Figure 1

Homoacetogens

H₂

Acetyl-CoA

Ethanol

Wood-
Ljungdahl
pathway

Butyrate

Acetate

CO₂

H₂

Butyrate

C₄

Microorganisms

Butyrogens

H₂

Syngas

N-Butanol

(57) Abstract: This invention provides compositions for the production of butanol. Specifically, the compositions of the present invention use syntrophic co-cultures for the production of butanol from syngas.
APPLICATION FOR A UNITED STATES PATENT

UNITED STATES PATENT AND TRADEMARK OFFICE

Case No. : 2116

Title: SYNTROPHIC CO-CULTURE OF ANAEROBIC MICROORGANISM FOR PRODUCTION OF N-BUTANOL FROM SYNGAS

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FIELD OF THE INVENTION

[0001] The invention provides a composition for the production of n-butanol and other C4-containing products from syngas using a syntrophic co-culture of anaerobic microorganisms.

BACKGROUND OF THE INVENTION

[0002] Butanol is an important industrial chemical with a wide range of applications. It can be used as a motor fuel particularly in combination with gasoline to which it can be added in all proportions. Isobutanol can also be used as a precursor to Methyl Tertiary Butyl Ether (MTBE). Currently the world production of n-Butanol is 3.5 million tons /yr. (7.7 billion lb /yr). Furthermore, conversion of alcohols to long chain linear hydrocarbons that would be suitable for jet fuel use are being developed and demonstrated, which could further increase the demand for n-Butanol (The Naval Air Warfare Center - Weapons Division, (2012) Cobalt and Abermarle). Fermentation of carbohydrates to acetone, butanol and ethanol (ABE) is well known and was commercially practiced worldwide from around 1915 to 1955 (Beesch, S.C. (1953) A Microbiological process Report – Applied Microbiology, 1, 85-95). With the advent of petrochemical processes and low cost petrochemical feedstocks the carbohydrate based processes became unattractive and were discontinued.

[0003] Further development and modernization of the ABE process was undertaken by several organizations. In the mid-1980s the Corn Products Corporation developed asporogenic strains and a multi-staged fermentation process that considerably improved the process economics (Marlatt, J.A. and R. Datta, (1986) Acetone-Butanol Fermentation Process, Biotechnology Progress (1986) 2, 1.23-28). Currently, two companies, Gevo and Butamax are engaged in conversion of several ethanol plants using recombinant microorganisms to produce
iso-butanol for new chemical uses. See US Patent No. 8,017,375 and US Patent No. 7,851,188. In all of these developments the primary feedstock is carbohydrate, primarily starch from corn.

[0004] The limitations for carbohydrate feedstocks are well known and some are fundamental. Starch and sugars from agricultural crops run into competing issues of food vs. energy/chemical production as well as the cost of the feedstocks and their availability. For lignocellulosic feedstocks such as woody biomass, grasses etc. the cost and yield from pretreatment and hydrolysis processes are very limiting. For example, typical woody biomass contains 50% cellulose while the remainder consists of hemicelluloses, lignin and other fractions. The chemical energy content of the fermentable fractions is often less than 50% of that of the feedstock, putting fundamental limitations on product yield.

[0005] Attempts have been made to improve the alcohol yield of bacterium that ferment a variety of sugars to acetate and butyrate. The art has sought to employ recombinant techniques to transform bacterium such as C. acetobutylicum (Green et al. (1996) Genetic manipulation of acid formation pathways, Green etal. Microbiology), 142, 2079-2086) and C. tyrobutyricum (X. Liu et al. (2006) Construction and Characterization of ack Deleted Mutant of Clostridium tyrobutyricum, Biotechnology Pref., 22, 1265-1275). However, such techniques have only resulted in transformation occurring at low frequencies.

[0006] Several microorganisms are able to use one-carbon compounds as carbon source and some even as an energy source. Synthesis gas is a common substrate for supplying the one carbon compounds such as CO and CO₂ as well as hydrogen. Synthesis gas can be produced by gasification of the whole biomass source without the need to unlock certain fractions. Synthesis gas can also be produced from other feedstocks via gasification of: (i) coal, (ii) municipal waste (iii) plastic waste, (iv) petcoke and (v) liquid residues from refineries or from the paper industry
(black liquor). Synthesis gas can also be produced from natural gas via steam reforming or autothermal reforming (partial oxidation).

When the syngas source is biomass, gasification technology converts all the components of the feedstock primarily to a mixture of CO, H₂, CO₂ and some residual CH₄, typically with 75 to 80% cold gas efficiency i.e. 75 to 80% of the chemical energy of the feedstock is available for further chemical or biological conversion to target products. The rest of the energy is available as heat that can be used to generate steam to provide some or all of the process energy required. Furthermore, a wide range of feedstocks, both renewable such as woody biomass, agricultural residues, municipal wastes etc. or non-renewable such as natural gas, can be gasified to produce these primary components.

[0007] Natural gas can be economically reformed to syngas with a wide variety of technologies using steam, oxygen, air or combinations thereof. This syngas has very good cold gas efficiency of approximately 85% to produce CO, H₂ and CO₂ with a wide range of target compositions.

[0008] Hence, syngas is a very economical feedstock that can be derived from a wide range of raw materials both renewable and non-renewable. Thus conversion of syngas to butanol with high yield and concentrations would lead to economical production of this important chemical.

[0009] The ability of anaerobic bacteria to produce n-butanol from the primary syngas components CO and H₂/CO₂ was discovered and reported in 1990/1991 by a team from the Michigan Biotechnology Institute, (A. Grethlein et al. (1991) Evidence of n-Butanol Production from Carbon Monoxide, Journal of Fermentation and Bioengineering, 72, 1, 58-60); (Grethlein et al. (1990) Continuous Production of Mixed Alcohols and Acids from Carbon Monoxide, Journal of Fermentation and Bioengineering, 24-25(1):875-885). Later, other organizations such as
University of Oklahoma and Oklahoma State University also isolated new organisms namely *Clostridium carboxydovoris* that also showed such conversion and n-butanol production (J.S. Liouet al. (2005) *Clostridium carboxydovoris* sp. nov. a solvent producing clostridium International Journal of Systematic and Evolutionary Microbiology 55(5):2085-2091). Subsequent fermentation development with these and other organisms in single culture fermentations have not been very successful - the n-butanol concentrations were achieved in the range of approximately 3 g/liter and the yield ranged from 20 to 45% of theoretical (% electrons to product) (see previous three references and Guillaume Bruante t al. (2010) Genomic Analysis of Carbon Monoxide Utilization and Butanol Production by *Clostridium carboxydovoris*, PLoS One, 5(9)). For a commercially successful process, the n-butanol concentration should be in the range of 8-10 g/liter and the yield should be in the 80% range, otherwise processing and separations costs become unattractive.

**[00010]** To overcome these barriers multi-stage fermentations with two or more organisms such as *Butyribacterium methylotrophicum* and *Clostridium acetobutylicum* have been proposed (Worden et al. (1991) Production of butanol and ethanol from synthesis gas via fermentation, Fuel, 70, 6154-619). The former would produce butyric acid and butanol at low concentrations from syngas and the latter would uptake these while converting carbohydrates to produce more butanol. Since *C. acetobutylicum* strains are able to produce 15 g/liter butanol the separations process would be viable. Such a combination could provide some increases in yield and product recovery, but it would be very cumbersome requiring two different types of feedstocks, syngas and carbohydrates as well as separate bioreactors one for gas conversion and another for carbohydrate conversion. Furthermore, in this scheme the carbohydrate feeding the *Clostridium acetobutylicum* is the primary feedstock and not the more economical syngas fed to the
Butyribacterium methyloptrophicum and all the limitations of carbohydrate feedstocks described above will be prevalent.

[00011] A more efficient conversion of syngas takes place when converting it to ethanol and acetate. The biochemical pathway of such synthesis gas conversion is described by the Wood-Ljungdahl Pathway. Fermentation of syngas to ethanol and acetate offers several advantages such as high specificity of the biocatalysts, lower energy costs (because of low pressure and low temperature bioconversion conditions), greater resistance to biocatalyst poisoning and nearly no constraint for a preset H2 to CO ratio (M. Bredwell et al. (1999) Reactor design issues for synthesis-gas fermentations, Biotechnology Progress 15, 834–844); (Klassen et al. (1992). Biological conversion of synthesis gas into fuels”, International Journal of Hydrogen Energy 17, p.281). Acetogens are a group of anaerobic bacteria able to convert syngas components, like CO, CO2 and H2 to acetate and ethanol via the reductive acetyl-CoA or the Wood-Ljungdahl pathway.

[00012] Several anaerobic bacteria have been isolated that have the ability to ferment syngas to ethanol, acetic acid and other useful end products. Clostridium ljungdahlii and Clostridium autoethanogenum, were two of the first known organisms to convert CO, CO2 and H2 to ethanol and acetic acid. Commonly known as homoacetogens, these microorganisms have the ability to reduce CO2 to acetate in order to produce required energy and to produce cell mass. The overall stoichiometry for the synthesis of ethanol using three different combinations of syngas components is as follows (J. Vega et al.(1989) The Biological Production of Ethanol from Synthesis Gas, Applied Biochemistry and Biotechnology, 20-1, p. 781):

\[
6 \text{CO} + 3 \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{OH} + 4 \text{CO}_2
\]

\[
2 \text{CO}_2 + 6 \text{H}_2 \rightarrow \text{CH}_3\text{CH}_2\text{OH} + 3 \text{H}_2\text{O}
\]
6 CO + 6 H₂ → 2 CH₃CH₂OH + 2 CO₂

[00013] The primary product produced by the fermentation of CO and/or H₂ and CO₂ by homoacetogens is ethanol principally according to the first two of the previously given reactions. Homoacetogens may also produce acetate. Acetate production occurs via the following reactions:

4CO + 2H₂O → CH₃COOH + 2CO₂

4H₂ + 2CO₂ → CH₃COOH + 2H₂O

[00014] *Clostridium ljungdahlii*, one of the first autotrophic microorganisms known to ferment synthesis gas to ethanol was isolated in 1987, as a homoacetogen it favors the production of acetate during its active growth phase (acetogenesis)) while ethanol is produced primarily as a non-growth-related product (solventogenesis) (K. Klasson et al. (1992) Biological conversion of synthesis gas into fuels, International Journal of Hydrogen Energy 17, p.281).

*Clostridium autoethanogenum* is a strictly anaerobic, gram-positive, spore-forming, rod-like, motile bacterium which metabolizes CO to form ethanol, acetate and CO₂ as end products, beside it ability to use CO₂ and H₂, pyruvate, xylose, arabinose, fructose, rhamnose and L-glutamate as substrates (J. Abrini, H. Naveau, E. Nyns.), “*Clostridium autoethanogenum*, Sp-Nov, an Anaerobic Bacterium That Produces Ethanol from Carbon-Monoxide”, Archives of Microbiology, 161(4), p. 345, 1994).

[00015] Anaerobic acetogenic microorganisms offer a viable route to convert waste gases, such as syngas, to useful products, such as ethanol, via a fermentation process. Such bacteria catalyze the conversion of H₂ and CO₂ and/or CO to acids and/or alcohols with higher specificity, higher yields and lower energy costs than can be attained by traditional production processes. While many of the anaerobic microorganisms utilized in the fermentation of ethanol
also produce butanol as a secondary product, to date, no single anaerobic microorganism has been described that can utilize the syngas fermentation process to produce high yields of butanol.

[00016] Therefore a need in the art remains for syntrophic co-cultures using microorganisms in the production of butanol using syngas as the primary fermentation substrate.

**SUMMARY OF THE INVENTION**

[00017] Provided herein is a microorganism co-culture for the conversion of at least one of CO or CO\textsubscript{2} and H\textsubscript{2} to butanol said co-culture comprising two or more microorganisms collectively having a nucleotide sequence identity at least 95% identical to SEQ ID No. 1 and a nucleotide sequence identity at least 70% identical to SEQ ID No. 2 or at least 65% identical to SEQ. ID No. 3. The new syntrophic co-culture of anaerobic microorganisms is defined by a unique set of nucleotide sequences and can produce butanol from a non-food substrate of CO or CO\textsubscript{2} and H\textsubscript{2} at much higher concentrations than previous methods for anaerobically producing butanol with microorganisms. In other particular embodiments the homocetogenic microorganism of the co-culture is cultured in a fermentor until it produces a concentration of ethanol of at least 1 g/L and the butyrogenic microorganism is added to the fermentor to produce the microorganism co-culture.

[00018] In other aspects of the invention a microorganism co-culture having a nucleotide sequence defining a gene for NADPH dependent Co reductase and a nucleotide sequence defining a gene for at least one of a Butyryl-CoA acetate transferase and Butyrate kinase is provided. In particular embodiments the co-culture has a nucleotide sequence defining a gene for Butyl acetate transferase and/or a gene for Butyrate kinase.
[00019] In particular embodiments the co-culture of the invention includes \textit{C. kluyveri}. In other embodiments the co-culture includes one or more homoacetogenic microorganisms selected from the group consisting of \textit{C. ljungdahlii, C. ragsdalei, C. authoethanongenum} and \textit{C. coskattii}. In yet other embodiments the co-culture comprises a mixture of a homoacetogenic microorganism and a butyrogenic microorganism.

[00020] In other particular embodiments the homocetogenic microorganism of the co-culture is cultured in a fermentor until it produces a concentration of ethanol of at least 1 g/L or at least 10 g/L and the butyrogenic microorganism is added to the fermentor to produce the microorganism co-culture.

[00021] In other aspects of the invention a syntrophic co-culture of anaerobic microorganisms for producing butanol from CO or \textit{CO}_2 and \textit{H}_2. In particular embodiments the co-culture of microorganisms contains at least one microorganism having at least one nucleotide sequence that encodes a gene to produce an NADPH dependent CoA reductase (NADPH CoAR) and at least one additional microorganism that encodes a gene for producing a Butyryl-CoA acetate transferase (BuCoAAT) or a Butyrate kinase (BuK) is provided. The co-culture is exposed to gaseous substrates selected from the group consisting of carbon monoxide, carbon dioxide and hydrogen or combinations thereof so that a C1-fixing microorganism containing an NADPH CoAR gene and a C4-producing microorganism containing at least one of the BuCoAAT or BuK gene under conditions effective for the co-culture to convert the gaseous substrate into butanol or/and into butyric acid so that the microorganism composition of the present invention can produce butanol. In most cases the gaseous substrate is syngas and the C4-producing microorganism is a butyrogen.
BRIEF DESCRIPTION OF THE DRAWINGS

[00022] These and other objects, features, and embodiments of the invention will be better understood from the following detailed description taken in conjunction with the drawings, wherein:

[00023] Figure 1 is a diagram of a schematic conversion path showing the production of n-butanol from a substrate input of syngas.

[00024] Figure 2 is a detailed diagram of the BuCoAAT pathway showing the conversion of acetate and ethanol conversion by a butyrogen to produce butyrate.

[00025] Figure 3 is a detailed diagram of the BuK pathway showing the conversion by a butyrogen to produce butyrate.

[00026] Figure 4 is a detailed diagram of the Wood-Ljundahl and Acetyl CoA conversion pathway showing the conversion of syngas by a homoacetogen to produce ethanol and acetate.

[00027] Figure 5(a) is a PCR screen using probes targeted to an NADPH CoAR (NADPH dependent CoA reductase) and a BuCoAAT (butyryl-CoA acetate transferase) for analysis of a syntrophic co-culture that includes C. autoethanogenum and a consortia of at least two butanol producing microorganisms.

[00028] Figure 5(b) is a PCR screen using probes targeted to an NADPH CoAR (NADPH dependent CoA reductase) and a BuCoAAT (butyryl-CoA acetate transferase) for analysis of a syntrophic co-culture that includes C. ragsdalei, C. Coskatii, and a butyrogenic consortia of microorganisms.

[00029] Figure 6(a) is a PCR screen using probes targeted to an NADPH CoAR (NADPH dependent CoA reductase) and BuK (butyrate kinase genes) for analysis of a syntrophic co-
culture that includes *C. autoethanogenum* and a consortia of two butanol producing microorganisms.

**[00030]** Figure 6(b) is a PCR screen using probes targeted to an NADPH CoAR (NADPH dependent CoA reductase) and aBuK (butyrate kinase genes) for analysis of a syntrophic co-culture that includes *C. ragsdalei, C. Coskatii*, and a butyrogenic consortia of microorganisms.

**[00031]** Figure 7 shows sequence IDs for three butyrate production genes identified in *C. carboxidivorans* and *C. kluyveri*.

**[00032]** Figure 7(a) shows a DNA sequence alignment of the BuCoAAT gene from *C. carboxidivorans* and the first gene from *C. kluyveri*

Figure 7(b) provides the DNA sequence of the BuCoAAT gene from *C. carboxidivorans*. Figure 7(c) shows a first DNA and a second DNA sequence of the Bu CoAAT from *C. kluyveri*.

**[00033]** Figure 8a shows butyrate production gene sequences identified in *C. carboxidivorans* for three BuK genes. Figure 8(b) provides an alignment of two *C. carboxidivorans* BuK genes. Figure 8(c) provides an alignment of two *C. carboxidivorans* BuK genes (Seq ID No. 3 and Seq ID No. 9).

**[00034]** Figure 9 shows gene sequences of the NADPH CoAR genes from four Clostridial homoacetogens with

**[00035]** Figure 9(a) showing the sequence alignment and strong homology of the four NADPH CoAR (NADPH dependent CoA reductase) gene sequences and Figure 9(b) showing raw NADPH CoAR (NADPH dependent CoA reductase) sequences of the four homoacetogens.

**[00036]** Figure 10 is a time plot of the butanol, acetate, butyrate and ethanol production from a 2 liter fermentation run using the discovered co-culture of microorganisms.
[00037] Figure 11 is a time plot of butanol and ethanol production and hydraulic retention time (HRT) from a 10,000 gallon fermentor using the discovered co-culture of this invention.

[00038] Figure 12 is an alignment of BCoATT C. kluwyeri(Ck) and C. carboxidivorans (Cc) over 145 bp probe region.

[00039] Figure 13 is an alignment of BCoATT C. kluwyeri and C. carboxidivorans over 101 bp probe region.

[00040] Figure 14 is an alignment of Seq. ID No. 4 C. carboxidivorans with Seq. No. 3 C. carboxidivorans over a 180 bp probe region.

[00041] Figure 15 is an alignment of C. carboxidivorans BuK-1 (Seq. ID No. 3) with C. difficile (Seq. ID No. 5) over entire gene.

DETAILED DESCRIPTION OF THE INVENTION

[00042] The invention provides a syntrophic co-culture of microorganisms for the production of butanol and other C4-containing products from syngas.

[00043] As used herein, synthesis gas (syngas) is a gas containing carbon monoxide, carbon dioxide and frequently hydrogen. “Syngas” includes streams that contain carbon dioxide in combination with hydrogen and that may include little or no carbon monoxide. “Syngas” may also include carbon monoxide gas streams that may have little or no hydrogen.

[00044] As used herein, the term “syntrophic” refers to the association of two or more different types (e.g. organisms, populations, strains, species, genera, families, etc.) of anaerobic microorganisms which are capable of forming a tightly associated metabolic relationship.

[00045] As used herein, the term "co-culture" of microorganisms refers to joint incubation or incubation together, of the syntrophic microorganisms. In the context of the present invention, the co-culture does not require cellular population growth during the joint incubation of the syntrophic microorganisms.
In one embodiment of the invention illustrated in Figure 1, two types of anaerobic microorganism are utilized to create the syntrophic co-cultures for production of butyrate and butanol. The first type of microorganism in the syntrophic co-culture is a primary C1- fixing homacetogenic microorganism, which utilizes syngas as the sole carbon and electron source and produces C1 compounds such as ethanol and acetate as the dissimilatory metabolite products.

The second type of microorganism in the syntrophic co-culture is capable of growing on the dissimilatory metabolites of the C1- fixing homacetogenic microorganism (ethanol and acetate) as its sole carbon and/or electron source to produce a C4-carbon molecule, such as butanol or butyric acid, as its primary product or together with syngas (as additional carbon and/or electron source) convert the metabolites of the C1-carbon fixing microorganism to C4-carbon molecules.

This second microorganism shall be referred to herein as the C4- butyrate producing microorganism. Advantageously, the C1-fixing homacetogenic microorganism may also be capable of converting the butyrate produced by the C4-producing microorganism into butanol and more often n-butanol. The term “butanol” refers to all four isomers of C4 alcohol (e.g. 2-butanol, isobutanol, 1-butanol and tert-butanol) and the term “n-butanol” refers to 1-butanol.

The C1- fixing microorganisms of the invention are also homoacetogens. Homoacetogens have the ability, under anaerobic conditions, to produce acetic acid and ethanol from the substrates, CO + H$_2$O, or H$_2$ + CO$_2$ or CO + H$_2$ +CO$_2$. The CO or CO$_2$ provide the carbon source and the H$_2$ or CO provide the electron source for the reactions producing acetic acid and ethanol.

The homoacetogen organism typically has the primary Wood Ljungdahl pathway to convert the CO and H$_2$/CO$_2$ from the syngas feed to ethanol and acetate which are then utilized by the butyrogens to produce butyrate. The homoacetogens can uptake the butyrate and very
efficiently convert it to n-butanol because of favored thermodynamics. Such symbiosis if preferably developed to form a very close association between the C₁ fixing and the C₄ producing microorganisms so that interspecies proton and electron transfer occur very efficiently across very short distances (approximately 1 micron). Such conditions achieve very good product concentrations (8-10 g/liter n-butanol) and yields (~ 80 % of electrons to n-butanol) in a single fermenter system. This combination of microorganism co-culture and substrates vastly improves the n-butanol production over that produced by a single culture fermentations. This discovery enables high yield production of butanol directly from syngas and leads to economical and efficient production processes for butanol from a wide range of feedstocks.

[00049] C₁-fixing microorganisms suitable for use in the inventive method include, without limitation, homoacetogens such as *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Clostridium ragsdalei*, and *Clostridium coskatai*. Additional C₁ fixing microorganisms that are suitable for the invention include *Alkalibaculum bacchi*, *Clostridium thermoaceticum*, and *Clostridium acetidum*.

[00050] In particular embodiments syntrophic C₄-producing microorganisms are a butyrogen capable of growing on ethanol and/or acetate as their primary carbon source. Butyrogens refers to any microorganism capable of converting syngas intermediates, such as ethanol and acetate, and some hydrogen to primarily n-butyrate. Butyrogens of the invention utilize at least one of two distinct pathways for butyrate production – the Butyrl CoA Acetate Transferase pathway (shown in Figure 2) and the Butyrl Kinase (BuK) pathway (shown in Figure 3). As can be seen
from the Figures 2 and 3, the Butyryl CoA Acetyl Transferase (BuCoAT) pathway converts ethanol and acetate to butyrate:

\[ \text{Ethanol} + \text{Acetate} \leftrightarrow \text{Butyrate} + \text{H}_2\text{O} \]

As shown in Figure 3 the BuK pathway converts acetate and hydrogen to Butyrate.

\[ 2\text{H}_2 + 2\text{Acetate} \leftrightarrow \text{Butyrate} + 2\text{H}_2\text{O} \]

In the BuCOAT pathway ethanol and acetate are converted to butyrate through a Butyryl CoA intermediate. Similarly acetate plus reducing equivalents through H\textsubscript{2} oxidation are converted to butyrate through a butyryl CoA intermediate. The pathways differ in their conversion steps from butyryl CoA to butyrate. The BuCoAT pathway converts butyryl CoA to butyrate through the BuCoAT enzyme while transferring the CoA moiety to acetate to form acetyl-CoA, which can later be used to form more butyrate. At the same time the BuK pathway converts butyryl CoA through a phosphotransbutyrylase and BuK enzyme. The NADPH-dependent CoA reductase converts butyryl-CoA directly into butanol in a 4 electron transfer reaction using NADPH.

Suitable butyrigenes for this invention include any microorganism that contains either or both of the BuCoAt pathway and BuK pathway and can grow on acetate and ethanol or on acetate and hydrogen as typically found in syngas. While many microorganism are known to produce butyrate from various carbohydrate sources (\textit{C. butyricum}, \textit{C. acetobutylicum}, \textit{C. tyrobutyricum}, \textit{C. beijerinckii}, \textit{C. pasteurianum}, \textit{C. barkeri}, \textit{C. thermobutyricum}, \textit{C. thermopalmarium}, \textit{Butyrribrio}, \textit{Sarcina}, \textit{Eubacterium}, \textit{Fusobacterium}, and \textit{Megasphaera}), only a few are known to grow exclusively on ethanol, acetate or syngas. The ones that have been identified so far are \textit{Clostridium kluwyeri}, \textit{Clostridium carboxidivorans}, and \textit{Butyrribacterium methylotrophicum}.

[00051] This invention can employ as the syntrophic co-culture a combination of microorganisms that provides unique and identifiable combination of genes that are not present
in organisms that can directly ferment syngas to butanol or in other butyrogenic that can utilize ethanol or acetate together with hydrogen to product butyrate.

[00052] The pairing of the homoacetogens with the butyrogenic provided herein demonstrates a vast improvement over the prior art. Table 1 shows a comparison of single culture production to the use of syntrophic cultures. As the results show, a four-fold increase in the concentration of n-butanol was achieved. Thus, in particular embodiments of the invention high yield production of butanol directly from syngas was achieved which leads to economical and efficient production processes for butanol from a wide range of feedstocks.

Table 1

<table>
<thead>
<tr>
<th>Bio-conversion method</th>
<th>n-Butanol concentration achieved (g/l)</th>
<th>n-Butanol yield achieved (% of electrons)</th>
<th>Ethanol by product (% of electrons)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. methylotrophicum (single culture)</td>
<td>2 to 3</td>
<td>40 to 45%</td>
<td>10 – 20%</td>
<td>See Grethlein et al. (1991)</td>
</tr>
<tr>
<td>C. carboxidivorans (single culture)</td>
<td>2 to 2.5</td>
<td>20 to 25%</td>
<td>20 to 25%</td>
<td>Liou et al. Guillaume et al.</td>
</tr>
<tr>
<td>Syntrophic Co-culture</td>
<td>8 to 9</td>
<td>60 to 80%</td>
<td>10 to 25%</td>
<td>Examples 1 and 2</td>
</tr>
</tbody>
</table>

The present invention provides a combination of the genes for an NADPH dependent CoA reductase and for the genes of a Butyryl-CoA acetate transferase and/or a Butyrate kinase such that this unique gene combination can make butanol from one or more syngas components.
NADPH dependent CoA reductase does not occur in the heteroacetogenic organisms nor do the Butyryl-CoA Acetate transferase or Butyrate kinase occur in the homocetogenic organism. The genetic novelty of these genes was established by identifying key genes in the syntrophic butyrate production pathway using targeted gene probes. The novelty of the butyryl-CoA transferase genes in the butanologenetic consortia appears to be a highly specific transferase reaction. Hence, unique combinations of genes exist in these syntrophic co-cultures that do not occur in other organisms that have been used to produce butanol.

It was also surprisingly found that this combination of genes existed no matter which homoacetogens or heteroacetogens were used and that the combination of genes, present in the syntrophic combination of the organisms containing these genes, will stay in close association without either of the organisms washing out from the co-culture in a fermentation. Most advanatageously this unique combination of genes produce butanol from syngas at high titers that were unachievable with other microorganisms absent the use of multiple substrates. (See Table 1.)

[00053] A successful syntrophic relationship between the different microorganisms of the present invention require that the homoacetogens and the butyrogens and are brought into close physical association with each other. In particular embodiments the C1 converting homoacetogens with the Wood Ljundahl pathway and the NADPH-dependent CoA reductase genes are brought together in an intimately mixed co-culture with the butyrogens having the BuCoAAT or the BuK genes. In another embodiment of the invention the C1 converting homoacetogens will have an NADPH CoAR (NADPH dependent CoA reductase) gene to further increase the production of butanol.
[00054] In one method of the invention, the syntrophic co-culture is formed by first growing a single species or a combination of known homoacetogen species on a syngas feed. Growth of the homoacetogens continues until they produce ethanol and acetate, normally at a concentration of at least 1 g/l and more typically in a moderate concentration range of 8 to 15 g/l and preferably at a concentration of 10 g/l and a cell concentration producing an optical density (O.D.) of about 2.0. Once the homoacetogens have produced a desired concentration of ethanol and acetate and the fermenter has reached a desired O.D., the homoacetogens are inoculated with one or more selected butyrogen species that are enriched from growth on acetate, ethanol and syngas. By maintaining growth and operating conditions such as pH, dilution rate, key nutrients etc., a stable syntrophic co-culture is developed that forms very close associations between the different microorganisms.

[00055] Those skilled in the art will be aware of other methods to initiate and grow the co-culture. Such methods may include the use of different substrates to first grow the butyrogen and then inoculate the fermentation medium containing the butyrogen with the homoacetogen. Another method for establishing a syntrophic association capable of converting syngas to butanol involves the growing of two or more defined cultures and establishing the pairing of these separate cultures.

[00056] Another method of pairing involves first growing the C4-producing butyrogen in a fermenter using ethanol and acetate as substrates until maximum productivity targets of butyric acid have been reached. Once the maximum productivity target has been reached a seed culture of the C1-fixing homoacetogen is added directly to the fermenter containing the butyrogen culture. Syngas mass transfer to the fermentation vessel is gradually increased to balance the gas consumption of the C1-fixing homoacetogen. The ethanol or acetate used to grow the
butyrogen are gradually decreased to zero as the C1-fixing homoacetogen begins to provide this substrate.

[00057] A modification of this last method of establishing a syntrophic culture involves first growing the C4-producing butyrogen culture in a fermenter with a biofilm support material that is either stationary or floating within the reactor. An example of such material is the Mutag Biochips. This method allows the butyrogen microorganism to first establish a biofilm on the carrier material thereby increasing the cell retention time versus the HRT of the fermenter. Again, target butyrogen productivity is reached before seeding the fermenter with the C1-fixing homoacetogen.

[00058] Another method to establish a syntrophic culture capable of producing butanol from syngas involves the initial mixing together of two or more cultures, one of which is a C1-fixing homoacetogen capable of growing on syngas and producing ethanol and acetate. The other culture(s) is a C4-producing butyrogen capable of converting ethanol or acetate to butyrate. Ethanol and acetate feed can gradually be decreased to zero as the production of these substrates by the C1-fixing homoacetogens increases to balance the substrate needs of the butyrogen production.

[00059] Suitable pairings of microorganisms for the syntrophic co-culture composition of this invention are identified by the presence of key genes in the syntrophic pathways for the homoacetogenic and butyrogenic microorganism. These pathway are typically identified by using targeted gene probes. The probes are targeted toward identifying the presence of genes in the syntrophic consortium that encode for an NADPH CoAR gene, at least one BuCoAAT gene or one BuK gene. The presence or absence of these genes can be further determined using
genomic DNA and suitable probes. Further description of the gene sequences are provided in the Examples.

[00060] The methods of the present invention can be performed in any of several types of fermentation apparatuses that are known to those of skill in the art, with or without additional modifications, or in other styles of operation equipment that are currently under development. Examples include but are not limited to conventional stirred tank fermenters (CSTRs), bubble column bioreactors (BCBR), membrane supported bioreactors (MSBR), two stage bioreactors, trickle bed reactors, membrane reactors, packed bed reactors containing immobilized cells, etc. Bioreactors may also include a column fermenter with immobilized or suspended cells, a continuous flow type reactor, a high pressure reactor, or a suspended cell reactor with cell recycle. Furthermore, reactors may be arranged in a series and/or parallel reactor system which contains any of the above-mentioned reactors. For example, multiple reactors can be useful for growing cells under one set of conditions and generating n-butanol (or other products) with minimal growth under another set of conditions.

[00061] Establishing the necessary close association of the co-culture may be influenced by the type of bioreactor employed for practice of the invention. For example in the case of planktonic type bioreactors the syntrophic co-culture may continue in a growth phase and be passaged up to larger fermentation vessels. In the case of an MSBR, an established co-culture from a planktonic fermenter may be used to inoculate the membranes. However, an MSBR may also be inoculated by a series of inoculations that alternate between addition of the homoacetogen and addition of the butyrogen.
[00062] These apparatuses will be used to develop and maintain the C1-fixing homoacetogen and butyrogen cultures used to establish the syntrophic metabolic association. The chief requirements of such an apparatus include:

a. Axenicity;

b. Anaerobic conditions;

c. Suitable conditions for maintenance of temperature, pressure, and pH;

d. Sufficient quantities of substrates are supplied to the culture;

e. Optimum mass transfer performance to supply the gases to the fermentation medium

e. The end products of the fermentation can be readily recovered from the bacterial broth.

[00063] Suitable gas sources of carbon and electrons are preferably added during the inoculation. In addition to those already described these gaseous sources come from a wide range of materials and include "waste" gases such as syngas, oil refinery waste gases, steel manufacturing waste gases, gases produced by steam, autothermal or combined reforming of natural gas or naphtha, biogas and products of biomass, coal or refinery residues gasification or mixtures of the latter. Sources also include gases (containing some H₂) which are produced by yeast, clostridial fermentations, and gasified cellulosic materials. Such gaseous substrates may be produced as byproducts of other processes or may be produced specifically for use in the methods of the present invention. Those of skill in the art will recognize that any source of substrate gas may be used in the practice of the present invention, so long as it is possible to provide the microorganisms of the co-culture with sufficient quantities of the substrate gases under conditions suitable for the bacterium to carry out the fermentation reactions.
In one embodiment of the invention, the source of CO, CO\textsubscript{2} and H\textsubscript{2} is syngas. Syngas for use as a substrate may be obtained, for example, as a gaseous product of coal or refinery residues gasification.

In addition to those sources as described, syngas can be produced by gasification of readily available low-cost agricultural raw materials expressly for the purpose of bacterial fermentation, thereby providing a route for indirect fermentation of biomass to alcohol. There are numerous examples of raw materials which can be converted to syngas, as most types of vegetation could be used for this purpose. Suitable raw materials include, but are not limited to, perennial grasses such as switchgrass, crop residues such as corn stover, processing wastes such as sawdust, byproducts from sugar cane harvesting (bagasse) or palm oil production, etc. Those of skill in the art are familiar with the generation of syngas from such starting materials. In general, syngas is generated in a gasifier from dried biomass primarily by pyrolysis, partial oxidation, and steam reforming, the primary products being CO, H\textsubscript{2} and CO\textsubscript{2}. The terms "gasification" and "pyrolysis" refer to similar processes; both processes limit the amount of oxygen to which the biomass is exposed. The term "gasification" is sometimes used to include both gasification and pyrolysis.

Combinations of sources for substrate gases fed into the fermentation process may also be utilized to alter the concentration of components in the feed stream to the bioreactor. For example, the primary source of CO, CO\textsubscript{2} and H\textsubscript{2} may be syngas, which typically exhibits a concentration ratio of 37% CO, 35% H\textsubscript{2}, and 18% CO\textsubscript{2}, but the syngas may be supplemented with gas from other sources to enrich the level of CO (i.e., steel mill waste gas is enriched in CO) or H\textsubscript{2}.
The syntrophic co-cultures of the present invention must be cultured and used under anaerobic conditions. As used herein, “anaerobic conditions” means the level of oxygen (O₂) is below 0.5 parts per million in the gas phase of the environment to which the microorganisms are exposed. One of skill in the art will be familiar with the standard anaerobic techniques for culturing these microorganisms (Balch and Wolfe (1976) Appl. Environ. Microbiol. 32:781-791; Balch et al., 1979, Microbiol. Rev. 43:260-296), which are incorporated herein by reference. Other operating conditions for the established co-culture will usually include a pH in a range of 5 to 7.

A suitable medium composition used to grow and maintain syntrophic co-cultures or separately grown cultures used for sequential fermentations, includes a defined media formulation. The standard growth medium is made from stock solutions which result in the following final composition per Liter of medium. The amounts given are in grams unless stated otherwise. Minerals: NaCl, 2; NH₄Cl, 25; KCl, 2.5; KH₂PO₄, 2.5; MgSO₄•7H₂O, 0.5; CaCl₂•2H₂O, 0.1. Trace metals: MnSO₄•H₂O, 0.01; Fe(NH₄)₂(SO₄)₂•6H₂O, 0.008; CoCl₂•6H₂O, 0.002; ZnSO₄•7H₂O, 0.01; NiCl₂•6H₂O, 0.002; Na₂MoO₄•2H₂O, 0.0002, Na₂SeO₄, 0.001, Na₂WO₄, 0.002. Vitamins (amount, mg): Pyridoxine HCl, 0.10; thiamine HCl, 0.05; riboflavin, 0.05; calcium pantothenate, 0.05; thiocetic acid, 0.05; p-aminobenzoic acid, 0.05; nicotinic acid, 0.05; vitamin B12, 0.05; mercaptoethane sulfonic acid, 0.05; biotin, 0.02; folic acid, 0.02. A reducing agent mixture is added to the medium at a final concentration of 0.1 g/L of cysteine (free base); and 0.1 Na₂S•2H₂O. Medium compositions can also be provided by yeast extract or corn steep liquor or supplemented with such liquids.

In general, fermentation of the syntrophic co-culture will be allowed to proceed until a desired level of butanol is produced in the culture media. Preferably, the level of butanol
produced is in the range of 2 grams/liters to 75 grams/liters and most preferably in the range of 6 grams/liter to 15 grams/liter. Alternatively, production may be halted when a certain rate of production is achieved, e.g. when the rate of production of a desired product has declined due to, for example, build-up of bacterial waste products, reduction in substrate availability, feedback inhibition by products, reduction in the number of viable bacteria, or for any of several other reasons known to those of skill in the art. In addition, continuous culture techniques exist which allow the continual replenishment of fresh culture medium with concurrent removal of used medium, including any liquid products therein (i.e. the chemostat mode). Also techniques of cell recycle may be employed to control the cell density and hence the volumetric productivity of the fermentor.

[00070] The products that are produced by the microorganisms of this invention can be removed from the culture and purified by any of several methods that are known to those of skill in the art. For example, butanol can be removed by distillation at atmospheric pressure or under vacuum, by adsorption or by other membrane based separations processes such as pervaporation, vapor permeation and the like.

[00071] This invention is more particularly described below and the Examples set forth herein are intended as illustrative only, as numerous modifications and variations therein will be apparent to those skilled in the art. As used in the description herein and throughout the claims that follow, the meaning of “a”, “an”, and “the” includes plural reference unless the context clearly dictates otherwise. The terms used in the specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Some terms have been more specifically defined to provide additional guidance to the practitioner regarding the description of the invention.
[00072] EXAMPLES

[00073] The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

**Example 1**

**Establishment of Stable Syntrophic Pairing of a Homoacetogen with Butyrogens.**

[00074] A 2-liter fermentation experiment was run in order to establish a syntrophic pairing of a type strain homoacetogen, *Clostridium autoethanogenum*, and a mixed culture of two butyrogens known to produce butyrate and have at least one gene for BuCoAAT and one gene for BuK. The mixed culture of *Clostridium autoethanogenum* was first grown to an O.D. of 1.7 on minimal media and syngas with a composition of H₂-56%, CO-22%, CO₂-5%, and CH₄-17% (mol%), 60mL/min. gas flow rate and agitation between 500-600 rpm. The ethanol and acetate concentrations were at 10 and 5 g/L respectively prior to the addition of 200 mL of the mixed butyrogen culture. Figure 10 shows the concentration of the ethanol, acetate, butyrate and butanol in the fermenter at the time of mixed butyrogen culture addition. The butyrate and butanol concentrations slowly increased and 6 days after inoculation with the butyrogens, butanol and butyrate concentration of 8.4 and 3.8 g/L, respectively were achieved. The increase in butanol and butyrate coincided with a decrease in ethanol and acetate to concentrations of 1.8 and 2.0, respectively. During this time period, more than 70% of the electrons consumed as syngas were being converted to butanol and butyrate.
Example 2

Establishment of Stable Syntrophic Pairing of two Homoacetogens with Butyrogenic consortia.

[00075] A fermentation experiment, similar to that of Example 1, was run. The main difference was that the syntrophic co-culture used two homoacetogens, *Clostridium ragsdali* and *Clostridium Coskattii*, in combination with an enriched consortium of butyrogens known to produce butyrate and having at least one of the genes for BuCoAAT and one of the genes for BuK. All of the conditions were the same as in Example 1 including the addition of butyrogen to the fermenter after establishing the homocetogens in the fermenters. The fermentation produced n-butanol by converting syngas with the syntrophic co-culture that included a suspended culture of the consortium.

Example 3

Molecular detection of NADPH-dependent CoA reductase and BuCoAAT genes in butanol-producing consortia.

[00076] High butanol-producing consortia were screened for the presence of key genetic targets using molecular probes. The PCR probes were designed to detect the presence of NADPH CoAR and BuCoAAT genes. The primer sequences for the NADPH CoAR gene were obtained from sequence alignments of the genes from four homoacetogen sequences. The forward and reverse primers used were: Forward, 5’-AAGCGGTGATTTTTACCAA-3’ and reverse 5’- GGGCCTTTTCAATATTTCCT-3’. The primers for amplifying the Butyryl-CoA acetate transferase gene(s) in butyrogens were obtained from a sequence alignment of the
*Clostridium kluyveri* BuCoATT genes. The primer sequences are: forward 5’-AAAAAAGGATYTDGGKATWCATTCC-3’ and reverse 5’-TCATAHARYTTYTTWGTWCCCAT-3’. Degeneracies were added to capture a broad range of butyrogens for quantitative studies. Figure 5(a) shows the results of PCR using genomic DNA taken from samples containing strain *C. autoethanogenum* and two butyrogenic consortia. Both consortia samples and the pure *C. autoethanogenum* DNA gave a PCR product of about 200 bp using probes targeted to the NADPH CoAR genes with lanes 1 and 2 showing the PCR result for two syntrophic co-cultures and lane 3 showing the result for a pure sample of *C. autoethanogenum*. Additionally, PCR cycling conditions consisted of 3 minutes at 94°C for template DNA denaturation followed by 30 cycles of 1 min. at 94°C, 30 sec. at 59°C, and 30 sec. at 72°C. All reaction mixes contained a 2x PCR dreamTaq master mix from Fermentas and the appropriate DNA template and primers at 50 nM final concentration.

[00077] Figure 5(a), lanes 4-6, show the gel results of PCR using the same two consortia and pure *C. autoethanogenum* DNA as shown in Fig. 5(a) but using a probe targeting the BCoATT gene(s). -Reactions were performed as described above. The butyrogenic consortia showed a product of about 150 bp using probes targeted to butyryl-CoA acetate transferase genes while *C. autoethanogenum* (lane 6) showed no PCR product for the BuCoAAT gene.

[00078] In Figure 5(b), lanes 1-3, the gel results are shown for PCR that was performed with the same DNA extracted from cultures in another reactor containing the DNA for pure *C. ragsdalei* and *C. coskattii* and the butyrogenic consortia. Reactions were performed as described above for the use of the NADPH CoAR probe. -The consortium DNA yielded an amplicon of about 200 bp using the NADPH CoAR probe, indicating that the homoacetogenic Clostridia
genetic targets are present in the consortia sample taken from the butyrogenic reactor. The pure
*C. ragsdalei* and *C. coskattii* DNA also gave an amplicon of the expected size (Figure 5b).

[00079] In Figure 5(b), lanes 4-6, the gel results are shown for PCR that used the same was
DNA extracted from the reactor containing the two homoacetogens, *C. ragsdalei* and *C. coskattii,
along with a butyrogenic consortium. Reactions were performed as described above for the use
of the NADPH CoAR probe (Figure 5(a) lanes 1-3.) The only difference in this case was the use
of the butyryl-CoA acetate transferase probe. As the gel results show the butyryl-CoA acetate
transferase probe only generated an amplicon with the consortia DNA and not with the pure *C.
ragsdalei* and *C. coskattii* DNA, indicating that the homoacetogenic Clostridia in the butyrogenic
reactor does not have BuCoAAT genes but the amplicon is solely due to the presence of
butyrogenic organisms.

**Example 4**

**Molecular detection of NADPH CoAR and BuK genes in butanol-producing consortia.**

[00080] Butyrogenic consortia by themselves do not make butanol without the NADPH
CoAR genes but can make butyrate using the butyrate kinase pathway. The butyrate can then be
converted to butanol by the acetyl-CoA reductase activity found in *C. autoethanogenum* and the
other homoacetogens.

[00081] In Figure 6(a), lanes 1-3, the gel results of PCR that was performed with DNA
extracted from cultures containing the DNA from *C. autoethanogenum* and two consortia
samples of butyrogens. Reactions were performed as described above for the use of the NADPH
CoAR probe. Consortia samples amplified NADPH CoAR genes indicating that in these
consortia samples acetyl-butyryl-CoA reductase genes were present and is contributing to
butanol production.
[00082] In Figure 6(a), lanes 4-6, the gel results are shown for PCR that was performed with DNA extracted from cultures containing the DNA from \textit{C. autoethanogenum} and two consortia samples of butyrogens. A PCR probe was designed to specifically amplify butyrate kinase genes in a wide variety of butyrogens and tested with consortium samples. The primers used were obtained from sequence alignments of \textit{C. carboxidivorans} genes. The forward primer was 5’-AAAGAGCTGGAAAAAGTTCC-3’ and the reverse 5’-CAAGCTTTGCTTTTTTCATCT-3’.

Reactions were performed as described above for the use of the NADPH CoAR probe (Figure 5(a) lanes 1-3.) The only difference in this case was the use of the BuK probe. Both consortium gave amplicons of about 180 bp, consistent with amplicons observed in control DNA. The results indicate that in both of these consortia samples the butyrate kinase and NADPH dependentCoA reductase gene (Fig. 6(a) lanes 1-3) are present and may both be contributing to butanol production.

[00083] In Figure 6(b), lanes 1-3, the gel results of PCR that was performed with DNA-the DNA of pure \textit{C. ragsdalei} and \textit{C. coskattii} and the butyrogenic consortia. Reactions were performed as described above for the use of the NADPH CoAR probe in Example 3A. As expected, the consortium DNA yielded an amplicon of about 200 bp using the NADPH CoAR probe, indicating that the homoacetogenic Clostridia organisms are present in the consortium sample taken from the butyrogenic reactor.

[00084] In Figure 6(b.), lanes 4-6, the gel results are shown for PCR that was performed with DNA extracted from cultures containing the DNA from pure \textit{C. ragsdalei} and \textit{C. coskattii} and the butyrogenic consortia. Reactions were performed as described above for the use of the NADPH CoAR probe (Figure 5(a) lanes 1-3.) The only difference in this case was the use of the BuK probe. The BuK probe only generated an amplicon with the co-culture DNA and not with the
pure *C. ragsdalei* and *C. coskatii* DNA thereby indicating that the homoacetogenic Clostridia in the butyrogenic reactor does not have BuK genes but the amplicon is solely due to the presence of butyrogenic organisms.

**Example 5**

**Clostridium carboxidivorans contains genes encoding BuK and BuCoAAT.**  
*Clostridium carboxidivorans* produces ethanol, acetate, butyrate and butanol when grown in the presence of syngas, which is largely a mixture of CO, H₂ and CO₂. Investigation of its genome sequence revealed two possible pathways to butyrate production with one predominating. The main route appears to be via the butyrate kinase pathway since there are three COGs annotated as such. The remaining part of this pathway is completely intact in *C. carboxidivorans*, that is, the phosphate transbutyrylase and all upstream genes to make butyrate and butanol are present. *C. carboxidivorans* also contains one gene that potentially allows the production of butyrate via the butyryl-CoA acetate transferase pathway gene and shows high homology to the genes from *C. kluyveri* (Figure 7a). The percent identity of the entire *C. kluyveri* and *C. carboxidivorans* BCoATT genes was 74%. These genes appear to be quite novel since most butyrate transferases are involved in the conversion of 4-hydroxybutyryl-CoA and acetoacetate to acetoacetyl-CoA, which then goes through the butyrate pathway. That reaction is involved in amino acid catabolic pathways. The novelty of the butyryl-CoA Acetate transferase genes in the butanologenic consortia appears to be a highly specific transferase reaction. The sequences for the one BuCoAAT gene in *C. carboxydovorans* is given in Figure 7(b) and for the two BuCoAAT genes in *C. kluyveri* are given in Figures 7(c).
The three butyrate kinase genes identified in *C. carboxidivorans* are shown in Fig. (8a). When the entire *C. carboxidivorans* butyrate kinase (Seq. ID No. 3) was aligned pairwise with the other two butyrate kinases (Seq. ID No. 4 and Seq. ID No. 5 Fig. 8b, 8c) the percent identities were 68% and 58%, respectively.

Interestingly, the *C. carboxidivorans* genome did not reveal the presence of the NADPH CoAR sequence, suggesting that these enzymes are only present in homoacetogenic Clostridia that produce ethanol from syngas. Furthermore, in contrast to the homoacetogenic Clostridia grown on syngas, *C. carboxidivorans* was unable to convert ketones such as acetone, butanone and pentanone to the corresponding secondary alcohols (data not shown), indicating that there is no cryptic short-chain fatty acid coenzyme A reductase activity in the cell.

**Example 6**

*Clostridium kluyveri* butyrate production genes.

*Clostridium kluyveri* contains a somewhat unique metabolic niche whereby it converts ethanol and acetate to butyrate and caproate. It doesn’t have the ability to convert syngas to butyrate since it lacks the Wood-Ljungdahl pathway. Examination of its genome sequence shows the presence of two butyryl-CoA acetate transferase genes and no butyrate kinases (Figure 7(c) indicating that the production of butyrate and caproate occurs via the transferase pathway.

*C. kluyveri* also lacks the NADPH CoAR gene sequence and enzymatic activity that’s been observed in homoacetogenic Clostridia and which is also lacking in *C. carboxidivorans*, a heteroacetogenic Clostridia described in Example 5.
Example 7

Homoacetogenic Clostridia containing NADPH CoAR sequences.

Examination of the genome sequences of Clostridia that exclusively produce C₂ alcohols and acids such as *C. autoethanogenum*, *C. ragsdalei*, *C. coskatii*, and *C. ljungdahlii* indicated the presence of a novel NADPH-dependent CoAR but not the butyrate kinase and butyryl-CoA transferase genes. The NADPH CoAR gene has been cloned and expressed in *E. coli* and has been shown to convert acetone, butanone and pentanone to their corresponding secondary alcohols indicating that it accommodates a variety of short-chained (C₂-C₅) ketones. This strain can also presumably convert the short-chain CoAs to their corresponding primary alcohols. These clostridia, when grown as a pure culture, produce ethanol and acetate but when in the presence of butyrate-producing organisms, are able to convert the acid in the CoA form to butanol in a 4-electron reduction. An alignment of the novel NADPH CoAR are shown for four syngas-utilizing homoacetogens (Fig. 9A). The raw sequences are shown in Figure 9b. When the NADPH-dependent CoAR were aligned pairwise with Seq. ID No. 1 the percent identities were very high. *C. ljungdahlii* CoAR was 100% identical to Seq. ID No. 1 and 100% identical to CoAR of *C. coskatii*. The CoAR gene of *C. ragsdalei* showed 97.2% identity to Seq. ID No. 1.

Example 8

Example of butanol production in single stage pilot scale BCBR

[00089] A 38,000 liter pilot scale Bubble Column BioReactor (BCBR) was first brought up to solventogenic conditions producing over 12 g/L of ethanol. The reactor was fed syngas as the only carbon and electron source to support the growth of the homoacetogen, *Clostridium autoethanogenum*. Composition of the syngas was on average, H₂-39, CO-29, CO₂-17, and CH₄-15 (mol%) and the rate of syngas addition varied from 35 to 144 lb/hr at a total fermenter volume
of 26,000 liters. The HRT of the fermentation vessel was slowly stepped down from 8 days at the start of the fermentation to 3.3 days by 800 hours. Figure 11 is a time plot of butanol and ethanol production and hydraulic retention time (HRT) from the 38,000 liter fermentor. After 800 hours, the ethanol producing fermentation was inoculated with a butyrogen culture.

[00090] The addition of the butyrogen culture and a further reduction of the HRT, showed an increase in the concentration of butanol (Fig 11). Once initial butanol production was observed the HRT was further dropped to 2.5 days. Butanol concentrations rose and remained above 4 g/L and progressively rose as high as 8 g/L for the next 1000 hours under these conditions. Increasing the HRT to 9 days further increased the butanol concentration to 9 g/L total. The butanol concentration was the highest when the fermentation was at 2.5 days HRT.

Electron flow from syngas consumption to fermentation products during this 1000 hour period show that 60-80% of the electrons ended up as butanol product.

Example 9

[00091] Alignment and percent identity of a BCoATT region covered by one of the detection probes

[00092] The PCR primers used in detecting butyrogens in different consortia (Figs. 5a and 5b) covered a 145 bp region in the C. kluyveri and C. carboxidivorans butyryl-CoA acetate transferase gene (BCoATT). When the two Clostridial DNA sequences of this region were aligned (Seq. ID No. 2 with Seq. ID No. 6) the identity was determined to be 80% (Fig. 12). This indicates that the probe targeted a well conserved region that will be useful for detecting not only C. kluyveri type butyrogens that use ethanol and acetate to make butyrate but also heteroacetogens which can use syngas as substrate to make C4 compounds. Several PCR reactions were run using pure C. kluyveri and pure C. carboxidivorans genomic DNA and both gave very strong amplicons of the expected size (data not shown).
Example 10

[00093] Alignment and percent identity of a BCoATT region covered by a second butyrogen detection probe

[00094] A second BCoATT detection probe was generated that covered a 101 bp region of BCoATT (Seq. ID No. 2) different from the one described in Example 9. Alignment of the two regions (Seq. ID No. 2 with Seq. ID No. 7) in the BCoATT genes showed the identity to be 91% (Fig. 13). This probe was also used to detect butyrrogens in consortium samples and with pure genomic DNA isolated from C. kluveri and C. carboxidivorans. All pure DNA and consortium samples gave a strong amplicon of the expected size (data not shown). This probe has been used along with the BCoATT probe described in Example 10 to provide a powerful detection tool for monitoring butyrogen populations in syntrophic butanol-producing reactors.

Example 11

[00095] Alignment and percent identity of a BuK region covered by one of the detection probes

[00096] The PCR primers used in detecting butyrrogens in different consortia in Figs. 6a and 6b covered a 180 bp region in the C. carboxidivorans butyrate kinase #1 gene (Buk) (Seq.ID No.3) When BuK#1 (Seq. ID #3) and the 180 bp region covered by the PCR probe (Seq. ID No. 8) were aligned the identity was determined to be 68% (Fig. 14). This indicates that the probe targets a well moderately conserved region that will be useful for detecting butyrate kinase-containing butyrrogens. Several PCR reactions were run using pure C. carboxidivorans genomic DNA and both gave very strong amplicons of the expected size (data not shown).
Example 12


[00098]  Alignment of butyrate kinase genes from *C. carboxidivorans* (BuK-2, Seq. ID No. 4) which is a syngas-fermenting butyrogen and *C. difficile* (Seq. ID No. 5), which is primarily a carbohydrate-fermenting organism shows a 70.8% identity (Fig. 15). This shows that the butyrate kinase has a relatively high degree of conserved nucleotides across two highly unrelated butyrogenic organisms.
Claims

1. A microorganism co-culture for the conversion of at least one of CO or CO₂ and H₂ to butanol said co-culture comprising two or more microorganisms collectively having a nucleotide sequence identity at least 95% identical to SEQ ID No. 1 and a nucleotide sequence identity at least 70% identical to SEQ ID No. 2 or at least 65% identical to SEQ. ID No. 3.

2. The co-culture of claim 1 wherein the co-culture has a nucleotide sequence identity at least 75% identical to SEQ ID No. 2.

3. The co-culture of claim 1 wherein the co-culture has a nucleotide sequence identity at least 75% identical to SEQ ID No. 2, and a nucleotide sequence identity at least 71% identical to SEQ. ID No. 3.

4. The co-culture of claim 1 wherein the co-culture includes C. kluyveri.

5. The co-culture of claim 1 wherein the co-culture includes one or more homoacetogenic microorganisms selected from the group consisting of C. ljungdahlii, C. ragsdaeli, C. authoethanongenum and C. coskatii

6. The co-culture of claim 5 wherein the co-culture comprises a mixture of a homoacetogenic microorganism and a butyrogenic microorganism.

7. The co-culture of claim 6 wherein the homocetogenic microorganism is cultured in a fermentor until it produces a concentration of ethanol of at least 1 g/L and the butyrogenic microorganism is added to the fermentor to produce the microorganism co-culture.
8. A microorganism co-culture having a nucleotide sequence defining a gene for NADPH dependent CoA reductase and a nucleotide sequence defining a gene for at least one of a Butyryl-CoA acetate transferase and Butyrate kinase.

9. The co-culture of claim 8 wherein the co-culture has a nucleotide sequence defining a gene for Butyryl-CoA acetate transferase.

10. The co-culture of claim 9 wherein the co-culture has a nucleotide sequence defining a gene for Butyryl-CoA acetate transferase and defining a gene for Butyrate kinase.

11. The co-culture of claim 8 wherein the co-culture includes *C. kluyveri*.

12. The co-culture of claim 8 wherein the co-culture includes one or more homoacetogenic microorganisms selected from the group consisting of *C. ljungdahlii*, *C. ragsdaeli*, *C. authoethanongenum* and *C. coskatii*

13. The co-culture of claim 12 wherein the co-culture comprises a mixture of a homoacetogenic microorganism and a butyrogenic microorganism.

14. The co-culture of claim 13 wherein the homoacetogenic microorganism is cultured in a fermentor until it produces a concentration of ethanol of at least 10 g/L and the butyrogenic microorganism is added to the fermentor to produce the microorganism co-culture.
Figure 1

N-Butanol

Butyrate

C4

Microorganisms

Butyrogens

H2

CO2

Syngas

H2

Wood-Ljungdahl pathway

Butyrate

Acetate

Acetyl-CoA

Ethanol

Homoacetogens
Figure 4

Carbonyl Branch

\[ CO \rightarrow [CO] \]

Methyl Branch

\[ CO_{2} \rightarrow HCOOH \rightarrow [CH_{3}]^{+} \rightarrow [CH_{3}-\text{Corrinoid-Protein}] \]

\[ CO_{2} \rightarrow 2[H] \rightarrow HCOOH \rightarrow [CH_{3}]^{+} \rightarrow [CH_{3}-\text{Corrinoid-Protein}] \]

Acetyl-CoA

Acetaldehyde

NADPH-dependent l-CoA reductase

Aldehyde ferredoxin oxidoreductase

Acetate

Ethanol
A. CLASSIFICATION OF SUBJECT MATTER
C12N 1/21(2006.01), C12P 7/16(2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N 1/21; C12P 7/16

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
eKOMPASS (KIPO internal) & Keywords: co-culture, microorganism, butanol, nadph dependent coa reductase, butyryl-coa acetate transferase, butyrate kinase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>A</td>
<td>ABBAS, KHAIDJA A., 'The synergistic effects of probiotic microorganisms on the</td>
<td>8-14</td>
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<td>microbial production of butyrate in vitro', 2009, Mcmair Scholars Research</td>
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<td>A</td>
<td>US 8296539 B2 (BURGARD, ANTHONY P.) 07 August 2012 See abstract; claims 53 and</td>
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<td>US 2010-0323418 A1 (BURGARD, ANTHONY P.) 23 December 2010 See claims 1 and 6.</td>
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<td>(BUELTEN, THOMAS et al.) 18 June 2009 See paragraphs [0237]-[238]; claims 1 and 3.</td>
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<td>A</td>
<td>DUNCAN, SYLVIA H. et al., 'Acetate utilization and butyryl coenzyme a (CoA):</td>
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<td>acetate-CoA transferase in Butyrate-producing bacterial from the human large</td>
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<td>intestine' October 2002, Applied and Environmental Microbiology, Vol.88, No.10,</td>
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<td>pp.5186-5190 See abstract.</td>
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</table>

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search: 25 April 2014 (25.04.2014)

Date of mailing of the international search report: 25 April 2014 (25.04.2014)

Name and mailing address of the ISA/KR
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HEO, Joo Hyung
Telephone No. +82-42-481-8150

Form PCT/ISA/210 (second sheet) (July 2009)
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/078238

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

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

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

2. ☒ Claims Nos.: 1-7
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   A sequence listing in electronic form was not furnished to this Authority. Therefore a meaningful search on claims 1-7 was not carried out.

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

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

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)
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Form PCT/ISA/210 (patent family annex) (July 2009)