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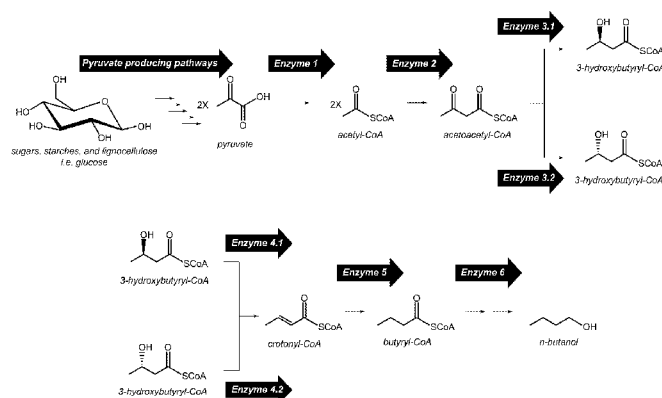
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Figure 1



(57) Abstract: The present disclosure provides optimized recombinant cells for the production of n-butanol. Methods for the use of these cells are also provided. Specifically, the utility of acylating aldehyde dehydrogenases and pyruvate:flavodoxin/ferredoxin-oxidoreductase for the improvement of w-butanol yields from recombinant cells is disclosed.

SYNTHETIC PATHWAYS FOR BIOFUEL SYNTHESIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application 61/354,129, filed June 11, 2010, the contents of which are hereby incorporated by reference in their entirety.

FIELD

[0002] The present disclosure relates to recombinant cells containing improved pathways for biofuel synthesis. In particular, recombinant cells and methods for the synthesis of *n*-butanol are provided.

BACKGROUND

[0003] Liquid fuels derived from plant biomass are renewable energy sources and the global demand for such biofuels is rising. Ethanol is the most widely used biofuel today, but its low energy return, high vaporizability and miscibility with water present major technical challenges. Alternative biofuels, such as *n*-butanol, more closely resemble gasoline and have the potential to replace ethanol as the predominant biofuel in the future.

[0004] While several microorganisms can produce ethanol as a fermentation product, only few natural microorganisms can produce *n*-butanol. Natural *n*-butanol producers, such as *Clostridium acetobutylicum* (*C. acetobutylicum*), can be used for industrial applications but are not as genetically tractable or robust fermentation hosts as, for example, *Escherichia coli* (*E. coli*) or *Saccharomyces cerevisiae* (*S. cerevisiae*). It is therefore attractive to engineer a recombinant pathway for biofuel production in such host as *E. coli* or *S. cerevisiae*.

[0005] *n*-Butanol biosynthesis typically includes several enzymatic steps, whereby different *n*-butanol synthesizing organisms can utilize different classes and combinations of enzymes to mediate the conversion from pyruvate to *n*-butanol. Generally, the startpoint of *n*-butanol synthesis, pyruvate, can be derived through the metabolism of various sugar substrates, including

glucose and xylose, but also starches and lignocellulosics. Pyruvate is then converted to acetyl-CoA. Acetyl-CoA is subsequently converted to acetoacetyl-CoA, which is itself converted to 3-hydroxybutyryl-CoA. 3-Hydroxybutyryl-CoA is converted to crotonyl-CoA. Crotonyl-CoA is converted to butyryl-CoA. Finally, butyryl-CoA is converted to *n*-butanol.

[0006] The *n*-butanol biosynthesis pathway of *C. acetobutylicum* converting acetyl-CoA to *n*-butanol can be lifted out and inserted into *E. coli*, thereby generating a recombinant cell that produces *n*-butanol (Inui, *et al.*, 2008, Appl. Microbiol. Biotechnol. 77, 1305-16; Atsumi *et al.*, 2008, Metab. Eng. 10, 305-11; Nielsen *et al.*, 2009, Metab. Eng. 11, 262-73). However, the *C. acetobutylicum* derived *n*-butanol biosynthesis pathway contains multiple bottlenecks that limit the yields of biofuel production.

[0007] In view of these facts and the growing global demand in biofuels, a significant need exists for more productive recombinant cells and improved methods for biofuel synthesis. Specifically, new recombinant cells are needed providing for robust and high-yielding *n*-butanol synthesis pathways.

BRIEF SUMMARY

[0008] Provided herein are recombinant cells for the production of *n*-butanol. Also provided are methods for producing *n*-butanol using the recombinant cells described herein.

[0009] Particularly, recombinant cells are provided including recombinant sequences encoding enzymes that constitute a synthetic pathway for *n*-butanol production. In one embodiment of the invention the enzymes include an acylating aldehyde dehydrogenase catalyzing the conversion of acetaldehyde to acetyl-CoA. In another embodiment the enzymes include a pyruvate:flavodoxin/ferredoxin-oxidoreductase catalyzing the conversion of pyruvate to acetyl-CoA. The acylating aldehyde dehydrogenase or pyruvate:flavodoxin/ferredoxin-oxidoreductase are combined with a keto-thiolase or acetyl-CoA acetyltransferase catalyzing the conversion of acetyl-CoA to acetoacetyl-CoA, an acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase catalyzing the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA, a crotonase catalyzing the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA a crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase catalyzing the conversion

of crotonyl-CoA to butyryl-CoA, and a butyraldehyde/butanol dehydrogenase catalyzing the conversion of butyryl-CoA to n-butanol.

[0010] Furthermore, methods for n-butanol production are provided. The methods include the step of growing a recombinant cell of the invention in the presence of a suitable carbon source.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **Figure 1**, shows the biosynthesis pathways for n-butanol. Enzyme 1 is a pyruvate dehydrogenase or a pyruvate dehydrogenase bypass consisting of a pyruvate decarboxyase and a acylating aldehyde dehydrogenase, or a pyruvate dehydrogenase bypass consisting of a pyruvate decarboxyase, a non-acylating aldehyde dehydrogenase, and an acetyl-CoA synthetase, or a pyruvate:flavodoxin/ferredoxin-oxidoreductase, or a pyruvate formate lyase and formate dehydrogenase; Enzyme 2 is a keto-thiolase or acetyl-CoA acetyltransferase; Enzyme 3 is an acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase; Enzyme 4 a crotonase; Enzyme 5 is a trans-2-enoyl-CoA reductase; Enzyme 6 is a butyraldehyde/butanol dehydrogenase. Subclasses of Enzymes 3 and 4, such Enzyme 3.1 and 3.2, may feature different stereoselectivities and produce different chiral intermediates. For example, the acetoacetyl-CoA reductase Hbd produces (*S*)-hydroxybutyryl-CoA while PhaB produces (*R*)-hydroxybutyryl-CoA.

[0012] **Figure 2** shows the production of *n*-butanol using different strains (1-6), promoters (6-12), enoyl-CoA reductase and ketoreductase/enoyl-CoA hydratase selection (10, 13-18) and overexpression of a pyruvate dehydrogenase (PDH) (19-20).

[0013] **Figure 3** shows the promoter optimization for Ccr expression and the S-tag analysis of Ccr solubility. Part A shows butanol production using pBT33-phaA,phaB-crt in combination with ccr-adhE2 and using promoters with variable strengths to transcribe the ccr-adhE2 operon. The plasmids used for expression of the ccr-adhE2 operon were pBAD33-ccr.adhE2, pTrc99a-ccr.adhE2, pCWori-ccr.adhE2, and pET29a-ccr.adhE2. Part B shows the relationship between n-butanol production (quantified by GC-MS) and soluble Ccr-Stag protein (quantified by S-Tag Rapid Assay Kit, Novagen).

[0014] **Figure 4** shows the trapping of pathway intermediates by Ter. Part A shows the reaction catalyzed by Crt is reversible in cell lysate after 2 hours. Part B shows that Ter is effectively irreversible in cell lysate with no observable reaction occurring within 2 hours.

[0015] **Figure 5** shows a NeighborNet graph of Ter from *T. denticola* (Tucci and Martin, 2007, FEBS Lett. 581 (2007) 1561-66). The scale bar at the lower right indicates estimated substitutions per site. Abbreviations are as follows: β and γ , proteobacteria; bactero, bacteroides; entero, enterobacteria; spiro, spirochete.

[0016] **Figure 6** shows the impact of replacing Ccr for Ter on *n*-butanol yields in recombinant *E. coli*. Elevating *E. coli* PDH levels in the presence of Ter results in further increases in *n*-butanol yields.

[0017] **Figure 7** shows *n*-butanol production in *E. coli* cell genetically modified to express the butanol biosynthetic pathway of Figure 1. The product retention time was compared to an authentic *n*-butanol standard in a chromatograph (left), and a product mass spectrum was compared to an authentic *n*-butanol standard (right) to confirm the identity of the fermentation product.

[0018] **Figure 8** compares the *n*-butanol production and the ethanol to butanol ratio in *E. coli* in the presence of basal levels of acetyl-CoA versus and after overexpression of the variants of *E. coli* PDH (pyruvate dehydrogenase complex), PFOR complex (pyruvate:flavodoxin/ferredoxin-oxidoreductase (YdbK), a flavodoxin-NADP reductase (Fpr), a ferredoxin (Fdx), and one of two flavodoxins (FldA or FldB) all of which are from *E. coli*), pyruvate formate lyase (Pfl) and formate dehydrogenase (Fdh), and PDH bypass (a pyruvate decarboxylase from *Z. mobilis*, an acylating aldehyde dehydrogenase from *E. coli*, and a pantothenate kinase from *E. coli*).

[0019] **Figure 9** shows total fuel (butanol and ethanol) titer in *E. coli* DH1 and a knockout strain in the presence of basal levels of acetyl-CoA versus expression of the PDHc bypass (a pyruvate decarboxylase from *Z. mobilis*, an acetylating aldehyde dehydrogenase from *E. coli*, and a pantetheinate kinase from *E. coli*).

[0020] **Figure 10** shows the four general pathways for the conversion of pyruvate to acetyl-CoA consisting of a pyruvate dehydrogenase complex, or a pyruvate:flavodoxin/ferrodoxin-oxidoreductase, or a pyruvate dehydrogenase bypass consisting of a pyruvate decarboxyase and acylating aldehyde dehydrogenase, or a pyruvate dehydrogenase bypass consisting of a pyruvate decarboxyase and a non-acylating aldehyde dehydrogenase and an acetyl-CoA synthetase, or pyruvate formate lyase and formate dehydrogenase.

[0021] **Figure 11** shows native fermentation pathways in *E.coli* that compete with fuel production under anaerobic and microaerobic conditions.

[0022] **Figure 12** shows n-butanol production in *S. cerevisiae*. Part A shows the recombinant pathway for n-butanol production in recombinant *S. cerevisiae* cells. Part B shows butanol production in *S. cerevisiae* BY4741 Δ adh. Column 1 shows background level production of butanol. Column 2 shows butanol titer by *Saccharomyces cerevisiae* BY4741 Δ adh strain harboring butanol production pathway and PDH bypass.

[0023] **Figure 13** shows the pentose phosphate pathway. The pentose phosphate pathway takes in C5 sugars, including xylose and arabinose, and using NADP⁺/H as a cofactor converts the sugars into molecules that can enter into glycolysis and then into the *n*-butanol producing pathway.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0024] The present disclosure relates to recombinant cells producing n-butanol and to methods of using these recombinant cells for the production of *n*-butanol from fermentable carbon sources.

n-Butanol Synthesis Pathway

[0025] *n*-Butanol can be produced by a recombinant cell containing recombinant sequences of at least six enzymes catalyzing the generation of acetyl-CoA and its stepwise conversion to *n*-

butanol (*Figures 1 and 10*). Acetyl-CoA can be generated from the glycolysis product pyruvate by means of a pyruvate dehydrogenase complex (PDHc), a pyruvate formate oxidoreductase (PFOR), the combined activities of a pyruvate formate lyase and a formate dehydrogenase (PFL-FDH), or a pyruvate dehydrogenase bypass pathway (PDH bypass). PDH bypass pathways can include a pyruvate dehydrogenase (PDC) in combination with an acylating aldehyde dehydrogenase (ALDH) or a non-acylating aldehyde dehydrogenase and an acetyl-CoA synthetase. The conversion of acetyl-CoA to *n*-butanol may proceed through the intermediates acetoacetyl-CoA, 3-hydroxybutyryl-CoA, crotonyl-CoA, and butyryl-CoA. The recombinant cells of this invention are engineered to contain efficient heterologous pathways for *n*-butanol production.

[0026] In one embodiment of the invention the recombinant cell contains recombinant sequences encoding i) an acylating aldehyde dehydrogenase catalyzing the conversion of acetaldehyde to acetyl-CoA (*Figure 1, Enzyme 1*), ii) a keto-thiolase or acetyl-CoA acetyltransferase catalyzing the conversion of acetyl-CoA to acetoacetyl-CoA (*Figure 1, Enzyme 2*), iii) an acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase catalyzing the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA (*Figure 1, Enzyme 3*), iv) a crotonase catalyzing the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA (*Figure 1, Enzyme 4*), v) a crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase catalyzing the conversion of crotonyl-CoA to butyryl-CoA (*Figure 1, Enzyme 5*), and vi) a butyraldehyde/butanol dehydrogenase catalyzing the conversion of butyryl-CoA to *n*-butanol (*Figure 1, Enzyme 6*).

[0027] In one specific embodiment the sequences encoding the acylating aldehyde dehydrogenase, the keto-thiolase or acetyl-CoA acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase are linked. In another specific embodiment the sequences are not linked.

[0028] Some organisms may not express an endogenous pyruvate decarboxylase or may express only low levels of pyruvate decarboxylase activity that limit the availability of acetaldehyde, the activity of the acylating aldehyde dehydrogenase, and the overall *n*-butanol

yields of the recombinant biosynthesis pathway. Therefore, in some embodiments the recombinant cell further contains a recombinant sequence encoding a pyruvate decarboxylase catalyzing the conversion of pyruvate to acetaldehyde. In another specific embodiment the pyruvate decarboxylase is derived from *Z. mobilis* or *S. cerevisiae*.

[0029] In one embodiment of the invention the recombinant cell contains recombinant sequences encoding i) a pyruvate:flavodoxin/ferredoxin-oxidoreductase catalyzing the conversion of pyruvate to acetyl-CoA (*Figure 1, Enzyme 1*), ii) a keto-thiolase or acetyl-CoA acetyltransferase catalyzing the conversion of acetyl-CoA to acetoacetyl-CoA (*Figure 1, Enzyme 2*), iii) an acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase catalyzing the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA (*Figure 1, Enzyme 3*), iv) a crotonase catalyzing the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA (*Figure 1, Enzyme 4*), v) a crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase catalyzing the conversion of crotonyl-CoA to butyryl-CoA (*Figure 1, Enzyme 5*), and vi) a butyraldehyde/butanol dehydrogenase catalyzing the conversion of butyryl-CoA to *n*-butanol (*Figure 1, Enzyme 6*).

[0030] In one specific embodiment the sequences encoding the pyruvate:flavodoxin/ferredoxin-oxidoreductase, the keto-thiolase or acetyl-CoA acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase are linked. In another specific embodiment the sequences are not linked.

[0031] In one specific embodiment the recombinant cell further comprising recombinant sequences encoding the ferredoxin-NADP reductase from *E. coli*, the ferredoxin *FdC* from *E. coli*, and the flavodoxins *FldA* and *FldB* from *E. coli*.

[0032] In one embodiment of the invention the recombinant cell produces *n*-butanol under aerobic conditions. In one embodiment of the invention the recombinant cell produces *n*-butanol under microaerobic conditions. Microaerobic conditions refer to an environment where the concentration of oxygen is less than that in the air. In one embodiment of the invention the

recombinant cell produces *n*-butanol under anaerobic conditions. In one specific embodiment the recombinant cell produces more *n*-butanol under anaerobic conditions than under aerobic or microaerobic conditions. In another specific embodiment the recombinant cell produces near quantitative yields of *n*-butanol under anaerobic conditions.

[0033] In one embodiment of the invention the recombinant cell produces *n*-butanol and ethanol under aerobic conditions. In one embodiment of the invention the recombinant cell produces *n*-butanol and ethanol under microaerobic conditions. In one embodiment of the invention the recombinant cell produces *n*-butanol and ethanol under anaerobic conditions. In one specific embodiment the recombinant cell produces more total levels of *n*-butanol and ethanol under anaerobic conditions than under aerobic or microaerobic conditions. In another specific embodiment the recombinant cell produces near quantitative yields of *n*-butanol and ethanol under anaerobic conditions.

[0034] In one embodiment of the invention the recombinant cell produces elevated levels of *n*-butanol compared to a wild-type cell under aerobic conditions. Elevated levels of *n*-butanol produced by the recombinant cell under aerobic conditions may be elevated by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%, 3-fold, 10-fold, 30-fold, 100-fold, 300-fold, 1,000-fold, 3,000-fold, 10,000-fold, 30,000-fold, 100,000-fold, 300,000-fold or 1,000,000-fold compared to the *n*-butanol levels produced by a wild-type cell under aerobic conditions. In specific embodiments the recombinant cell produces at least 0.01 g/L, at least 0.03 g/L, at least 0.1 g/L, at least 0.3 g/L, at least 1.0 g/L, at least 1.5 g/L, at least 2.0 g/L, at least 2.5 g/L, at least 3.0 g/L, at least 3.5 g/L, at least 4.0 g/L, at least 4.5 g/L, at least 5.0 g/L, at least 6.0 g/L, at least 7.0 g/L, at least 8.0 g/L, at least 9.0 g/L, at least 10.0 g/L, at least 15.0 g/L, at least 20.0 g/L, at least 30.0 g/L, at least 50.0 g/L, or at least 75.0 g/L *n*-butanol under aerobic conditions.

[0035] In one embodiment of the invention the recombinant cell produces elevated total levels of *n*-butanol and ethanol compared to a wild-type cell under aerobic conditions. Elevated total levels of *n*-butanol and ethanol produced by the recombinant cell under aerobic conditions may be elevated by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%, 3-fold, 10-fold, 30-fold, 100-fold, 300-fold, 1,000-fold, 3,000-fold, 10,000-fold, 30,000-fold, 100,000-fold, 300,000-fold or 1,000,000-fold compared to the total levels of *n*-butanol and ethanol produced

by a wild-type cell under aerobic conditions. In specific embodiments the recombinant cell produces under aerobic conditions total levels of *n*-butanol and ethanol of at least 0.01 g/L, at least 0.03 g/L, at least 0.1 g/L, at least 0.3 g/L, at least 1.0 g/L, at least 1.5 g/L, at least 2.0 g/L, at least 2.5 g/L, at least 3.0 g/L, at least 3.5 g/L, at least 4.0 g/L, at least 4.5 g/L, at least 5.0 g/L, at least 6.0 g/L, at least 7.0 g/L, at least 8.0 g/L, at least 9.0 g/L, at least 10.0 g/L, at least 15.0 g/L, at least 20.0 g/L, at least 30.0 g/L, at least 50.0 g/L, or at least 75.0 g/L.

[0036] In one embodiment of the invention the recombinant cell produces elevated levels of *n*-butanol compared to a wild-type cell under anaerobic conditions. Elevated levels of *n*-butanol produced by the recombinant cell under anaerobic conditions may be elevated by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%, 3-fold, 10-fold, 30-fold, 100-fold, 300-fold, 1,000-fold, 3,000-fold, 10,000-fold, 30,000-fold, 100,000-fold, 300,000-fold or 1,000,000-fold compared to the *n*-butanol levels produced by a wild-type cell under anaerobic conditions. In specific embodiments the recombinant cell produces at least 0.01 g/L, at least 0.03 g/L, at least 0.1 g/L, at least 0.3 g/L, at least 1.0 g/L, at least 1.5 g/L, at least 2.0 g/L, at least 2.5 g/L, at least 3.0 g/L, at least 3.5 g/L, at least 4.0 g/L, at least 4.5 g/L, at least 5.0 g/L, at least 6.0 g/L, at least 7.0 g/L, at least 8.0 g/L, at least 9.0 g/L, at least 10.0 g/L, at least 15.0 g/L, at least 20.0 g/L, at least 30.0 g/L, at least 50.0 g/L, or at least 75.0 g/L *n*-butanol under anaerobic conditions.

[0037] In one embodiment of the invention the recombinant cell produces elevated total levels of *n*-butanol and ethanol compared to a wild-type cell under anaerobic conditions. Elevated total levels of *n*-butanol and ethanol produced by the recombinant cell under anaerobic conditions may be elevated by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%, 3-fold, 10-fold, 30-fold, 100-fold, 300-fold, 1,000-fold, 3,000-fold, 10,000-fold, 30,000-fold, 100,000-fold, 300,000-fold or 1,000,000-fold compared to the total levels of *n*-butanol and ethanol produced by a wild-type cell under anaerobic conditions. In specific embodiments the recombinant cell produces under anaerobic conditions total levels of *n*-butanol and ethanol of at least 0.01 g/L, at least 0.03 g/L, at least 0.1 g/L, at least 0.3 g/L, at least 1.0 g/L, at least 1.5 g/L, at least 2.0 g/L, at least 2.5 g/L, at least 3.0 g/L, at least 3.5 g/L, at least 4.0 g/L, at least 4.5 g/L, at least 5.0 g/L, at least 6.0 g/L, at least 7.0 g/L, at least 8.0 g/L, at least 9.0 g/L, at least 10.0 g/L, at least 15.0 g/L, at least 20.0 g/L, at least 30.0 g/L, at least 50.0 g/L, or at least 75.0 g/L.

Enzyme 1: Acetyl-CoA Generation

[0038] Recombinant cells of this invention contain at least one recombinant pathway for the production of acetyl-CoA (*Figure 10*). In one embodiment of the invention the recombinant cell contains recombinant sequences encoding a pyruvate dehydrogenase complex (PDH). In a specific embodiment the PDH is Pdh from *E.coli*. In another embodiment the recombinant cell contains recombinant sequences encoding a pyruvate formate lyase (PFL) and a formate dehydrogenase (FDH).

[0039] In another embodiment the recombinant cell contains recombinant sequences encoding a pyruvate formate oxidoreductase complex (PFOR). In one specific embodiment PFOR includes a pyruvate:flavodoxin/ferredoxin-oxidoreductase, a flavodoxin-NADP reductase, a ferredoxin, and at least one flavodoxins. In another specific embodiment the recombinant sequences encoding PFOR includes YdbK (SEQ ID NOs: 472, 473), Fpr (SEQ ID NOs: 464, 465), Fdx (SEQ ID NOs: 466, 467), and FldA (SEQ ID NOs: 468, 469), or FldB (SEQ ID NOs: 470, 471) from *E.coli*.

[0040] In another embodiment the recombinant cell contains recombinant sequences encoding a pyruvate dehydrogenase bypass (PDH bypass). In one specific embodiment the PDHc bypass includes recombinant sequences encoding a pyruvate decarboxylase (PDC). In another specific embodiment the PDHc bypass includes recombinant sequences encoding a non-acylating aldehyde dehydrogenase (AIDH). In another specific embodiment the PDH bypass includes recombinant sequences encoding an acetyl-CoA synthetase (ACS). In another specific embodiment the PDHc bypass includes recombinant sequences encoding a PDC, a non-acylating AIDH, and an ACS. In another specific embodiment the PDHc bypass includes recombinant sequences encoding an acetylating AIDH. In a preferred embodiment the PDHc bypass includes recombinant sequences encoding a PDC and an acylating AIDH. In another preferred embodiment the PDHc bypass includes recombinant sequences encoding a PDC from *Z. mobilis* and an acylating aldehyde dehydrogenase from *E. coli*. In another preferred embodiment the PDHc bypass contains recombinant sequences encoding Pdc from *Z. mobilis* and EutEA from *E. coli*.

[0041] Recombinant sequences encoding PDHc, PFOR, PFL, FDH, acylating AIDH and non-acylating AIDH enzymes may be derived from all prokaryotic organisms, including proteobacterial, archaeobacterial, bacteroidal, enterobacterial, spirochetal organisms, and all eukaryotic organisms, including mammalian, insect, fungal and yeast organisms. Preferred examples include, but are not limited to: *E. coli* Pdh, which is composed of the three genes aceE (SEQ ID NOs: 1, 2), aceF (SEQ ID NOs: 3, 4), and lpdA (SEQ ID NOs: 5,6), the *E. faecalis* Pdh, which is composed of the four genes pdhA (SEQ ID NOs: 7, 8), pdhB (SEQ ID NOs: 9, 10), aceF (SEQ ID NOs: 11, 12), and lpdA (SEQ ID NOs: 13, 14), the *E. coli* Pfor genes ydbK (SEQ ID NOs: 35, 36), fpr (SEQ ID NOs: 37, 38), fdx (SEQ ID NOs: 39, 40), fldA (SEQ ID NOs: 41, 42), and fldB (SEQ ID NOs: 43, 44), the *Z. mobilis* pdc gene (SEQ ID NOs: 474, 475), and the *E. coli* acetylating aldehyde dehydrogenase gene eutE (SEQ ID NOs: 476, 477).

Enzyme 2: Keto-Thiolase or Acetyl-CoA Acetyltransferase

[0042] Recombinant sequences encoding the keto-thiolase or acetyl-CoA acetyltransferase may be derived from all prokaryotic organisms, including proteobacterial, archaeobacterial, bacteroidal, enterobacterial, spirochetal organisms, and all eukaryotic organisms, including mammalian, insect, fungal and yeast organisms. Preferred examples include, but are not limited to: the *Rastonia eutrophus* acetoacetyl-CoA thiolase/synthase phaA (SEQ ID NOs: 15, 16) and related enzymes from cells that make polyhydroxyalkanoates, *C. acetobutylicum* acetoacetyl-CoA thiolase/synthase thI, and *E. coli* acetoacetyl-CoA thiolase/synthase atoB.

Enzyme 3: Acetoacetyl-CoA Reductase or Hydroxybutyryl-CoA Dehydrogenase

[0043] Recombinant sequences encoding acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase may be derived from all prokaryotic organisms, including proteobacterial, archaeobacterial, bacteroidal, enterobacterial, spirochetal organisms, and all eukaryotic organisms, including mammalian, insect, fungal and yeast organisms. Preferred examples include, but are not limited to: the *R. eutrophus* 3-hydroxybutyryl-CoA dehydrogenase phaB (SEQ ID NOs: 17, 18), the *C. acetobutylicum* acetoacetyl-CoA reductase hbd (SEQ ID NOs: 19, 20).

Enzyme 4: Crotonase

[0044] Recombinant sequences encoding crotonase may be derived from all prokaryotic organisms, including proteobacterial, archaeobacterial, bacteroid, enterobacterial, spirochetal organisms, and eukaryotic organisms, including mammalian, insect, fungal and yeast organisms. Preferred examples include, but are not limited to: the *C. acetobutylicum* crotonase crt (SEQ ID NOs: 21, 22) or the *A. cavaie* crotonase phaJ (SEQ ID NOs: 478, 479).

Enzyme 5: Crotonyl-CoA Reductase or Trans-enoyl-CoA Reductase

[0045] Recombinant sequences encoding crotonyl-CoA reductase or trans-enoyl-CoA reductase may be derived from all prokaryotic organisms, including proteobacterial, archaeobacterial, bacteroid, enterobacterial, spirochetal organisms, and all eukaryotic organisms, including mammalian, insect, fungal and yeast organisms. Preferred examples include, but are not limited to: *T. denticola* (SEQ ID NOs: 29, 30), *E. gracilis* (SEQ ID NOs: 31, 32), *Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia cepacia*, *Methylobacillus flagellatus*, *Xylella fastidiosa*, *Xanthomonas campestris*, *Xanthomonas cryzae*, *Pseudomonas putida*, *Pseudomonas entomophila*, *Marinomonas* sp., *Psychromonas ingrahamii*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio splendidus*, *Vibrio* sp., *Shewanella frigidimarina*, *Oceanospirillum* sp., *Aeromonas hydrophila* subsp., *Serratia proteamaculans*, *Saccharophagus degradans*, *Colwellia psychrerythraea*, *Reinekea* sp., *Idiomarina loihiensis*, *Streptomyces avermitilis*, *Coxiella burnetii* Dugway, *Polaribacter irgensii*, *Flavobacterium johnsoniae*, *Cytophaga hutchisonii*, *E. coli*, *R. eutrophus*, *A. caviae*, or *C. acetobutylicum*.

[0046] The disclosure includes examples for the use of Ters from *T. denticola* and *Euglena gracilis* (*E. gracilis*), the polypeptide sequences of which are 48% homologous.

[0047] In a specific embodiment the recombinant sequence encoding the crotonyl-CoA reductase is derived from *Streptomyces collinus* (*S. collinus*). In another specific embodiment the recombinant sequence encoding the trans-enoyl-CoA reductase (TER) is derived from *T. denticola*. In another specific embodiment the crotonyl-CoA reductase is ccr from *S. collinus*. In another specific embodiment the trans-enoyl-CoA reductase is ter from *T. denticola*.

Enzyme 6: Butyraldehyde/Butanol Dehydrogenase

[0048] Recombinant sequences encoding the butyraldehyde/butanol dehydrogenase may be derived from all prokaryotic organisms, including proteobacterial, archaeobacterial, bacteroid, enterobacterial, spirochetal organisms, and all eukaryotic organisms, including mammalian, insect, fungal and yeast organisms. Preferred examples include, but are not limited to: the *C. acetobutylicum* butyraldehyde/butanol dehydrogenases adhE2 (SEQ ID NOs: 33, 34) or aad (SEQ ID NOs: X, Y) and related sequences from *Clostridia* sp, including but not limited to adhE1, bdhA, bdhB from *C. acetobutylicum*; and aldH from *Clostridium perfringens*, *Clostridium botulinum* A, *Clostridium beijerinckii*, and *Clostridium difficile*. In another specific embodiment the butyraldehyde/butanol dehydrogenase is the butyryl-CoA dehydrogenase bcd from *C. acetobutylicum*.

Cofactor Specificity

[0049] Biomass degradation, and especially the degradation of hemicellulose, yields both C6 sugars such as glucose and C5 sugars such as xylose. Whereas C6 sugars are typically metabolized through the NAD⁺/NADH-dependent Embden-Meyerhof-Parnas pathway (the most common glycolytic pathway), C5 sugars are typically metabolized through the Pentose Phosphate Pathway, which is NADP⁺/NADPH-dependent (Figure 13). NADP⁺/NADPH-dependent enzymes of the Pentose Phosphate Pathway include a glucose dehydrogenase, such as gcd of *E.coli*, and a 2-keto-D-gluconate reductase, such as tiaE of *E.coli*. Applicants do not wish to be bound by theory. However, when producing *n*-butanol from hemicellulose-derived carbon sources it is believed to be beneficial to integrate NADPH-specific enzymes, such as the 3-hydroxybutyryl-CoA dehydrogenase PhaB from *R. eutrophus*, in the *n*-butanol synthesis pathway to rebalance the NADP⁺ required for continued C5 sugar assimilation.

[0050] Because the metabolism of different carbon sources may differently affect cellular NAD⁺/NADH- and NADP⁺/NADPH-redox systems, without wishing to be bound by theory, it is further believed that it is beneficial to tailor recombinant *n*-butanol synthesis pathways to contain an optimized number of either NAD⁺/NADH-dependent or NADP⁺/NADPH-dependent enzymes. This tailoring allows for an optimal rebalancing of the respective redox systems and

ultimately leads to optimized carbon source utilization and *n*-butanol yields. For example, when metabolizing a hexose-rich carbon source, recombinant cells containing a greater number of NAD⁺/NADH-dependent enzymes are preferred. On the contrary, when metabolizing a pentose-rich carbon source recombinant cells containing a greater number of NADP⁺/NADPH-dependent enzymes are preferred. When metabolizing a carbon source yielding a mix of hexoses and pentoses, such as hemicellulose, recombinant cells containing a mix of NAD⁺/NADH-dependent and NADP⁺/NADPH-dependent enzymes within the recombinant *n*-butanol pathway are preferred.

[0051] In one embodiment of the invention the recombinant *n*-butanol synthesis pathway uses NADH, but no NADPH. In one specific embodiment, the recombinant *n*-butanol synthesis pathway (Figure 1, Enzymes 1-6) uses 4 moles of NADH for the production of one mole of *n*-butanol. Such a recombinant *n*-butanol synthesis pathway includes the *C. acetobutylicum* acetoacetyl-CoA reductase Hbd and the *C. acetobutylicum* crotonase Crt. In another embodiment of the invention the recombinant *n*-butanol synthesis pathway uses both NADH and NADPH. In one specific embodiment, the recombinant *n*-butanol synthesis pathway uses 3 moles of NADH and 1 mole of NADPH for the production of one mole of *n*-butanol. Such a recombinant *n*-butanol synthesis pathway includes the *R. eutrophus* 3-hydroxybutyryl-CoA dehydrogenase PhaB and the *A. cavaie* crotonase PhaJ. In a preferred embodiment the recombinant *n*-butanol synthesis pathway using 3 moles of NADH and 1 mole of NADPH includes the acetyl-CoA acetyltransferase PhaA, the *R. eutrophus* 3-hydroxybutyryl-CoA dehydrogenase PhaB, the *A. cavaie* crotonase PhaJ and the trans-enoyl-coA reductase Ter from *T. denticola*.

Coenzyme A Synthesis

[0052] In one embodiment the recombinant cell further contains recombinant sequences encoding one or more enzymes of the coenzyme A biosynthesis pathway.

[0053] In one embodiment the recombinant cell further contains a recombinant sequence encoding a pantothenate kinase catalyzing the conversion of pantothenate to 4'-phosphopantothenate. In one specific embodiment the pantothenate kinase is derived from

E.coli. In another specific embodiment the pantothenate kinase is PanK/CoaA (SEQ ID NOs: 455, 456), or CoaX SEQ ID NOs: 457, 458).

[0054] In another embodiment the recombinant cell further contains a recombinant sequence encoding a phosphopantothenoylcysteine synthetase catalyzing the conversion of 4'-phosphopantothenate to 4'-phosphopantothenoylcysteine. In a specific embodiment the phosphopantothenoylcysteine synthetase is derived from *E.coli*. In another specific embodiment the phosphopantothenoylcysteine synthetase is Ppcs or CoaB (SEQ ID NOs: 459, 460).

[0055] In another embodiment the recombinant cell further contains a recombinant sequence encoding phosphopantothenoylcysteine decarboxylase catalyzing the conversion of 4'-phosphopantothenoylcysteine to 4'-phosphopantetheine. In a specific embodiment the phosphopantothenoylcysteine decarboxylase is derived from *E.coli*. In another specific embodiment the phosphopantothenoylcysteine decarboxylase is Ppcdc or CoaC (SEQ ID NOs: 459, 460).

[0056] In another embodiment the recombinant cell further contains a recombinant sequence encoding phosphopantetheine adenylyl transferase catalyzing the transfer of an adenylyl group from ATP to 4'-phosphopantetheine. In a specific embodiment the phosphopantetheine adenylyl transferase is derived from *E.coli*. In another specific embodiment the phosphopantetheine adenylyl transferase is Ppat or CoaD (SEQ ID NOs: 461, 462).

[0057] In another embodiment the recombinant cell further contains a recombinant sequence encoding dephosphocoenzyme A kinase catalyzing the phosphorylation of dephospho-CoA. In a specific embodiment the dephosphocoenzyme A kinase is derived from *E.coli*. In another specific embodiment the dephosphocoenzyme A kinase is CoaE (SEQ ID NOs: 463, 464).

[0058] Recombinant sequences encoding pantothenate kinase, phosphopantothenoylcysteine synthetase, phosphopantothenoylcysteine decarboxylase, phosphopantetheine adenylyl transferase, or dephosphocoenzyme A kinase may be derived from all prokaryotic organisms, including proteobacterial, archaeobacterial, bacteroid, enterobacterial, spirochetal organisms, and all eukaryotic organisms, including mammalian, insect, fungal and yeast organisms.

Competing Pathways

[0059] In one embodiment of the invention the recombinant cell further contains mutations reducing or eliminating the activity of enzymes in pathways that utilize pyruvate or acetyl-CoA to synthesize products other than *n*-butanol (*Figure 11*). In one specific embodiment enzyme activities are reduced or eliminated in a pathway synthesizing lactate from pyruvate. In another specific embodiment enzyme activities are reduced or eliminated in a pathway synthesizing acetate from pyruvate. In another specific embodiment enzyme activities are reduced or eliminated in a pathway synthesizing acetate from acetyl-CoA. In another specific embodiment enzyme activities are reduced or eliminated in a pathway synthesizing ethanol from acetyl-CoA.

[0060] In one embodiment the recombinant cell contains a lactate dehydrogenase that catalyzes the conversion of pyruvate to lactate with reduced or eliminated activity. In a specific embodiment the lactate dehydrogenase is *ldhA* from *E.coli*. In another embodiment the recombinant cell contains a pyruvate oxidase that catalyzes the conversion of pyruvate to acetate with reduced or eliminated activity. In a specific embodiment the pyruvate oxidase is *poxB* from *E.coli*. In another embodiment the recombinant cell contains an alcohol dehydrogenase that catalyzes the conversion of acetyl-CoA to ethanol with reduced or eliminated activity. In a specific embodiment the alcohol dehydrogenase is *adhE* from *E. coli*. In another embodiment the recombinant cell contains an acetate kinase that catalyzes the conversion of acetyl-CoA to acetate with reduced or eliminated activity. In a specific embodiment the acetate kinase is *ackA*. In another embodiment the recombinant cell contains a phosphotransacetylase that catalyzes the conversion of acetyl-CoA to acetate with reduced or eliminated activity. In a specific embodiment the phosphotransacetylase is *pta*. In another embodiment the recombinant cell contains a fumarate dehydrogenase that catalyzes the conversion of succinate to fumarate with reduced or eliminated activity. In a specific embodiment the phosphotransacetylase is *frd* from *E. coli*.

[0061] The activity of an enzyme having reduced or eliminated activity may be reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% relative to a wild type enzyme, the activity of which is not reduced. Mutations reducing or eliminating the activity of enzymes may include point mutations that cause amino acid changes in the enzymes, deletion mutations,

nonsense mutations, frameshift mutations, sequence duplications or inversions and insertions. Mutations may be introduced in a targeted or non-targeted manner. Mutations may be introduced by molecular biology means, such as homologous recombinations, antisense technologies or RNA interference, or by chemical means, such as treatments with DNA intercalators or DNA methylating agents.

[0062] In one embodiment the recombinant cell is a yeast cell. In a specific embodiment the yeast cell further contains mutations reducing or eliminating the activity of enzymes in pathways that utilize pyruvate or acetyl-CoA to synthesize products other than *n*-butanol. In another specific embodiment the enzymes may include the alcohol dehydrogenase *adh1*, the NAD-dependent glycerol-3-phosphate dehydrogenases *gpd1* or *gpd2*, the NADP-dependent glutamate dehydrogenase *gdh1*, the aquaglyceroporin *fps1*, the pyruvate decarboxylases *pdc1*, *pdc2*, *pdc3*, *pdc4*, and *pdc5*, the acetyl-CoA synthetases *acs1* and *acs2*, and the acetaldehyde dehydrogenases *ALDH1*, *ADLH2*, *ALDH3*, *ALDH4*, *ALDH5*, *ALDH6*.

[0063] In another specific embodiment the recombinant cell further contains recombinant sequences encoding the glutamate synthase *glt1* or the glutamine synthetase *gln1*.

Cells

[0064] Recombinant cells of the invention may include all prokaryotic –including proteobacterial, archaeobacterial, bacteroidal, enterobacterial, spirochetal- and eukaryotic –including mammalian, insect, fungal and yeast- cell types. Preferred embodiments of the invention include, but are not limited to *E. coli* cells, *Zymomonas mobilis* (*Z. mobilis*) cells, *Bacillus subtilis* (*B. subtilis*) cells, yeast cells including *S. cerevisiae* cells and *S. pombe* cells, cyanobacterial cells such as *Synechocystis* sp. and *Synechococcus* sp., photosynthetic cells such as *Rhodospirillum* sp., solvent producing cells such as *Clostridium* sp. (including but not limited to *Clostridium acetobutylicum* and *Clostridium beijerinckii*), chemoautotrophic cells such as *Ralstonia* sp., in general and *Ralstonia eutrophus* in particular, aromatic-degrading cells such as *Pseudomonas* sp. and *Rhodococcus* sp., thermophilic cells such as *Thermoanaerobacterium saccharolyticum* (*T. saccharolyticum*) and *Thermotoga* sp., cellulytic cells such as *Trichoderma*

reesei (*T. reesei*) cells, and *Aspergillus niger* (*A. niger*) cells, and lignocellulytic cells such as *Phanerochaete chrysosporium* (*P. chrysosporium*), CHO cells, SF9 cells.

General Methods

[0065] Metabolites and products formed as part of the recombinant biofuel pathway can be identified and quantified using standard HPLC chromatography and mass spectrometry techniques. Enzymatic activities can be determined using traditional spectrophotometric activity assays relying on the detection of NAD(P)H cofactor consumption.

[0066] The nucleic acids may be synthesized, isolated, or manipulated using standard molecular biology techniques such as those described in Sambrook, J. et al. 2000. Molecular Cloning: A Laboratory Manual (Third Edition). Techniques may include cloning, expression of cDNA libraries, and amplification of mRNA or genomic DNA.

[0067] The nucleic acids of the present disclosure, or subsequences thereof, may be incorporated into a cloning vehicle comprising an expression cassette or vector. The cloning vehicle can be a viral vector, a plasmid, a phage, a phagemid, a cosmid, a fosmid, a bacteriophage, or an artificial chromosome. The viral vector can comprise an adenovirus vector, a retroviral vector, or an adeno-associated viral vector. The cloning vehicle can comprise a bacterial artificial chromosome (BAC), a plasmid, a bacteriophage P1-derived vector (PAC), a yeast artificial chromosome (YAC), or a mammalian artificial chromosome (MAC).

[0068] The nucleic acids may be operably linked to a promoter. The promoter can be a viral, prokaryotic, or eukaryotic promoter. The promoter can be a constitutive promoter, an inducible promoter, a tissue-specific promoter, or an environmentally regulated or a developmentally regulated promoter.

Methods for Producing *n*-Butanol

[0069] In one embodiment of the invention the method for the production of *n*-butanol includes the step of growing a recombinant cell of the invention in the presence of a suitable carbon source.

[0070] Suitable carbon sources may include, but are not limited to glucose, glycerol, sugars, starches, and lignocellulosics, including but not limited to glucose derived from cellulose and C₅ sugars derived from hemicellulose, such as xylose.

[0071] In one specific embodiment the recombinant cell of the invention is grown under aerobic conditions. In another specific embodiment the recombinant cell of the invention is grown under microaerobic conditions. In another specific embodiment the recombinant cell of the invention is grown under anaerobic conditions. In another specific embodiment the recombinant cell of the invention is grown under conditions wherein it produces more *n*-butanol under anaerobic conditions than under aerobic or microaerobic conditions. In another specific embodiment the recombinant cell of the invention is grown under conditions wherein it produces more total levels of *n*-butanol and ethanol under anaerobic conditions than under aerobic or microaerobic conditions. In another specific embodiment the recombinant cell of the invention is grown under anaerobic conditions wherein it produces near quantitative yields of *n*-butanol. In another specific embodiment the recombinant cell of the invention is grown under anaerobic conditions wherein it produces near quantitative yields of *n*-butanol and ethanol.

[0072] In one specific embodiment the recombinant cell of the invention is grown under aerobic conditions wherein it produces elevated levels of *n*-butanol compared to a wild-type cell grown under aerobic conditions. Total levels of *n*-butanol produced by the recombinant cell of the invention under aerobic conditions may be elevated by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%, 3-fold, 10-fold, 30-fold, 100-fold, 300-fold, 1,000-fold, 3,000-fold, 10,000-fold, 30,000-fold, 100,000-fold, 300,000-fold or 100,000-fold compared to the *n*-butanol levels produced by a wild-type cell under aerobic conditions. In specific embodiments the recombinant cell of the invention is grown under aerobic conditions wherein it produces at least 0.01 g/L, at least 0.03 g/L, at least 0.1 g/L, at least 0.3 g/L, at least 1.0 g/L, at least 1.5 g/L, at least 2.0 g/L, at least 2.5 g/L, at least 3.0 g/L, at least 3.5 g/L, at least 4.0 g/L, at least 4.5 g/L, at least 5.0 g/L, at least 6.0 g/L, at least 7.0 g/L, at least 8.0 g/L, at least 9.0 g/L, at least 10.0 g/L, at least 15.0 g/L, at least 20.0 g/L, at least 30.0 g/L, at least 50.0 g/L, or at least 75.0 g/L *n*-butanol.

[0073] In one specific embodiment the recombinant cell of the invention is grown under aerobic conditions wherein it produces elevated total levels of *n*-butanol and ethanol compared to

a wild-type cell grown under aerobic conditions. Total levels of *n*-butanol and ethanol produced by the recombinant cell of the invention under aerobic conditions may be elevated by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%, 3-fold, 10-fold, 30-fold, 100-fold, 300-fold, 1,000-fold, 3,000-fold, 10,000-fold, 30,000-fold, 100,000-fold, 300,000-fold or 100,000-fold compared to the total levels of *n*-butanol and ethanol produced by a wild-type cell under aerobic conditions. In specific embodiments the recombinant cell of the invention is grown under aerobic conditions wherein it produces total levels of *n*-butanol and ethanol of at least 0.01 g/L, at least 0.03 g/L, at least 0.1 g/L, at least 0.3 g/L, at least 1.0 g/L, at least 1.5 g/L, at least 2.0 g/L, at least 2.5 g/L, at least 3.0 g/L, at least 3.5 g/L, at least 4.0 g/L, at least 4.5 g/L, at least 5.0 g/L, at least 6.0 g/L, at least 7.0 g/L, at least 8.0 g/L, at least 9.0 g/L, at least 10.0 g/L, at least 15.0 g/L, at least 20.0 g/L, at least 30.0 g/L, at least 50.0 g/L, or at least 75.0 g/L.

[0074] In one specific embodiment the recombinant cell of the invention is grown under anaerobic conditions wherein it produces elevated levels of *n*-butanol compared to a wild-type cell grown under anaerobic conditions. Total levels of *n*-butanol produced by the recombinant cell of the invention under anaerobic conditions may be elevated by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%, 3-fold, 10-fold, 30-fold, 100-fold, 300-fold, 1,000-fold, 3,000-fold, 10,000-fold, 30,000-fold, 100,000-fold, 300,000-fold or 100,000-fold compared to the *n*-butanol levels produced by a wild-type cell under anaerobic conditions. In specific embodiments the recombinant cell of the invention is grown under anaerobic conditions wherein it produces at least 0.01 g/L, at least 0.03 g/L, at least 0.1 g/L, at least 0.3 g/L, at least 1.0 g/L, at least 1.5 g/L, at least 2.0 g/L, at least 2.5 g/L, at least 3.0 g/L, at least 3.5 g/L, at least 4.0 g/L, at least 4.5 g/L, at least 5.0 g/L, at least 6.0 g/L, at least 7.0 g/L, at least 8.0 g/L, at least 9.0 g/L, at least 10.0 g/L, at least 15.0 g/L, at least 20.0 g/L, at least 30.0 g/L, at least 50.0 g/L, or at least 75.0 g/L *n*-butanol.

[0075] In one specific embodiment the recombinant cell of the invention is grown under anaerobic conditions wherein it produces elevated total levels of *n*-butanol and ethanol compared to a wild-type cell grown under anaerobic conditions. Total levels of *n*-butanol and ethanol produced by the recombinant cell of the invention under anaerobic conditions may be elevated by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 100%, 3-fold, 10-fold, 30-fold, 100-fold, 300-fold, 1,000-fold, 3,000-fold, 10,000-fold, 30,000-fold, 100,000-fold, 300,000-fold or

100,000-fold compared to the total levels of *n*-butanol and ethanol produced by a wild-type cell under anaerobic conditions. In specific embodiments the recombinant cell of the invention is grown under anaerobic conditions wherein it produces total levels of *n*-butanol and ethanol of at least 0.01 g/L, at least 0.03 g/L, at least 0.1 g/L, at least 0.3 g/L, at least 1.0 g/L, at least 1.5 g/L, at least 2.0 g/L, at least 2.5 g/L, at least 3.0 g/L, at least 3.5 g/L, at least 4.0 g/L, at least 4.5 g/L, at least 5.0 g/L, at least 6.0 g/L, at least 7.0 g/L, at least 8.0 g/L, at least 9.0 g/L, at least 10.0 g/L, at least 15.0 g/L, at least 20.0 g/L, at least 30.0 g/L, at least 50.0 g/L, or at least 75.0 g/L.

[0076] The methods described herein can be practiced in combination with other methods useful for the production of *n*-butanol, such as methods for the conversion of lignocellulosic materials into biofuels.

[0077] For example, plant material may be subjected to pretreatment including ammonia fiber expansion (AFEX), steam explosion, treatment with alkaline aqueous solutions, acidic solutions, organic solvents, ionic liquids (IL), electrolyzed water, phosphoric acid, and combinations thereof. Pretreatments that remove lignin from the plant material may increase the overall amount of sugar released from the hemicellulose.

[0078] Because hemicellulose degradation yields both C6 sugars (e.g., glucose) and C5 sugars (e.g., xylose) a combination of recombinant *n*-butanol biosynthesis pathways with optimized recombinant glycolysis pathways (for C6 sugar assimilation) or optimized recombinant pentose phosphate pathways (for C5 sugar assimilation) may be useful for the achievement of optimal biomass utilization and *n*-butanol yields.

Preferred Embodiments

[0079] In one preferred embodiment of the invention the recombinant cell contains recombinant sequences encoding the pyruvate decarboxylase Pdc from *Z. mobilis*, the acylating aldehyde dehydrogenase EutE from *E. coli*, the keto-thiolase PhaA from *R. eutrophus*, the hydroxybutyryl-CoA dehydrogenase Hbd from *C. acetobutylicum*, the crotonase Crt from *C. acetobutylicum*, the crotonyl-CoA reductase Ter from *T. denticola*, and the alcohol dehydrogenase AdhE2 from *C. acetobutylicum*. In another preferred embodiment the recombinant cell contains recombinant sequences encoding the pyruvate:flavodoxin/ferredoxin-

oxidoreductase YdbK from *E.coli*, the keto-thiolase PhaA from *R. eutrophus*, the hydroxybutyryl-CoA dehydrogenase Hbd from *C. acetobutylicum*, the crotonase Crt from *C. acetobutylicum*, the crotonyl-CoA reductase Ter from *T. denticola*, and the alcohol dehydrogenase AdhE2 from *C. acetobutylicum*. In another preferred embodiment the recombinant cell is a *S. cerevisiae* cell, an *E. coli* cell, a *C. acetobutylicum* cell, or a *C. beijerinckii* cell.

[0080] In another preferred embodiment the recombinant cell further contains a recombinant sequence encoding a component of an acetyl-CoA synthesis pathway, including pantothenate kinase (PanK, CoaA, CoaX), phosphopantothenoylcysteine synthetase (Ppcs, CoaB), phosphopantothenoylcysteine decarboxylase (Ppcdc, CoaC), and phosphopantetheine adenylyl transferase (Ppat, CoaD), and dephosphocoenzyme A kinase (CoaE).

[0081] In another preferred embodiment the recombinant cell further contains reduced or eliminated activities of at least one enzyme of a biosynthesis pathways utilizing pyruvate or acetyl-CoA for other purposes than n-butanol biosynthesis, such as lactate dehydrogenase, pyruvate oxidase, alcohol dehydrogenase, acetate kinase, or phosphotransacetylase.

[0082] In another preferred embodiment a preferred recombinant cell of the invention is grown in the presence of a suitable carbon source. In another preferred embodiment the preferred cell of the invention is grown under anaerobic conditions. In another preferred embodiment the preferred cell of the invention is grown under conditions wherein the cell produces total levels of n-butanol and ethanol of at least 5.0 g/L.

EXAMPLES

[0083] The following Examples are merely illustrative and are not meant to limit any aspects of the present disclosure in any way.

Summary of Examples

[0084] Example 1: Production of n-butanol in recombinant *E. coli*

- [0085] Example 2: Identification of bottleneck in recombinant n-butanol synthesis pathway
- [0086] Example 3: Ter increases n-butanol production in recombinant cells
- [0087] Example 4: Elevation of PDH and PFOR activities further increase n-butanol yields
- [0088] Example 5: Efficient production of n-butanol in a recombinant cell
- [0089] Example 6: Construction of a recombinant *S. cerevisiae* cell for n-butanol production

Materials and methods.

[0090] Terrific Broth (TB), LB Broth Miller (LB), LB Agar Miller, sulfuric acid and glycerol were purchased from EMD Biosciences (Darmstadt, Germany). Isopropyl β -D-1-thiogalactopyranoside (IPTG) D-glucose, Dithiothreitol (DTT), Tris-HCl, phenylmethanesulfonyl fluoride (PMSF), carbenicillin (Cb), ammonium acetate, streptomycin sulfate and HPLC-grade acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). L-arabinose, chloramphenicol (Cm), kanamycin (Km), coenzyme A (CoASH), acetyl-CoA, acetoacetyl-CoA, crotonyl-CoA, butyryl-CoA, butyraldehyde, N,N,N',N'-Tetramethylethylenediamine (TEMED), NADH, NADPH, and NAD⁺ were purchased from Sigma-Aldrich (St. Louis, MO). Polyacrylamide, Protein Assay reagent, electrophoresis grade sodium dodecyl sulfate (SDS), and ammonium persulfate were purchased from Bio-Rad Laboratories (Hercules, CA). All PCR amplifications were carried out with Phusion polymerase (New England BioLabs; Ipswich, MA), unless otherwise noted. Deoxynucleotides (dNTPs) and Platinum Taq High-Fidelity polymerase (Pt Taq HF) were purchased from Invitrogen (Carlsbad, CA). All restriction enzymes, antarctic phosphatase, polynucleotide kinase, T4 Polymerase and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). DNA was isolated using the QIAprep Spin Miniprep Kit, QIAquick PCR Purification Kit, and QIAquick Gel Extraction Kit (QIAGEN; Valencia, CA) as appropriate. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and resuspended at a stock concentration of 100 μ M in 10 mM Tris-HCl, pH 8.5. Codon optimization and back-translation were carried out using Gene Designer 2.0 (DNA 2.0; Menlo Park, CA). All synthetic genes and inserts were sequenced using the sequencing primers for the appropriate gene(s) following plasmid construction by the

UC Berkeley Sequencing Facility, Sequetech (Mountain View, CA), or Quintara Biosciences (Berkeley, CA). All absorbance readings were taken on a DU-800 spectrometer (Beckman-Coulter; Fullerton, CA) or a SpectraMax M2 plate reader (Molecular Devices; Toronto, Canada).

Bacterial strains.

[0091] *E. coli* DH10B-T1R, DH10B-T1R(de3), DH1, DH1(de3), and BL21(de3), and were used for protein and n-butanol production studies. DH10B-T1R and DH1 were lysogenized using λ DE3 Lysogenization Kit from Novagen (San Diego, CA). Additional strain optimization in *E. coli* DH1 was achieved by knocking out metabolic genes to divert carbon flux from organic acid metabolites to the synthetic butanol pathway (Table 1, Figure 9).

Cell culture.

[0092] *E. coli* strains were transformed by electroporation using the appropriate plasmids. A single colony from a fresh transformation was then used to seed an overnight culture grown in Terrific Broth (TB) supplemented with 0.5% glucose and appropriate antibiotics at 37°C in a rotary shaker (200 rpm). Antibiotics were used at a concentration of 50 µg/mL for strains with a single resistance marker. For strains with multiple resistance markers, kanamycin (Km) and chloramphenicol (Cm) were used at 25 µg/mL and carbenicillin (Cb) was used at 50 µg/mL.

Example 1: Production of n-butanol in *E. coli*

[0093] A recombinant pathway for n-butanol synthesis in *E. coli* was constructed in the form of a two plasmid system in *E. coli* BL21(de3) cells comprising the *R. eutrophus* genes phaA and phaB, the *C. acetobutylicum* genes crt and adh2 and the *S. cinnamonensis* gene ccr (Figure 2). Although n-butanol formation could be observed by gas chromatography-mass spectrometry, the titer achieved in *E. coli* BL21(de3) cells was low (~2 mg/L).

Gene synthesis

[0094] Synthetic genes encoding PhaA (SEQ ID NO 15), PhaB (SEQ ID NO 16), Crt (SEQ ID NO 21), Ccr (SEQ ID NO 23), and AdhE2 (SEQ ID NO 33) were optimized for *E. coli* class II

codon usage and obtained from Epoch Biosciences (Sugar Land, TX). Gene2Oligo (<http://berry.engin.umich.edu/gene2oligo>) was used to convert the gene sequence into primer sets using default optimization settings (Gene Construction Primers: Ter (*E. gracilis*) – SEQ ID NOs 45-112; Ter (*T. denticola*) – SEQ ID NOs 113-184; Ccr (*S. cinnamonensis*) – SEQ ID NOs 185-260; Hbd (*C. acetobutylicum*) - SEQ ID NOs 261-314). To assemble the synthetic gene, each primer was added at a final concentration of 1 μ M to the first PCR reaction (50 μ L) containing 1 $\times$ Pl Taq HF buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), MgSO₄ (1.5 mM), dNTPs (250 μ M each), and Pt Taq HF (5 U). The following thermocycler program was used for the first assembly reaction: 95 °C for 5 min; 95 °C for 30 s; 55 °C for 2 min; 72 °C for 10 s; 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 72 °C for 20 s plus 3 s/cycle; these cycles were followed by a final incubation at 72 °C for 5 min. The second assembly reaction (50 μ L) contained 16 μ L of the unpurified first PCR reaction with standard reagents for Pt Taq HF. The thermocycler program for the second PCR was: 95 °C for 30 s; 55 °C for 2 min; 72 °C for 10 s; 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 72 °C for 80 s; these cycles were followed by a final incubation at 72 °C for 5 min. The second PCR reaction (16 μ L) was transferred again into fresh reagents and run using the same program. Following gene construction, the DNA smear at the appropriate size was gel purified and used as a template for the rescue PCR (50 μ L) with Pt Taq HF and rescue primers (TdTer F1 and R1) under standard conditions. The resulting rescue product was either inserted directly in the appropriate vector or first cloned into pCR2.1-TOPO using a TOPO TA Cloning Kit from Invitrogen.

Construction of plasmids

[0095] Standard molecular biology techniques were used to carry out plasmid construction using *E. coli* DH10B-T1R as the cloning host. Primers are listed in SEQ ID NOs 315-334. Annealed inserts were generated by phosphorylating each primer (1.5 pmol) individually with polynucleotide kinase in T4 DNA ligase buffer followed by incubation at 37 °C for 30 min and heat inactivation at 65 °C for 20 min. The phosphorylated primers were then mixed in 1 $\times$ annealing buffer (100mM NaCl, 50mM HEPES, pH 7.4) and annealed using the following program and used immediately once the reaction reached 25 °C: 90 °C for 4 min, 70 °C for 10 min, ramped to 37 °C at 0.5 °C/s, 37 °C for 15 min, ramped to 25 °C at 0.5 °C/s.

pBT33-phaAB-crt. The phaAB operon was amplified from pCR2.1-phaA2.phaB using the phaA2 F2 and phaB R2 primers and inserted into the SacI-XbaI restriction sites of pBAD33 to generate pBAD33-phaAB. The pTrc99a-crt cloning intermediate was made by inserting the synthetic crt gene into the NcoI-XmaI restriction sites of pTrc99a using the crt F2 and crt R2 primers to amplify the insert. The resulting PTrc.crt.rnB cassette was amplified from pTrc99a-crt using the pTrc99a F4 and pTrc99a R4 primers and inserted non-directionally into the BglII site of pBAD33-phaAB to produce pBT33-phaABcrt. Sequencing showed the coding strand of the phaAB operon was on the same strand as the crt gene. pBT33-phaB-hbd. The pCR2.1-phaA.hbd cloning intermediate was constructed by amplification of the synthetic hbd gene from pCR2.1-hbd with the hbd F1 and hbd R1 primers and insertion into the EcoRIHindIII restriction sites of pCR2.1-phaA2.phaB. The phaAB operon of pBT33-phaAB-crt was then replaced with a new multiple cloning site by digestion with NdeI and XhoI and insertion of a linker using sequence and ligation independent cloning (SLIC) (Li and Elledge, 2007, Nature Methods. 4, 251-56). The insert was made by amplifying the rrnB terminator from pBAD33 using primers rrnB SLIC F1 and rrnB SLIC R1. The amplified fragment and digested vector were independently treated with 0.5 U T4 polymerase for 30 min and the reaction was quenched with the addition of dATP. The insert and vector were incubated in 1x ligation buffer for 30 min at 37°C and transformed immediately.

[0096] *pCWOri-ccr.adhE2*. pCWOri-ccr.adhE2 was made by inserting the ccr-adhE2 operon from pET29accr. adhE2 into the NdeI-HindIII sites of pCWOri. The primers used to amplify the operon were ccr F1 and adhE2 R1.

In vivo production of n-butanol

[0097] For production of n-butanol production in baffled flasks, the overnight cultures were grown for 12-16 h and used to inoculate TB (50 mL) with either 2% glucose or 2% glycerol replacing the standard glycerol supplement and appropriate antibiotics in a 250 mL-baffled flask to a starting OD600 = 0.05. The cultures were grown at 37°C in a rotary shaker (200 rpm) and induced with IPTG (1.0 mM) and L-arabinose (0.2%) when appropriate at OD600 = 0.35-0.45. At this time the growth temperature was reduced to 30°C. Upon induction and following all daily samplings, flasks were sealed with Parafilm M (Pechiney Plastic Packaging, Chicago, IL).

For production of n-butanol production in culture tubes, the overnight cultures were grown for 22-26 h and used to inoculate (1%, 50 μ L) precultures in TB with 0.5% glucose (5 mL). After incubation at 37 °C in rotary shaker (250 rpm) for 16 h, precultures were back-diluted 8 to OD₆₀₀=0.4 in TB with 2.5% glucose replacing the standard glycerol supplement (5 mL) in anaerobic tubes (20 mm; Bellco Glass; Vineland, NJ) and induced with IPTG (1.0 mM) and L-arabinose (0.2%). The growth temperature was then reduced to 30°C and the culture tubes sealed with aluminum seals using butyl rubber septa (Bellco Glass) unless otherwise noted. For anaerobic growth, the headspace of the cultures was deoxygenated with Ar gas after backdilution and induction. Semi-anaerobic growth was performed with cultures in sealed tubes without degassing with Ar and aerobic growth was performed in unsealed tubes. Extraction and quantification of n-butanol. Samples (2 mL) were removed from cell culture and cleared of biomass by centrifugation at 20817 $\times$ g for 2 min using an Eppendorf 5417R centrifuge (Hamburg, Germany). The supernatant or cleared media sample was then mixed 1:1 with an aqueous solution containing the isobutanol internal standard (1000 mg/L). These samples were then analyzed on a Trace GC Ultra (Thermo Scientific; Waltham, MA) using an HP-5MS column (0.25 mm $\times$ 30 m, 0.25 μ M film thickness, J & W Scientific). The oven program was as follows: 75 °C for 3 min, ramp to 300 °C at 45 °C/min, 300 °C for 1 min. n-Butanol was quantified using by flame ionization detection (FID) (using flow of 350 ml/min air, 35 ml/min H₂, and 30ml/min He). Samples containing n-butanol levels below 500 mg/L were then re-quantified with a DSQII single-quadrupole mass spectrometer (Thermo Scientific; Waltham, MA) using single ion monitoring (m/z 41 and 56) concurrent with full scan mode (m/z 35-80) for samples with n-butanol levels lower than 500 mg/L. Samples were quantified relative to a standard curve of 2, 5, 10, 25, 50, and 100 mg/L n-butanol for MS detection or 62.5, 125, 250, 500, 1000, 2000, 4000 mg/L n-butanol for FID detection. Standard curves were prepared freshly during each run and normalized for injection volume using the internal isobutanol standard

Example 2: Identification of bottleneck in recombinant n-butanol synthesis pathway

[0098] The initial n-butanol yields obtained with the recombinant cellular system of Example 1 were subsequently improved ~60-fold by promoter and host cell optimization (Figures 2 and 3A).

A correlation was observed between n-butanol yields and solubility of the Ccr protein, which pointed to a bottleneck in the n-butanol biosynthesis pathway at the conversion step of crotonyl-CoA to butyryl-CoA (*Figure 3B*).

Construction of plasmids

[0099] pBAD33-*ccr.adhE2*. The *ccr-adhE2* operon was amplified from pET29a-*ccr.adhE2* using the *ccr* F1 and *adhE2* R17 primers and inserted into the NdeI-SalI sites of pBAD33-*phaAB*, the insert was digested using NdeI and XhoI.

[00100] pTrc99a-*ccr.adhE2*. pTrc99a-*ccr.adhE2* was made by inserting the *ccr-adhE2* operon from pET29a-*ccr.adhE2* into the NcoI-SacI sites. The primers used to amplify the operon were *ccr* F15 and *adhE2* R2.

[00101] pCWOri-*ter.adhE2*. The *ter* gene was amplified from pET16b-His-*ter* with TdTer F1 and TdTer R102 and inserted directly into the NdeI-EcoRI restriction sites of pCWOri-*ccr.adhE2*.

[00102] pET29a-*ccr.adhE2*. The *ccr* gene was amplified using the *ccr* F1 and *ccr* R2 primers and inserted into the NdeI-EcoRI sites of pET29a. pET29a-*ccr.adhE2* was constructed by insertion of the *adhE2* gene into the EcoRI-SacI restriction sites of pET29a-*ccr* after amplification using the *adhE2* F1 and *adhE2* R2 primers.

Example 3: Ter increases n-butanol production in recombinant cells

[00103] In an experiment similar to Example 1, the replacement of the *S. cinnamomensis* gene *ccr* for *ter* genes from *E. gracilis* and *T. denticola* resulted in significantly increased n-butanol yields, where the recombinant biosynthesis pathway further comprised the *R. eutrophus* gene *phaA*, and the *C. acetobutylicum* genes *hbd*, *crt* and *adh2* (*Figure 5*). This experiment thus demonstrates that the incorporation of Ter enzymes into the recombinant biosynthesis pathway for n-butanol relieves a bottleneck at the stage of crotonyl-CoA to butyryl-CoA conversion.

Example 4: Elevation of PDH and PDHc bypass activities further increase n-butanol yields

[00104] Acetyl-CoA is the building block for the production of advanced fuels ranging from short-, medium-, and long-chain length fatty alcohols, fatty acids, fatty acid esters, and alkanes. A major challenge in the production of these molecules is the bottleneck from the endpoint of glycolysis, the conversion of pyruvate to acetyl-CoA. Four classes of enzymes were identified that can relieve this bottleneck: pyruvate dehydrogenase PDH, PDHc bypass comprised of two enzymes (pdc and eutE), *E. coli* pyruvate formate oxido-reductase (PFOR), and *E. coli* pyruvate formate lyase with *C. boidinii* formate dehydrogenase (pfl and fdh).

[00105] In an experiment similar to Example 4, the elevation of PDH activity further increased n-butanol yields beyond the yields observed in the presence of Ter alone (*Figures 5 and 6*). This finding demonstrates that a second bottleneck existed in the n-butanol biosynthesis pathway at the initial conversion of pyruvate to acetyl-CoA. Increasing the concentration of acetyl-CoA by increasing the turnover of pyruvate relieved this second bottleneck and resulted in higher n-butanol yields.

[00106] The third route to generate acetyl-CoA from pyruvate is catalyzed by PDHc bypass that is composed of two enzymes, pyruvate decarboxylase and acetylating aldehyde dehydrogenase. Acetaldehyde is generated by pyruvate decarboxylase from pyruvate and then oxidized to acetyl-CoA, coupled with the reduction of NAD⁺ to balance the reducing equivalent required for butanol synthesis. In the presence of these enzymes, and under anaerobic conditions, n-butanol yield can increase by 50% (Fig 8).

Example 5: Efficient production of n-butanol in a recombinant cell

[00107] Through the use of Ter from *T. denticola* and overexpression of the *E. coli* pyruvate dehydrogenase complex or the pyruvate decarboxylase of *Z. mobilis* and the acetylating aldehyde dehydrogenase of *E. coli* in a pathway otherwise comprising the *R. eutrophus* gene phaA, and the *C. acetobutylicum* genes hbd, crt and adh2 it was possible to engineer a highly efficient recombinant cell for the production of n-butanol.

Table 1: Knockout *E. coli* DH1 host strains for the production of n-butanol.

Strain	Genotype
<i>E. coli</i> DH1	<i>endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17(rK- mK+) λ-</i>
MC001	<i>E. coli</i> DH1 $\Delta adhE$
MC002	<i>E. coli</i> DH1 $\Delta adhE, \Delta ldhA$
MC003	<i>E. coli</i> DH1 $\Delta adhE, \Delta ldhA, \Delta ackA-pta$
MC004	<i>E. coli</i> DH1 $\Delta adhE, \Delta ldhA, \Delta poxB$
MC005	<i>E. coli</i> DH1 $\Delta adhE, \Delta ldhA, \Delta ackA-pta, \Delta poxB$
MC006	<i>E. coli</i> DH1 $\Delta adhE, \Delta ldhA, \Delta ackA-pta, \Delta poxB, \Delta frdBC$

Example 6: *n*-Butanol production in a recombinant *S. cerevisiae* cell

[00108] *S. cerevisiae* is another preferred host for a recombinant *n*-butanol production pathway and well suited to support industrial fuel production. The preferred recombinant *n*-butanol synthesis pathway was inserted into *S. cerevisiae* (Figure 12A). The recombinant pathway includes the pyruvate decarboxylase Pdc from *Z. mobilis*, the acylating aldehyde dehydrogenase EutE from *E. coli*, the keto-thiolase PhaA from *R. eutrophus*, the hydroxybutyryl-CoA dehydrogenase Hbd from *C. acetobutylicum*, the crotonase Crt from *C. acetobutylicum*, the crotonyl-CoA reductase Ter from *T. denticola*, and the alcohol dehydrogenase AdhE2 from *C. acetobutylicum* (Figure 12A). The DNA constructs shown in Figure 12A for both plasmid-based and chromosomal gene expression were made using standard methods described above and one-step isothermal DNA assembly as described by Gibson, *et al.*, *Nat. Methods.* (2009) **6**, p. 343.

[00109] To optimize production of *n*-butanol, pyruvate decarboxylase pdc (mutant cell: Δpdc) and the alcohol dehydrogenase adh1 (mutant cell: $\Delta adh1$) were targeted for deletion in *S. cerevisiae* because these enzymes are involved in competing, acetyl-CoA consuming pathways other than *n*-butanol production. (See also Figure 11 for analogous *E. coli* pathways). Wild-type *S. cerevisiae* as well as Δpdc and $\Delta adh1$ strains bearing a plasmid-based *n*-butanol genetic

system were prepared using standard molecular biology techniques. Recombinant *S. cerevisiae* cells with the preferred *n*-butanol pathway were shown to produce at least 10 mg/L *n*-butanol. For example, a $\Delta adh1$ mutant cell, *S. cerevisiae* BY4741 Δadh , containing the *n*-butanol production pathway (Figure 12A) was shown to produce greater than 12 mg/L *n*-butanol (Figure 12B, column 2), whereas the background level of *n*-butanol production of *S. cerevisiae* BY4741 Δadh was only about 2 mg/L (Figure 12B, column 1).

CLAIMS

What is claimed:

1. A recombinant cell for the synthesis of n-butanol, the cell comprising:
 - i. a recombinant sequence encoding an acylating aldehyde dehydrogenase catalyzing the conversion of acetaldehyde to acetyl-CoA,
 - ii. a recombinant sequence encoding a keto-thiolase or acetyl-CoA acetyltransferase catalyzing the conversion of acetyl-CoA to acetoacetyl-CoA,
 - iii. a recombinant sequence encoding an acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase catalyzing the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA,
 - iv. a recombinant sequence encoding a crotonase catalyzing the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA,
 - v. a recombinant sequence encoding a crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase catalyzing the conversion of crotonyl-CoA to butyryl-CoA, and
 - vi. a recombinant sequence encoding a butyraldehyde/butanol dehydrogenase catalyzing the conversion of butyryl-CoA to n-butanol.
2. The recombinant cell of claim 1, wherein the sequences encoding the acylating aldehyde dehydrogenase, the keto-thiolase or acetyl-CoA acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase are linked.
3. The recombinant cell of claim 1, wherein the sequences encoding the acylating aldehyde dehydrogenase, the keto-thiolase or acetyl-CoA acetyltransferase, the

acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase are not linked.

4. The recombinant cell of claim 1 further comprising a recombinant sequence encoding a pyruvate decarboxylase catalyzing the conversion of pyruvate to acetaldehyde.
5. The recombinant cell of claim 4, wherein the pyruvate decarboxylase is derived from *Z. mobilis* or *S. cerevisiae*.
6. A recombinant cell for the synthesis of n-butanol, the cell comprising:
 - i. a recombinant sequence encoding an pyruvate:flavodoxin/ferredoxin-oxidoreductase catalyzing the conversion of pyruvate to acetyl-CoA,
 - ii. a recombinant sequence encoding a keto-thiolase or acetyl-CoA acetyltransferase catalyzing the conversion of acetyl-CoA to acetoacetyl-CoA,
 - iii. a recombinant sequence encoding an acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase catalyzing the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA,
 - iv. a recombinant sequence encoding a crotonase catalyzing the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA,
 - v. a recombinant sequence encoding a crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase catalyzing the conversion of crotonyl-CoA to butyryl-CoA, and
 - vi. a recombinant sequence encoding a butyraldehyde/butanol dehydrogenase catalyzing the conversion of butyryl-CoA to n-butanol.
7. The recombinant cell of claim 6, wherein the sequences encoding the pyruvate:flavodoxin/ferredoxin-oxidoreductase, the keto-thiolase or acetyl-CoA

acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase are linked.

8. The recombinant cell of claim 6, wherein the sequences encoding the pyruvate:flavodoxin/ferredoxin-oxidoreductase, the keto-thiolase or acetyl-CoA acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase are not linked.
9. The recombinant cell of claim 6, wherein the pyruvate:flavodoxin/ferredoxin-oxidoreductase is derived from *E. coli*, *S. cerevisiae*, *E. faecalis*, or *Z. mobilis*.
10. The recombinant cell of claims 1 or 6, wherein the acylating aldehyde dehydrogenase is derived from *E.coli*.
11. The recombinant cell of claim 10, wherein the acylating aldehyde dehydrogenase is EutE.
12. The recombinant cell of claims 1 or 6, wherein the keto-thiolase or acetyl-CoA acetyltransferase is derived from *E.coli*, *R. eutrophus*, *A. caviae*, *T. denticola* or *C. acetobutylicum*.
13. The recombinant cell of claim 12, wherein the keto-thiolase or acetyl-CoA acetyltransferase is PhaA, AtoB, FadA, or Thl.
14. The recombinant cell of claims 1 or 6, wherein the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase is derived from *E.coli*, *R. eutrophus*, *A. caviae*, *T. denticola*, or *C. acetobutylicum*.

15. The recombinant cell of claim 14, wherein the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase is Hbd or PhaB.
16. The recombinant cell of claims 1 or 6, wherein the crotonase is derived from *E.coli*, *R. eutrophus*, *A. caviae*, *T. denticola*, or *C. acetobutylicum*.
17. The recombinant cell of claim 16, wherein the crotonase is Crt or PhaJ.
18. The recombinant cell of claims 1 or 6, wherein the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase is derived from *E.coli*, *R. eutrophus*, *A. caviae*, *T. denticola*, *C. acetobutylicum* or *S. collinus*.
19. The recombinant cell of claim 18, wherein the crotonyl-CoA reductase, butyryl-CoA dehydrogenase, or trans-enoyl-CoA reductase is Ter or Ccr.
20. The recombinant cell of claims 1 or 6, wherein the butyraldehyde/butanol dehydrogenase is derived from *E.coli*, *R. eutrophus*, *A. caviae*, *T. denticola*, or *C. acetobutylicum*.
21. The recombinant cell of claim 20, wherein the butyraldehyde/butanol dehydrogenase is Bcd, Aad, or AdhE2.
22. The recombinant cell of claim 4, wherein the pyruvate decarboxylase is Pdc from *Z. mobilis*, the acylating aldehyde dehydrogenase is EutE from *E. coli*, the keto-thiolase is PhaA from *R. eutrophus*, the hydroxybutyryl-CoA dehydrogenase is Hbd from *C. acetobutylicum*, the crotonase is Crt from *C. acetobutylicum*, the crotonyl-CoA reductase is Ter from *T. denticola*, and the alcohol dehydrogenase is AdhE2 from *C. acetobutylicum* and wherein the recombinant cell is a *S. cerevisiae* cell or an *E. coli* cell.
23. The recombinant cell of claim 6, wherein the pyruvate:flavodoxin/ferredoxin-oxidoreductase is YdbK from *E.coli*, the keto-thiolase is PhaA from *R. eutrophus*, the hydroxybutyryl-CoA dehydrogenase is Hbd from *C. acetobutylicum*, the crotonase is

Crt from *C. acetobutylicum*, the crotonyl-CoA reductase is Ter from *T. denticola*, and the alcohol dehydrogenase is AdhE2 from *C. acetobutylicum* and wherein the recombinant cell is a *S. cerevisiae* cell or an *E. coli* cell.

24. The recombinant cell of claim 23 further comprising recombinant sequences encoding the ferredoxin-NADP reductase from *E. coli*, the ferredoxin *FdC* from *E. coli*, and the flavodoxins *FldA* and *FldB* from *E. coli*.
25. The recombinant cell of claim 1, wherein the recombinant sequences encoding the acylating aldehyde dehydrogenase, the keto-thiolase or acetyl-CoA acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase encode enzymes that require 4 moles of NADH for the production of one mole of n-butanol.
26. The recombinant cell of claim 6, wherein the recombinant sequences encoding the pyruvate:flavodoxin/ferredoxin-oxido-reductase, the keto-thiolase or acetyl-CoA acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase encode enzymes that require require 4 moles of NADH for the production of one mole of n-butanol.
27. The recombinant cell of claims 25 or 26, wherein acetoacetyl-CoA reductase is the *C. acetobutylicum* acetoacetyl-CoA reductase Hbd and crotonase is the *C. acetobutylicum* crotonase Crt.
28. The recombinant cell of claim 1, wherein the recombinant sequences encoding the acylating aldehyde dehydrogenase, the keto-thiolase or acetyl-CoA acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA

reductase, and the butyraldehyde/butanol dehydrogenase acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase encode enzymes that require 3 moles of NADH and 1 mole of NADPH for the production of one mole of n-butanol.

29. The recombinant cell of claim 1, wherein the recombinant sequences encoding the pyruvate:flavodoxin/ferredoxin-oxidoreductase, the keto-thiolase or acetyl-CoA acetyltransferase, the acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase, the crotonase, the crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-enoyl-CoA reductase, and the butyraldehyde/butanol dehydrogenase acetoacetyl-CoA reductase or hydroxybutyryl-CoA dehydrogenase encode enzymes that require require 3 moles of NADH and 1 mole of NADPH for the production of one mole of n-butanol.
30. The recombinant cell of claims 28 or 29, wherein the 3-hydroxybutyryl-CoA dehydrogenase is the *R. eutrophus* 3-hydroxybutyryl-CoA dehydrogenase phaB and the crotonase is the *A. cavaie* crotonase PhaJ.
31. The recombinant cell of claims 28 or 29, wherein the acetyl-CoA acetyltransferase is the *R. eutrophus* acetyltransferase PhaA, the 3-hydroxybutyryl-CoA dehydrogenase is the *R. eutrophus* 3-hydroxybutyryl-CoA dehydrogenase phaB, the crotonase is the *A. cavaie* crotonase PhaJ and the transenoyl-coA reductase is the *T. denticola* transenoyl-coA reductase Ter.
32. The recombinant cell of claims 1 or 6, further comprising a recombinant sequence encoding a pantothenate kinase catalyzing the conversion of pantothenate to 4'-phosphopantothenate.
33. The recombinant cell of claim 32, wherein the pantothenate kinase is derived from *E.coli*.
34. The recombinant cell of claim 32, wherein the pantothenate kinase is PanK, CoaA, or CoaX

35. The recombinant cell of claims 1 or 6, further comprising a recombinant sequence encoding a phosphopantothenoylcysteine synthetase catalyzing the conversion of 4'-phosphopantothenate to 4'-phosphopantothenoylcysteine.
36. The recombinant cell of claim 35, wherein the phosphopantothenoylcysteine synthetase is derived from *E.coli*.
37. The recombinant cell of claim 35, wherein the phosphopantothenoylcysteine synthetase is Ppcs or CoaB.
38. The recombinant cell of claims 1 or 6, further comprising a recombinant sequence encoding phosphopantothenoylcysteine decarboxylase catalyzing the conversion of 4'-phosphopantothenoylcysteine to 4'-phosphopantetheine.
39. The recombinant cell of claim 38, wherein the phosphopantothenoylcysteine decarboxylase is derived from *E.coli*.
40. The recombinant cell of claim 38, wherein the phosphopantothenoylcysteine decarboxylase is Ppcdc or CoaC.
41. The recombinant cell of claims 1 or 6, further comprising a recombinant sequence encoding phosphopantetheine adenylyl transferase catalyzing the transfer of an adenylyl group from ATP to 4'-phosphopantetheine.
42. The recombinant cell of claim 41, wherein the phosphopantetheine adenylyl transferase is derived from *E.coli*.
43. The recombinant cell of claim 41, wherein the phosphopantetheine adenylyl transferase is Ppat or CoaD.
44. The recombinant cell of claims 1 or 6, further comprising a recombinant sequence encoding dephosphocoenzyme A kinase catalyzing the phosphorylation of dephospho-CoA.

45. The recombinant cell of claim 44, wherein the dephosphocoenzyme A kinase is derived from *E.coli*.
46. The recombinant cell of claim 44, wherein the dephosphocoenzyme A kinase is CoaE.
47. The recombinant cell of claims 1 or 6, wherein the activity of a lactate dehydrogenase catalyzing the conversion of pyruvate to lactate is reduced or eliminated.
48. The recombinant cell of claim 47, wherein the pyruvate oxidase is LdhA.
49. The recombinant cell of claims 1 or 6, wherein the activity of a pyruvate oxidase catalyzing the conversion of pyruvate to acetate is reduced or eliminated.
50. The recombinant cell of claim 49, wherein the pyruvate oxidase is PoxB.
51. The recombinant cell of claims 1 or 6, wherein the activity of an alcohol dehydrogenase catalyzing the conversion of acetyl-CoA to ethanol is reduced or eliminated.
52. The recombinant cell of claim 51, wherein the alcohol dehydrogenase is AdhE2.
53. The recombinant cell of claims 1 or 6, wherein the activity of an acetate kinase catalyzing the conversion of acetyl-CoA to acetate is reduced or eliminated.
54. The recombinant cell of claim 53, wherein the acetate kinase is AckA.
55. The recombinant cell of claims 1 or 6, wherein the activity of a phosphotransacetylase catalyzing the conversion of acetyl-CoA to acetate is reduced or eliminated.
56. The recombinant cell of claim 55, wherein the phosphotransacetylase is Pta.
57. The recombinant cell of claims 1 or 6, wherein the cell is a prokaryotic cell or a eukaryotic cell.

58. The recombinant cell of claims 1 or 6, wherein the cell is a proteobacterial, archaeobacterial, bacteroidal, enterobacterial, spirochetel, mammalian, insect, fungal, yeast, *E. coli*, *Z. mobilis*, *B. subtilis*, *S. cerevisiae*, *S. pombe*, *Synechocystis* sp., *Synechococcus* sp., *Rhodospirillum* sp., *Ralstonia* sp., *Pseudomonas* sp., *Rhodococcus* sp., *T. saccharolyticum*, *Thermotoga* sp., *T. reesei*, *A. niger*, *P. chrysosporium*, CHO, or SF9 cell.
59. The recombinant cell of claims 1 or 6, wherein the cell produces elevated levels of n-butanol compared to a wild-type cell.
60. The recombinant cell of claims 1 or 6, wherein the cell produces at least 4.0g/L n-butanol.
61. The recombinant cell of claims 1 or 6, wherein the cell produces elevated total levels of n-butanol and ethanol compared to a wild-type cell.
62. The recombinant cell of claims 1 or 6, wherein the cell produces total levels of n-butanol of at least 4.0 g/L.
63. A method for the production of n-butanol, the method comprising growing a recombinant cell of claims 1 or 6 in the presence of a suitable carbon source.
64. The method of claim 63, wherein the recombinant cell is grown under microaerobic conditions.
65. The method of claim 63, wherein the recombinant cell is grown under anaerobic conditions.
66. The method of claim 63, wherein the recombinant cell is grown under conditions wherein the cell produces elevated levels of n-butanol compared to a wild-type cell.
67. The method of claim 63, wherein the recombinant cell is grown under conditions wherein the cell produces at least 4.0 g/L n-butanol.

68. The method of claim 63, wherein the recombinant cell is grown under conditions wherein the cell produces elevated total levels of n-butanol and ethanol compared to a wild-type cell.
69. The method of claim 63, wherein the recombinant cell is grown under conditions wherein the cell produces total levels of n-butanol and ethanol of at least 4.0 g/L.
70. The recombinant cell of claims 1 or 6, wherein the recombinant cell is an *E.coli* cell.

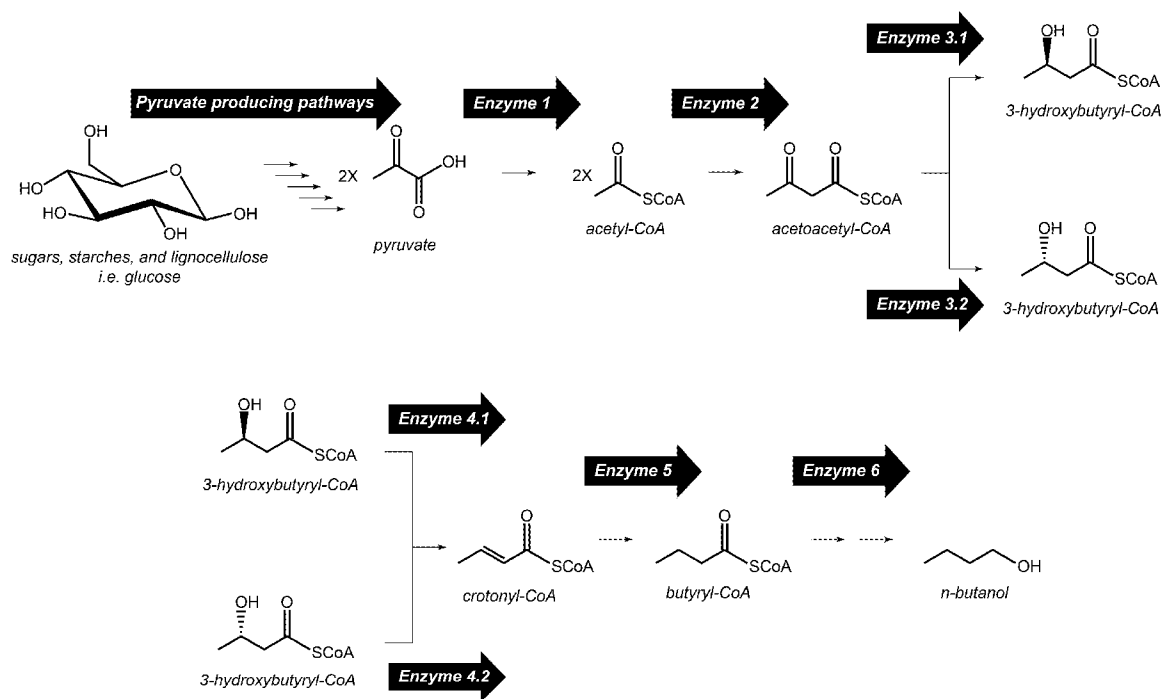
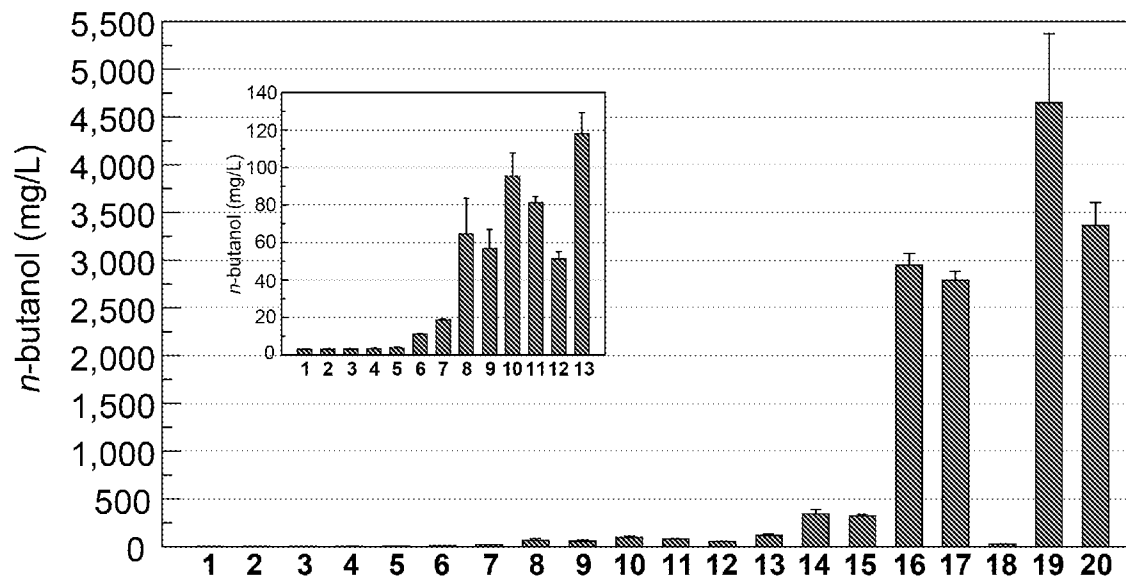
Figure 1

Figure 2

host strain	plasmid 1	plasmid 2	plasmid 3	n-butanol (mg L ⁻¹)
1 BL21(de3)	pBad33-Bu1	pET-ccr.adhE2	—	3.0 ± 0.1
2 W3110(de3)	pBad33-Bu1	pET-ccr.adhE2	—	3.0 ± 0.3
3 MG1655(de3)	pBad33-Bu1	pET-ccr.adhE2	—	3.1 ± 0.2
4 DH10B(de3)	pBad33-Bu1	pET-ccr.adhE2	—	3.3 ± 0.6
5 DH5a(de3)	pBad33-Bu1	pET-ccr.adhE2	—	3.8 ± 0.4
6 DH1(de3)	pBad33-Bu1	pET-ccr.adhE2	—	11.1 ± 0.4
7 DH1(de3)	pBad33-Bu1	pBad-ccr.adhE2	—	18.8 ± 0.3
8 DH1(de3)	pBad33-Bu1	pTrc-ccr.adhE2	—	65 ± 19
9 DH1(de3)	pBad33-Bu1	pCWori-ccr.adhE2	—	57 ± 10
10 DH1	pBT33-Bu1	pCWori-ccr.adhE2	—	95 ± 12
11 DH1	pTT33-Bu1	pCWori-ccr.adhE2	—	81 ± 3
12 DH1	pT5T33-Bu1	pCWori-ccr.adhE2	—	51 ± 4
13 DH1	pBT33-Bu2	pCWori-ccr.adhE2	—	120 ± 10
14 DH1	pBT33-Bu1	pCWori-ter.adhE2	—	340 ± 50
15 DH1	pBT33-Bu1	pCWori-ter.adhE2	pBBR1	320 ± 20
16 DH1	pBT33-Bu2	pCWori-ter.adhE2	pBBR1	2,950 ± 130
17 DH1	pBT33-Bu3	pCWori-ter.adhE2	pBBR1	2,790 ± 100
18 DH1	pBT33-Bu4	pCWori-ter.adhE2	pBBR1	27 ± 2
19 DH1	pBT33-Bu2	pCWori-ter.adhE2	pBBR1-aceEF.lpd	4,650 ± 720
20 DH1	pBT33-Bu3	pCWori-ter.adhE2	pBBR1-aceEF.lpd	3,360 ± 240

Figure 3A

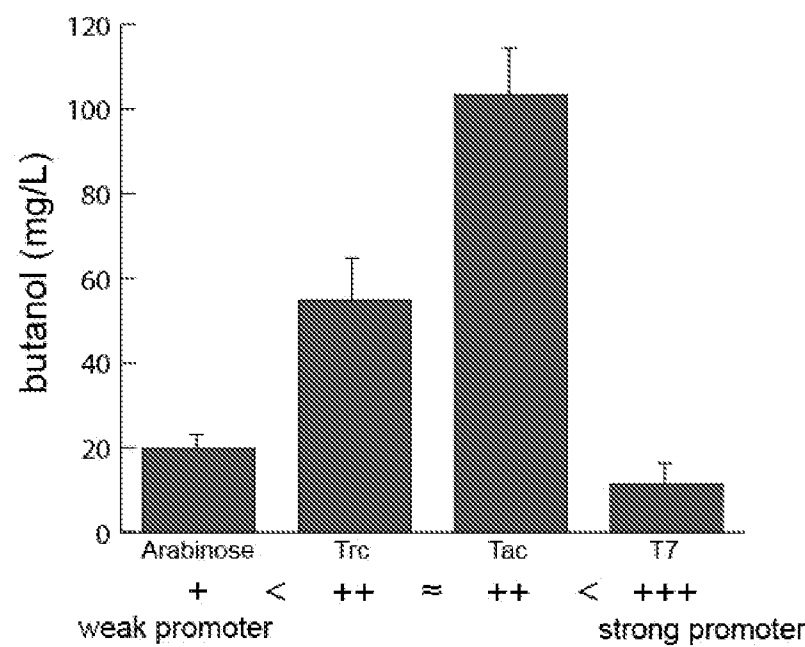


Figure 3B

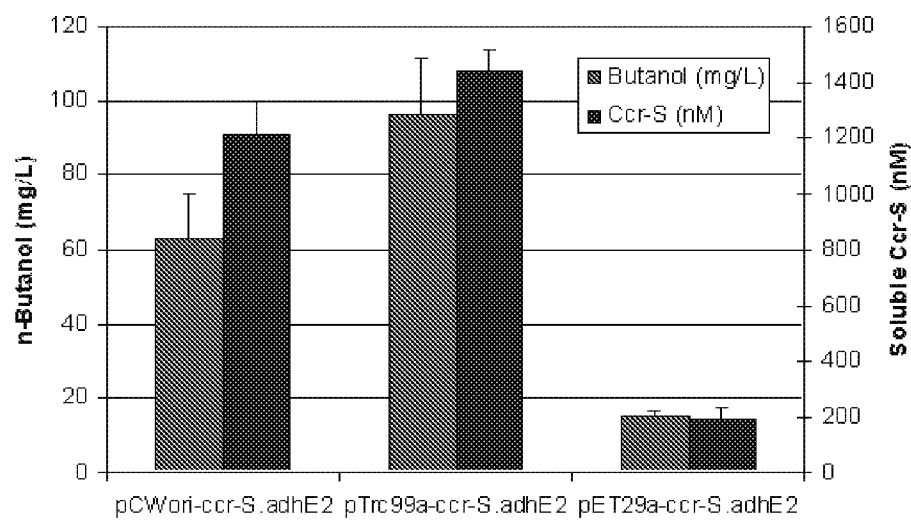


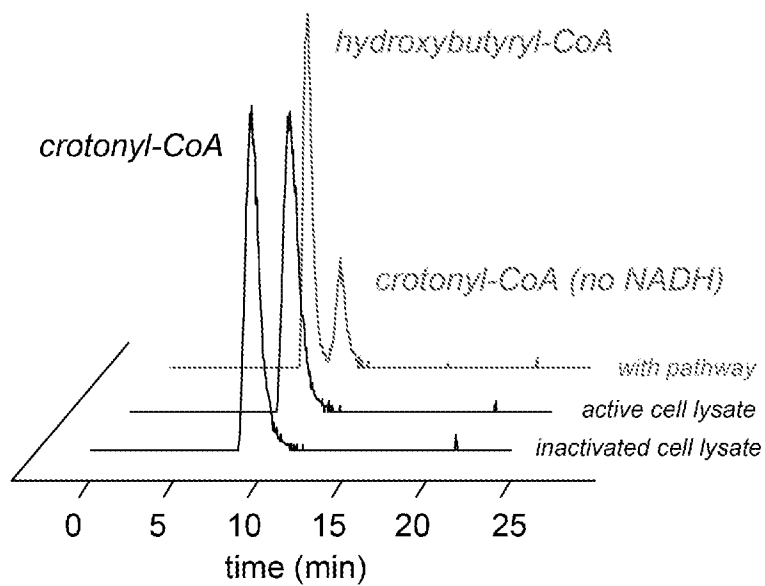
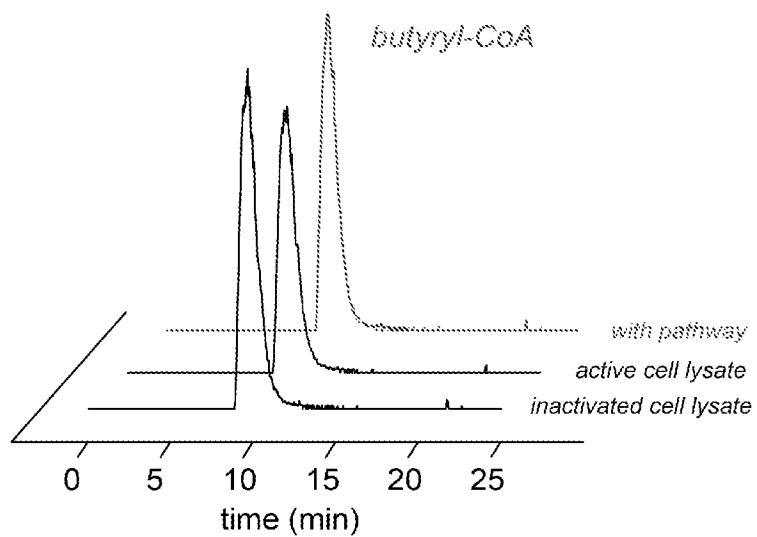
Figure 4A**Figure 4B**

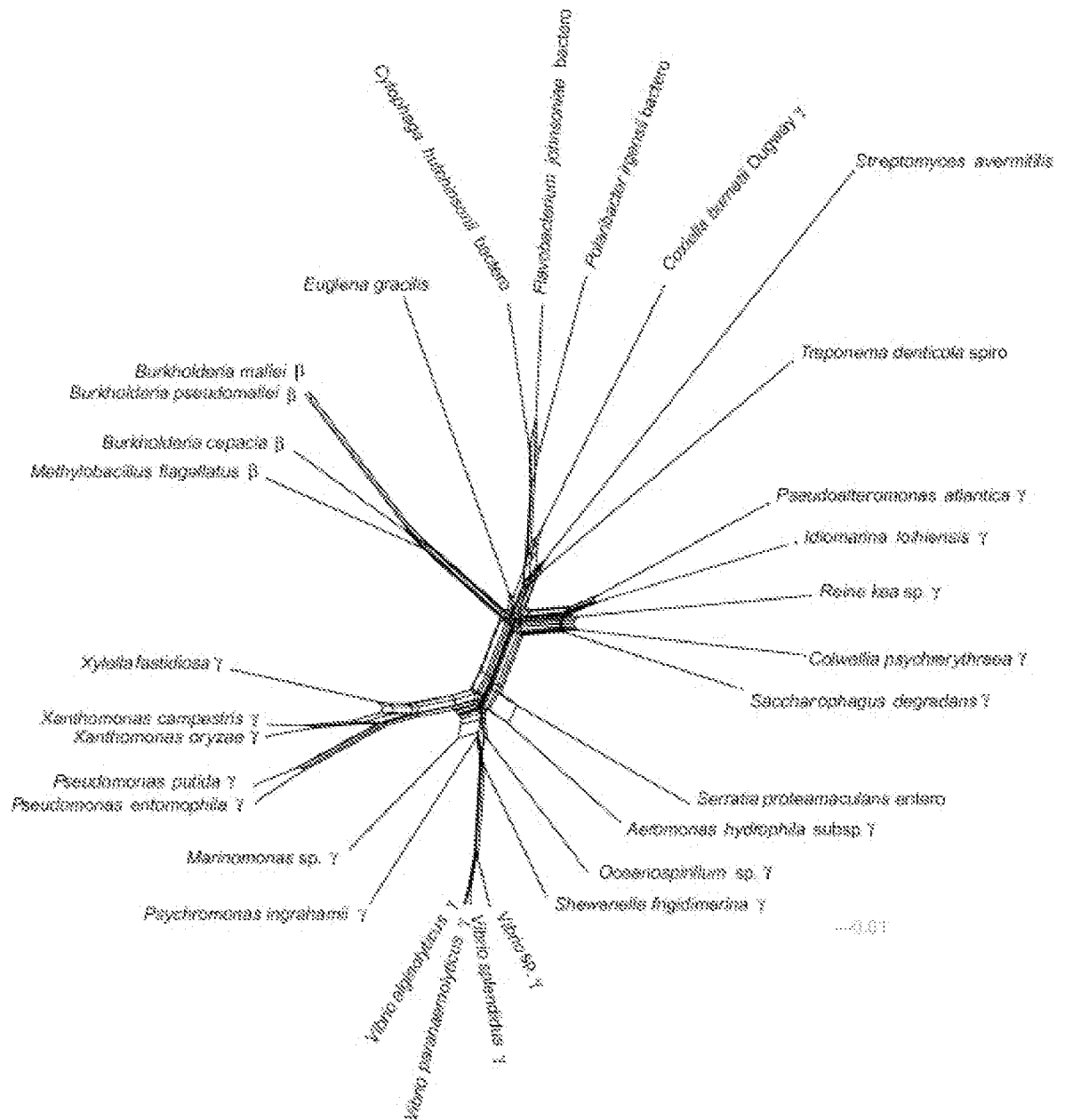
Figure 5

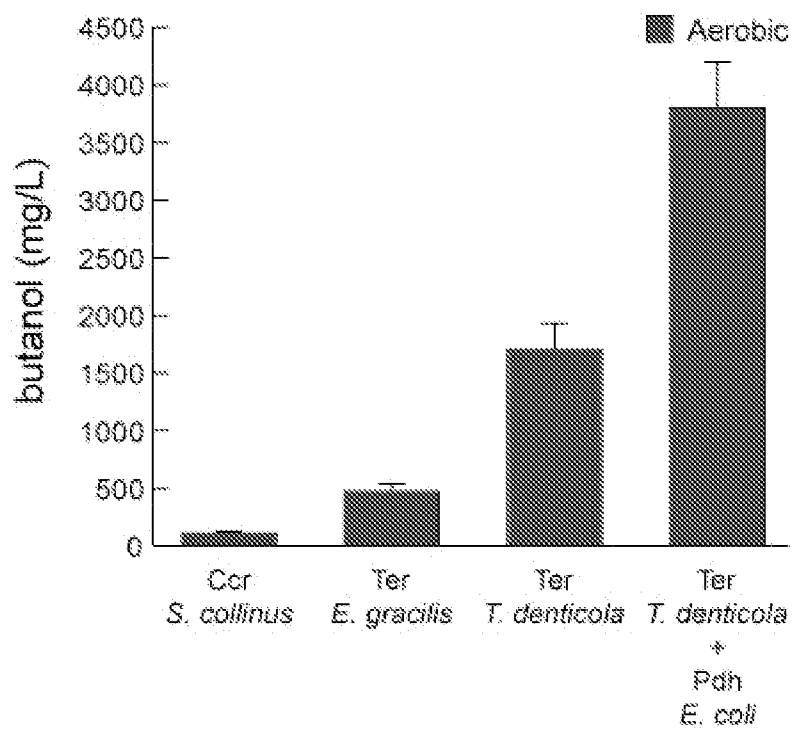
Figure 6

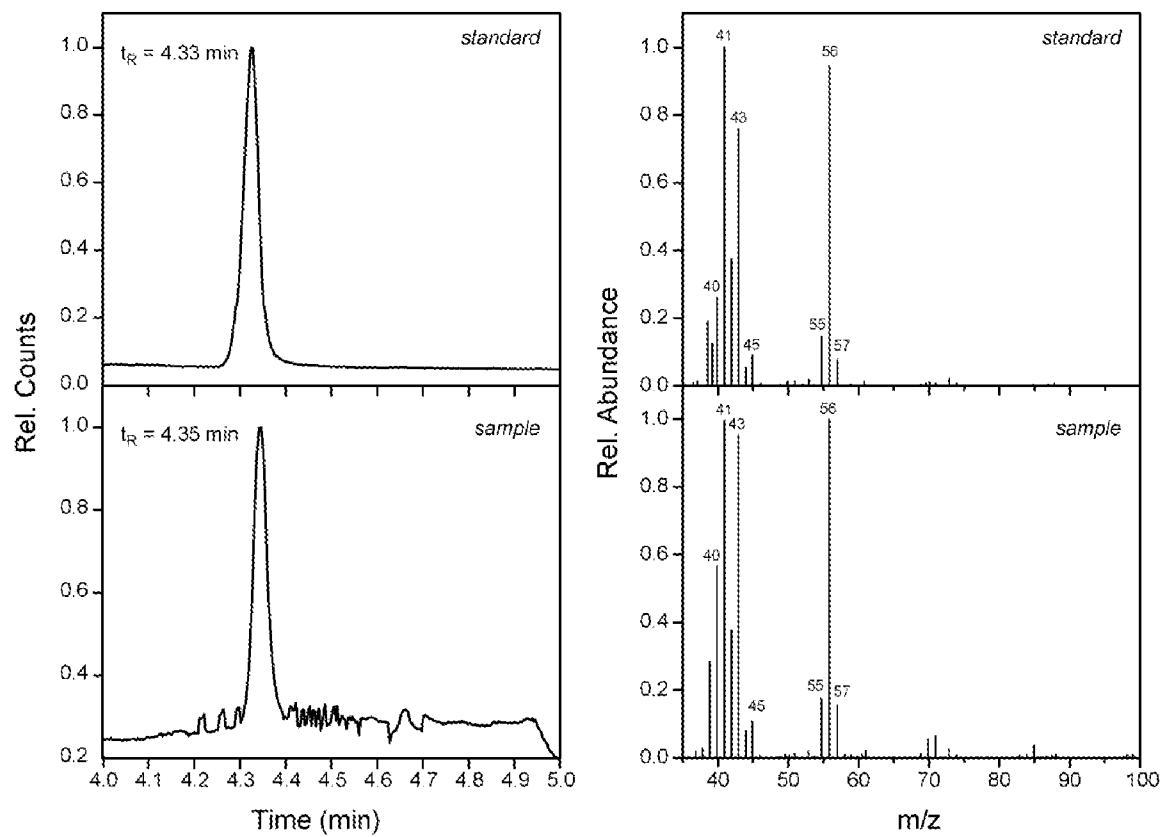
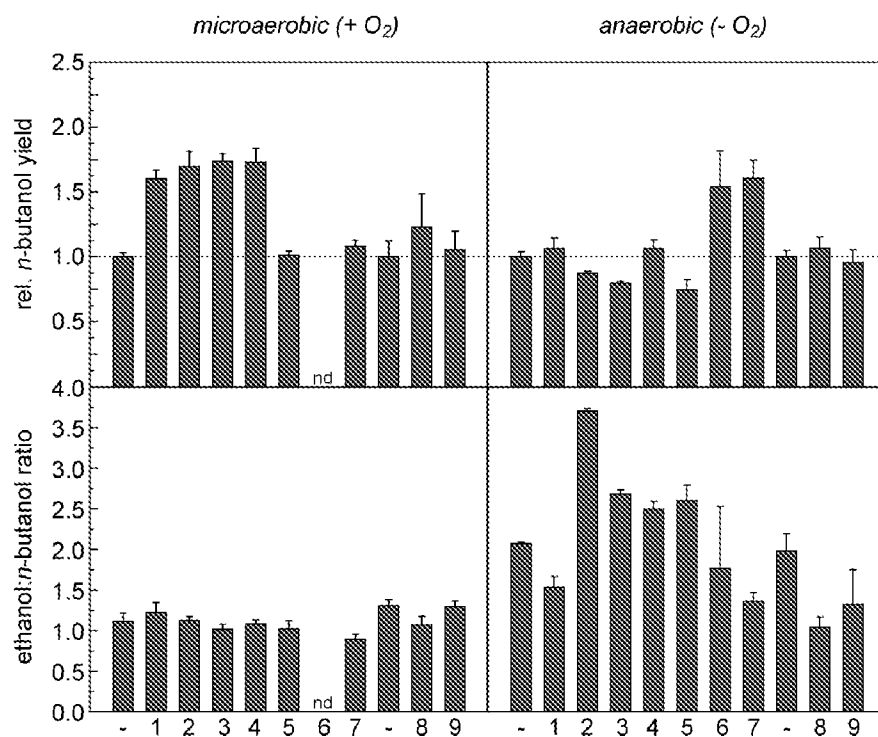
Figure 7

Figure 8

strain	plasmid	description
-	pBBR2-(empty)	negative control
1	pBBR2-aceEF.lpd (<i>E. coli</i>)	PDHc (<i>E. coli</i>)
2	pBBR2-aceEF.lpd H322Y (<i>E. coli</i>)	PDHc (<i>E. coli</i>) NADH regulatory site mutant
3	pBBR2-aceEF.lpd E354K (<i>E. coli</i>)	PDHc (<i>E. coli</i>) NADH regulatory site mutant
4	pBBR2-aceEF.lpd H322Y E354K (<i>E. coli</i>)	PDHc (<i>E. coli</i>) NADH regulatory site mutant
5	pBBR2-aceEF.lpd (<i>E. faecalis</i>)	PDHc (<i>E. faecalis</i>)
6	pBBR2-fdh-pflAB	PFL-FDH
7	pBBR2-pdc.eutA.coaA	PDHc bypass (acetylating AldH)
-	pCOLADuet-(empty)	negative control
8	pCOLADuet-ydbK.fpr-fdx.fldA	PFOR
9	pCOLADuet-ydbK.fpr-fdx.fldB	PFOR

Figure 9

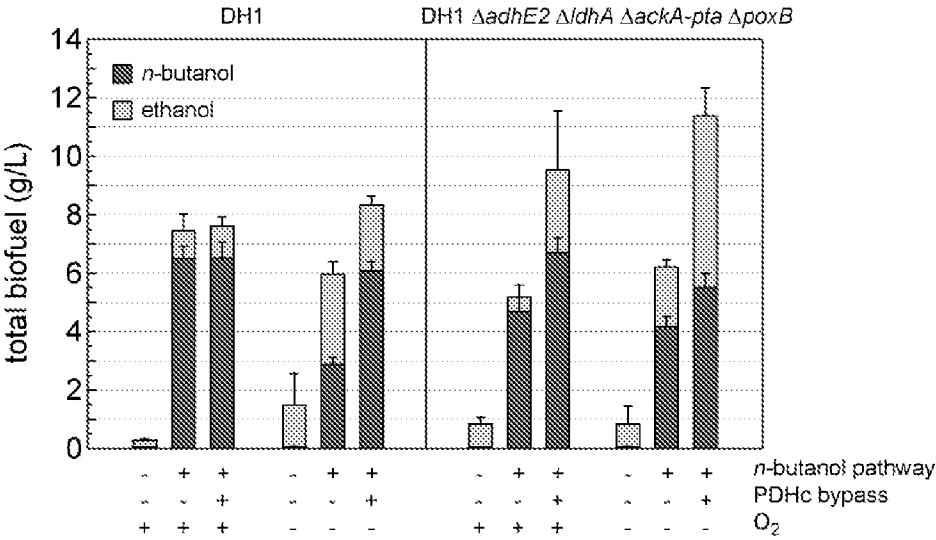


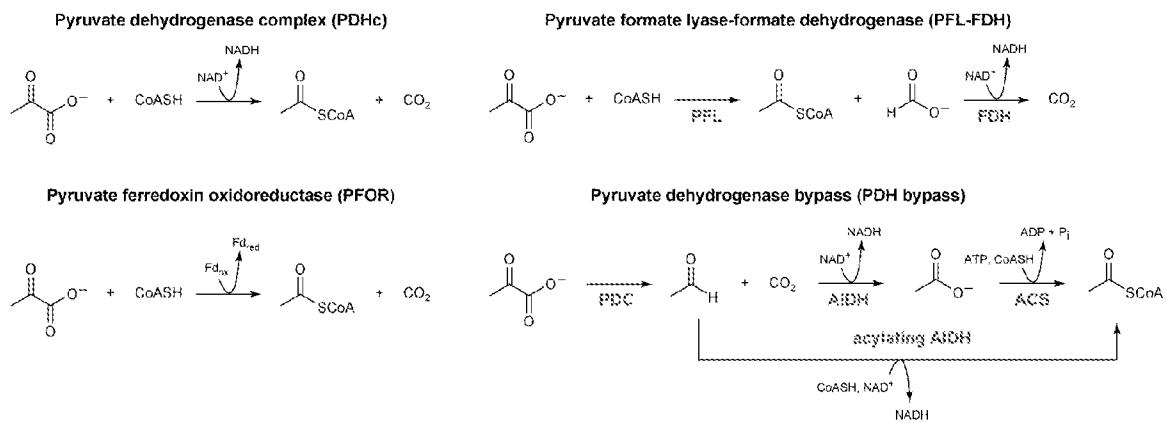
Figure 10

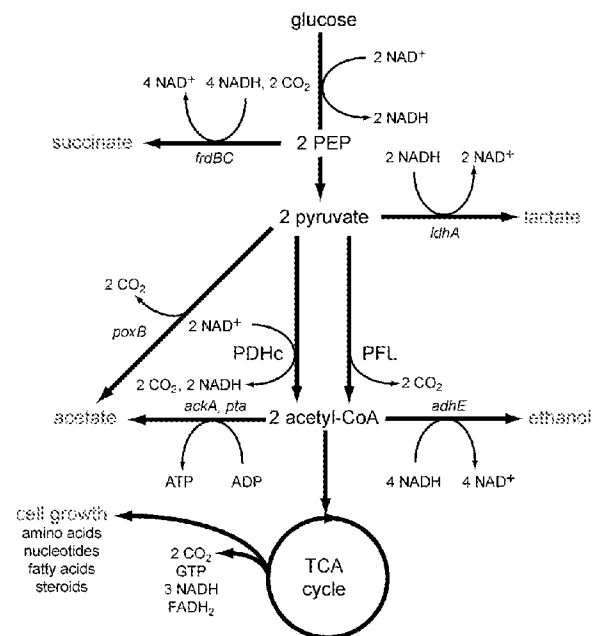
Figure 11

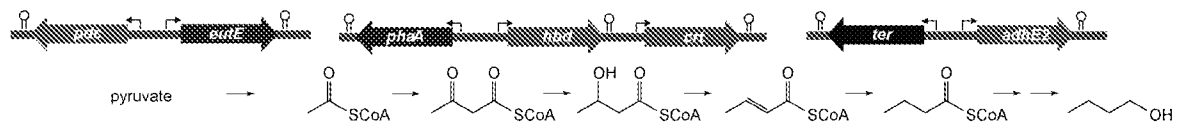
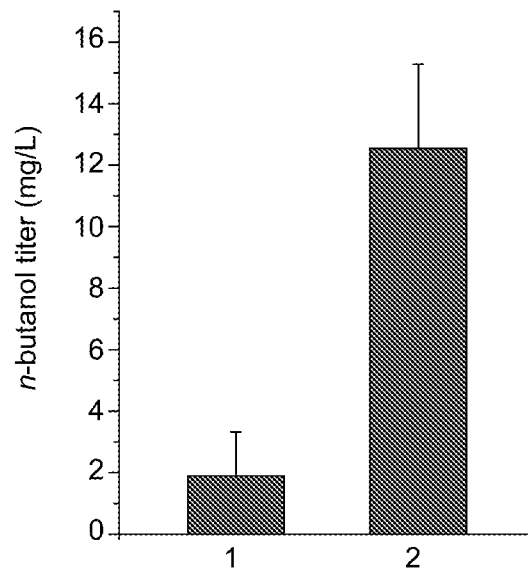
Figure 12A**Figure 12B**

Figure 13