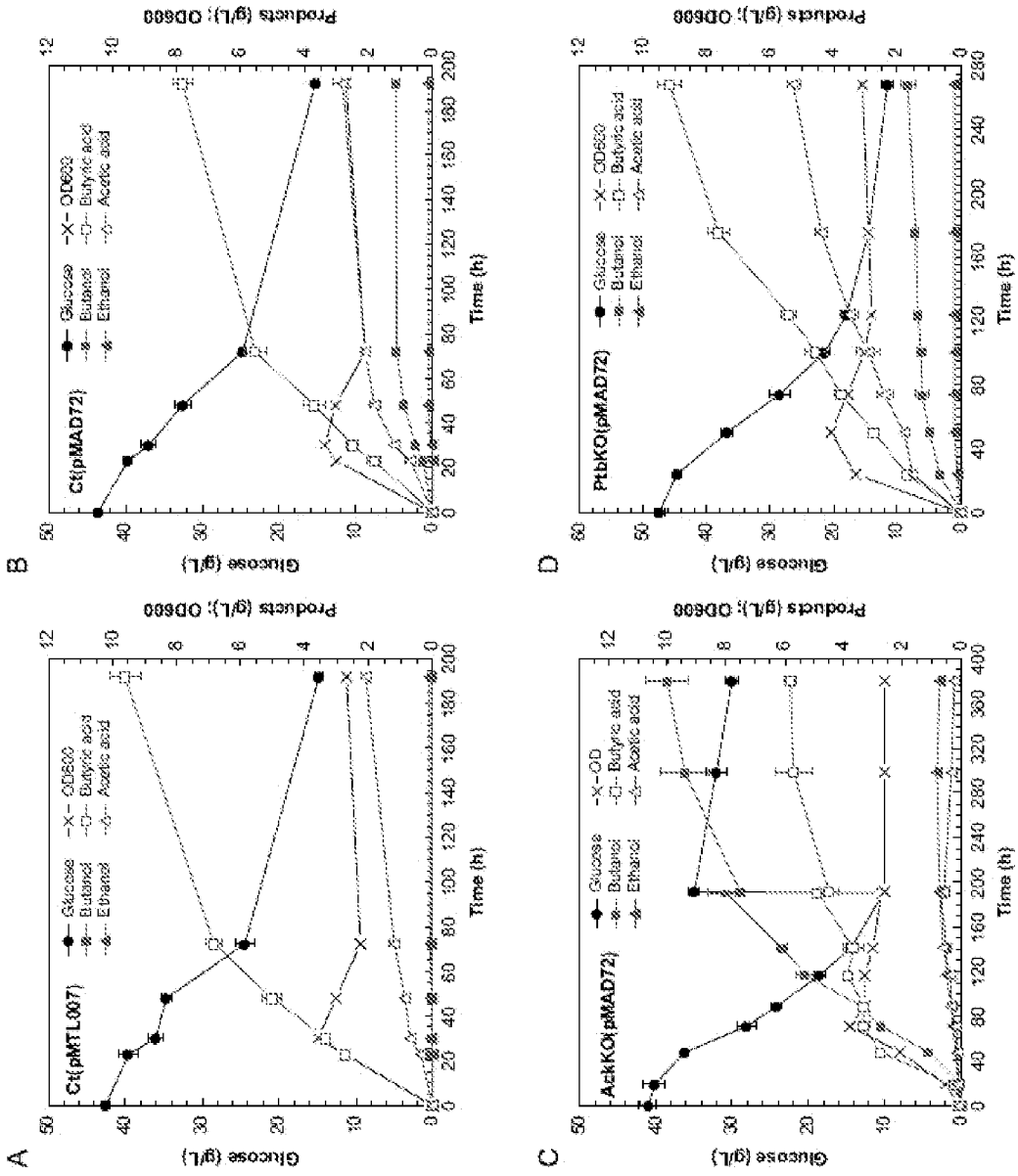
A
Ct(pMTL007)
Glucose
Butanol
Ethanol
OD600
Butyric acid
Acetic acid
Glucose (g/L)
Products (g/L); OD600
Time (h)
B
Ct(pMAD72)
C
AckKO(pMAD72)
OD
D
PtbKO(pMAD72)

FIG. 7

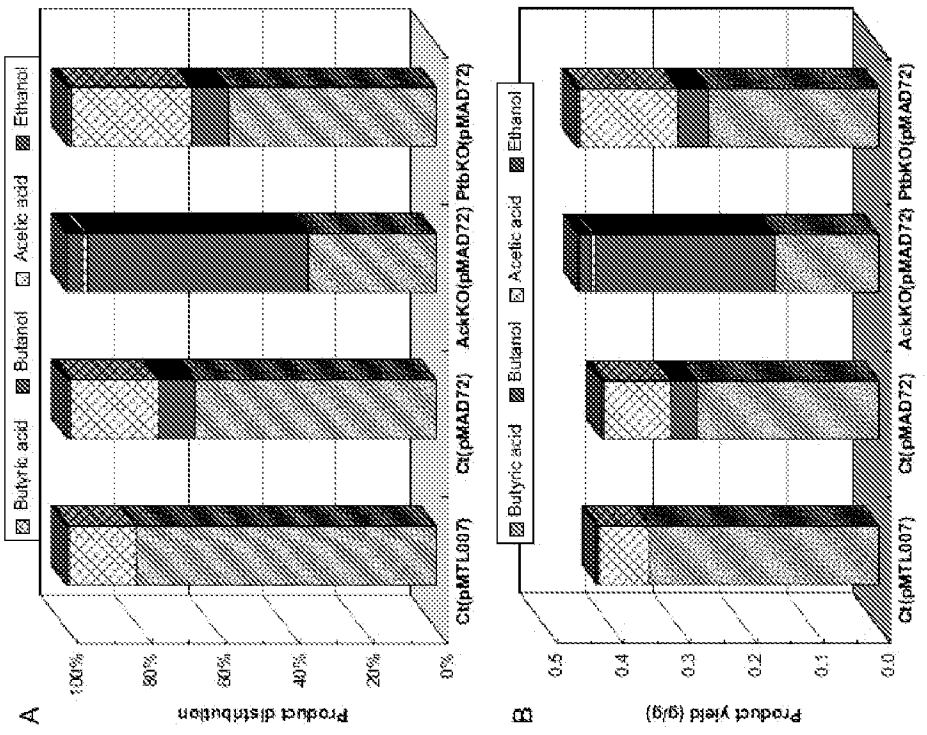
A
Product distribution
100%
80%
60%
40%
20%
0%
Butyric acid
Butanol
Acetic acid
Ethanol
Ct(pMTL007)
Ct(pMAD72)
AckKO(pMAD72)
PtbKO(pMAD72)
B
Product yield (g/g)
0.5
0.4
0.3
0.2
0.1
0.0
Butyric acid
Butanol
Acetic acid
Ethanol
Ct(pMTL007)
Ct(pMAD72)
AckKO(pMAD72)
PtbKO(pMAD72)

FIG. 8

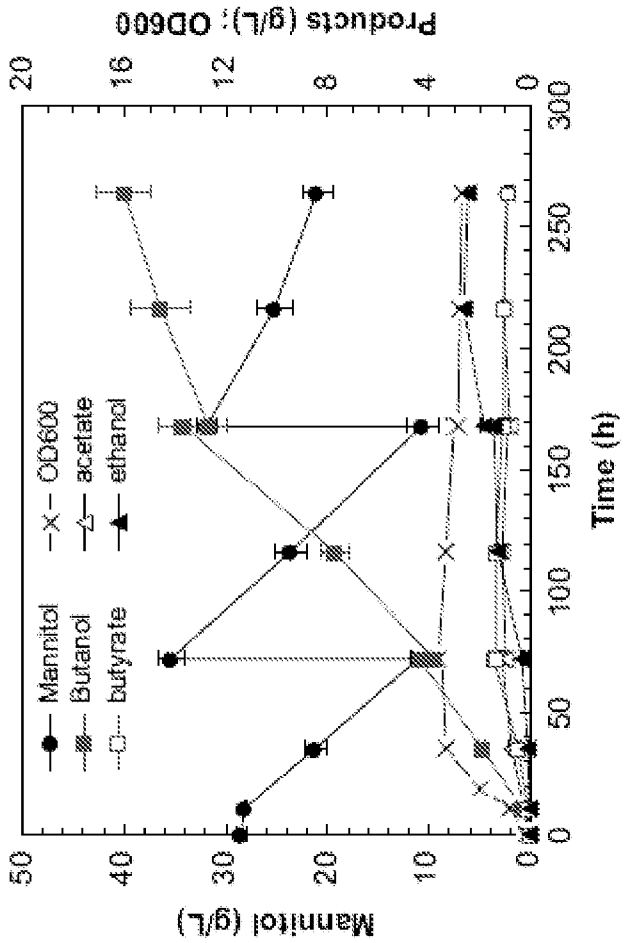
Products (g/L); OD600
Mannitol (g/L)
Time (h)
Mannitol
Butanol
butyrate
OD600
acetate
ethanol

FIG. 9

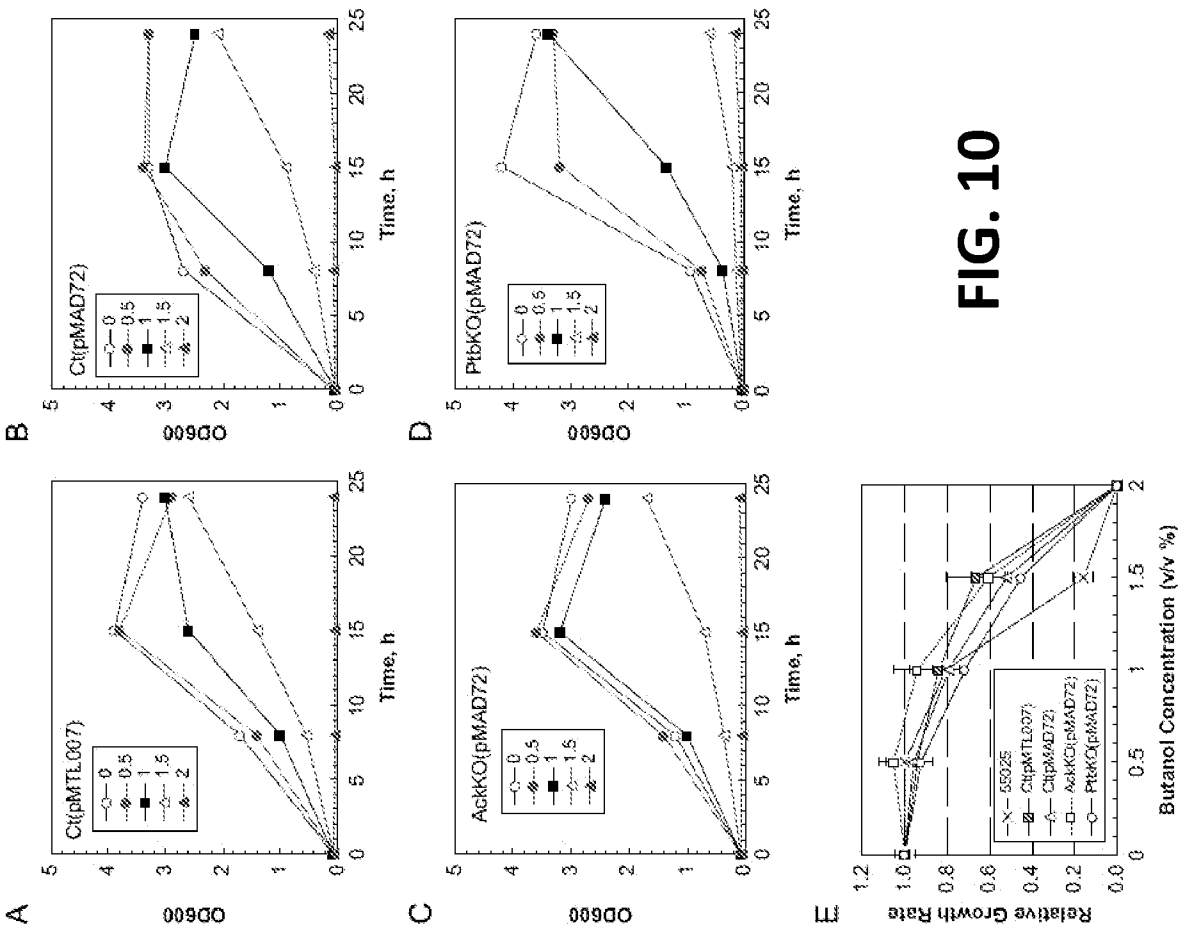
A
Ct(pMTL007)
0
0.5
1
1.5
2
OD600
Time, h
B
Ct(pMAD72)
C
AckKO(pMAD72)
D
PtbKO(pMAD72)
E
Relative Growth Rate
Butanol Concentration (v/v %)
55025
Ct(pMTL007)
Ct(pMAD72)
AckKO(pMAD72)
PtbKO(pMAD72)

FIG. 10