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(54) **Title:** PRETREATMENT OF BIOMASS

(57) **Abstract:** Compositions and methods for producing alcohols and other fermentation end-products by fermenting alkaline or acid pretreated plant material feedstocks with a microorganism are disclosed herein.

## PRETREATMENT OF BIOMASS

### CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 61/221.591, filed June 29, 2009, which is incorporated herein by reference in its entirety.

### BACKGROUND OF THE INVENTION

[0002] Increasing cost of petroleum-based transportation fuels, dwindling petroleum reserves and concerns over the environmental impact of petroleum-fuel combustion are driving a strong demand for viable alternatives to replace petroleum-based fuels. In particular, recent years have highlighted the promise of producing biofuels through bio-conversion of a variety of biomass material, such as lignocellulosic material, starch, or agriculture waste/byproducts, in combination with enzymes and yeast/bacterial systems. A particular challenge is developing technology with the potential to economically convert polysaccharide containing materials such as woody or non-woody plant material, as well as waste materials and side products from the processing of plant matter into high value transportation fuels and other energy forms or chemical feedstocks. Various examples of these polysaccharide containing materials include cellulosic, lignocellulosic, and hemicellulosic material; pectin containing material; starch; wood; corn stover; switchgrass; paper; and paper pulp sludge.

[0003] Some processes for converting these polysaccharide containing materials into biofuels such as ethanol require first the conversion of pretreated biomass substrates such as starch or cellulose containing materials into simple sugars (saccharification) through, for example, enzymatic hydrolysis, and the subsequent conversion (fermentation) of these simple sugars into biofuels such as ethanol through fermentation by yeasts. However, current bioconversion technologies have faced problems of high production costs and diversion of agricultural products from the food supply. These problems, and others, have limited the usefulness of the current technology.

[0004] In some fermentations for production of ethanol, a simple sugar, such as sucrose, is obtained and fermented directly into ethanol. Such processes are used, for example, in Brazil to convert cane sugar to fuel grade ethanol. These processes are limited geographically to where simple sugar sources are inexpensive, such as in sugarcane growing regions. Additionally, these processes carry the undesirable aspect of diverting a valuable food source, such as sugar, to industrial rather than food uses.

[0005] Some fermentations for the production of ethanol utilize material that first requires hydrolysis, or conversion into less complex or lower molecular weight sugars prior to the conversion to ethanol. Such processes are frequently described for the production of corn ethanol, with the starch derived from corn being broken down, for example by added enzymes, and then finally converted to ethanol with organisms such as a *Saccharomyces* or *Zymomonas* species. Use of other materials, such as cellulosic, hemicellulosic or lignocellulosic materials also frequently require hydrolysis with added enzymes or by

other chemicals/thermal means, which has been the subject of much research, but little historical success.

[0006] The use of enzymes which are added to the process is undesirable from both a cost standpoint and due to the fact that the processor is generally limited to those enzymes which are commercially readily available. Historically, the enzymes commercially available have been selected for processes such as conversion of starch to simple sugars such as glucose or fructose, laundry applications, and cereal foods. They are generally highly specialized, meaning that a single enzyme generally cannot be used with the widely varying feed material. Instead a number of enzymes are frequently used and combined into an "enzyme cocktail." Broader activity is achieved with such mixtures. However, this broader activity can come with a significantly higher price tag, as only a portion of the enzymes being added may be useful with the particular substrate being used in any one particular batch. Other enzymes, which are a part of the cocktail, may not be active on one substrate but are included in the mixture to provide usefulness for other feed substrates that may be used. As a result, in any one particular batch at least a portion of the enzymes added may not significantly contribute to the processing and are wasted.

[0007] Fermentation of biomass to produce biofuels such as alcohols (*e.g.* methanol, ethanol, butanol, or propanol) can provide much needed solutions for the world energy problem. Lignocellulosic biomass has cellulose and hemicellulose as two major components. Hydrolysis of these components results in both hexose (C6) as well as pentose (C5) sugars. Biomass conversion efficiency is highly dependent on the range of carbohydrates that can be utilized by the organism used in the biomass to fuel conversion process. In particular, an inability to utilize both hexose (*e.g.* cellobiose, glucose) and pentose (*e.g.* arabinose, xylose) sugars for conversion into ethanol can dramatically limit the total amount of biofuel or other chemicals that can be generated from a given quantity of biomass. Therefore, to obtain a high conversion efficiency of lignocellulosic biomass to ethanol (yield), it is important to be able to successfully ferment both hexose and pentose sugars into ethanol.

[0008] However, fermentation of pentose sugars (xylose and arabinose) can be a technological bottleneck for ethanol production from biomass. This limitation can lead to ethanol production at low efficiencies, low maximum achievable biofuel titer in a fermentation reaction, and low biofuel productivity. Further, much of the carbohydrate content of biomass can be lost through the solubilization of pentose sugars during pretreatment. Generally, lower yields and low productivity result in higher production costs, which can translate into competitive disadvantages which may not be offset by other characteristics of the microorganism.

[0009] Therefore, a fermentation process for producing ethanol or other desirable products from various feedstocks with high yield and productivity is desirable.

## SUMMARY OF THE INVENTION

[0010] In one aspect, described herein is a product for biofuel production comprising: a biomass feedstock pretreated under low severity conditions wherein said pretreated feedstock comprises a higher amount of oligosaccharides after said pretreatment than before; and a microorganism that is capable of directly fermenting and scarifying said oligosaccharides to produce a fermentation end-product. In one embodiment the pretreated feedstock comprises a greater percentage of oligosaccharides than monomer saccharides. In another embodiment the microorganism produces an enzyme which saccharifys a portion of said feedstock, and wherein said enzyme is selected from the group consisting of a cellulase, a hemicellulase, an amylase, a protease, a chitinase, a pectinase, a keratinase, or a combination thereof. In another embodiment the feedstock comprises a greater amount of five carbon monomer saccharides than six carbon monomer saccharides. In another embodiment the five carbon monomer sugars comprise xylose, arabinose, or a combination thereof. In another embodiment the six-carbon sugars are glucose, galactose, mannose, or a combination thereof. In another embodiment the low severity conditions comprise treating said feedstock with a dilute acid. In another embodiment the dilute acid hydrolysis comprises treating said feedstock with peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, or hydrochloric acid. In another embodiment the microorganism is ferments five-carbon saccharides and six-carbon saccharides. In another embodiment the microorganism is a *Clostridium*. In another embodiment the microorganism is *Clostridium phytofermentans*. In another embodiment the microorganism is *Clostridium* sp. Q.D. In another embodiment the microorganism is genetically modified. In another embodiment the low severity conditions comprises incubating said feedstock at a temperature of from about 80<sup>0</sup>C to about 170<sup>0</sup>C. In another embodiment the low severity conditions comprises incubating said feedstock at a temperature of 140<sup>0</sup>C. In another embodiment the product further comprises a second substance that neutralizes an acidic pH of said feedstock. In another embodiment the second substance is ammonium hydroxide. In another embodiment the neutralized feedstock comprises substantially more oligosaccharides than monomer five-carbon or six-carbon sugars. In another embodiment the feedstock is treated with sulfuric acid to decrease its pH to between 6.1 to 6.9.

[0011] In another aspect, described herein is a method of producing a fermentation end-product, comprising: treating a feedstock with an acid for a first period of time at about 120<sup>0</sup>C to 180<sup>0</sup>C; treating said feedstock for a second period of time at about 180<sup>0</sup>C to 200<sup>0</sup>C; neutralizing the pH of said feedstock with a base; and incubating said neutralized feedstock with a microorganism that directly ferments and saccharifys said feedstock to produce a fermentation end-product. In one embodiment the acid is peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, or hydrochloric acid. In another embodiment the acid is sulfuric acid. In another embodiment the sulfuric acid is provided in an aqueous solution at a concentration of about 0.5% to about 2% by volume. In another embodiment the base is ammonium hydroxide. In another embodiment the ammonium hydroxide is added at a concentration of about 0.5% to about 2% by weight of said feedstock. In another embodiment the pH of

said feedstock in step a) is decreased to between 6.1 to 6.9. In another embodiment the fermentation end-product is an alcohol. In another embodiment the fermentation end-product is ethanol. In another embodiment the microorganism is a *Clostridium*. In another embodiment the microorganism is *Clostridium phytofermentans*. In another embodiment the microorganism is *Clostridium* sp. Q.D. In another embodiment the microorganism is genetically modified. In another embodiment the neutralized feedstock comprises a greater percentage of oligosaccharides than monomer saccharides. In another embodiment the neutralized feedstock comprises a greater amount of five carbon monomer saccharides than six carbon monomer saccharides. In another embodiment the five-carbon sugars are xylose, arabinose, or a combination thereof. In another embodiment the six-carbon sugars are glucose, galactose, mannose, or a combination thereof. In another embodiment the treatment is performed at a temperature of from about 120<sup>0</sup>C to about 140<sup>0</sup>C. In another embodiment the temperature is at 140<sup>0</sup>C. In another embodiment the neutralized feedstock comprises more oligosaccharides than five-carbon or six-carbon monomer saccharides. In another embodiment the second period of time is greater than said first period of time. In another embodiment the first period of time is thirty minutes. In another embodiment the pretreated feedstock is not detoxified. In another embodiment the second period of time is an hour and a half.

[0012] In another aspect, described herein is a method of producing a fermentation end-product, comprising: Pretreating a biomass feedstock comprising: treating a feedstock with sulfuric acid for a first period of time at about 140<sup>0</sup>C; treating said feedstock for a second period of time at about 190<sup>0</sup>C, wherein said second period of time is greater than said first period of time; and neutralizing the pH of said feedstock with ammonium hydroxide, wherein said feedstock is not is not detoxified; and incubating said pretreated feedstock with *Clostridium phytofermentans* to produce ethanol.

[0013] In another aspect, described herein is a method of producing a fermentation end-product, comprising: Pretreating a biomass feedstock comprising: treating a feedstock with a first acid for a first period of time at about 130<sup>0</sup>C to 180<sup>0</sup>C; treating said feedstock for a second period of time at about 180<sup>0</sup>C to 200<sup>0</sup>C; treating said feedstock for a third period of time with a second acid and one or more enzymes; and neutralizing the pH of said feedstock with a base, wherein said neutralized feedstock comprises substantially more monomer saccharides than oligosaccharides; and incubating said neutralized feedstock with a microorganism that directly ferments and saccarifys said feedstock to produce a fermentation end-product. In one embodiment the first or second acid is peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, or hydrochloric acid. The method of claim 45, wherein said first or second acid is sulfuric acid. The method of claim 47, wherein said sulfuric acid is provided in an aqueous solution at a concentration of about 0.5% to about 2% by volume. The method of claim 45, wherein said base is ammonium hydroxide. The method of claim 49, wherein said ammonium hydroxide is added at a concentration of about 0.5% to about 2% by weight of said feedstock. The method of claim 45, wherein the pH of said feedstock in step a) is decreased to between 6.1 to 6.9. The method of claim 45, wherein said fermentation end-product is an alcohol. The method of

claim 45, wherein said fermentation end-product is ethanol. The method of claim 45, wherein said microorganism is a *Clostridium*. The method of claim 45, wherein said microorganism is *Clostridium phytofermentans*. The method of claim 45, wherein said microorganism is *Clostridium* sp. Q.D. The method of claim 45, wherein said microorganism is a yeast. The method of claim 45, wherein said microorganism is *E. coli*. The method of claim 45, wherein said microorganism is *Z. mobilis*. The method of claim 45, wherein said microorganism is genetically modified. The method of claim 45, wherein after step a) and b) said neutralized feedstock comprises a greater percentage of monomer saccharides than oligosaccharides.

[0014] In another aspect, described herein is a method of producing a fermentation end-product, comprising: Pretreating a biomass feedstock comprising: treating a feedstock with an acid for a first period of time at about 130<sup>0</sup>C to 180<sup>0</sup>C; treating said feedstock for a second period of time at about 180<sup>0</sup>C to 200<sup>0</sup>C, wherein said pretreatment produces a pretreated feedstock with substantially more oligosaccharides than monomer saccharides; and incubating said feedstock with a microorganism that directly ferments and saccharifys said feedstock to produce a fermentation end-product.

[0015] In another aspect, described herein is a method of producing a pretreated biomass feedstock comprising: treating a feedstock with sulfuric acid for a first period of time at about 140<sup>0</sup>C; treating said feedstock for a second period of time at about 190<sup>0</sup>C, wherein said second period of time is greater than said first period of time; and neutralizing the pH of said feedstock with ammonium hydroxide, wherein said feedstock is not detoxified.

[0016] In another aspect, described herein is a product for biofuel production comprising: at least two different types of feedstock that are pretreated with at least one acidic substance to maintain an acidic pH during said pretreatment; and a microorganism that is capable of directly fermenting and saccharifying either of said two different types of feedstock to produce a biofuel at a substantially similar yield and/or rate. In one embodiment, said microorganism produces an enzyme which saccharifies a portion of said feedstock, and wherein said enzyme is selected from the group consisting of a cellulase, a hemicellulase, an amylase, a protease, a chitinase, a pectinase, a keratinase, or a combination thereof. In another embodiment, said feedstock comprises cellulose, starch, xylan, pectin, chitin, keratin or a combination thereof. In another embodiment, said acidic substance is selected from the group consisting of peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, and hydrochloric acid. In another embodiment, said microorganism is capable of fermenting a five-carbon sugar and a six-carbon sugar. In another embodiment, said bacterium is a member of the genus *Clostridium*. In another embodiment, said bacterium is *Clostridium phytofermentans*. In another embodiment, said pretreatment further comprises maintaining at a temperature of from about 80<sup>0</sup>C to about 120<sup>0</sup>C. In another embodiment, said feedstock further comprises a second substance that neutralizes said acidic pH of said feedstock. In another embodiment, said second substance is NaOH. In another embodiment, said neutralized feedstock comprises substantially more oligosaccharides than five-carbon or six-carbon sugars.

[0017] In another aspect, described herein is a method of producing ethanol, comprising: pretreating a feedstock with a first substance which adjust pH of said feedstock to an acidic level; adding a second substance adjusting pH of said pretreated feedstock to neutral pH; contacting said neutralized feedstock with a microorganism; and producing five-carbon and six-carbon sugars from said feedstock fermenting said sugars to ethanol by said microorganism. In one embodiment, said five-carbon sugars are xylose, arabinose, or a combination thereof. In another embodiment, said six-carbon sugars are glucose, galactose, mannose, or a combination thereof. In another embodiment, said first substance is selected from the group consisting of peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, and hydrochloric acid. In another embodiment, said second substance is NaOH. In another embodiment, said NaOH is added at a concentration of about 0.5% to about 2% by weight of said feedstock. In another embodiment, said pretreating is performed at a temperature of from about 80°C to about 120°C. In another embodiment, said bacterium is a member of the genus *Clostridium*. In another embodiment, said bacterium is *Clostridium phytofermentans*. In another embodiment, said neutralized feedstock comprises substantially more oligosaccharides than five-carbon or six-carbon sugars.

[0018] In another aspect, described herein is a method of producing ethanol comprising pretreating a biomass comprising solids and liquid with sulfuric acid, neutralizing said pretreated biomass with NaOH, contacting said neutralized biomass comprising substantially more oligosaccharides than monosaccharides with *Clostridium phytofermentans* and fermenting oligosaccharides and monosaccharides by said *Clostridium phytofermentans*.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0019] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of compositions and methods described herein will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0020] **Figure 1** shows a graph of ethanol concentration over time for different fermentation conditions of pH.

[0021] **Figure 2** is a map of the plasmid pIMPT1029 used to transform *Clostridium phytofermentans*.

[0022] **Figure 3** shows a graph of ethanol concentration over time for fermentation of different feedstocks.

[0023] **Figure 4** shows a graph of differential expression of enzymes specific to a feedstock comprising xylan (panel A) and cellulose (panel B).

[0024] **Figure 5** shows a three-dimensional graph of enhanced ethanol production from fermentation of feedstocks pretreated with an alkaline agent and increased temperature.

[0025] **Figure 6** shows a graph of ethanol production over time for fermentation of alkaline-treated corn stover.

[0026] **Figure 7** shows a graph demonstrating the ability of *Clostridium phytofermentans* to uptake 5-carbon and 6-carbon sugars.

[0027] **Figure 8** shows a graph of ethanol production over time from the fermentation of 5-carbon sugars.

[0028] **Figure 9** shows a graph of ethanol production over time from the fermentation of municipal liquid waste.

[0029] **Figure 10** shows a graph of fermentation of multiple feedstocks by *Clostridium phytofermentans*, including 5% corn stover (panel A), 5% bagasse (panel B), 5% switchgrass (panel C), and 5% poplar (panel D). The different lines in each panel represent untreated feedstock (bottom line), lime air treated feedstock (middle line) and 1% sodium hydroxide treated feed stock (top line).

[0030] **Figure 11** shows a graph of the fermentation of organic acids to ethanol by *Clostridium phytofermentans*.

[0031] **Figure 12** depicts a method for producing fermentation end products from biomass by first treating biomass with an acid at elevated temperature and pressure in a hydrolysis unit.

[0032] **Figure 13** depicts a method for producing fermentation end products from biomass by charging biomass to a consolidated bioprocessing unit.

[0033] **Figure 14** illustrates pretreatments that produce hexose or pentose saccharides or oligomers (i.e., oligosaccharides) that are then unprocessed or processed further and either fermented separately or together.

[0034] **Figure 15** illustrates thermochemical severity factors applied to a feedstock.

[0035] **Figure 16** illustrates the percentages of oligomeric sugar contents of samples treated under various severity factors.

[0036] **Figure 17** illustrates a plant process for direct fermentation of pretreated samples with high oligomeric contents and fermentation of monomeric sugars.

[0037] **Figure 18** illustrates the content of a sample containing substantially more oligomeric sugars than monomeric sugars.

[0038] **Figure 19** illustrates quantitation of ethanol produced by the plant process of figure 18.

[0039] **Figure 20** illustrates the content of a sample containing substantially more monomeric sugars than oligomeric sugars.

#### **INCORPORATION BY REFERENCE**

[0040] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

#### **DETAILED DESCRIPTION OF THE INVENTION**

[0041] The following description and examples illustrate embodiments of compositions and methods described herein in detail. Those of skill in the art will recognize that there are numerous variations and

modifications of this invention that are encompassed within its scope. Accordingly, the description of a preferred embodiment should not be deemed to limit the scope of compositions and methods described herein.

### **Definitions**

[0042] Unless characterized differently, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0043] "About" means a referenced numeric indication plus or minus 10% of that referenced numeric indication. For example the term about 4 would include a range of 3.6 to 4.4.

[0044] The term "biomass" as used herein has its ordinary meaning as known to those skilled in the art and may include one or more biological material that can be converted into a biofuel, chemical or other product. One exemplary source of biomass is plant matter. Plant matter can be, for example, woody plant matter, non-woody plant matter, cellulosic material, lignocellulosic material, hemicellulosic material, carbohydrates, pectin, starch, inulin, fructans, glucans, sugar cane, grasses, switchgrass, bamboo, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, poplar, pine, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, paper, shrubs and bushes, vegetables, fruits, flowers, and material derived from these. Plant matter can be further described by reference to the chemical species present, such as proteins, polysaccharides and oils. Polysaccharides include polymers of various monosaccharides and derivatives of monosaccharides including glucose, fructose, lactose, galacturonic acid, rhamnose, etc. Plant matter also includes agricultural waste byproducts or side streams such as pomace, corn steep liquor, corn steep solids, distillers grains, peels, pits, fermentation waste, straw, lumber, sewage, garbage and food leftovers. These materials can come from farms, forestry, industrial sources, households, etc. Other examples are aquatic or marine biomass which include, but are not limited to, kelp, other seaweed, algae, and marine microflora, microalgae, sea grass, salt marsh grasses such as *Spartina* sp. or *Phragmites* and the like. Biomass can be derived from a single source, or biomass can comprise a mixture derived from more than one source. For example, biomass could comprise a mixture of bagasse and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, including animal manure, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Another non-limiting example of biomass is animal matter, including, for example milk, meat, fat, animal processing waste, and animal waste. "Feedstock" is frequently used to refer to biomass being used for a process, such as those described herein.

[0045] The term "broth" as used herein has its ordinary meaning as known to those skilled in the art and can include the entire contents of the combination of soluble and insoluble matter, suspended matter, cells and medium.

[0046] The terms "conversion efficiency" or "yield" as used herein have their ordinary meaning as known to those skilled in the art and can include the mass of product made from a mass of substrate. The term can be expressed as a percentage yield of the product from a starting mass of substrate. For the production of ethanol from glucose, the net reaction is generally accepted as:  $C_6H_{12}O_6 \rightarrow 2 C_2H_5OH + 2 CO_2$  and the theoretical maximum conversion efficiency or yield is 51% (wt.). Frequently, the conversion efficiency will be referenced to the theoretical maximum, for example, "80% of the theoretical maximum." In the case of conversion of glucose to ethanol, this statement would indicate a conversion efficiency of 41% (wt.). The context of the phrase will indicate the substrate and product intended to one of skill in the art. For substrates comprising a mixture of different carbon sources such as found in biomass (xylan, xylose, glucose, cellobiose, arabinose cellulose, hemicellulose etc.), the theoretical maximum conversion efficiency of the biomass to ethanol is an average of the maximum conversion efficiencies of the individual carbon source constituents weighted by the relative concentration of each carbon source. In some cases, the theoretical maximum conversion efficiency is calculated based on an assumed saccharification efficiency. By way of example only, given carbon source comprising 10g of cellulose, the theoretical maximum conversion efficiency can be calculated by assuming saccharification of the cellulose to the assimilable carbon source glucose of about 75% by weight. In this example, 10g of cellulose can provide 7.5g of glucose which can provide a maximum theoretical conversion efficiency of about  $7.5g \times 51\%$  or 3.8g of ethanol. In other cases, the efficiency of the saccharification step can be calculated or determined (i.e. measured). Saccharification efficiencies anticipated by the methods disclosed herein include about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99% or about 100% for any carbohydrate carbon sources larger than a single monosaccharide subunit.

[0047] The term "dry cell weight" is used herein to refer to a method of determining the cell content of a broth or inoculum, and the value so determined. Generally, the method includes rinsing or washing a volume of broth followed by drying and weighing the residue. In some cases, a sample of broth is simply centrifuged with the layer containing cells collected, dried, and weighed. Frequently, the broth is centrifuged, then resuspended in water or a mixture of water and other ingredients, such as a buffer, ingredients to create an isotonic condition or ingredients to control any change in osmotic pressure. The centrifuge-resuspend steps can be repeated, if desired. Different resuspending solutions can be used prior to the final centrifuging and drying. When an insoluble medium component is present, the presence of the insoluble component can be ignored, with the value determined as above. Preferred methods when insoluble medium components are present include those where the insoluble component is reacted to a soluble form, dissolved or extracted into a different solvent that can include water, or separated by an appropriate method, such as by centrifugation, gradient centrifugation, flotation, filtration, or other suitable technique or combination of techniques.

[0048] The term "fatty acid comprising material" as used herein has its ordinary meaning as known to those skilled in the art and can comprise one or more chemical compounds that include one or more

fatty acid moieties as well as derivatives of these compounds and materials that comprise one or more of these compounds. Common examples of compounds that include one or more fatty acid moieties include triacylglycerides, diacylglycerides, monoacylglycerides, phospholipids, lysophospholipids, free fatty acids, fatty acid salts, soaps, fatty acid comprising amides, esters of fatty acids and monohydric alcohols, esters of fatty acids and polyhydric alcohols including glycols (*e.g.* ethylene glycol, or propylene glycol), esters of fatty acids and polyethylene glycol, esters of fatty acids and polyethers, esters of fatty acids and polyglycol, esters of fatty acids and saccharides, or esters of fatty acids with other hydroxyl-containing compounds. A fatty acid comprising material can be one or more of these compounds in an isolated or purified form. It can be a material that includes one or more of these compounds that is combined or blended with other similar or different materials. It can be a material where the fatty acid comprising material occurs with or is provided with other similar or different materials, such as vegetable and animal oils; mixtures of vegetable and animal oils; vegetable and animal oil byproducts; mixtures of vegetable and animal oil byproducts; vegetable and animal wax esters; mixtures, derivatives and byproducts of vegetable and animal wax esters; seeds; processed seeds; seed byproducts; nuts; processed nuts; nut byproducts; animal matter; processed animal matter; byproducts of animal matter; corn; processed corn; corn byproducts; distiller's grains; beans; processed beans; bean byproducts; soy products; lipid containing plant, fish or animal matter; processed lipid containing plant or animal matter; byproducts of lipid containing plant, fish or animal matter; lipid containing microbial material; processed lipid containing microbial material; and byproducts of lipid containing microbial matter. Such materials can be utilized in liquid or solid forms. Solid forms include whole forms, such as cells, beans, and seeds; ground, chopped, slurried, extracted, flaked, or milled. The fatty acid portion of the fatty acid comprising compound can be a simple fatty acid, such as one that includes a carboxyl group attached to a substituted or un-substituted alkyl group. The substituted or unsubstituted alkyl group can be straight or branched, saturated or unsaturated. Substitutions on the alkyl group can include hydroxyls, phosphates, halogens, alkoxy, or aryl groups. The substituted or unsubstituted alkyl group can have 7 to 29 carbons and preferably 11 to 23 carbons (*e.g.*, 8 to 30 carbons and preferably 12 to 24 carbons counting the carboxyl group) arranged in a linear chain with or without side chains and/or substitutions. Addition of the fatty acid comprising compound can be by way of adding a material comprising the fatty acid comprising compound.

[0049] The term "fed-batch" or "fed-batch fermentation" as used herein has its ordinary meaning as known to those skilled in the art and can include a method of culturing microorganisms where nutrients, other medium components, or biocatalysts (including, for example, enzymes, fresh organisms, extracellular broth, etc.) are supplied to the fermentor during cultivation, but culture broth is not harvested from the fermentor until the end of the fermentation. It can optionally include "self seeding" or "partial harvest" techniques where a portion of the fermentor volume is harvested and then fresh medium is added to the remaining broth in the fermentor with at least a portion of the inoculum being the broth that was left in the fermentor. In some embodiments, a fed-batch process might be referred to

with a phrase such as, "fed-batch with cell augmentation." This phrase can include an operation where nutrients and microbial cells are added or one where microbial cells with no substantial amount of nutrients are added. The more general phrase "fed-batch" encompasses these operations as well. The context where any of these phrases is used will indicate to one of those skilled in the art the techniques being considered.

[0050] The term "fermentable sugars" as used herein has its ordinary meaning as known to those skilled in the art and may include one or more sugars and/or sugar derivatives that can be utilized as a carbon source by the microorganism, including monomers, dimers, and polymers of sugars and/or sugar derivatives including two or more of sugars and/or sugar derivatives. In some cases, the organism may break down these polymers, such as by hydrolysis, prior to incorporating the broken down material. Exemplary fermentable sugars include, but are not limited to glucose, xylose, arabinose, galactose, mannose, rhamnose, cellobiose, lactose, sucrose, maltose, and fructose.

[0051] The term "fermentation" as used herein has its ordinary meaning as known to those skilled in the art and can include culturing of a microorganism or a group of microorganisms in or on a suitable medium for the microorganisms. The microorganisms can be aerobes, anaerobes, facultative anaerobes, heterotrophs, autotrophs, photoautotrophs, photoheterotrophs, chemoautotrophs, and/or chemoheterotrophs. The cellular activity, including cell growth can be growing aerobic, microaerophilic, or anaerobic. The cells can be in any phase of growth, including lag (or conduction), exponential, transition, stationary, dormant, vegetative, or sporulating phase.

[0052] "Fermentive end-product" is used herein to include biofuels, chemicals, compounds suitable as liquid fuels, gaseous fuels, reagents, chemical feedstocks, chemical additives, processing aids, food additives, and other products. Examples of fermentive end-products include but are not limited to 1,4 diacids (succinic, fumaric and malic), 2,5 furan dicarboxylic acid, 3 hydroxy propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, xylitol/arabinitol, butanediol, butanol, methane, methanol, ethane, ethene, ethanol, n-propane, 1-propene, 1-propanol, propanal, acetone, propionate, n-butane, 1-butene, 1-butanol, butanal, butanoate, isobutanal, isobutanol, 2-methylbutanal, 2-methylbutanol, 3-methylbutanal, 3-methylbutanol, 2-butene, 2-butanol, 2-butanone, 2,3-butanediol, 3-hydroxy-2-butanone, 2,3-butanedione, ethylbenzene, ethenylbenzene, 2-phenylethanol, phenylacetaldehyde, 1-phenylbutane, 4-phenyl-1-butene, 4-phenyl-2-butene, 1-phenyl-2-butene, 1-phenyl-2-butanol, 4-phenyl-2-butanol, 1-phenyl-2-butanone, 4-phenyl-2-butanone, 1-phenyl-2,3-butandiol, 1-phenyl-3-hydroxy-2-butanone, 4-phenyl-3-hydroxy-2-butanone, 1-phenyl-2,3-butanedione, n-pentane, ethylphenol, ethenylphenol, 2-(4-hydroxyphenyl)ethanol, 4-hydroxyphenylacetaldehyde, 1-(4-hydroxyphenyl) butane, 4-(4-hydroxyphenyl)-1-butene, 4-(4-hydroxyphenyl)-2-butene, 1-(4-hydroxyphenyl)-1-butene, 1-(4-hydroxyphenyl)-2-butanol, 4-(4-hydroxyphenyl)-2-butanol, 1-(4-hydroxyphenyl)-2-butanone, 4-(4-hydroxyphenyl)-2-butanone, 1-(4-hydroxyphenyl)-2,3-butandiol, 1-(4-hydroxyphenyl)-3-hydroxy-2-butanone, 4-(4-hydroxyphenyl)-3-hydroxy-2-butanone, 1-(4-hydroxyphenyl)-2,3-butanedione, indolyethane, indolyethene, 2-(indole-

3-)ethanol, n-pentane, 1-pentene, 1-pentanol, pentanal, pentanoate, 2-pentene, 2-pentanol, 3-pentanol, 2-pentanone, 3-pentanone, 4-methylpentanal, 4-methylpentanol, 2,3-pentanediol, 2-hydroxy-3-pentanone, 3-hydroxy-2-pentanone, 2,3-pentanedione, 2-methylpentane, 4-methyl-1-pentene, 4-methyl-2-pentene, 4-methyl-3-pentene, 4-methyl-2-pentanol, 2-methyl-3-pentanol, 4-methyl-2-pentanone, 2-methyl-3-pentanone, 4-methyl-2,3-pentanediol, 4-methyl-2-hydroxy-3-pentanone, 4-methyl-3-hydroxy-2-pentanone, 4-methyl-2,3-pentanedione, 1-phenylpentane, 1-phenyl-1-pentene, 1-phenyl-2-pentene, 1-phenyl-3-pentene, 1-phenyl-2-pentanol, 1-phenyl-3-pentanol, 1-phenyl-2-pentanone, 1-phenyl-3-pentanone, 1-phenyl-2,3-pentanediol, 1-phenyl-2-hydroxy-3-pentanone, 1-phenyl-3-hydroxy-2-pentanone, 1-phenyl-2,3-pentanedione, 4-methyl-1-phenylpentane, 4-methyl-1-phenyl-1-pentene, 4-methyl-1-phenyl-2-pentene, 4-methyl-1-phenyl-3-pentene, 4-methyl-1-phenyl-3-pentanol, 4-methyl-1-phenyl-2-pentanol, 4-methyl-1-phenyl-3-pentanone, 4-methyl-1-phenyl-2-pentanone, 4-methyl-1-phenyl-2,3-pentanediol, 4-methyl-1-phenyl-2,3-pentanedione, 4-methyl-1-phenyl-3-hydroxy-2-pentanone, 4-methyl-1-phenyl-2-hydroxy-3-pentanone, 1-(4-hydroxyphenyl) pentane, 1-(4-hydroxyphenyl)-1-pentene, 1-(4-hydroxyphenyl)-2-pentene, 1-(4-hydroxyphenyl)-3-pentene, 1-(4-hydroxyphenyl)-2-pentanol, 1-(4-hydroxyphenyl)-3-pentanol, 1-(4-hydroxyphenyl)-2-pentanone, 1-(4-hydroxyphenyl)-3-pentanone, 1-(4-hydroxyphenyl)-2,3-pentanediol, 1-(4-hydroxyphenyl)-2-hydroxy-3-pentanone, 1-(4-hydroxyphenyl)-3-hydroxy-2-pentanone, 1-(4-hydroxyphenyl)-2,3-pentanedione, 4-methyl-1-(4-hydroxyphenyl) pentane, 4-methyl-1-(4-hydroxyphenyl)-2-pentene, 4-methyl-1-(4-hydroxyphenyl)-3-pentene, 4-methyl-1-(4-hydroxyphenyl)-1-pentene, 4-methyl-1-(4-hydroxyphenyl)-3-pentanol, 4-methyl-1-(4-hydroxyphenyl)-2-pentanol, 4-methyl-1-(4-hydroxyphenyl)-3-pentanone, 4-methyl-1-(4-hydroxyphenyl)-2-pentanone, 4-methyl-1-(4-hydroxyphenyl)-2,3-pentanediol, 4-methyl-1-(4-hydroxyphenyl)-2,3-pentanedione, 4-methyl-1-(4-hydroxyphenyl)-3-hydroxy-2-pentanone, 4-methyl-1-(4-hydroxyphenyl)-2-hydroxy-3-pentanone, 1-indole-3-pentane, 1-(indole-3)-1-pentene, 1-(indole-3)-2-pentene, 1-(indole-3)-3-pentene, 1-(indole-3)-2-pentanol, 1-(indole-3)-3-pentanol, 1-(indole-3)-2-pentanone, 1-(indole-3)-3-pentanone, 1-(indole-3)-2,3-pentanediol, 1-(indole-3)-2-hydroxy-3-pentanone, 1-(indole-3)-3-hydroxy-2-pentanone, 1-(indole-3)-2,3-pentanedione, 4-methyl-1-(indole-3)-pentane, 4-methyl-1-(indole-3)-2-pentene, 4-methyl-1-(indole-3)-3-pentene, 4-methyl-1-(indole-3)-1-pentene, 4-methyl-2-(indole-3)-3-pentanol, 4-methyl-1-(indole-3)-2-pentanol, 4-methyl-1-(indole-3)-3-pentanone, 4-methyl-1-(indole-3)-2-pentanone, 4-methyl-1-(indole-3)-2,3-pentanediol, 4-methyl-1-(indole-3)-2,3-pentanedione, 4-methyl-1-(indole-3)-3-hydroxy-2-pentanone, 4-methyl-1-(indole-3)-2-hydroxy-3-pentanone, n-hexane, 1-hexene, 1-hexanol, hexanal, hexanoate, 2-hexene, 3-hexene, 2-hexanol, 3-hexanol, 2-hexanone, 3-hexanone, 2,3-hexanediol, 2,3-hexanedione, 3,4-hexanediol, 3,4-hexanedione, 2-hydroxy-3-hexanone, 3-hydroxy-2-hexanone, 3-hydroxy-4-hexanone, 4-hydroxy-3-hexanone, 2-methylhexane, 3-methylhexane, 2-methyl-2-hexene, 2-methyl-3-hexene, 5-methyl-1-hexene, 5-methyl-2-hexene, 4-methyl-1-hexene, 4-methyl-2-hexene, 3-methyl-3-hexene, 3-methyl-2-hexene, 3-methyl-1-hexene, 2-methyl-3-hexanol, 5-methyl-2-hexanol, 5-methyl-3-hexanol, 2-methyl-3-hexanone, 5-methyl-2-hexanone, 5-methyl-3-hexanone, 2-methyl-3,4-hexanediol, 2-methyl-

3,4-hexanedione, 5-methyl-2,3-hexanediol, 5-methyl-2,3-hexanedione, 4-methyl-2,3-hexanediol, 4-methyl-2,3-hexanedione, 2-methyl-3-hydroxy-4-hexanone, 2-methyl-4-hydroxy-3-hexanone, 5-methyl-2-hydroxy-3-hexanone, 5-methyl-3-hydroxy-2-hexanone, 4-methyl-2-hydroxy-3-hexanone, 4-methyl-3-hydroxy-2-hexanone, 2,5-dimethylhexane, 2,5-dimethyl-2-hexene, 2,5-dimethyl-3-hexene, 2,5-dimethyl-3-hexanol, 2,5-dimethyl-3-hexanone, 2,5-dimethyl-3,4-hexanediol, 2,5-dimethyl-3,4-hexanedione, 2,5-dimethyl-3-hydroxy-4-hexanone, 5-methyl-1-phenylhexane, 4-methyl-1-phenylhexane, 5-methyl-1-phenyl-1-hexene, 5-methyl-1-phenyl-2-hexene, 5-methyl-1-phenyl-3-hexene, 4-methyl-1-phenyl-1-hexene, 4-methyl-1-phenyl-2-hexene, 4-methyl-1-phenyl-3-hexene, 5-methyl-1-phenyl-2-hexanol, 5-methyl-1-phenyl-3-hexanol, 4-methyl-1-phenyl-2-hexanol, 4-methyl-1-phenyl-3-hexanol, 5-methyl-1-phenyl-2-hexanone, 5-methyl-1-phenyl-3-hexanone, 4-methyl-1-phenyl-2-hexanone, 4-methyl-1-phenyl-3-hexanone, 5-methyl-1-phenyl-2,3-hexanediol, 4-methyl-1-phenyl-2,3-hexanediol, 5-methyl-1-phenyl-3-hydroxy-2-hexanone, 5-methyl-1-phenyl-2-hydroxy-3-hexanone, 4-methyl-1-phenyl-3-hydroxy-2-hexanone, 4-methyl-1-phenyl-2-hydroxy-3-hexanone, 5-methyl-1-phenyl-2,3-hexanedione, 4-methyl-1-phenyl-2,3-hexanedione, 4-methyl-1-(4-hydroxyphenyl)hexane, 5-methyl-1-(4-hydroxyphenyl)-1-hexene, 5-methyl-1-(4-hydroxyphenyl)-2-hexene, 5-methyl-1-(4-hydroxyphenyl)-3-hexene, 4-methyl-1-(4-hydroxyphenyl)-1-hexene, 4-methyl-1-(4-hydroxyphenyl)-2-hexene, 4-methyl-1-(4-hydroxyphenyl)-3-hexene, 5-methyl-1-(4-hydroxyphenyl)-2-hexanol, 5-methyl-1-(4-hydroxyphenyl)-3-hexanol, 4-methyl-1-(4-hydroxyphenyl)-2-hexanol, 4-methyl-1-(4-hydroxyphenyl)-3-hexanol, 5-methyl-1-(4-hydroxyphenyl)-2-hexanone, 5-methyl-1-(4-hydroxyphenyl)-3-hexanone, 4-methyl-1-(4-hydroxyphenyl)-2,3-hexanediol, 4-methyl-1-(4-hydroxyphenyl)-2,3-hexanediol, 5-methyl-1-(4-hydroxyphenyl)-3-hydroxy-2-hexanone, 5-methyl-1-(4-hydroxyphenyl)-2-hydroxy-3-hexanone, 4-methyl-1-(4-hydroxyphenyl)-3-hydroxy-2-hexanone, 4-methyl-1-(4-hydroxyphenyl)-2-hydroxy-3-hexanone, 5-methyl-1-(4-hydroxyphenyl)-2,3-hexanedione, 4-methyl-1-(4-hydroxyphenyl)-2,3-hexanedione, 4-methyl-1-(indole-3-)hexane, 5-methyl-1-(indole-3)-1-hexene, 5-methyl-1-(indole-3)-2-hexene, 5-methyl-1-(indole-3)-3-hexene, 4-methyl-1-(indole-3)-1-hexene, 4-methyl-1-(indole-3)-2-hexene, 4-methyl-1-(indole-3)-3-hexene, 5-methyl-1-(indole-3)-2-hexanol, 5-methyl-1-(indole-3)-3-hexanol, 4-methyl-1-(indole-3)-2-hexanol, 4-methyl-1-(indole-3)-3-hexanol, 5-methyl-1-(indole-3)-2-hexanone, 5-methyl-1-(indole-3)-3-hexanone, 4-methyl-1-(indole-3)-2-hexanone, 4-methyl-1-(indole-3)-3-hexanone, 5-methyl-1-(indole-3)-2,3-hexanediol, 4-methyl-1-(indole-3)-2,3-hexanediol, 5-methyl-1-(indole-3)-3-hydroxy-2-hexanone, 5-methyl-1-(indole-3)-2-hydroxy-3-hexanone, 4-methyl-1-(indole-3)-3-hydroxy-2-hexanone, 4-methyl-1-(indole-3)-2-hydroxy-3-hexanone, 5-methyl-1-(indole-3)-2,3-hexanedione, 4-methyl-1-(indole-3)-2,3-hexanedione, n-heptane, 1-heptene, 1-heptanol, heptanal, heptanoate, 2-heptene, 3-heptene, 2-heptanol, 3-heptanol, 4-heptanol, 2-heptanone, 3-heptanone, 4-heptanone, 2,3-heptanediol, 2,3-heptanedione, 3,4-heptanediol, 3,4-heptanedione, 2-hydroxy-3-heptanone, 3-hydroxy-2-heptanone, 3-hydroxy-4-heptanone, 4-hydroxy-3-heptanone, 2-methylheptane, 3-methylheptane, 6-methyl-2-heptene, 6-methyl-3-heptene, 2-

methyl-3-heptene, 2-methyl-2-heptene, 5-methyl-2-heptene, 5-methyl-3-heptene, 3-methyl-3-heptene, 2-methyl-3-heptanol, 2-methyl-4-heptanol, 6-methyl-3-heptanol, 5-methyl-3-heptanol, 3-methyl-4-heptanol, 2-methyl-3-heptanone, 2-methyl-4-heptanone, 6-methyl-3-heptanone, 5-methyl-3-heptanone, 3-methyl-4-heptanone, 2-methyl-3,4-heptanediol, 2-methyl-3,4-heptanedione, 6-methyl-3,4-heptanediol, 6-methyl-3,4-heptanedione, 5-methyl-3,4-heptanediol, 5-methyl-3,4-heptanedione, 2-methyl-3-hydroxy-4-heptanone, 2-methyl-4-hydroxy-3-heptanone, 6-methyl-3-hydroxy-4-heptanone, 6-methyl-4-hydroxy-3-heptanone, 5-methyl-3-hydroxy-4-heptanone, 5-methyl-4-hydroxy-3-heptanone, 2,6-dimethylheptane, 2,5-dimethylheptane, 2,6-dimethyl-2-heptene, 2,6-dimethyl-3-heptene, 2,5-dimethyl-2-heptene, 2,5-dimethyl-3-heptene, 3,6-dimethyl-3-heptene, 2,6-dimethyl-3-heptanol, 2,6-dimethyl-4-heptanol, 2,5-dimethyl-3-heptanol, 2,5-dimethyl-4-heptanol, 2,6-dimethyl-3,4-heptanediol, 2,6-dimethyl-3,4-heptanedione, 2,5-dimethyl-3,4-heptanediol, 2,5-dimethyl-3,4-heptanedione, 2,6-dimethyl-3-hydroxy-4-heptanone, 2,6-dimethyl-4-hydroxy-3-heptanone, 2,5-dimethyl-3-hydroxy-4-heptanone, 2,5-dimethyl-4-hydroxy-3-heptanone, n-octane, 1-octene, 2-octene, 1-octanol, octanal, octanoate, 3-octene, 4-octene, 4-octanol, 4-octanone, 4,5-octanediol, 4,5-octanedione, 4-hydroxy-5-octanone, 2-methyloctane, 2-methyl-3-octene, 2-methyl-4-octene, 7-methyl-3-octene, 3-methyl-3-octene, 3-methyl-4-octene, 6-methyl-3-octene, 2-methyl-4-octanol, 7-methyl-4-octanol, 3-methyl-4-octanol, 6-methyl-4-octanol, 2-methyl-4-octanone, 7-methyl-4-octanone, 3-methyl-4-octanone, 6-methyl-4-octanone, 2-methyl-4,5-octanediol, 2-methyl-4,5-octanedione, 3-methyl-4,5-octanediol, 3-methyl-4,5-octanedione, 2-methyl-4-hydroxy-5-octanone, 2-methyl-5-hydroxy-4-octanone, 3-methyl-4-hydroxy-5-octanone, 3-methyl-5-hydroxy-4-octanone, 2,7-dimethyloctane, 2,7-dimethyl-3-octene, 2,7-dimethyl-4-octene, 2,7-dimethyl-4-octanol, 2,7-dimethyl-4-octanone, 2,7-dimethyl-4,5-octanediol, 2,7-dimethyl-4,5-octanedione, 2,7-dimethyl-4-hydroxy-5-octanone, 2,6-dimethyloctane, 2,6-dimethyl-3-octene, 2,6-dimethyl-4-octene, 3,7-dimethyl-3-octene, 2,6-dimethyl-4-octanol, 3,7-dimethyl-4-octanol, 2,6-dimethyl-4-octanone, 3,7-dimethyl-4-octanone, 2,6-dimethyl-4,5-octanediol, 2,6-dimethyl-4,5-octanedione, 2,6-dimethyl-4-hydroxy-5-octanone, 2,6-dimethyl-5-hydroxy-4-octanone, 3,6-dimethyloctane, 3,6-dimethyl-3-octene, 3,6-dimethyl-4-octene, 3,6-dimethyl-4-octanol, 3,6-dimethyl-4-octanone, 3,6-dimethyl-4,5-octanediol, 3,6-dimethyl-4,5-octanedione, 3,6-dimethyl-4-hydroxy-5-octanone, n-nonane, 1-nonene, 1-nonanol, nonanal, nonanoate, 2-methylnonane, 2-methyl-4-nonene, 2-methyl-5-nonene, 8-methyl-4-nonene, 2-methyl-5-nonanol, 8-methyl-4-nonanol, 2-methyl-5-nonanone, 8-methyl-4-nonanone, 8-methyl-4,5-nonanediol, 8-methyl-4,5-nonanedione, 8-methyl-4-hydroxy-5-nonanone, 8-methyl-5-hydroxy-4-nonanone, 2,8-dimethylnonane, 2,8-dimethyl-3-nonene, 2,8-dimethyl-4-nonene, 2,8-dimethyl-5-nonene, 2,8-dimethyl-4-nonanol, 2,8-dimethyl-5-nonanol, 2,8-dimethyl-4-nonanone, 2,8-dimethyl-5-nonanone, 2,8-dimethyl-4,5-nonanediol, 2,8-dimethyl-4,5-nonanedione, 2,8-dimethyl-4-hydroxy-5-nonanone, 2,8-dimethyl-5-hydroxy-4-nonanone, 2,7-dimethylnonane, 3,8-dimethyl-3-nonene, 3,8-dimethyl-4-nonene, 3,8-dimethyl-5-nonene, 3,8-dimethyl-4-nonanol, 3,8-dimethyl-5-nonanol, 3,8-dimethyl-4-nonanone, 3,8-dimethyl-5-nonanone, 3,8-dimethyl-4,5-nonanediol, 3,8-dimethyl-4,5-nonanedione, 3,8-dimethyl-4-hydroxy-5-nonanone, 3,8-dimethyl-5-

hydroxy-4-nonanone, n-decane, 1-decene, 1-decanol, decanoate, 2,9-dimethyldecane, 2,9-dimethyl-3-decene, 2,9-dimethyl-4-decene, 2,9-dimethyl-5-decanol, 2,9-dimethyl-5-decanone, 2,9-dimethyl-5,6-decanediol, 2,9-dimethyl-6-hydroxy-5-decanone, 2,9-dimethyl-5,6-decanedionen-undecane, 1-undecene, 1-undecanol, undecanal, undecanoate, n-dodecane, 1-dodecene, 1-dodecanol, dodecanal, dodecanoate, n-dodecane, 1-decdecene, 1-dodecanol, ddodecanal, dodecanoate, n-tridecane, 1-tridecene, 1-tridecanol, tridecanal, tridecanoate, n-tetradecane, 1-tetradecene, 1-tetradecanol, tetradecanal, tetradecanoate, n-pentadecane, 1-pentadecene, 1-pentadecanol, pentadecanal, pentadecanoate, n-hexadecane, 1-hexadecene, 1-hexadecanol, hexadecanal, hexadecanoate, n-heptadecane, 1-heptadecene, 1-heptadecanol, heptadecanal, heptadecanoate, n-octadecane, 1-octadecene, 1-octadecanol, octadecanal, octadecanoate, n-nonadecane, 1-nonadecene, 1-nonadecanol, nonadecanal, nonadecanoate, eicosane, 1-eicosene, 1-eicosanol, eicosanal, eicosanoate, 3-hydroxypropanal, 1,3-propanediol, 4-hydroxybutanal, 1,4-butanediol, 3-hydroxy-2-butanone, 2,3-butanediol, 1,5-pentane diol, homocitrate, homoisocitrate, b-hydroxy adipate, glutarate, glutarsemialdehyde, glutaraldehyde, 2-hydroxy-1-cyclopentanone, 1,2-cyclopentenediol, cyclopentanone, cyclopentanol, (S)-2-acetolactate, (R)-2,3-Dihydroxy-isovalerate, 2-oxoisovalerate, isobutyryl-CoA, isobutyrate, isobutyraldehyde, 5-amino pentaldehyde, 1,10-diaminodecane, 1,10-diamino-5-decene, 1,10-diamino-5-hydroxydecane, 1,10-diamino-5-decanone, 1,10-diamino-5,6-decanediol, 1,10-diamino-6-hydroxy-5-decanone, phenylacetaldehyde, 1,4-diphenylbutane, 1,4-diphenyl-1-butene, 1,4-diphenyl-2-butene, 1,4-diphenyl-2-butanol, 1,4-diphenyl-2-butanone, 1,4-diphenyl-2,3-butanediol, 1,4-diphenyl-3-hydroxy-2-butanone, 1-(4-hydroxyphenyl)-4-phenylbutane, 1-(4-hydroxyphenyl)-4-phenyl-1-butene, 1-(4-hydroxyphenyl)-4-phenyl-2-butene, 1-(4-hydroxyphenyl)-4-phenyl-2-butanol, 1-(4-hydroxyphenyl)-4-phenyl-2-butanone, 1-(4-hydroxyphenyl)-4-phenyl-2,3-butanediol, 1-(4-hydroxyphenyl)-4-phenyl-3-hydroxy-2-butanone, 1-(indole-3)-4-phenylbutane, 1-(indole-3)-4-phenyl-1-butene, 1-(indole-3)-4-phenyl-2-butene, 1-(indole-3)-4-phenyl-2-butanol, 1-(indole-3)-4-phenyl-2-butanone, 1-(indole-3)-4-phenyl-2,3-butanediol, 1-(indole-3)-4-phenyl-3-hydroxy-2-butanone, 4-hydroxyphenylacetaldehyde, 1,4-di(4-hydroxyphenyl)butane, 1,4-di(4-hydroxyphenyl)-1-butene, 1,4-di(4-hydroxyphenyl)-2-butene, 1,4-di(4-hydroxyphenyl)-2-butanol, 1,4-di(4-hydroxyphenyl)-2-butanone, 1,4-di(4-hydroxyphenyl)-2,3-butanediol, 1,4-di(4-hydroxyphenyl)-3-hydroxy-2-butanone, 1-(4-hydroxyphenyl)-4-(indole-3)-butane, 1-(4-hydroxyphenyl)-4-(indole-3)-1-butene, 1-di(4-hydroxyphenyl)-4-(indole-3)-2-butene, 1-(4-hydroxyphenyl)-4-(indole-3)-2-butanol, 1-(4-hydroxyphenyl)-4-(indole-3)-2-butanone, 1-(4-hydroxyphenyl)-4-(indole-3)-2,3-butanediol, 1-(4-hydroxyphenyl)-4-(indole-3)-3-hydroxy-2-butanone, indole-3-acetaldehyde, 1,4-di(indole-3)-butane, 1,4-di(indole-3)-1-butene, 1,4-di(indole-3)-2-butene, 1,4-di(indole-3)-2-butanol, 1,4-di(indole-3)-2-butanone, 1,4-di(indole-3)-2,3-butanediol, 1,4-di(indole-3)-3-hydroxy-2-butanone, succinate semialdehyde, hexane-1,8-dicarboxylic acid, 3-hexene-1,8-dicarboxylic acid, 3-hydroxy-hexane-1,8-dicarboxylic acid, 3-hexanone-1,8-dicarboxylic acid, 3,4-hexanediol-1,8-dicarboxylic acid, 4-hydroxy-3-hexanone-1,8-dicarboxylic acid, fucoidan, iodine, chlorophyll, carotenoid, calcium, magnesium, iron,

sodium, potassium, phosphate, lactic acid, acetic acid, formic acid, isoprenoids, or polyisoprenes. Further, such products can include succinic acid, pyruvic acid, enzymes such as cellulases, polysaccharases, lipases, proteases, ligninases, and hemicellulases and may be present as a pure compound, a mixture, or an impure or diluted form.

[0053] The term "fuel, biofuel and/or other chemicals" as used herein has its ordinary meaning as known to those skilled in the art and can include one or more compounds suitable as liquid fuels, gaseous fuels, reagents, chemical feedstocks, chemical additives, processing aids, food additives, and other uses that chemicals can be put to. The term includes, but is not limited to, hydrocarbons, hydrogen, methane, hydroxy compounds such as alcohols (*e.g.* ethanol, butanol, propanol, methanol, etc.), carbonyl compounds such as aldehydes and ketones (*e.g.* acetone, formaldehyde, 1-propanal, etc.), organic acids, derivatives of organic acids such as esters (*e.g.* wax esters, glycerides, etc.) and other functional compounds including, but not limited to, 1, 2-propanediol, 1, 3-propanediol, lactic acid, formic acid, acetic acid, succinic acid, pyruvic acid, enzymes such as cellulases, polysaccharases, lipases, proteases, ligninases, and hemicellulases and may be present as a pure compound, a mixture, or an impure or diluted form.

[0054] The term "pH modifier" as used herein has its ordinary meaning as known to those skilled in the art and can include any material that will tend to increase, decrease or hold steady the pH of the broth or medium. A pH modifier can be an acid, a base, a buffer, or a material that reacts with other materials present to serve to raise, lower, or hold steady the pH. In some embodiments, more than one pH modifier can be used, such as more than one acid, more than one base, one or more acid with one or more bases, one or more acids with one or more buffers, one or more bases with one or more buffers, or one or more acids with one or more bases with one or more buffers. In some embodiments, a buffer can be produced in the broth or medium or separately and used as an ingredient by at least partially reacting in acid or base with a base or an acid, respectively. When more than one pH modifiers are utilized, they can be added at the same time or at different times. In some embodiments, one or more acids and one or more bases can be combined, resulting in a buffer. In some embodiments, media components, such as a carbon source or a nitrogen source can also serve as a pH modifier; suitable media components include those with high or low pH or those with buffering capacity. Exemplary media components include acid- or base-hydrolyzed plant polysaccharides having residual acid or base, ammonia fiber explosion (AFEX) treated plant material with residual ammonia, lactic acid, corn steep solids or liquor.

[0055] The term "plant polysaccharide" as used herein has its ordinary meaning as known to those skilled in the art and may comprise one or more polymers of sugars and sugar derivatives as well as derivatives of sugar polymers and/or other polymeric materials that occur in plant matter. Exemplary plant polysaccharides include lignin, cellulose, starch, pectin, and hemicellulose. Others are chitin, sulfonated polysaccharides such as alginic acid, agarose, carrageenan, porphyran, furcelleran and funoran. Generally, the polysaccharide can have two or more sugar units or derivatives of sugar units. The sugar units and/or derivatives of sugar units may repeat in a regular pattern, or otherwise. The

sugar units can be hexose units or pentose units, or combinations of these. The derivatives of sugar units can be sugar alcohols, sugar acids, amino sugars, etc. The polysaccharides can be linear, branched, cross-linked, or a mixture thereof. One type or class of polysaccharide can be cross-linked to another type or class of polysaccharide.

[0056] The term "Pretreatment" or "pretreated" as used herein refers to any mechanical, chemical, thermal, biochemical process or combination of these processes whether in a combined step or performed sequentially, that achieves disruption or expansion of the biomass so as to render the biomass more susceptible to attack by enzymes and/or microbes. In some embodiments, pretreatment can include removal or disruption of lignin so as to make the cellulose and hemicellulose polymers in the plant biomass more available to cellulolytic enzymes and/or microbes, for example, by treatment with acid or base. In some embodiments, pretreatment can include the use of a microorganism of one type to render plant polysaccharides more accessible to microorganisms of another type. In some embodiments, pretreatment can also include disruption or expansion of cellulosic and/or hemicellulosic material. Steam explosion, and ammonia fiber expansion (or explosion) (AFEX) are well known thermal/chemical techniques. Hydrolysis, including methods that utilize acids and/or enzymes can be used. Other thermal, chemical, biochemical, enzymatic techniques can also be used.

[0057] The term "productivity" as used herein has its ordinary meaning as known to those skilled in the art and can include the mass of a material of interest produced in a given time in a given volume. Units can be, for example, grams per liter-hour, or some other combination of mass, volume, and time. In fermentation, productivity is frequently used to characterize how fast a product can be made within a given fermentation volume. The volume can be referenced to the total volume of the fermentation vessel, the working volume of the fermentation vessel, or the actual volume of broth being fermented. The context of the phrase will indicate the meaning intended to one of skill in the art. Productivity is different from "titer" in that productivity includes a time term, and titer is analogous to concentration.

[0058] The term "saccharification" as used herein has its ordinary meaning as known to those skilled in the art and can include conversion of plant polysaccharides to lower molecular weight species that can be utilized by the organism at hand. For some organisms, this includes conversion to monosaccharides, disaccharides, trisaccharides, and oligosaccharides of up to about seven monomer units, as well as similar sized chains of sugar derivatives and combinations of sugars and sugar derivatives. For some organisms, the allowable chain-length may be longer and for some organisms the allowable chain-length may be shorter.

[0059] The term "sugar compounds" as used herein has its ordinary meaning as known to those skilled in the art and can include monosaccharide sugars, hexoses and pentoses; sugar alcohols; sugar acids; sugar amines; compounds containing two or more of these linked together directly or indirectly through covalent or ionic bonds; and mixtures thereof. Also included within this description are disaccharides; trisaccharides; oligosaccharides; polysaccharides; and sugar chains, branched and/or linear, of any length.

## Introduction

[0060] Biomass is a renewable source of energy, which can be biologically fermented to produce an end-product such as a fuel (*e.g.* alcohol, ethanol, organic acid, acetic acid, lactic acid, methane, or hydrogen) for mobile engines or a chemical compound for other commercial purposes. Biomass includes agricultural residues (*e.g.*, corn stalks, grass, straw, grain hulls, or bagasse), animal waste (*e.g.*, manure from cattle, poultry, and hogs), algae, woody materials (*e.g.*, wood or bark, sawdust, timber slash, or mill scrap), municipal waste (*e.g.*, waste paper, recycled toilet papers, or yard clippings), and energy crops (*e.g.*, poplars, willows, switchgrass, alfalfa, or prairie bluestem). Lignocellulosic biomass has cellulose and hemicellulose as two major components. To obtain a high fermentation efficiency of lignocellulosic biomass to end-product (*i.e.*, high yield), it can be important to provide an appropriate pretreatment for removing and/or detoxifying at least a portion of the lignin content and for making cellulose and hemicelluloses more amendable to enzymatic hydrolysis.

[0061] Recently, the conversion to ethanol of polymeric hexose and pentose sugars in cellulose and hemicellulose has been achieved. See U.S. Pat. No. 4,349,628 to English et al; see also U.S. Pat. No. 4,400,470 to Zeikus et al; U.S. Pat. No. 5,000,000 to Ingram et al; U.S. Pat. No. 5,028,539 to Ingram et al; and U.S. Pat. No. 5,162,516 to Ingram et al, all of which are incorporated herein by reference.

[0062] Compositions and methods described herein are directed to saccharification and fermentation of disparate types of feedstock with a microorganism that is capable of direct saccharification and fermentation of cellulosic biomass, and result in fermenting such feedstock to produce a fermentive product.

## Microorganisms

[0063] Various embodiments of the invention offer benefits relating to improving the titer and/or productivity of alcohol production by a microorganism, by pretreating a feedstock to be fermented with an alkaline substance. Any microorganism capable of fermenting feedstocks can be utilized in compositions and methods described herein. Any of numerous microbes known to ferment biomass can be utilized, including but not limited to bacteria, or fungi. Non-limiting examples of organisms useful for compositions and methods described herein include species within the genera *Aspergillus*, *Bacillus*, *Cellulomonas*, *Chlorella*, *Clostridium*, *Fibrobacter*, *Neocallimasterix*, *Nocardiopsis*, *Ruminococcus*, *Schizophyllum*, *Streptomyces*, *Thermomonospora*, *Thermomyces*, and *Trichoderma*.

[0064] A microorganism can be a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans*. A microorganism can be recombinant or wild-type. For example, a recombinant organism can be genetically modified to enhance activity of one or more proteins associated with saccharification or fermentation (*e.g.*, cellulase or hydrolytic enzymes).

[0065] In some embodiments, microorganism capable of direct saccharification and fermentation of a hemicellulosic, lignocellulosic or cellulosic feedstock is utilized. Such an organism can be recombinant

or wild type. Furthermore, the organism can be a bacterium. In a further embodiment, the bacterium is from the genus *Clostridium*. In yet a further embodiment, the organism is *C. phytofermentans*.

[0066] *C. phytofermentans* ("Q microbe") includes, but is not limited to, American Type Culture Collection 700394T. For clarity, the term "Q microbe" is a generic reference to *C. phytofermentans* and should not be construed to be limited to a specific strain. Aspects of the invention generally include systems, methods, and compositions for producing fuels, such as ethanol, and/or other useful organic products involving, for example, strain 700394T and/or any other strain of the species *Clostridium phytofermentans*, including those which may be derived from strain ISDg<sup>T</sup>, including genetically modified strains, or strains separately isolated or cultured to develop a particular characteristic that is advantageous to saccharification and fermentation of one or more different biomass. Some exemplary species can be defined using standard taxonomic considerations (Stackebrandt and Goebel, International Journal of Systematic Bacteriology, 44:846-9, 1994): Strains with 16S rRNA sequence homology values of 97% and higher as compared to the type strain (ISDg<sup>T</sup>), and strains with DNA re-association values of at least about 70% can be considered *Clostridium phytofermentans*.

[0067] Considerable evidence exists to indicate that many microbes which have 70% or greater DNA re-association values also have at least 96% DNA sequence identity and share phenotypic traits defining a species. Analyses of the genome sequence of *Clostridium phytofermentans* strain ISDg<sup>T</sup> indicate the presence of large numbers of genes and genetic loci that are likely to be involved in mechanisms and pathways for plant polysaccharide fermentation, giving rise to the unusual fermentation properties of this microbe which can be found in all or nearly all strains of the species *Clostridium phytofermentans*. *Clostridium phytofermentans* strains can be natural isolates, or genetically modified strains.

[0068] The "Q" microbe provides useful advantages for the conversion of biomass to ethanol and other products. One advantage is the ability to produce enzymes capable of hydrolyzing polysaccharides and higher saccharides to lower molecular weight saccharides, oligosaccharides, disaccharides, and monosaccharides. Furthermore, Q microbe can produce a wide spectrum of hydrolytic enzymes that can facilitate fermenting of various biomass materials, including cellulosic, hemicellulosic, lignocellulosic materials; pectins; starches; wood; paper; agricultural products; forest waste; tree waste; yard waste; tree bark; leaves; grasses; sawgrass; woody plant matter; non-woody plant matter; carbohydrates; inulin; fructans; glucans; corn; sugar cane; grasses; bamboo, and material derived from these materials.

[0069] The Q microbe cells used for the seed inoculum or for cell augmentation can be prepared or treated in ways that relate to their ability to produce enzymes useful for hydrolyzing the components of the production medium. For example, in one embodiment, the Q microbe cells can produce useful enzymes after they are transferred to the production medium or production fermentor. In another embodiment, the Q microbe cells can have already produced useful enzymes prior to transfer to the production medium or the production fermentor. In another embodiment, the Q microbe cells can be ready to produce useful enzymes once transferred to the production medium or the production

fermentor, or the Q microbe cells can have some combination of these enzyme production characteristics. In one embodiment, the seed can be grown initially in a medium containing a simple sugar source, such as corn syrup, and then transitioned to the production medium carbon source prior to transfer to the production medium. In another embodiment, the seed is grown on a combination of simple sugars and production medium carbon source prior to transfer to the production medium. In another embodiment, the seed is grown on the production medium carbon source from the start. In another embodiment, the seed is grown on one production medium carbon source and then transitioned to another production medium carbon source prior to transfer to the production medium. In another embodiment, the seed is grown on a combination of production medium carbon sources prior to transfer to the production medium. In another embodiment, the seed is grown on a carbon source that favors production of hydrolytic enzymes with activity toward the components of the production medium.

[0070] As demonstrated in Figure 3, *C. phytofermentans* ferments feedstocks containing different fermentable polysaccharides. For Figure 3, fermentation reactions were performed utilizing media containing the individual fermentable feedstocks. Furthermore, Figure 9 demonstrates the ability of *C. phytofermentans* to convert liquid municipal waste to ethanol, and Figure 10 demonstrates the ability of *C. phytofermentans* to produce ethanol by fermenting multiple materials in a mixed-source feedstock. For Figure 10, different feedstocks were either untreated (bottom line in all panels) treated with lime air (middle line in all panels), or treated with 1% sodium hydroxide (top line in all panels) prior to fermentation.

[0071] The organism can usually produce the enzymes to break down different feedstock components and metabolic processes as needed (Figure 4), frequently without excessive production of unnecessary hydrolytic enzymes, or in some embodiments, one or more enzymes can be added to further improve the organism's production capability. This ability to produce a very wide range of saccharolytic enzymes gives *C. phytofermentans* and the associated technology distinct advantages in biomass fermentation, especially those fermentations not utilizing simple sugars as the feedstock. Various fermentation conditions can enhance the activities of the organism, resulting in higher yields, higher productivity, greater product selectivity, and/or greater conversion efficiency. In some embodiments, fermentation conditions can include fed batch operation and fed batch operation with cell augmentation; addition of complex nitrogen sources such as corn steep powder or yeast extract; addition of specific amino acids including proline, glycine, isoleucine, and/or histidine; addition of a complex material containing one or more of these amino acids; addition of other nutrients or other compounds such as phytate, proteases enzymes, or polysaccharase enzymes. In some embodiments, the addition of one material may provide supplements that fit into more than one category, such as providing amino acids and phytate.

[0072] In some embodiments, *C. phytofermentans* can be used to hydrolyze various higher saccharides present in biomass to lower saccharides, such as in preparation for fermentation to produce ethanol, hydrogen, or other chemicals such as organic acids including formic acid, acetic acid, and lactic acid.

Another advantage of *C. phytofermentans* is its ability to hydrolyze polysaccharides and higher saccharides that contain hexose sugar units, that contain pentose sugar units, and that contain both, into lower saccharides and in some cases monosaccharides. *C. phytofermentans* can also uptake both hexose and pentose sugars and convert both to ethanol (Figure 7 and Figure 8). Figure 7 shows simultaneous uptake of hexose and pentose sugars from a medium containing glucose, cellobiose and xylose. Figure 8 shows the ability of *C. phytofermentans* to convert a pentose (xylose) to ethanol. These enzymes and/or the hydrolysate can be used in fermentations to produce various products including fuels, and other chemicals. Another advantage of *C. phytofermentans* is its ability to produce ethanol, hydrogen, and other fuels or compounds such as organic acids including acetic acid, formic acid, and lactic acid from lower sugars such as monosaccharides. Another advantage of *C. phytofermentans* is its ability to perform the combined steps of hydrolyzing a higher molecular weight biomass containing sugars and/or higher saccharides or polysaccharides to lower sugars and fermenting these lower sugars into desirable products including ethanol, hydrogen, and other compounds such as organic acids including formic acid, acetic acid, and lactic acid. Still another advantage of *C. phytofermentans* is its ability to ferment organic acids which may be present in the biomass, or may be produced during fermentation of complex carbohydrates present in a feedstock (Figure 11).

[0073] Another advantage of *C. phytofermentans* is its ability to grow under conditions that include elevated ethanol concentration, high sugar concentration, low sugar concentration, utilize insoluble carbon sources, and/or operate under anaerobic conditions. These characteristics, in various combinations, can be used to achieve operation with long fermentation cycles and can be used in combination with batch fermentations, fed batch fermentations, self-seeding/partial harvest fermentations, and recycle of cells from the final fermentation as inoculums.

[0074] Compositions and methods described herein provides methods for use of microorganisms, such as *Clostridium phytofermentans* or other *Clostridium* species, which in some embodiments have the capability of simultaneously hydrolyzing and fermenting lignocellulosic biomass. In one embodiment a microorganism simultaneously ferments both hexose and pentose fractions to produce a fermentation end-product. In another embodiment *Clostridium phytofermentans* or other *Clostridium* species can provide useful advantages for the conversion of biomass to ethanol or other fermentation end-products (e.g. alcohol, organic acid, acetic acid, lactic acid, methane, or hydrogen) by its ability to produce enzymes capable of hydrolyzing polysaccharides and higher saccharides to lower molecular weight saccharides, oligosaccharides, disaccharides, and monosaccharides. In some embodiments, a microorganism (such as *Clostridium phytofermentans* or other *Clostridium* species) can be utilized in methods described herein to perform the combined steps of hydrolyzing a higher molecular weight biomass containing sugars and/or higher saccharides or polysaccharides to lower sugars and fermenting oligosaccharides, disaccharides, and monosaccharides from both cellulose as well as hemicelluloses into one or more fermentation end-products (including, but not limited to ethanol, methane, hydrogen, and other compounds such as organic acids including formic acid, acetic acid, and lactic acid). Methods

described herein further provide for the growth, culturing, or fermenting of a microorganism, such as *Clostridium phytofermentans* or another *Clostridium* species under conditions that include elevated ethanol concentration, high sugar concentration, low sugar concentration, utilization of insoluble carbon sources, and anaerobic conditions.

[0075] In some embodiments, the Q microbe or *Clostridium* sp. Q.D is used to hydrolyze various higher saccharides present in biomass to lower saccharides, such as in preparation for fermentation to produce ethanol, hydrogen, or other chemicals such as organic acids including formic acid, acetic acid, and lactic acid. In another embodiment, a Q microbe or *Clostridium* sp. Q.D is used to hydrolyze polysaccharides and higher saccharides such as hexose saccharides. In another embodiment, a Q microbe or *Clostridium* sp. Q.D is used to hydrolyze polysaccharides and higher saccharides such as pentose saccharides. In another embodiment, a Q microbe or *Clostridium* sp. Q.D is used to hydrolyze polysaccharides and higher saccharides that contain both hexose and pentose sugar units. In another embodiment, a Q microbe or *Clostridium* sp. Q.D is used to hydrolyze polysaccharides and higher saccharides into lower saccharides or monosaccharides. In another embodiment, hydrolysate from Q microbe or *Clostridium* sp. Q.D treatment is used in a fermentation process to produce one or more fermentation products such as a biofuel. In another embodiment, a Q microbe or *Clostridium* sp. Q.D is used to produce ethanol, hydrogen, or compounds such as organic acids including acetic acid, formic acid, and lactic acid from a lower sugar such as monosaccharide or a disaccharide. In another embodiment, a Q microbe or *Clostridium* sp. Q.D is used to perform the combined steps of hydrolyzing a higher molecular weight biomass containing sugars and/or higher saccharides or polysaccharides to lower sugars and fermenting these lower sugars into desirable products including ethanol, hydrogen, and other compounds such as organic acids including formic acid, acetic acid, and lactic acid, or other fermentation end products.

[0076] Many bacterial species are known to ferment feedstocks with varying compositions. Selection of an optimal bacterial strain for fermentation depends on the composition of a particular feedstock, fermenting environment such as tolerance to ethanol, alkaline treatment, and other physical parameters of fermentation (e.g., pH, pressure, or temperature). Methods disclosed herein are applicable and readily adaptable to any bacterial strain that anaerobically ferments feedstock. A bacterial strain useful for the methods disclosed herein can be isolated from the group consisting of *Acetivibrio cellulolyticus*, *Acetivibrio cellulosolvens*, *Acetivibrio ethanolgigriens*, *Acetivibrio multivorans*, *Acetoanaerobium noterae*, *Acetofilamentum rigidum*, *Acetogenium kivui*, *Acetomicrobium faecale*, *Acetomicrobium flavidum*, *Acetothermus paucivorans*, *Acidaminobacter hydrogeniformans*, *Anaerobiospirillum succiniciproducens*, *Anaerobiospirillum thomasi*, *Anaerorhabdus furcosa*, *Anaerovibrio burkinabensis*, *Anaerovibrio glycerini*, *Anaerovibrio lipolyticus*, *Atopobium fossor*, *Atopobium minutum*, *Atopobium parvulum*, *Atopobium rima*, *Atopobium vaginae*, *Bacteroides acidifaciens*, *Bacteroides amylophilus*, *Bacteroides asaccharolyticus*, *Bacteroides bivius*, *Bacteroides buccae*, *Bacteroides buccalis*, *Bacteroides caccae*, *Bacteroides capillosus*, *Bacteroides capillus*, *Bacteroides cellulosolvens*,

*Bacteroides coagulans*, *Bacteroides corporis*, *Bacteroides denticola*, *Bacteroides disiens*, *Bacteroides distasonis*, *Bacteroides eggerthii*, *Bacteroides endodontalis*, *Bacteroides forsythus*, *Bacteroides fragilis*, *Bacteroides furcosus*, *Bacteroides galacturonicus*, *Bacteroides gingivalis*, *Bacteroides gracilis*, *Bacteroides helcogenes*, *Bacteroides heparinolyticus*, *Bacteroides hypermegas*, *Bacteroides intermedius*, *Bacteroides levii*, *Bacteroides loescheii*, *Bacteroides macacae*, *Bacteroides melaninogenicus*, *Bacteroides melaninogenicus subsp. intermedius*, *Bacteroides melaninogenicus subsp. macaca*, *Bacteroides melaninogenicus subsp. melaninogenicus*, *Bacteroides merdae*, *Bacteroides microfusis*, *Bacteroides multiacidus*, *Bacteroides nodosus*, *Bacteroides ochraceus*, *Bacteroides oralis*, *Bacteroides oris*, *Bacteroides oulorum*, *Bacteroides ovatus*, *Bacteroides pectinophilus*, *Bacteroides pentosaceus*, *Bacteroides pneumosintes*, *Bacteroides polypragmatus*, *Bacteroides praeacutus*, *Bacteroides putredinis*, *Bacteroides pyogenes*, *Bacteroides ruminicola*, *Bacteroides ruminicola subsp. brevis*, *Bacteroides ruminicola subsp. ruminicola*, *Bacteroides salivus*, *Bacteroides splanchnicus*, *Bacteroides stercoris*, *Bacteroides succinogenes*, *Bacteroides suis*, *Bacteroides tectus*, *Bacteroides termitidis*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, *Bacteroides ureolyticus*, *Bacteroides veroralis*, *Bacteroides vulgatus*, *Bacteroides xylanolyticus*, *Bacteroides zoogloformans*, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium animalis*, *Bifidobacterium animalis subsp. animalis*, *Bifidobacterium animalis subsp. lactis*, *Bifidobacterium asteroides*, *Bifidobacterium bifidum*, *Bifidobacterium bourn*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium choerinum*, *Bifidobacterium coryneforme*, *Bifidobacterium cuniculi*, *Bifidobacterium denticolens*, *Bifidobacterium dentium*, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacterium globosum*, *Bifidobacterium indicum*, *Bifidobacterium infantis*, *Bifidobacterium inopinatum*, *Bifidobacterium lactis*, *Bifidobacterium longum*, *Bifidobacterium magnum*, *Bifidobacterium merycicum*, *Bifidobacterium minimum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum*, *Bifidobacterium pseudolongum subsp. globosum*, *Bifidobacterium pseudolongum subsp. pseudolongum*, *Bifidobacterium psychraerophilum*, *Bifidobacterium pullorum*, *Bifidobacterium ruminantium*, *Bifidobacterium saeculare*, *Bifidobacterium scardovii*, *Bifidobacterium subtile*, *Bifidobacterium suis*, *Bifidobacterium thermacidophilum*, *Bifidobacterium thermacidophilum subsp. porcinum*, *Bifidobacterium thermacidophilum subsp. thermacidophilum*, *Bifidobacterium thermophilum*, *Bilophila wadsworthia*, *Butyrivibrio cjoossotus*, *Butyrivibrio fibrisolvens*, *Butyrivibrio hungatei*, *Campylobacter butzleri*, *Campylobacter cinaedi*, *Campylobacter coli*, *Campylobacter concisus*, *Campylobacter cryaerophilus*, *Campylobacter curvus*, *Campylobacter fennelliae*, *Campylobacter fetus*, *Campylobacter fetus subsp. fetus*, *Campylobacter fetus subsp. venerealis*, *Campylobacter gracilis*, *Campylobacter helveticus*, *Campylobacter hominis*, *Campylobacter hyoilei*, *Campylobacter hyointestinalis*, *Campylobacter hyointestinalis subsp. hyointestinalis*, *Campylobacter hyointestinalis subsp. lawsonii*, *Campylobacter insulaenigrae*, *Campylobacter jejuni*, *Campylobacter jejuni subsp. doylei*, *Campylobacter jejuni subsp. jejuni*, *Campylobacter lanienae*, *Campylobacter lari*, *Campylobacter mucosalis*, *Campylobacter inustetae*,

'&' *Campylobacter nitrofigilis*, *Campylobacter pylori*, *Campylobacter pylori* subsp. *mustelae*, *Campylobacter pylori* subsp. *pylori*, *Campylobacter rectus*, *Campylobacter showae*, *Campylobacter sputorum*, *Campylobacter sputorum* subsp. *bubulus*, *Campylobacter sputorum* subsp. *mucosalis*, *Campylobacter sputorum* subsp. *sputorum*, *Campylobacter upsaliensis*, *Catonella morbi*, *Centipeda periodontii*, *Dialister invisus*, *Dialister pneumosintes*, *Dichelobacter nodosus*, *Fervidobacterium gondwanens*, *Feiyidobacterium islandicum*, *Fervidobacterium nodosum*, *Fervidobacterium pennivorans*, *Fibrobacter intestinalis*, *Fibrobacter succinogenes*, *Fibrobacter succinogenes* subsp. *Elongatus*, *Fibrobacter succinogenes* subsp. *succinogenes*, *Fusobacterium alocis*, *Fusobacterium canifelinum*, *Fusobacterium equinum*, *Fusobacterium gonidiaformans*, *Fusobacterium mortiferum*, *Fusobacterium naviforme*, *Fusobacterium necrogenes*, *Fusobacterium necrophorum*, *Fusobacterium necrophorum* subsp. *funduliforme*, *Fusobacterium necrophorum* subsp. *necrophorum*, *Fusobacterium nucleatum*, *Fusobacterium nucleatum* subsp. *animalis*, *Fusobacterium nucleatum* subsp. *fusiforme*, *Fusobacterium nucleatum* subsp. *nucleatum*, *Fusobacterium nucleatum* subsp. *polymorphum*, *Fusobacterium nucleatum* subsp. *vincentii*, *Fusobacterium perfoetens*, *Fusobacterium periodonticum*, *Fusobacterium plautii*, *Fusobacterium polysaccharolyticum*, *Fusobacterium prausnitzii*, *Fusobacterium pseudonecrophorum*, *Fusobacterium russii*, *Fusobacterium simiae*, *Fusobacterium sulci*, *Fusobacterium ulcerans*, *Fusobacterium variuni*, *Halanaerobacter chitinivorans*, *Halanaerobacter lacunarum*, *Halanaerobacter salinarius*, *Halanaerobium acetihylicum*, *Halanaerobium alcaliphilum*, *Halanaerobium congolense*, *Halanaerobium fermentans*, *Halanaerobium kushneri*, *Halanaerobium lacusrosei*, *Halanaerobium praevalens*, *Halanaerobium saccharolyticum*, *Halanaerobium saccharolyticum* subsp. *Saccharolyticum*, *Halanaerobium saccharolyticum* subsp. *senegalense*, *Halanaerobium salsuginis*, *Ilyobacter delafieldii*, *Ilyobacter insuetus*, *Hyobacter polytropus*, *Ilyobacter tartaricus*, *Johnsonella ignava*, *Lachnobacterium bovis*, *Leptotrichia buccalis*, *Leptotrichia goodfellowii*, *Leptotrichia hofstadii*, *Leptotrichia shahii*, *Leptotrichia trevisanii*, *Leptotrichia wadei*, *Malonomonas rubra*, *Megamonas hypermegale*, *Mitsuokella dentalis*, *Mitsuokellalaludini*, *Mitsuokella multacida*, *Oxalobacterformigenes*, *Oxalobacter vibrioformis*, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, *Pectinatus portalensis*, *Pelobacter acetylenicus*, *Pelobacter acidigallici*, *Pelobacter carbinolicus*, *Pelobacter massiliensis*, *Pelobacter propionicus*, *Pelobacter venetianus*, *Porphyromonas asaccharolytica*, *Porphyromonas cangingivalis*, *Porphyromonas canons*, *Porphyromonas cansulci*, *Poiphyromonas catoniae*, *Porphyromonas circumdentaria*, *Porphyromonas crevioricanis*, *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *Porphyromonas gingivicanis*, *Porphyromonas gulae*, *Poiphyromonas levii*, *Porphyromonas macacae*, *Porphyromonas salivosa*, *Poiphyromonas uenonis*, *Prevotella albensis*, *Prevotella bivia*, *Prevotella brevis*, *Prevotella bryantii*, *Prevotella buccae*, *Prevotella buccalis*, *Prevotella corporis*, *Prevotella dentalis*, *Prevotella denticola*, *Prevotella disiens*, *Prevotella enoeca*, *Prevotella heparinolytica*, *Prevotella intermedia*, *Prevotella loescheii*, *Prevotella melaninogenica*, *Prevotella multiformis*, *Prevotella nigrescens*, *Prevotella oralis*, *Prevotella oris*, *Prevotella oulorum*, *Prevotella pollens*, *Prevotella ruminicola*, *Prevotella salivae*,

*Prevotella shahii*, *Prevotella tanneriae*, *Prevotella veroralis*, *Prevotella zoogloformans*, *Propionibacterium acidipropionici*, *Propionibacterium acnes*, *Propionibacterium australiense*,  
*Propionibacterium avidum*, *Propionibacterium cyclohexanicum*, *Propionibacterium freudenreichii*,  
*Propionibacterium freudenreichii* subsp. *Freudenreichii*, *Propionibacterium freudenreichii* subsp.  
*shermanii*, *Propionibacterium granulosum*, *Propionibacterium innocuum*, *Propionibacterium jensenii*,  
*Propionibacterium lymphophilum*, *Propionibacterium microaerophilum*, *Propionibacterium propionicum*, *Propionibacterium thoenii*, *Propionigenium maris*, *Propionigenium modestum*,  
*Propionispira arboris*, *Rikenella microfusus*, *Roseburia cecicola*, *Roseburia intestinalis*, *Ruminobacter amylophilus*, *Sebaldella termitidis*, *Selenomonas acidaminovorans*, *Selenomonas artemidis*,  
*Selenomonas diana*, *Selenomonas flueggei*, *Selenomonas infelix*, *Selenomonas lactificifex*, *Selenomonas lipolytica*, *Selenomonas ruminantium*, *Selenomonas ruminantium* subsp.  
*lactilytica*, *Selenomonas ruminantium* subsp. *ruminantium*, *Selenomonas sputigena*, *Sporomusa acidovorans*, *Sporomusa aerivorans*, *Sporomusa malonica*, *Sporomusa ovata*, *Sporomusa paucivorans*,  
*Sporomusa silvacetica*, *Sporomusa sphaeroides*, *Sporomusa termitida*, *Succinimonas amylolytica*,  
*Succinivibrio dextrinosolvens*, *Syntrophobacter fumaroxidans*, *Syntrophobacter pfennigii*,  
*Syntrophobacter wolinii*, *Syntrophomonas curvata*, *Syntrophomonas erecta*, *Syntrophomonas sapovorans*, *Syntrophomonas wolfei*, *Syntrophomonas wolfei*, *Sutterella stercoricanis*, *Sutterella wadsworthensi*, *Saponavida*, *Thermobacteroides acetoethylicus*, *Thermobacteroides leptospartum*,  
*Thermobacteroides proteolyticus*, *Thermosiphon africanus*, *Thermosiphon atlanticus*, *Thermosiphon geolei*, *Thermosiphon japonicus*, *Thermosiphon melanesiensis*, *Thermotoga elfi*, *Thermotoga hypogea*,  
*Thermotoga lettingae*, *Thermotoga maritima*, *Thermotoga naphthophila*, *Thermotoga neapolitana*,  
*Thermotoga petrophila*, *Thermotoga subterranea*, *Thermotoga thermarum*, *Tissierella creatinini*,  
*Tissierella creatinophila*, *Tissierella praeacuta*, *Wolinella curva*, *Wolinella recta*, *Wolinella succinogenes*, *Zymophilus paucivorans*, *Zymophilus raffinosisivorans*, *Desulfobacter curvatus*,  
*Desulfobacter halotolerans*, *Desulfobacter hydrogenophilus*, *Desulfobacter latus*, *Desulfobacter postgatei*, *Desulfobacter vibrioformis*, *Desulfobacterium anilini*, *Desulfobacterium autotrophicum*,  
*Desulfobacterium catecholicum*, *Desulfobacterium cetonicum*, *Desulfobacterium indolicum*,  
*Desulfobacterium macestii*, *Desulfobacterium phenolicum*, *Desulfobulbus elongatus*, *Desulfobulbus mediterraneus*, *Desulfobulbus propionicus*, *Desulfobulbus rhabdiformis*, *Desulfococcus biacutus*,  
*Desulfococcus multivorans*, *Desulfomicrobium apsheronum*, *Desulfomicrobium baculatum*,  
*Desulfomicrobium escambiense*, *Desulfomicrobium macestii*, *Desulfomicrobium norvegicum*,  
*Desulfomicrobium orale*, *Desulfomonas pigra*, *Desulfomonas limimaris*, *Desulfomonas tiedjei*,  
*Desulfonema ishimotonii*, *Desulfonema limicola*, *Desulfonema magnum*, *Desulfosarcina variabilis*,  
*Desulfotomaculum acetoxidans*, *Desulfotomaculum aeronauticum*, *Desulfotomaculum alkaliphilum*,  
*Desulfotomaculum antarcticum*, *Desulfotomaculum auripigmentum*, *Desulfotomaculum austriacum*,  
*Desulfotomaculum geothermicum*, *Desulfotomaculum gibsoniae*, *Desulfotomaculum guttoideum*,  
*Desulfotomaculum halophilum*, *Desulfotomaculum kuznetsovii*, *Desulfotomaculum luciae*,

*Desulfotomaculum nigrificans*, *Desulfotomaculum orientis*, *Desulfotomaculum putei*,  
*Desulfotomaculum ruminis*, *Desulfotomaculum sapomandens*, *Desulfotomaculum solfataricum*,  
*Desulfotomaculum thermoacetoxidans*, *Desulfotomaculum thermobenzoicum*, *Desulfotomaculum*  
*thermobenzoicum subsp. thermobenzoicum*, *Desulfotomaculum thermobenzoicum subsp.*  
*thermosyntrophicum*, *Desulfotomaculum thermocisternum*, *Desulfotomaculum thermosapovorans*,  
*Desulfovibrio acrylicus*, *Desulfovibrio aespoeensis*, *Desulfovibrio africanus*, *Desulfovibrio alaskensis*,  
*Desulfovibrio alcoholivorans*, *Desulfovibrio aminophilus*, *Desulfovibrio baarsii*, *Desulfovibrio*  
*baculatus*, *Desulfovibrio bastinii*, *Desulfovibrio burkinensis*, *Desulfovibrio carbinolicus*, *Desulfovibrio*  
*cuneatus*, *Desulfovibrio dechloracetivorans*, *Desulfovibrio desulfuricans*, *Desulfovibrio desulfuricans*  
*subsp. aestuarii*, *Desulfovibrio desulfuricans subsp. desulfuricans*, *Desulfovibrio fructosivorans*,  
*Desulfovibrio furfur alis*, *Desulfovibrio gabonensis*, *Desulfovibrio giganteus*, *Desulfovibrio gigas*,  
*Desulfovibrio gracilis*, *Desulfovibrio halophilus*, *Desulfovibrio hydrothermalis*, *Desulfovibrio*  
*indonesiensis*, *Desulfovibrio inopinatus*, *Desulfovibrio intestinalis*, *Desulfovibrio litoralis*,  
*Desulfovibrio longreachensis*, *Desulfovibrio longus*, *Desulfovibrio magneticus*, *Desulfovibrio*  
*mexicanus*, *Desulfovibrio oxyclinae*, *Desulfovibrio piger*, *Desulfovibrio profundus*, *Desulfovibrio*  
*putealis*, *Desulfovibrio salexigens*, *Desulfovibrio sapovorans*, *Desulfovibrio senezii*, *Desulfovibrio*  
*simplex*, *Desulfovibrio sulfodismutans*, *Desulfovibrio termitidis*, *Desulfovibrio thermophilus*,  
*Desulfovibrio vietnamensis*, *Desulfovibrio vulgaris*, *Desulfovibrio vulgaris subsp. oxamicus*,  
*Desulfovibrio vulgaris subsp. vulgaris*, *Desulfovibrio zosterae*, *Desulfurella acetivorans*, *Desulfurella*  
*kamchatkensis*, *Desulfurella multipotens*, *Desulfurella propionica*, *Desulfuromonas acetexigens*,  
*Desulfuromonas acetoxidans*, *Desulfuromonas chloroethenica*, *Desulfuromonas palmitatis*,  
*Desulfuromonas thiophila*, *Thermodesulfobacterium commune*, *Thermodesulfobacterium*  
*hveragerdense*, *Thermodesulfobacterium hydrogeniphilum*, *Thennodesulfobacterium thermophilum*,  
*Acidaminococcus fermentans*, *Megasphaera cerevisiae*, *Megasphaera elsdenii*, *Megasphaera*  
*micronuciformis* *Syntrophococcus sucromutans*, *Veillonella alcalescens*, *Veillonella alcalescens subsp.*  
*alcalescens*, *Veillonella alcalescens subsp. criceti*, *Veillonella alcalescens subsp. dispar*, *Veillonella*  
*alcalescens subsp. ratti*, *Veillonella atypica*, *Veillonella caviae*, *Veillonella criceti*, *Veillonella dispar*,  
*Veillonella niontpellierensis*, *Veillonella parvula*, *Veillonella parvula subsp. atypical*, *Veillonella*  
*parvula subsp. parvula*, *Veillonella parvula subsp. rodentium*, *Veillonella ratti*, *Veillonella rodentium*,  
*Coprococcus catu*, *Coprococcus comes*, *Coprococcus eutactus*, *Peptococcus asaccharolyticus*,  
*Peptococcus glycinophilus*, *Peptococcus heliotrinreducens*, *Peptococcus indolicus*, *Peptococcus*  
*magnus*, *Peptococcus niger*, *Peptococcus prevotii*, *Peptococcus saccharolyticus*, *Peptostreptococcus*  
*anaerobius*, *Peptostreptococcus asaccharolyticus*, *Peptostreptococcus barnesae*, *Peptostreptococcus*  
*harei*, *Peptostreptococcus heliotrinreducens*, *Peptostreptococcus hydrogenalis*, *Peptostreptococcus*  
*indolicus*, *Peptostreptococcus ivorii*, *Peptostreptococcus lacrimalis*, *Peptostreptococcus lactolyticus*,  
*Peptostreptococcus magnus*, *Peptostreptococcus micros*, *Peptostreptococcus octavius*,  
*Peptostreptococcus parvulus*, *Peptostreptococcus prevotii*, *Peptostreptococcus productus*,

*Peptostreptococcus tetradius*, *Peptostreptococcus vaginalis*, *Ruminococcus albus*, *Ruminococcus bromii*, *Ruminococcus callidus*, *Ruminococcus flavefaciens*, *Ruminococcus gnavus*, *Ruminococcus hansenii*, *Ruminococcus hydrogenotrophicus*, *Ruminococcus lactaris*, *Ruminococcus luti*, *Ruminococcus obeum*, *Ruminococcus palustris*, *Ruminococcus pasteurii*, *Ruminococcus productus*, *Ruminococcus schinkii*, *Ruminococcus torques*, *Sarcina maxima*, *Sarcina ventriculi*, *Clostridium absonum*, *Clostridium aceticum*, *Clostridium acetireducens*, *Clostridium acetobutylicum*, *Clostridium acidisoli*, *Clostridium aciduria*, *Clostridium aerotolerans*, *Clostridium akagii*, *Clostridium aldrichii*, *Clostridium algidicarnis*, *Clostridium algidixylanolyticum*, *Clostridium aminophilum*, *Clostridium aminovalericum*, *Clostridium amygdalinum*, *Clostridium arcticum*, *Clostridium argentinense*, *Clostridium aurantibutyricum*, *Clostridium baratii*, *Clostridium barkeri*, *Clostridium bartlettii*, *Clostridium beijerinckii*, *Clostridium bifementans*, *Clostridium boltea*, *Clostridium botulinum*, *Clostridium bowmanii*, *Clostridium bryantii*, *Clostridium butyricum*, *Clostridium cadaveris*, *Clostridium caminithermale*, *Clostridium carnis*, *Clostridium celatum*, *Clostridium celerecrescens*, *Clostridium cellobioparum*, *Clostridium cellulifementans*, *Clostridium cellulolyticum*, *Clostridium cellulose*, *Clostridium cellulovorans*, *Clostridium chartatabidum*, *Clostridium chauvoei*, *Clostridium clostridioforme*, *Clostridium coccoides*, *Clostridium cochlearium*, *Clostridium cocleatum*, *Clostridium colicanis*, *Clostridium colinum*, *Clostridium collagenovorans*, *Clostridium cylindrosporum*, *Clostridium difficile*, *Clostridium dioli*, *Clostridium disporicum*, *Clostridium durum*, *Clostridium estertheticum*, *Clostridium estertheticum subsp. estertheticum*, *Clostridium estertheticum subsp. laramiense*, *Clostridium fallax*, *Clostridium felsineum*, *Clostridium fervidum*, *Clostridium fimetarium*, *Clostridium formicaceticum*, *Clostridium frigidicarnis*, *Clostridium frigoris*, *Clostridium gasigenes*, *Clostridium ghonii*, *Clostridium glycolicum*, *Clostridium grantii*, *Clostridium haemolyticum*, *Clostridium halophilum*, *Clostridium hastiforme*, *Clostridium hathewayi*, *Clostridium herbivorans*, *Clostridium hiranonis*, *Clostridium histolyticum*, *Clostridium homopropionicum*, *Clostridium hungatei*, *Clostridium hydroxybenzoicum*, *Clostridium hylemonae*, *Clostridium jejuense*, *Clostridium indolis*, *Clostridium innocuum*, *Clostridium intestinale*, *Clostridium irregulare*, *Clostridium isatidis*, *Clostridium josui*, *Clostridium kluyveri*, *Clostridium lactatifermentans*, *Clostridium lacusfityxellense*, *Clostridium laramiense*, *Clostridium lentocellum*, *Clostridium lentoputrescens*, *Clostridium leptum*, *Clostridium limosum*, *Clostridium litorale*, *Clostridium lituseburense*, *Clostridium ljungdahlii*, *Clostridium lortetii*, *Clostridium magnum*, *Clostridium malenominatum*, *Clostridium manganotii*, *Clostridium mayombei*, *Clostridium methoxybenzovorans*, *Clostridium methylpentosum*, *Clostridium neopropionicum*, *Clostridium oceanicum*, *Clostridium orbiscindens*, *Clostridium oroticum*, *Clostridium oxalicum*, *Clostridium papyrosolvans*, *Clostridium paradoxum*, *Clostridium parapaperfringens*, *Clostridium paraputrificum*, *Clostridium pascui*, *Clostridium pasteurianum*, *Clostridium peptidivorans*, *Clostridium perenne*, *Clostridium paperfringens*, *Clostridium pfennigii*, *Clostridium phytofermentans*, *Clostridium piliforme*, *Clostridium polysaccharolyticum*, *Clostridium populeti*, *Clostridium propionicum*, *Clostridium proteoclasticum*, *Clostridium proteolyticum*, *Clostridium psychrophilum*,

*Clostridium puniceum*, *Clostridium purinolyticum*, *Clostridium putrefaciens*, *Clostridium putrificum*,  
*Clostridium quercicolum*, *Clostridium quinii*, *Clostridium ramosum*, *Clostridium rectum*, *Clostridium*  
*roseum*, *Clostridium saccharobutylicum*, *Clostridium saccharolyticum*, *Clostridium*  
*saccharoperbutylaceticum*, *Clostridium sardiniense*, *Clostridium sartagoforme*, *Clostridium*  
*scatologenes*, *Clostridium scindens*, *Clostridium septicum*, *Clostridium sordellii*, *Clostridium*  
*sphenoides*, *Clostridium spiroforme*, *Clostridium sporogenes*, *Clostridium sporosphaeroides*,  
*Clostridium stercorarium*, *Clostridium stercorarium subsp. leptospartum*, *Clostridium stercorarium*  
*subsp. stercorarium*, *Clostridium stercorarium subsp. ihermolacticum*, *Clostridium sticklandii*,  
*Clostridium straminisolvens*, *Clostridium subterminale*, *Clostridium symbiosum*, *Clostridium termitidis*,  
*Clostridium tertium*, *Clostridium tetani*, *Clostridium tetanomorphum*, *Clostridium ihermaceticum*,  
*Clostridium thermautotrophicum*, *Clostridium thermoalkaliphilum*, *Clostridium thermobutyricum*,  
*Clostridium ihermocellum*, *Clostridium ihermocopriae*, *Clostridium thermohydrosulfuricum*,  
*Clostridium thermolacticum*, *Clostridium thermopalmarium*, *Clostridium thermopapyrolyticum*,  
*Clostridium thermosuccinogenes*, *Clostridium thermosulfurigenes*, *Clostridium thiosulfatireducens*,  
*Clostridium tyrobutyricum*, *Clostridium uliginosum*, *Clostridium ultunense*, *Clostridium villosum*,  
*Clostridium vincentii*, *Clostridium viride*, *Clostridium xylanolyticum*, *Clostridium xylanovorans*,  
*Amoebobacter pedioformis*, *Amoebobacter pendens*, *Amoebobacter purpureus*, *Amoebobacter roseus*,  
*Chromatium buderi*, *Chromatium glycolicum*, *Chromatium gracile*, *Chromatium minus*, *Chromatium*  
*minutissimum*, *Chromatium okenii*, *Chromatium purpuratum*, *Chromatium salexigens*, *Chromatium*  
*tepidum*, *Chromatium vinosum*, *Chromatium violascens*, *Chromatium warmingii*, *Chromatium weissei*,  
*Lamprobacter modestohalophilus*, *Lamprocystis purpurea*, *Lamprocystis roseopersicina*, *Thiocapsa*  
*halophila*, *Thiocapsa litoralis*, *Thiocapsa marina*, *Thiocapsa penden*, *Thiocapsa rosea*, *Thiocapsa*  
*roseopersicina*, *Thiocystis gelatinosa*, *Thiocystis minor*, *Thiocystis violacea*, *Thiocystis violascens*,  
*Thiodictyon bacillosum*, *Thiodictyon elegans*, *Thiopedia rosea*, *Thiospirillumjenense*,  
*Ectothiorhodospira abdelmalekii*, *Ectothiorhodospira haloalkaliphila*, *Ectothiorhodospira halochloris*,  
*Ectothiorhodospira halophila*, *Ectothiorhodospira marina*, *Ectothiorhodospira marismortui*,  
*Ectothiorhodospira mobilis*, *Ectothiorhodospira shaposhnikovii*, *Ectothiorhodospira vacuolata*,  
*Rhodobacter adriaticus*, *Rhodobacter azotoformans*, *Rhodobacter blasticus*, *Rhodobacter capsulatus*,  
*Rhodobacter euryhalinus*, *Rhodobacter sphaeroides*, *Rhodobacter sulfidophilus*, *Rhodobacter*  
*veldkampii*, *Rhodocyclus gelatinosus*, *Rhodocyclus purpureus*, *Rhodocyclus tenuis*, *Rhodomicrobium*  
*vanniellii*, *Rhodopila globiformis*, *Rhodopseudomonas acidophila*, *Rhodopseudomonas adriatica*,  
*Rhodopseudomonas blastica*, *Rhodopseudomonas capsulata*, *Rhodopseudomonas faecalis*,  
*Rhodopseudomonas gelatinosa*, *Rhodopseudomonas globiformis*, *Rhodopseudomonas Julia*,  
*Rhodopseudomonas marina*, *Rhodopseudomonas palustris*, *Rhodopseudomonas rhenobacensis*,  
*Rhodopseudomonas rosea*, *Rhodopseudomonas rutila*, *Rhodopseudomonas sphaeroides*,  
*Rhodopseudomonas sulfidophila*, *Rhodopseudomonas sulfoviridis*, *Rhodopseudomonas viridis*,  
*Rhodospirillum centenum*, *Rhodospirillumfulvum*, *Rhodospirillum molischianum*, *Rhodospirillum*

*photometricum*, *Rhodospirillum rubrum*, *Rhodospirillum salexigens*, *Rhodospirillum salinarum*,  
*Rhodospirillum sodomense*, *Rhodospirillum tenue*, *Erythrobacter aquimaris*, *Erythrobacter citreus*,  
*Erythrobacter flavus*, *Erythrobacter gaetbuli*, *Erythrobacter litoralis*, *Erythrobacter longus*,  
*Erythrobacter seohaensis*, *Methanobacterium aarhusense*, *Methanobacterium alcaliphilum*,  
*Methanobacterium arbophilicum*, *Methanobacterium beijingense*, *Methanobacterium bryantii*,  
*Methanobacterium congolense*, *Methanobacterium defluvii*, *Methanobacterium espanolae*,  
*Methanobacterium formicicum*, *Methanobacterium ivanovii*, *Methanobacterium mobile*,  
*Methanobacterium oryzae*, *Methanobacterium ruminantium*, *Methanobacterium subterraneum*,  
*Methanobacterium thermaggregans*, *Methanobacterium thermalcaliphilum*, *Methanobacterium*  
*thermautotrophicum*, *Methanobacterium thermoflexum*, *Methanobacterium thermoformicicum*,  
*Methanobacterium thermophilum*, *Methanobacterium uliginosum*, *Methanobacterium wolfei*,  
*Methanobrevibacter acididurans*, *Methanobrevibacter arboriphilus*, *Methanobrevibacter curvatus*,  
*Methanobrevibacter cuticularis*, *Methanobrevibacter filiformis*, *Methanobrevibacter gottschalkii*,  
*Methanobrevibacter oralis*, *Methanobrevibacter ruminantium*, *Methanobrevibacter smithii*,  
*Methanobrevibacter thaueri*, *Methanobrevibacter woesei*, *Methanobrevibacter wolinii*, *Methanococcus*  
*delta*, *Methanococcus fervens*, *Methanococcus frisius*, *Methanococcus halophilus*, *Methanococcus*  
*igneus*, *Methanococcus infernus*, *Methanococcus jannaschii*, *Methanococcus maripaludis*,  
*Methanococcus mazei*, *Methanococcus thermolithotrophicus*, *Methanococcus vannielii*, *Methanococcus*  
*voltae*, *Methanococcus vulcanius*, *Methanococcoides burtonii*, *Methanococcoides methylutens*,  
*Methanolobus bombayensis*, *Methanolobus oregonensis*, *Methanolobus siciliae*, *Methanolobus taylorii*,  
*Methanolobus tindarius*, *Methanolobus vulcani*, *Methanolacinia paynteri*, *Methanomicrobium mobile*,  
*Methanomicrobium paynteri*, *Methanogenium aggregans*, *Methanogenium bourgense*, *Methanogenium*  
*cariaci*, *Methanogenium frigidum*, *Methanogenium frittonii*, *Methanogenium liminatans*,  
*Methanogenium marinum*, *Methanogenium marisnigri*, *Methanogenium olentangyi*, *Methanogenium*  
*organophilum*, *Methanogenium tationis*, *Methanogenium thermophilicum*, *Methanospirillum hungatei*,  
*Methanoplanus endosymbiosus*, *Methanoplanus limicola*, *Methanoplanus petrolearius*, *Methanothrix*  
*concilii*, *Methanothrix soehngeni*, *Methanothrix thermoacetophila*, *Methanothrix thermophila*,  
*Methanothermus fervidus*, *Methanothermus sociabilis*, *Methanocorpusculum aggregans*,  
*Methanocorpusculum bavaricum*, *Methanocorpusculum labreanum*, *Methanocorpusculum parvum*,  
*Methanocorpusculum sinense*, *Methanoculleus bourgensis*, *Methanoculleus chikugoensis*,  
*Methanoculleus marisnigri*, *Methanoculleus oldenburgensis*, *Methanoculleus olentangyi*,  
*Methanoculleus palmolei*, *Methanoculleus submarinus*, *Methanoculleus thermophilus*,  
*Methanohalobium evestigatum*, *Methanohalophilus halophilus*, *Methanohalophilus mahii*,  
*Methanohalophilus oregonensis*, *Methanohalophilus portucalensis*, *Methanohalophilus zhilinae*,  
*Methanosarcina acetivorans*, *Methanosarcina baltica*, *Methanosarcina barkeri*, *Methanosarcina frisia*,  
*Methanosarcina lacustris*, *Methanosarcina methanica*, *Methanosarcina semesia*, *Methanosarcina*  
*siciliae*, *Methanosarcina thermophila*, *Methanosarcina vacuolata*, *Methanosphaera cuniculi*,

*Methanospaera stadtmannae*, *Eubacterium acidaminophilum*, *Eubacterium aerofaciens*, *Eubacterium aggregans*, *Eubacterium alactolyticum*, *Eubacterium angustum*, *Eubacterium barken*, *Eubacterium bifforme*, *Eubacterium brachy*, *Eubacterium budayi*, *Eubacterium callanderi*, *Eubacterium cellulosolvens*, *Eubacterium combesii*, *Eubacterium contortum*, *Eubacterium coprostanoligenes*, *Eubacterium cylindroids*, *Eubacterium desmolans*, *Eubacterium dolichum*, *Eubacterium eligens*, *Eubacterium exiguum*, *Eubacterium fissicatena*, *Eubacterium formicigenerans*, *Eubacterium fossor*, *Eubacterium hadrum*, *Eubacterium hallii*, *Eubacterium infirmum*, *Eubacterium lentum*, *Eubacterium limosum*, *Eubacterium minutum*, *Eubacterium moniliforme*, *Eubacterium multifonne*, *Eubacterium nitritogenes*, *Eubacterium nodatum*, *Eubacterium oxidoreducens*, *Eubacterium plautii*, *Eubacterium plexicaudatum*, *Eubacterium pyruvativorans*, *Eubacterium ramulus*, *Eubacterium rectale*, *Eubacterium ruminantium*, *Eubacterium saburreum*, *Eubacterium saphenum*, *Eubacterium siraeum*, *Eubacterium suis*, *Eubacterium sulci*, *Eubacterium tarantellae*, *Eubacterium tardum*, *Eubacterium tenue*, *Eubacterium timidum*, *Eubacterium tortuosum*, *Eubacterium uniforme*, *Eubacterium ventriosum*, *Eubacterium xylanophilum*, *Eubacterium yurii*, *Eubacterium yurii subsp. margaretiae*, *Eubacterium Yurii*, *Abiotrophia adiacens*, *Abiotrophia balaenopterae*, *Abiotrophia defectiva*, *Abiotrophia elegans*, *Atopobiumfossor*, *Atopobium minutum*, *Atopobium parvulum*, *Atopobium rima*, *Atopobium vaginae*, *Gemella bergeri*, *Gemella cuniculi*, *Gemella haemolysans*, *Gemella morbillorum*, *Gemella palaticanis*, *Gemella sanguinis*, *Granulicatella adiacens*, *Granulicatella balaenopterae*, *Granulicatella elegans*, *Finegoldia magna*, *Lactobacillus acetotolerans*, *Lactobacillus acidifarinae*, *Lactobacillus acidipiscis*, *Lactobacillus acidophilus*, *Lactobacillus acidophilus*, *Lactobacillus agilis*, *Lactobacillus algidus*, *Lactobacillus alimentarius*, *Lactobacillus amylolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus antri*, *Lactobacillus arizonensis*, *Lactobacillus aviarius*, *Lactobacillus aviarius subsp. Araffinosus*, *Lactobacillus aviarius subsp. Aviarius*, *Lactobacillus bavaricus*, *Lactobacillus bif fermentans*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus bulgaricus*, *Lactobacillus carnis*, *Lactobacillus casei*, *Lactobacillus casei subsp. alactosus*, *Lactobacillus casei subsp. casei*, *Lactobacillus casei subsp. pseudopantarum*, *Lactobacillus casei subsp. rhamnosus*, *Lactobacillus casei subsp. tolerans*, *Lactobacillus catenaformis*, *Lactobacillus cellobiosus*, *Lactobacillus coleohominis*, *Lactobacillus collinoide*, *Lactobacillus confuses*, *Lactobacillus coryniformis*, *Lactobacillus coryniformis subsp. coryniformis*, *Lactobacillus coryniformis subsp. torquens*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus curvatus subsp. curvatus*, *Lactobacillus curvatus subsp. melibiosus*, *Lactobacillus cypricasei*, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus delbrueckii subsp. delbrueckii*, *Lactobacillus delbrueckii subsp. indicus*, *Lactobacillus delbrueckii subsp. lactis*, *Lactobacillus diolivorans*, *Lactobacillus divergens*, *Lactobacillus durianis*, *Lactobacillus equi*, *Lactobacillus far ciminis*, *Lactobacillus ferintoshensis*, *Lactobacillus fermentum*, *Lactobacillus fornicalis*, *Lactobacillus fructivorans*, *Lactobacillus fructosus*, *Lactobacillus frumenti*, *Lactobacillus fuchuensis*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus gastricus*, *Lactobacillus gramini*, *Lactobacillus*

*halotolerans*, *Lactobacillus hammesii*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus heterohiochii*, *Lactobacillus hilgardii*, *Lactobacillus homohiochii*, *Lactobacillus iners*, *Lactobacillus ingluviei*, *Lactobacillus intestinalis*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kalixensis*, *Lactobacillus kandleri*, *Lactobacillus kefir anofaciens*, *Lactobacillus kefir anofaciens subsp. kefir anofaciens*, *Lactobacillus kefir anofaciens subsp. kefirgranum*, *Lactobacillus kefirgranum*, *Lactobacillus kefir*, *Lactobacillus kimchii*, *Lactobacillus kitasatonis*, *Lactobacillus kunkeei*, *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus mindensis*, *Lactobacillus minor*, *Lactobacillus mucosae*, *Lactobacillus murinus*, *Lactobacillus nagelii*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus panther is*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus par acasei subsp. par acasei*, *Lactobacillus paracasei subsp. tolerans*, *Lactobacillus par acollinoides*, *Lactobacillus parakefiri*, *Lactobacillus par alimentarius*, *Lactobacillus parapantarum*, *Lactobacillus pentosus*, *Lactobacillus perolens*, *Lactobacillus piscicola*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus psittaci*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus rimae*, *Lactobacillus rogosae*, *Lactobacillus rossii*, *Lactobacillus ruminis*, *Lactobacillus saerimneri*, *Lactobacillus sakei*, *Lactobacillus sakei subsp. carnosus*, *Lactobacillus sakei subsp. sakei*, *Lactobacillus salivarius*, *Lactobacillus salivarius subsp. salicinius*, *Lactobacillus salivarius subsp. salivarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus satsumensis*, *Lactobacillus sharpeae*, *Lactobacillus spicheri*, *Lactobacillus suebicus*, *Lactobacillus suntoryeus*, *Lactobacillus ihermotolerans*, *Lactobacillus trichodes*, *Lactobacillus uli*, *Lactobacillus ultunensis*, *Lactobacillus vaccinostercus*, *Lactobacillus vaginalis*, *Lactobacillus versmoldensis*, *Lactobacillus viridescens*, *Lactobacillus vitulinu*, *Lactobacillus xylosus*, *Lactobacillus yamanashiensis*, *Lactobacillus yamanashiensis subsp. mali*, *Lactobacillus yamanashiensis subsp. yamanashiensis*, *Lactobacillus zaeae*, *Lactobacillus zymae*, *Actinomyces bernardiae*, *Actinomyces bovis*, *Actinomyces bowdeni*, *Actinomyces cardiffensis*, *Actinomyces catuli*, *Actinomyces colecanis*, *Actinomyces dentalis*, *Actinomyces denticolens*, *Actinomyces europaeus*, *Actinomyces funkei*, *Actinomyces Georgia*, *Actinomyces gerencseriae*, *Actinomyces graevenitzii*, *Actinomyces hongkongensis*, *Actinomyces hordeovulneris*, *Actinomyces howellii*, *Actinomyces humiferus*, *Actinomyces hyovaginalis*, *Actinomyces israelii*, *Actinomyces marimammalium*, *Actinomyces meyeri*, *Actinomyces naeslundii*, *Actinomyces nasicola*, *Actinomyces neuui*, *Actinomyces neuui subsp. anitratus*, *Actinomyces neuui subsp. neuui*, *Actinomyces odontolyticus*, *Actinomyces oricola*, *Actinomyces pyogenes*, *Actinomyces pyogenes*, *Actinomyces radidentis*, *Actinomyces radingae*, *Actinomyces slackii*, *Actinomyces suimastitidis*, *Actinomyces suis*, *Actinomyces turicensis*, *Actinomyces urogenitalis*, *Actinomyces vaccimaxillae*, *Actinomyces viscosus*, *Arcanobacterium bernardiae*, *Arcanobacterium haemolyticum*, *Arcanobacterium hippocoleae*, *Arcanobacterium phocae*, *Arcanobacterium pluranimalium*, *Arcanobacterium pyogenes*, *Actinobaculum schaalii*, *Actinobaculum suis*, *Actinobaculum urinale*, *Bulleidia extructa*, *Collinsella aerofaciens*, *Collinsella intestinalis*, *Collinsella*

*stercoris*, *Cryptobacterium curtum*, *Holdemania filiformis*, *Rothia aerea*, *Rothia amarae*, *Rothia dentocariosa*, *Rothia mucilaginoso*, *Rothia nasitnuriu*m, *Pseudoramibacter alactolyticus*, *Mogibacterium diversum*, *Mogibacterium neglectum*, *Mogibacterium pumilum*, *Mogibacterium timidum*, *Mogibacterium vescum*, *Slackia exigua*, *Slackia heliotrinireducens*, and *Eggerthella lenta*.

[0077] In one embodiment, one or more microorganisms used for the fermentation of biomass are a *Clostridium phytofermentans*. In another embodiment, one or more microorganisms used for the fermentation of biomass are a *Clostridium* sp. Q.D. In another embodiment, *Clostridium* sp. Q.D. has the NRRL patent deposit designation NRRL B-50361. *Clostridium* sp. Q.D consists of motile rods that form terminal spores. These Gram-positive rods were isolated from a 0.3% Maltose, 5% Azo-CM-Cellulose, QM plate comprising a mutated pool of *Clostridium phytofermentans* and were cultured in liquid QM media. Endoglucases activity was noted on the plate after four days' incubation at 35° C. The *Clostridium* sp. Q.D bacterium was distinguishable from *Clostridium phytofermentans* in that Q.D is a faster-growing colony of larger size having a larger clearing zone in the presence of glucose and 5% Azo-CM-Cellulose plates. It also displays a color modification in the presence of higher concentrations of glucose (2-3% glucose), changing to an orange color. Percent identity values for Q.D bacterium compared with representative members of the *Clostridium* sp. ranged from 99.8% with *Clostridium xylanolyticum* to 99.7% with *Clostridium algidixylanolyticum*. Also *C. xylanolyticum* has terminal endospores whereas *C. algidixylanolyticum* has subterminal endospores.

In one embodiment, one or more microorganisms used for the fermentation of biomass is a wild-type microorganism. Wild-type microorganisms are those microorganisms that are substantially similar to an isolate obtained from a natural environment and include isolates that have been propagated in a laboratory environment. In another embodiment, one or more of the microorganisms is bred and/or selected for a desirable trait. Methods of selection or breeding can include growth with a medium comprising a carbon source that is or approximates the carbon source to be utilized in the production of fermentation end-products. Desirable traits include but are not limited to increased biomass saccharification, increased production of a specific fermentation end-product, increased ethanol production, increased tolerance to ethanol, increased enzyme synthesis or decreased sporulation. A method of selection can further include the use of mutagenesis (*e.g.* by chemical or irradiation means) to generate a desired populations of microorganisms. Mutagenized microorganisms can then be selected for desired traits, leading to a higher frequency of desirable isolates. In another embodiment, one or more microorganisms used for fermentation can be a recombinant microorganism. A Recombinant microorganism comprises one or more changes to its nucleic acids in comparison to a respective wild-type microorganism that did not arise by spontaneous (*i.e.* natural) mutation. In one embodiment a recombinant microorganisms comprises an exogenous polynucleic acid from another species( such as another microorganism), a synthetic polynucleic acid, or a polynucleic acid isolated or derived from the same species.

### **Genetic modification of microorganism**

[0078] In another aspect, compositions and methods are provided to produce a fermentation end-product such as one or more alcohols, *e.g.*, ethanol, by the creation and use of a genetically modified microorganism. In one embodiment the genetically modified microorganism is *Clostridium phytofermentans*. In one embodiment the genetic modification is to a nucleic acid sequence that regulates or encodes a protein related to a fermentative biochemical pathways, expression of saccharolytic enzymes, or increasing tolerance of environmental conditions during fermentation. In another embodiment, the genetic modification is to a nucleic acid sequence in a microorganism. In one embodiment, the microorganism is transformed with polynucleotides encoding one or more genes for a pathway, enzyme, or protein of interest. In another embodiment, the microorganism is transformed to produce multiple copies of one or more genes for a pathway, enzyme, or protein of interest. In some embodiments, the polynucleotide transformed into the microorganism is heterologous. In other embodiments, the polynucleotide is derived from microorganism. In one embodiment, the microorganism is transformed with heterologous polynucleotides encoding one or more genes encoding enzymes for the fermentation of a hexose, wherein said genes are expressed at sufficient levels to confer upon said microorganism transformant the ability to produce ethanol at increased concentrations, productivity levels or yields compared to a microorganism that is not transformed. In another embodiment, the microorganism is transformed with heterologous polynucleotides encoding one or more genes encoding enzymes for the fermentation of a pentose, wherein said genes are expressed at sufficient levels to confer upon said microorganism transformant the ability to produce ethanol or other end-products at increased concentrations, productivity levels or yields compared to a microorganism that is not transformed. In still other embodiments, the microorganism is transformed with a combination of enzymes for fermentation of hexose and pentose saccharides. In some embodiments, an enhanced rate of end-product production can be achieved by genetic modification. In another embodiment, the microorganism is transformed with heterologous polynucleotides encoding one or more genes encoding saccharolytic enzymes for the saccharification of a polysaccharide, wherein said genes are expressed at sufficient levels to confer upon the transformed microorganism an ability to saccharify a polysaccharide to mono-, di- or oligosaccharides at increased concentrations, rates of saccharification or yields of mono-, di- or oligosaccharides compared to a microorganism that is not transformed.

[0079] In another embodiment the genetic modification is to a nucleic acid sequence a *Clostridium phytofermentans*. In one embodiment, the *Clostridium phytofermentans* is transformed with polynucleotides encoding one or more genes for a pathway, enzyme, or protein of interest. In another embodiment, the *Clostridium phytofermentans* is transformed to produce multiple copies of one or more genes for the pathway, enzyme, or protein of interest. In some embodiments, the polynucleotide transformed into the *Clostridium phytofermentans* is heterologous. In other embodiments, the polynucleotide is derived from *Clostridium phytofermentans*. In one embodiment, the *Clostridium*

*phytofermentans* is transformed with heterologous polynucleotides encoding one or more genes encoding enzymes for the fermentation of a hexose, wherein said genes are expressed at sufficient levels to confer upon said *Clostridium phytofermentans* transformant the ability to produce ethanol at increased concentrations, productivity levels or yields compared to a *Clostridium phytofermentans* that is not transformed. In another embodiment, the *Clostridium phytofermentans* is transformed with heterologous polynucleotides encoding one or more genes encoding enzymes for the fermentation of a pentose, wherein said genes are expressed at sufficient levels to confer upon said *Clostridium phytofermentans* transformant the ability to produce ethanol or other end-products at increased concentrations, productivity levels or yields compared to a *Clostridium phytofermentans* that is not transformed. In still other embodiments, the *Clostridium phytofermentans* is transformed with a combination of enzymes for fermentation of hexose and pentose saccharides. In some embodiments, an enhanced rate of end-product production can be achieved.

[0080] In another embodiment further modifications can be made to enhance the end-product (*e.g.*, ethanol) production by a recombinant microorganism. In one embodiment, a recombinant microorganism can further comprise an additional heterologous DNA segment, the expression product of which is a protein involved in the transport of mono- and/or oligosaccharides into the recombinant host. Likewise, additional genes from the glycolytic pathway can be incorporated into the host. In such ways, an enhanced rate of ethanol production can be achieved.

[0081] In order to improve the production of fermentation end-products (*e.g.* ethanol), modifications can be made in transcriptional regulators, genes for the formation of organic acids, carbohydrate transporter genes, sporulation genes, genes that influence the formation/regenerate of enzymatic cofactors, genes that influence ethanol tolerance, genes that influence salt tolerance, genes that influence growth rate, genes that influence oxygen tolerance, genes that influence catabolite repression, genes that influence hydrogen production, genes that influence resistance to heavy metals, genes that influence resistance to acids or genes that influence resistance to aldehydes.

[0082] Those skilled in the art will appreciate that a number of modifications can be made to the methods exemplified herein. For example, a variety of promoters can be utilized to drive expression of the heterologous genes in the recombinant *Clostridium phytofermentans* host. The skilled artisan, having the benefit of the instant disclosure, will be able to readily choose and utilize any one of the various promoters available for this purpose. Similarly, skilled artisans, as a matter of routine preference, can utilize a higher copy number plasmid. In another embodiment, constructs can be prepared for chromosomal integration of the desired genes. Chromosomal integration of foreign genes can offer several advantages over plasmid-based constructions, the latter having certain limitations for commercial processes. Ethanologenic genes have been integrated chromosomally in *E. coli* B; see Ohta *et al.* (1991) *Appl. Environ. Microbiol.* 57:893-900. In general, this is accomplished by purification of a DNA fragment containing (1) the desired genes upstream from an antibiotic resistance gene and (2) a fragment of homologous DNA from the target organism. This DNA can be ligated to form circles

without replicons and used for transformation. Thus, the gene of interest can be introduced in a heterologous host such as *E. coli*, and short, random fragments can be isolated and ligated in *Clostridium phytofermentans* to promote homologous recombination.

[0083] In other embodiments, *Clostridium phytofermentans* isolates can be obtained without the use of recombinant DNA techniques that exhibit desirable properties such as increased productivity, increased yield, or increased titer. For example, mutagenesis, or random mutagenesis can be performed by chemical means or by irradiation of the microorganism. The population of mutagenized microorganisms can then be screened for beneficial mutations that exhibit one or more desirable properties. Screening can be performed by growing the mutagenized organisms on substrates that comprise carbon sources that will be utilized during the generation of end-products by fermentation. Screening can also include measuring the production of end-products during growth of the organism, or measuring the digestion or assimilation of the carbon source(s). The isolates so obtained can further be transformed with recombinant polynucleotides or used in combination with any of the methods and compositions provided herein to further enhance biofuel production.

### **Feedstock**

[0084] Feedstocks containing cellulosic, hemicellulosic, and/or lignocellulosic material can be derived from, for example, agricultural crops, crop residues, trees, woodchips, sawdust, paper, cardboard, grasses, and other sources. Feedstocks with a relatively high level of hemicellulose (*e.g.*, 40% or more prior to pretreatment) can be utilized for compositions and methods described herein compositions and methods described herein. In some instances, feedstock can be from a single source, or be a combination of materials (*e.g.*, bagasse and corn stover). Organisms useful in compositions and methods described herein are capable of fermenting the one or more materials comprising the feedstock. In some instances, the organisms produce a fermentative product (*e.g.*, ethanol) from all materials comprising the feedstock. In still other instances, the organisms are capable of fermenting the feedstock materials from different sources at substantially the same yield coefficient.

[0085] Cellulose is a linear polymer of glucose where the glucose units are connected via  $\beta(1\rightarrow4)$  linkages. Hemicellulose is a branched polymer of a number of sugar monomers including glucose, xylose, mannose, galactose, rhamnose and arabinose. Hemicellulose can have sugar acids such as mannuronic acid and galacturonic acid present as well. Lignin is a cross-linked, racemic macromolecule of mostly  $\beta$ -coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These three polymers occur together in lignocellulosic materials in plant biomass. The different characteristics of the three polymers can make hydrolysis of the combination difficult as each polymer tends to shield the others from enzymatic attack.

[0086] Described herein provide for compositions for producing alcohol, *e.g.*, ethanol, comprising a culture of a fermentative microorganism (*e.g.*, *C. phytofermentans*) in a medium comprising one or more feedstocks, *e.g.*, cellulosic, hemicellulosic, lignocellulosic materials. Such feedstocks can include materials as agricultural crops, crop residues, yard waste, trees, wood chips, sawdust, paper, cardboard,

algae or other materials containing cellulose, hemicellulosic, lignocellulose, pectin, polyglucose, polyfructose, and/or hydrolyzed forms of these materials. Additional nutrients can be present including sulfur- and nitrogen-containing compounds such as amino acids, proteins, hydrolyzed proteins, ammonia, urea, nitrate, nitrite, soy, soy derivatives, casein, casein derivatives, milk powder, milk derivatives, whey, yeast extract, hydrolyze yeast, autolyzed yeast, corn steep liquor, corn steep solids, monosodium glutamate, and/or other fermentation nitrogen sources, vitamins, cofactors and/or mineral supplements. The feedstock can be pretreated or not, such as described in U.S. Provisional Application No. 61/032,048, filed February 27, 2008 or U.S. Provisional Application No. 61/158,581, filed March 9, 2009, incorporated herein by reference in their entireties.

[0087] In some embodiments, a microorganism, (such as *Clostridium phytofermentans*) is contacted with pretreated or non-pretreated feedstock containing cellulosic, hemicellulosic, and/or lignocellulosic material. Additional nutrients can be present or added to the biomass material to be processed by the microorganism including nitrogen-containing compounds such as amino acids, proteins, hydrolyzed proteins, ammonia, urea, nitrate, nitrite, soy, soy derivatives, casein, casein derivatives, milk powder, milk derivatives, whey, yeast extract, hydrolyze yeast, autolyzed yeast, corn steep liquor, corn steep solids, monosodium glutamate, and/or other fermentation nitrogen sources, vitamins, and/or mineral supplements. In some embodiments, one or more additional lower molecular weight carbon sources can be added or be present such as glucose, sucrose, maltose, corn syrup, Distillers Dried Solubles (DDS), Distillers Dried Grains (DDG), Condensed Distillers Solubles (CDS), Distillers Wet Grains (DWG), Distillers Dried Grains with Solubles (DDGS), lactic acid, etc.

[0088] Such lower molecular weight carbon sources can serve multiple functions including providing an initial carbon source at the start of the fermentation period, help build cell count, control the carbon/nitrogen ratio, remove excess nitrogen, or some other function. In some embodiments another medium supplement is added, such as pH modifier, a lipid (*e.g.*, a fatty acid), a surfactant or a chelating agent.

[0089] In various embodiments, particular medium components can have beneficial effects on the performance of the fermentation, such as increasing the titer of desired products, or increasing the rate that the desired products are produced. Specific compounds can be supplied as a specific, pure ingredient, such as a particular amino acid, or it can be supplied as a component of a more complex ingredient, such as using a microbial, plant or animal product as a medium ingredient to provide a particular amino acid, promoter, cofactor, or other beneficial compound. In some cases, the particular compound supplied in the medium ingredient can be combined with other compounds by the organism resulting in a fermentation-beneficial compound. One example of this situation would be where a medium ingredient provides a specific amino acid which the organism uses to make an enzyme beneficial to the fermentation. Other examples can include medium components that are used to generate growth or product promoters. In such cases, it can be possible to obtain a fermentation-beneficial result by supplementing the enzyme, promoter, growth factor, etc. or by adding the precursor.

In some situations, the specific mechanism whereby the medium component benefits the fermentation is not known, only that a beneficial result is achieved.

[0090] In one embodiment, beneficial fermentation results can be achieved by adding corn steep powder to the fermentation. In another embodiment a typical composition for corn steep powder is shown in Tables 1-2. The addition of the corn steep powder can result in increased ethanol titer in batch fermentation, improved productivity and reduced production of side products such as organic acids. In another embodiment beneficial results with corn steep powder can be achieved in the methods of the embodiments at usage levels of about 3 to about 20 g/L, about 5 to about 15 g/L, or about 8 to about 12 g/L. In another embodiment beneficial results with steep powder can be achieved at a level of about 3 g/L, 3.1 g/L, 3.2 g/L, 3.3 g/L, 3.4 g/L, 3.5 g/L, 3.6 g/L, 3.7 g/L, 3.8 g/L, 3.9 g/L, 4 g/L, 4.1 g/L, 4.2 g/L, 4.3 g/L, 4.4 g/L, 4.5 g/L, 4.6 g/L, 4.7 g/L, 4.8 g/L, 4.9 g/L, 5 g/L, 5.1 g/L, 5.2 g/L, 5.3 g/L, 5.4 g/L, 5.5 g/L, 5.6 g/L, 5.7 g/L, 5.8 g/L, 5.9 g/L, 6 g/L, 6.1 g/L, 6.2 g/L, 6.3 g/L, 6.4 g/L, 6.5 g/L, 6.6 g/L, 6.7 g/L, 6.8 g/L, 6.9 g/L, 7 g/L, 7.1 g/L, 7.2 g/L, 7.3 g/L, 7.4 g/L, 7.5 g/L, 7.6 g/L, 7.7 g/L, 7.8 g/L, 7.9 g/L, 8 g/L, 8.1 g/L, 8.2 g/L, 8.3 g/L, 8.4 g/L, 8.5 g/L, 8.6 g/L, 8.7 g/L, 8.8 g/L, 8.9 g/L, 9 g/L, 9.1 g/L, 9.2 g/L, 9.3 g/L, 9.4 g/L, 9.5 g/L, 9.6 g/L, 9.7 g/L, 9.8 g/L, 9.9 g/L, 10 g/L, 10.1 g/L, 10.2 g/L, 10.3 g/L, 10.4 g/L, 10.5 g/L, 10.6 g/L, 10.7 g/L, 10.8 g/L, 10.9 g/L, 11 g/L, 11.1 g/L, 11.2 g/L, 11.3 g/L, 11.4 g/L, 11.5 g/L, 11.6 g/L, 11.7 g/L, 11.8 g/L, 11.9 g/L, 12 g/L, 12.1 g/L, 12.2 g/L, 12.3 g/L, 12.4 g/L, 12.5 g/L, 12.6 g/L, 12.7 g/L, 12.8 g/L, 12.9 g/L, 13 g/L, 13.1 g/L, 13.2 g/L, 13.3 g/L, 13.4 g/L, 13.5 g/L, 13.6 g/L, 13.7 g/L, 13.8 g/L, 13.9 g/L, 14 g/L, 14.1 g/L, 14.2 g/L, 14.3 g/L, 14.4 g/L, 14.5 g/L, 14.6 g/L, 14.7 g/L, 14.8 g/L, 14.9 g/L, 15 g/L, 15.1 g/L, 15.2 g/L, 15.3 g/L, 15.4 g/L, 15.5 g/L, 15.6 g/L, 15.7 g/L, 15.8 g/L, 15.9 g/L, 16 g/L, 16.1 g/L, 16.2 g/L, 16.3 g/L, 16.4 g/L, 16.5 g/L, 16.6 g/L, 16.7 g/L, 16.8 g/L, 16.9 g/L, 17 g/L, 17.1 g/L, 17.2 g/L, 17.3 g/L, 17.4 g/L, 17.5 g/L, 17.6 g/L, 17.7 g/L, 17.8 g/L, 17.9 g/L, 18 g/L, 18.1 g/L, 18.2 g/L, 18.3 g/L, 18.4 g/L, 18.5 g/L, 18.6 g/L, 18.7 g/L, 18.8 g/L, 18.9 g/L, 19 g/L, 19.1 g/L, 19.2 g/L, 19.3 g/L, 19.4 g/L, 19.5 g/L, 19.6 g/L, 19.7 g/L, 19.8 g/L, 19.9 g/L, or 20 g/L.

[0091] In one embodiment corn steep powder can also be fed throughout the course of the entire fermentation or a portion of the fermentation, continuously or delivered at intervals. In another embodiment usage levels include maintaining a nitrogen concentration of about 0.05 g/L to about 3g/L (as nitrogen), where at least a portion of the nitrogen is supplied from corn steep powder; about 0.3g/L to 1.3g/L; or about 0.4 g/L to about 0.9 g/L. In another embodiment the nitrogen level is about 0.05 g/L, 0.06 g/L, 0.07 g/L, 0.08 g/L, 0.09 g/L, 0.1 g/L, 0.11 g/L, 0.12 g/L, 0.13 g/L, 0.14 g/L, 0.15 g/L, 0.16 g/L, 0.17 g/L, 0.18 g/L, 0.19 g/L, 0.2 g/L, 0.21 g/L, 0.22 g/L, 0.23 g/L, 0.24 g/L, 0.25 g/L, 0.26 g/L, 0.27 g/L, 0.28 g/L, 0.29 g/L, 0.3 g/L, 0.31 g/L, 0.32 g/L, 0.33 g/L, 0.34 g/L, 0.35 g/L, 0.36 g/L, 0.37 g/L, 0.38 g/L, 0.39 g/L, 0.4 g/L, 0.41 g/L, 0.42 g/L, 0.43 g/L, 0.44 g/L, 0.45 g/L, 0.46 g/L, 0.47 g/L, 0.48 g/L, 0.49 g/L, 0.5 g/L, 0.51 g/L, 0.52 g/L, 0.53 g/L, 0.54 g/L, 0.55 g/L, 0.56 g/L, 0.57 g/L, 0.58 g/L, 0.59 g/L, 0.6 g/L, 0.61 g/L, 0.62 g/L, 0.63 g/L, 0.64 g/L, 0.65 g/L, 0.66 g/L, 0.67 g/L, 0.68 g/L, 0.69 g/L, 0.7 g/L, 0.71 g/L, 0.72 g/L, 0.73 g/L, 0.74 g/L, 0.75 g/L, 0.76 g/L, 0.77 g/L, 0.78 g/L, 0.79

g/L, 0.8 g/L, 0.81 g/L, 0.82 g/L, 0.83 g/L, 0.84 g/L, 0.85 g/L, 0.86 g/L, 0.87 g/L, 0.88 g/L, 0.89 g/L, 0.9 g/L, 0.91 g/L, 0.92 g/L, 0.93 g/L, 0.94 g/L, 0.95 g/L, 0.96 g/L, 0.97 g/L, 0.98 g/L, 0.99 g/L, 1 g/L, 1.01 g/L, 1.02 g/L, 1.03 g/L, 1.04 g/L, 1.05 g/L, 1.06 g/L, 1.07 g/L, 1.08 g/L, 1.09 g/L, 1.1 g/L, 1.11 g/L, 1.12 g/L, 1.13 g/L, 1.14 g/L, 1.15 g/L, 1.16 g/L, 1.17 g/L, 1.18 g/L, 1.19 g/L, 1.2 g/L, 1.21 g/L, 1.22 g/L, 1.23 g/L, 1.24 g/L, 1.25 g/L, 1.26 g/L, 1.27 g/L, 1.28 g/L, 1.29 g/L, 1.3 g/L, 1.31 g/L, 1.32 g/L, 1.33 g/L, 1.34 g/L, 1.35 g/L, 1.36 g/L, 1.37 g/L, 1.38 g/L, 1.39 g/L, 1.4 g/L, 1.41 g/L, 1.42 g/L, 1.43 g/L, 1.44 g/L, 1.45 g/L, 1.46 g/L, 1.47 g/L, 1.48 g/L, 1.49 g/L, 1.5 g/L, 1.51 g/L, 1.52 g/L, 1.53 g/L, 1.54 g/L, 1.55 g/L, 1.56 g/L, 1.57 g/L, 1.58 g/L, 1.59 g/L, 1.6 g/L, 1.61 g/L, 1.62 g/L, 1.63 g/L, 1.64 g/L, 1.65 g/L, 1.66 g/L, 1.67 g/L, 1.68 g/L, 1.69 g/L, 1.7 g/L, 1.71 g/L, 1.72 g/L, 1.73 g/L, 1.74 g/L, 1.75 g/L, 1.76 g/L, 1.77 g/L, 1.78 g/L, 1.79 g/L, 1.8 g/L, 1.81 g/L, 1.82 g/L, 1.83 g/L, 1.84 g/L, 1.85 g/L, 1.86 g/L, 1.87 g/L, 1.88 g/L, 1.89 g/L, 1.9 g/L, 1.91 g/L, 1.92 g/L, 1.93 g/L, 1.94 g/L, 1.95 g/L, 1.96 g/L, 1.97 g/L, 1.98 g/L, 1.99 g/L, 2 g/L, 2.01 g/L, 2.02 g/L, 2.03 g/L, 2.04 g/L, 2.05 g/L, 2.06 g/L, 2.07 g/L, 2.08 g/L, 2.09 g/L, 2.1 g/L, 2.11 g/L, 2.12 g/L, 2.13 g/L, 2.14 g/L, 2.15 g/L, 2.16 g/L, 2.17 g/L, 2.18 g/L, 2.19 g/L, 2.2 g/L, 2.21 g/L, 2.22 g/L, 2.23 g/L, 2.24 g/L, 2.25 g/L, 2.26 g/L, 2.27 g/L, 2.28 g/L, 2.29 g/L, 2.3 g/L, 2.31 g/L, 2.32 g/L, 2.33 g/L, 2.34 g/L, 2.35 g/L, 2.36 g/L, 2.37 g/L, 2.38 g/L, 2.39 g/L, 2.4 g/L, 2.41 g/L, 2.42 g/L, 2.43 g/L, 2.44 g/L, 2.45 g/L, 2.46 g/L, 2.47 g/L, 2.48 g/L, 2.49 g/L, 2.5 g/L, 2.51 g/L, 2.52 g/L, 2.53 g/L, 2.54 g/L, 2.55 g/L, 2.56 g/L, 2.57 g/L, 2.58 g/L, 2.59 g/L, 2.6 g/L, 2.61 g/L, 2.62 g/L, 2.63 g/L, 2.64 g/L, 2.65 g/L, 2.66 g/L, 2.67 g/L, 2.68 g/L, 2.69 g/L, 2.7 g/L, 2.71 g/L, 2.72 g/L, 2.73 g/L, 2.74 g/L, 2.75 g/L, 2.76 g/L, 2.77 g/L, 2.78 g/L, 2.79 g/L, 2.8 g/L, 2.81 g/L, 2.82 g/L, 2.83 g/L, 2.84 g/L, 2.85 g/L, 2.86 g/L, 2.87 g/L, 2.88 g/L, 2.89 g/L, 2.9 g/L, 2.91 g/L, 2.92 g/L, 2.93 g/L, 2.94 g/L, 2.95 g/L, 2.96 g/L, 2.97 g/L, 2.98 g/L, 2.99 g/L, or 3 g/L.

[0092] In another embodiment, other related products can be used, such as corn steep liquor or corn steep solids. When corn steep liquor is used, the usage rate would be approximately the same as for corn steep solids on a solids basis. In another embodiment, the corn steep powder (or solids or liquor) is added in relation to the amount of carbon substrate that is present or that will be added. When added in this way, beneficial amounts of corn steep powder (or liquor or solids) can include about 1:1 to about 1:6 g/g carbon, about 1:1 to about 1:5 g/g carbon, or about 1:2 to about 1:4 g/g carbon. In another embodiment ratios as high as about 1.5:1 g/g carbon or about 3:1 g/g carbon or as low as about 1:8 g/g carbon or about 1:10 g/g carbon are used. In another embodiment the ratio is 2:1 g/g carbon, 1.9:1 g/g carbon, 1.8:1 g/g carbon, 1.7:1 g/g carbon, 1.6:1 g/g carbon, 1.5:1 g/g carbon, 1.4:1 g/g carbon, 1.3:1 g/g carbon, 1.2:1 g/g carbon, 1.1:1 g/g carbon, 1:1 g/g carbon, 1:1.1 g/g carbon, 1:1.2 g/g carbon, 1:1.3 g/g carbon, 1:1.4 g/g carbon, 1:1.5 g/g carbon, 1:1.6 g/g carbon, 1:1.7 g/g carbon, 1:1.8 g/g carbon, 1:1.9 g/g carbon, 1:2 g/g carbon, 1:2.1 g/g carbon, 1:2.2 g/g carbon, 1:2.3 g/g carbon, 1:2.4 g/g carbon, 1:2.5 g/g carbon, 1:2.6 g/g carbon, 1:2.7 g/g carbon, 1:2.8 g/g carbon, 1:2.9 g/g carbon, 1:3 g/g carbon, 1:3.1 g/g carbon, 1:3.2 g/g carbon, 1:3.3 g/g carbon, 1:3.4 g/g carbon, 1:3.5 g/g carbon, 1:3.6 g/g carbon, 1:3.7 g/g carbon, 1:3.8 g/g carbon, 1:3.9 g/g carbon, 1:4 g/g carbon, 1:4.1 g/g carbon, 1:4.2 g/g carbon, 1:4.3 g/g carbon, 1:4.4 g/g carbon, 1:4.5 g/g carbon, 1:4.6 g/g carbon, 1:4.7 g/g carbon, 1:4.8

g/g carbon, 1:4.9 g/g carbon, 1:5 g/g carbon, 1:5.1 g/g carbon, 1:5.2 g/g carbon, 1:5.3 g/g carbon, 1:5.4 g/g carbon, 1:5.5 g/g carbon, 1:5.6 g/g carbon, 1:5.7 g/g carbon, 1:5.8 g/g carbon, 1:5.9 g/g carbon, 1:6 g/g carbon, 1:6.1 g/g carbon, 1:6.2 g/g carbon, 1:6.3 g/g carbon, 1:6.4 g/g carbon, 1:6.5 g/g carbon, 1:6.6 g/g carbon, 1:6.7 g/g carbon, 1:6.8 g/g carbon, 1:6.9 g/g carbon, 1:7 g/g carbon, 1:7.1 g/g carbon, 1:7.2 g/g carbon, 1:7.3 g/g carbon, 1:7.4 g/g carbon, 1:7.5 g/g carbon, 1:7.6 g/g carbon, 1:7.7 g/g carbon, 1:7.8 g/g carbon, 1:7.9 g/g carbon, 1:8 g/g carbon, 1:8.1 g/g carbon, 1:8.2 g/g carbon, 1:8.3 g/g carbon, 1:8.4 g/g carbon, 1:8.5 g/g carbon, 1:8.6 g/g carbon, 1:8.7 g/g carbon, 1:8.8 g/g carbon, 1:8.9 g/g carbon, 1:9 g/g carbon, 1:9.1 g/g carbon, 1:9.2 g/g carbon, 1:9.3 g/g carbon, 1:9.4 g/g carbon, 1:9.5 g/g carbon, 1:9.6 g/g carbon, 1:9.7 g/g carbon, 1:9.8 g/g carbon, 1:9.9 g/g carbon, or 1:10 g/g carbon.

[0093] In one embodiment, beneficial fermentation results can be achieved by adding corn steep powder in combination with yeast extract to the fermentation. Beneficial results with corn steep powder in combination with yeast extract can be achieved in the methods of the embodiments at corn steep powder usage levels of about 3 to about 20 g/L, about 5 to about 15 g/L, or about 8 to about 12 g/L and yeast extract usage levels of about 3 to 50 g/L, about 5 to about 30 g/L, or about 10 to about 30 g/L. The corn steep powder and yeast extract can also be fed throughout the course of the entire fermentation or a portion of the fermentation, continuously or delivered at intervals.

[0094] In other embodiments, the beneficial compounds from corn steep powder and/or yeast extract, such as glycine, histidine, isoleucine, proline, or phytate as well as combinations of these compounds can be added to the medium or broth to obtain a beneficial effect.

[0095] Table 1. Compositional characteristics of corn steep powder (source (except as noted): product datasheet for spray dried corn steep liquor, Roquette, Solulys 095E).

| Parameter                      | Value   |
|--------------------------------|---|
| Loss on drying                 | 5.5% maximum  |
| pH in solution                 | 3.9-4.5   |
| total acidity (as lactic acid) | 14-20%  |
| reducing sugars                | 1.5% maximum  |
| amino nitrogen                 | 1.5-3.5%  |
| total nitrogen                 | 7.0-8.5%  |
| Ash                            | 13.5-17.5%  |
| phosphorus (as P)              | 2.4-3.2%  |
| protein content (N x 6.25)     | 48% (approximately)   |
| Phytic acid (dry weight basis) | 8% (source: WO1997035489 19971002; A Process for Obtaining Phytic Acid and Lactic Acid) |

[0096] Table 2. Typical amino acid content in corn steep liquor (source: J. Nielsen, "Physiological Engineering Aspects of Penicillium Chrysogenum," Table 8.3, p. 243 (World Scientific 1997)).

| Amino Acid    | Free<br>g/kg dry weight | Total<br>g/kg dry weight |
|---------------|-------------------------|--------------------------|
| Alanine       | 40.7                    | 54.5                     |
| Arginine      | 2.4                     | 20.3                     |
| Aspartate     | 2.2                     | 19.9                     |
| Cysteine      | 0                       | 1.3                      |
| Glutamate     | 7.7                     | 40.2                     |
| Glycine       | 6.6                     | 26.8                     |
| Histidine     | 0                       | 31.8                     |
| Isoleucine    | 11.2                    | 17.3                     |
| Leucine       | 35.5                    | 39.3                     |
| Lysine        | 0                       | 14.8                     |
| Methionine    | 6.5                     | 6.9                      |
| Phenylalanine | 26.2                    | 27.4                     |
| Proline       | 27.7                    | 48.2                     |
| Serine        | 10.7                    | 19.0                     |
| Threonine     | 9.3                     | 20.7                     |
| Tyrosine      | 1.3                     | 6.5                      |
| Valine        | 20.1                    | 30.5                     |

### Pretreatment of Feedstock

[0097] In one aspect of the invention, methods are provided for the pretreatment of feedstock used in the fermentation and production of biofuels and ethanol. The pretreatment steps can include mechanical, thermal, pressure, chemical, thermochemical, and/or biochemical tests pretreatment prior to being used in a bioprocess for the production of fuels and chemicals, but untreated biomass material can be used in the process as well.

[0098] Provided herein are compositions and method for the pretreatment of feedstock used in the fermentation and production of biofuels and ethanol. In some instances, the feedstock is comprised of material with a relatively high content of hemicellulose. In some embodiments, such pretreatment increases the yield of a fermentive product (*e.g.*, ethanol) by fermentation of the pretreated feedstock. For example Figure 5 and Figure 6 demonstrate an increase in ethanol production by hydrolysis and fermentation of a feedstock disclosed herein that has been pretreated in the presence of an alkaline catalyst (*e.g.*, NaOH). For Figure 5, quarter inch chop corn stover from a single agricultural source was sieved to 2 mm and used as the standardized feedstock for all fermentation reactions. In addition, the fermentive mixture can be maintained at a temperature of from about 80°C to about 140°C.

[0099] Furthermore, a feedstock or combination of feedstocks can be manipulated mechanically prior to, during, or after contacting with an alkaline catalyst and/or heat. Mechanical processes can reduce the

particle size of the biomass material so that it can be more conveniently handled in the bioprocess and can increase the surface area of the feedstock to facilitate contact with

chemicals/biochemicals/biocatalysts or organisms. Mechanical processes can also separate one type of biomass material from another. The biomass material can also be subjected to thermal and/or chemical pretreatments to render plant polymers more accessible. Multiple steps of treatment can also be used.

[00100] Mechanical processes include, are not limited to, washing, soaking, milling, size reduction, screening, shearing, size classification and density classification processes. Chemical processes include, but are not limited to, bleaching, oxidation, reduction, acid treatment, base treatment, sulfite treatment, acid sulfite treatment, basic sulfite treatment, ammonia treatment, and hydrolysis. Thermal processes include, but are not limited to, sterilization, ammonia fiber expansion or explosion ("AFEX"), steam explosion, holding at elevated temperatures in the presence or absence of water, and freezing.

[00101] Various embodiments of the invention offer benefits relating to improving the titer and/or productivity of alcohol production by *Clostridium phytofermentans* by culturing the organism in a medium comprising one or more compounds comprising particular fatty acid moieties and/or culturing the organism under conditions of controlled pH.

[00102] In one embodiment, the pretreatment of biomass comprises the addition of an alkaline agent, including but not limited to ammonium hydroxide, sodium hydroxide, potassium hydroxide, calcium hydroxide, magnesium hydroxide, calcium carbonate, lye, and other basic salts may be added to the feedstock, alone or in combination. In some embodiments, alkaline agents are added to a feedstock at a concentration sufficient to maintain an alkaline pH, including but not limited to, pH 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5 or higher. In other embodiments, alkaline agents are added at a percentage of the weight of the feedstock to be treated in order to obtain and/or maintain an alkaline pH of the treated feedstock. For example, an alkaline agent may be added at 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1.0%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, 2.9%, 3.0%, 3.1%, 3.2%, 3.3%, 3.4%, 3.5%, 3.6%, 3.7%, 3.8%, 3.9%, 4.0%, or higher. The addition of a percentage weight of an alkaline substance may vary based on the pH of the feedstock (e.g., a feedstock with a lower pH may require the addition of more alkaline substance than a feedstock with a higher pH).

[00103] In one embodiment, Q microbe is fermented with a substrate at about pH 5-8.5 In one embodiment a Q microbe is fermented at pH of about 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, or 8.5.

[00104] Production of high levels of alcohol requires both the ability for the organism to thrive generally in the presence of elevated alcohol levels and the ability to continue to produce alcohol without undue inhibition or suppression by the alcohol and/or other components present. Frequently, different metabolic pathways will be implicated for each of these. For example, pathways related to cell

growth generally include those related to protein production, membrane production as well as the production of all of the cellular subsystems necessary for the cell to survive. Pathways related to alcohol production will frequently be more specific, such as those pathways related to the metabolism of sugars leading to production of alcohol and the enzymes that are necessary for the production of alcohol and intermediates. The pathway for one alcohol, *e.g.*, ethanol, can share some similar enzymes, but will also have enzymes and substrates unique to that pathway. While there can be some overlap between these sets of pathways, it is not expected that enhancement of one will automatically result in the enhancement of the other.

[00105] In some embodiments, alcohol intolerance or alcohol-induced toxicity can be related to permeabilization of the cell membrane by elevated levels of alcohol, leading to leakage of intracellular enzymes and nutrients. In some other cases, alcohol tolerance and the ability to produce high alcohol titers is related to the ability of intracellular enzymes to withstand denaturing by the alcohol present, *e.g.*, within the cell, whether due to production by the cell itself or from transport across the cell membrane. In some cases, a more robust membrane will allow a higher alcohol gradient to be present across the membrane, thus allowing the cells to grow and/or continue to produce alcohol at higher external alcohol concentrations. It has been demonstrated with *Clostridium phytofermentans* that in some fermentation processes an ethanol concentration attains a plateau of about 15 g/L after about 36 - 48 hours of batch fermentation, with carbon substrate remaining in the broth. In one embodiment lowering the fermentation pH to about 6.5 and/or adding unsaturated fatty acids resulted in a significant increase in the amount of ethanol produced by the organism, with about 35 g/L of ethanol observed in the broth following a 72-hour fermentation. In another embodiment it was observed that the productivity of the organism was higher (to about 0 g/L-d) when the ethanol titer was low and lower (to about 2 g/L-d) when the ethanol concentration was higher. Fermentation at reduced pH (optionally with the addition of fatty acids) resulted in about a five fold increase in the ethanol production rate.

[00106] In another aspect, the invention provides methods of producing alcohol; *e.g.*, ethanol, comprising culturing a strain of *Clostridium phytofermentans* in a medium under conditions of controlled pH. In one embodiment, a strain of *Clostridium phytofermentans* is cultured at a pH less than 7 (an acidic pH). In another embodiment a strain of *Clostridium phytofermentans* is cultured at a pH more than 7 (an alkaline pH).

[00107] In one embodiment, a strain of *Clostridium phytofermentans* is cultured at an acidic pH. In one embodiment the strain of *Clostridium phytofermentans* is cultured in a medium that comprises a carbon source such as agricultural crops, crop residues, trees, wood chips, sawdust, paper, cardboard, or other materials containing cellulose, hemicellulosic, lignocellulose, pectin, polyglucose, polyfructose, and/or hydrolyzed forms of these (collectively, "Feedstock"). Additional nutrients can be present including sulfur- and nitrogen-containing compounds such as amino acids, proteins, hydrolyzed proteins, ammonia, urea, nitrate, nitrite, soy, soy derivatives, casein, casein derivatives, milk powder, milk derivatives, whey, yeast extract, hydrolyze yeast, autolyzed yeast, corn steep liquor, corn steep solids,

monosodium glutamate, and/or other fermentation nitrogen sources, vitamins, cofactors and/or mineral supplements. The Feedstock can be pretreated or not, such as described in U.S. Provisional Patent Application No. 61/032048, filed February 27, 2008 or U.S. Provisional Application No. 61/158,581, filed on March 9, 2009, which are herein incorporated by reference in their entireties. The procedures and techniques for growing the organism to produce a fuel or other desirable chemical such as described in Provisional U.S. Patent Application Nos. 61/032048 or U.S. Provisional Application filed on March 9, 2009, No. 61/158,581 are herein incorporated by reference in their entireties.

**[00108]** In one embodiment a strain of *Clostridium phytofermentans* is cultured in a medium, wherein the pH of the medium is controlled at less than about pH 7.2 for at least a portion of the fermentation. In one embodiment, the pH is controlled within a range of about pH 3.0 to about 7.1 or about pH 4.5 to about 7.1, pH 4.5 to about 6.9, or about pH 5.0 to about 6.3, or about pH 5.5 to about 6.3, or about pH 6.0 to about 6.5, or about pH 5.5 to about 6.9 or about pH 6.2 to about 6.7. In another embodiment the pH is controlled at about 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.8, or 6.9. The pH can be controlled by the addition of a pH modifier. In some embodiments, a pH modifier can be an acid, a base, a buffer, or a material that reacts with other materials present to serve to raise or lower the pH. In some embodiments, more than one pH modifier can be used, such as more than one acid, more than one base, one or more acid with one or more bases, one or more acids with one or more buffers, one or more bases with one or more buffers, or one or more acids with one or more bases with one or more buffers. When more than one pH modifiers are utilized, they can be added at the same time or at different times. In some embodiments, one or more acids and one or more bases can be combined, resulting in a buffer. In some embodiments, media components, such as a carbon source or a nitrogen source can also serve as a pH modifier; suitable media components include those with high or low pH or those with buffering capacity. Exemplary media components include acid- or base-hydrolyzed plant polysaccharides having with residual acid or base, AFEX treated plant material with residual ammonia, lactic acid, corn steep solids or liquor.

**[00109]** In some embodiments, the pH modifier can be added as a part of the medium components prior to inoculation with the *Clostridium phytofermentans*. In other embodiments, the pH modifier can also be added after inoculation with the *Clostridium phytofermentans*. In some embodiments, sufficient buffer capacity can be added to the seed fermentation by way of various pH modifiers and/or other medium components and/or metabolites to maintain a desired pH provide adequate pH control during the final fermentation stage. In other cases, pH modifier can be added only to the final fermentation stage. In still other cases, pH modifier can be added to both the seed stage and the final stage. In one embodiment, the pH is monitored throughout the fermentation and is adjusted in response to changes in the fermentation. In one embodiment, the pH modifier added to the medium of a fermentation changes the pH value of the medium by about 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 or more. In another embodiment, the pH modifier is added whenever the alcohol content of the fermentation is about 0.5 g/L, 1.0 g/L, 2.0 g/L, or 5.0 g/L or more. In some embodiments different types of pH modifiers can be

utilized at different stages or points in the fermentation, such as a buffer being used at the seed stage, and base and/or acid added in the final fermenter, or an acid being used at one time and a base at another time.

[00110] In some embodiments, a constant pH can be utilized throughout the fermentation. In some embodiments, it can be advantageous to start the fermentation at one pH, and then to lower the pH during the course of the fermentation. In embodiments where the pH is lowered, the pH can be lowered in a stepwise fashion or a more gradual fashion. Suitable times for lowering the pH include during a lag phase of cellular growth, during an exponential phase of cellular growth, during a stationary phase of cellular growth, during a death phase of cellular growth, or before or during periods of cell proliferation. In some embodiments the pH can be lowered during more than one phase of growth. While in some embodiments, the pH can be lowered in a stepwise fashion, such as with the change occurring over a period of about 10 minutes or less, advantageous growth can be achieved in some embodiments by lowering the pH more gradually, such as over a period of about 10 minutes to about six hours or longer. In some embodiments, the timing and/or amount of pH reduction can be related to the growth conditions of the cells, such as in relation to the cell count, the alcohol produced, the alcohol present, or the rate of alcohol production. In some embodiments, the pH reduction can be made in relation to physical or chemical properties of the fermentation, such as viscosity, medium composition, gas production, or off gas composition.

[00111] Non-limiting examples of suitable buffers include salts of phosphoric acid, including monobasic, dibasic, and tribasic salts, mixtures of these salts and mixtures with the acid; salts of citric acid, including the various basic forms, mixtures and mixtures with the acid; and salts of carbonate.

[00112] Suitable acids and bases that can be used as pH modifiers include any liquid or gaseous acid or base that is compatible with the organism. Examples include ammonia, ammonium hydroxide, sulfuric acid, lactic acid, citric acid, phosphoric acid, sodium hydroxide, and HCl. In some cases, the selection of the acid or base can be influenced by the compatibility of the acid or base with equipment being used for fermentation. In some embodiments, both an acid addition, to lower pH or consume base, and a base addition, to raise pH or consume acid, can be used in the same fermentation.

[00113] The timing and amount of pH modifier to add can be determined from a measurement of the pH of the contents of the fermentor, such as by grab sample or by a submerged pH probe, or it can be determined based on other parameters such as the time into the fermentation, gas generation, viscosity, alcohol production, titration, etc. In some embodiments, a combination of these techniques can be used.

[00114] In one embodiment, the pH of the pretreatment is initiated at a neutral pH and then is reduced to an acidic pH. In one embodiment, the pH of the pretreatment is initiated at a neutral pH and then is reduced to an acidic pH and raised to neutral pH again prior to fermentation. In one embodiment, the pH of the fermentation is initiated at a neutral pH and then is reduced to an acidic pH. In another embodiment, the pH of the fermentation is initiated at a neutral pH and then is reduced to an acidic pH when the production of alcohol is detected. In another embodiment, the pH of the fermentation is

initiated at an acidic pH and is maintained at an acidic pH until the fermentation reaches a stationary phase of growth.

**[00115]** In another embodiment, a feedstock is pretreated at about pH of 8 to 12 to obtain a high concentration of hemicellulose and a low concentration of lignins in the pretreated feedstock. In some embodiments, a feedstock is pretreated at about pH of 8 to 12 to maintain a high concentration of hemicellulose and a low concentration of lignins such that concentration of the components in the pretreated stock is optimal for fermentation with a microbe such as *C. phytofermentans*. Other parameters such as temperature and time can be changed to obtain the desired results. For example, in some embodiments a feedstock is pretreated at about pH of 8 to 12 at a low temperature for a long time to maintain a high concentration of hemicellulose and a low concentration of lignins in the pretreated feedstock.

**[00116]** In some embodiments, the feedstock is treated with NaOH such that the concentration of the components in the pretreated stock is optimal for fermentation with *C. phytofermentans*. The NaOH pretreatment can be performed in combination with agents such as hydrogen peroxide or urea. The NaOH pretreatment, alone or in combination with hydrogen peroxide or urea, can be performed at 60°C, 80°C, 90°C, 100°C, 120°C, 140°C, 160°C or 180°C. The NaOH pretreatment, alone or in combination with hydrogen peroxide or urea, can be performed for 10, 15, 20, 30, 35, 40, 50 minutes or 1, 5, 7, 9, 10, 11, 15, 20, 25, 30, 35 or 36 hours.

**[00117]** In some embodiments, the feedstock is treated with KOH such that the concentration of the components in the pretreated stock is optimal for fermentation with *C. phytofermentans*. The KOH pretreatment can be performed in combination with agents such as hydrogen peroxide or urea. The KOH pretreatment, alone or in combination with hydrogen peroxide or urea, can be performed at 60°C, 80°C, 90°C, 100°C, 120°C, 140°C, 160°C or 180°C. The KOH pretreatment, alone or in combination with hydrogen peroxide or urea, can be performed for 10, 15, 20, 30, 35, 40, 50 minutes or 1, 5, 7, 9, 10, 11, 15, 20, 25, 30, 35 or 36 hours.

**[00118]** In some embodiments, the feedstock is treated with Ca(OH)<sub>2</sub> such that the concentration of the components in the pretreated stock is optimal for fermentation with *C. phytofermentans*. The Ca(OH)<sub>2</sub> pretreatment can be performed in combination with agents such as hydrogen peroxide or urea. The Ca(OH)<sub>2</sub> pretreatment, alone or in combination with hydrogen peroxide or urea, can be performed at 60°C, 80°C, 90°C, 100°C, 120°C, 140°C, 160°C or 180°C. The Ca(OH)<sub>2</sub> pretreatment, alone or in combination with hydrogen peroxide or urea, can be performed for 10, 15, 20, 30, 35, 40, 50 minutes or 1, 5, 7, 9, 10, 11, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 96 hours.

**[00119]** In some instances, pretreatment comprises the addition of an acid or a base and water to the biomass to form a mixture, and maintaining the mixture at a relatively high temperature. For example, pretreatment can be performed at a temperature above 60°C, above 70°C, above 80°C, above 90°C, above 100°C, above 110°C, above 120°C, above 130°C, above 140°C, above 150°C, above 160°C, above 170°C, above 180°C, above 190°C, above 200°C or higher. In some embodiments, the

temperature during pretreatment is maintained, at least for a portion of the pretreatment at between 60°C and 140°C, between 80°C and 120°C, or between 90°C and 110°C. In one embodiment, an oxidizing agent, selected from the group consisting of oxygen and oxygen-containing gasses, may be added under pressure to the mixture. In one embodiment, pretreatment involves the addition of a base in combination with increased temperature. In one embodiment, pretreatment involves the addition of an acid in combination with increased temperature.

[00120] In another embodiment, one or more chelators are added to the feedstock during pretreatment. As used herein, the term "chelator" refers to "chemicals that form soluble, complex molecules with certain metal ions, inactivating the ions so that they cannot normally react with other elements or ions." Numerous chelators could be used as appropriate for the feedstock to be treated. Non-limiting examples of chelators include, dicarboxymethylglutamic acid, ethylenediaminedisuccinic acid (EDDS), ethylenediaminetriacetic acid (EDTA), methylamine, certain proteins and non-polar amino acids (*e.g.*, pyocyanin, pyoverdin, enterobactin, methionine), certain polysaccharides (*e.g.*, phytochelatin), ascorbic acid, citric acid, malic acid, nitrilotriacetic acid, oxalic acid, and siderophores (*e.g.*, desferoxamine B). The choice of which chelator, or combination of chelators, to utilize can be determined based on the specificity of the chelator (*i.e.*, which metal(s) are chelated) and/or by the ability of the chelator to function in the pretreatment environment. For example, where an alkaline pH is maintained by the addition of an alkaline agent to the feedstock, the chelator(s) chosen would be capable of functioning at alkaline pH. Additionally, where high temperature is utilized during pretreatment, the chelator(s) chosen would be capable of functioning at high temperature.

[00121] In another embodiment, one or more biochemical processes can be utilized during pretreatment. Biochemical processes include, but are not limited to, treatment with enzymes and treatment with microorganisms. Various enzymes that can be utilized include cellulase, amylase,  $\beta$ -glucosidase, xylanase, gluconase, and other polysaccharases; lysozyme; laccase, and other lignin-modifying enzymes; lipoxygenase, peroxidase, and other oxidative enzymes; proteases; and lipases. One or more of the mechanical, chemical, thermal, thermochemical, and biochemical processes can be combined or used separately. Such combined processes can also include those used in the production of paper, cellulose products, microcrystalline cellulose, and cellulotics; and can include pulping, kraft pulping, acidic sulfite processing. The feedstock can be a side stream or waste stream from a facility that utilizes one or more of these processes on a biomass material, such as cellulosic, hemicellulosic or lignocellulosic material. Examples include paper plants, cellulotics plants, cotton processing plants, and microcrystalline cellulose plants. The feedstock can also include cellulose-containing or cellulosic containing waste materials. The feedstock can also be biomass materials, such as wood, grasses, corn, starch, or sugar, produced or harvested as an intended feedstock for production of ethanol or other products such as by *C. phytofermentans*.

[00122] In another embodiment, an enzyme can directly convert the polysaccharide to monosaccharides. In some instances, an enzyme can hydrolyze the polysaccharide to oligosaccharides and the enzyme or another enzyme can hydrolyze the oligosaccharides to monosaccharides.

[00123] In one embodiment, the enzymes present in the fermentation can be produced separately and then added to the fermentation or they can be produced by microorganisms present in the fermentation.

[00124] In another embodiment, a method of the invention may utilize pretreatment processes disclosed in U.S. Patents and Patent Applications US20040 152881, US20040171 136, US20040 168960, US20080121359, US20060069244, US20060188980, US20080176301, 5693296, 6262313, US20060024801, 5969189, 6043392, US20020038058, US5865898, US5865898, US6478965, 5986133, US20080280338, each of which is incorporated by reference herein in its entirety.

[00125] In another embodiment, the AFEX process can be used for pretreatment of feedstocks. The AFEX process can be used in the preparation of cellulosic, hemicellulosic or lignocellulosic materials for fermentation to ethanol or other products. The process generally includes combining the feedstock with ammonia, heating under pressure, and suddenly releasing the pressure. Water can be present in various amounts. The AFEX process has been the subject of numerous patents and publications.

[00126] In another embodiment, pretreatment of biomass comprises dilute acid hydrolysis. Example of dilute acid hydrolysis treatment are disclosed in T. A. Lloyd and C. E Wyman, *Bioresource Technology*, (2005) 96, 1967), incorporated by reference herein in its entirety. In other embodiments, pretreatment of biomass comprises pH controlled liquid hot water treatment. Examples of pH controlled liquid hot water treatments are disclosed in N. Mosier et al., *Bioresource Technology*, (2005) 96, 1986, incorporated by reference herein in its entirety. In other embodiments, pretreatment of biomass comprises aqueous ammonia recycle process (ARP). Examples of aqueous ammonia recycle process are described in T. H. Kim and Y. Y. Lee, *Bioresource Technology*, (2005)96, 2007, incorporated by reference herein in its entirety.

[00127] In another embodiment, the above-mentioned methods have two steps: a pretreatment step that leads to a wash stream, and an enzymatic hydrolysis step of pretreated-biomass that produces a hydrolyzate stream. In the above methods, the pH at which the pretreatment step is carried out increases progressively from dilute acid hydrolysis to hot water pretreatment to alkaline reagent based methods (AFEX, ARP, and lime pretreatments). Dilute acid and hot water treatment methods solubilize mostly hemicellulose, whereas methods employing alkaline reagents remove most lignin during the pretreatment step. As a result, the wash stream from the pretreatment step in the former methods contains mostly hemicellulose-based sugars, whereas this stream has mostly lignin for the high-pH methods. The subsequent enzymatic hydrolysis of the residual feedstock leads to mixed sugars (C5 and C6) in the alkali-based pretreatment methods, while glucose is the major product in the hydrolysate from the low and neutral pH methods. The enzymatic digestibility of the residual biomass is somewhat better for the high-pH methods due to the removal of lignin that can interfere with the accessibility of cellulase enzyme to cellulose. In some instances, pretreatment results in removal of 30%, 40%, 50%,

60%, 70% or more of the lignin component of the feedstock. In other instances, more than 40%, 50%, 60%, 70%, 80% or more of the hemicellulose component of the feedstock remains after pretreatment. In some embodiments, the microorganism (*e.g.*, *C. phytofermentans*) is capable of fermenting both five-carbon and six-carbon sugars, which can be present in the feedstock, or can result from the enzymatic degradation of components of the feedstock.

[00128] In another embodiment, pretreatment of biomass comprises ionic liquid pretreatment. Biomass can be pretreated by incubation with an ionic liquid, followed by extraction with a wash solvent such as alcohol or water. The treated biomass can then be separated from the ionic liquid/wash-solvent solution by centrifugation or filtration, and sent to the saccharification reactor or vessel. Examples of ionic liquid pretreatment are disclosed in US publication No. 2008/0227162, incorporated herein by reference in its entirety.

[00129] Examples of pretreatment methods are disclosed in U.S. Patent No. 4600590 to Dale, U.S. Patent No. 4644060 to Chou, U.S. Patent No. 5037663 to Dale, U.S. Patent No. 5171592 to Holtzapple, et al., et al., U.S. Patent No. 5939544 to Karstens, et al., U.S. Patent No. 5473061 to Bredereck, et al., U.S. Patent No. 6416621 to Karstens., U.S. Patent No. 6106888 to Dale, et al., U.S. Patent No. 6176176 to Dale, et al., PCT publication WO2008/020901 to Dale, et al., Felix, A., et al., Anim. Prod. 51, 47-61 (1990), Wais, A.C., Jr., et al., Journal of Animal Science, 35, No. 1,109-1 12 (1972), which are incorporated herein by reference in their entireties.

[00130] In some embodiments, after pretreatment by any of the above methods the feedstock contains cellulose, hemicellulose, soluble oligomers, simple sugars, lignins, volatiles and/or ash. The parameters of the pretreatment can be changed to vary the concentration of the components of the pretreated feedstock. For example, in some embodiments a pretreatment is chosen so that the concentration of hemicellulose and/or soluble oligomers is high and the concentration of lignins is low after pretreatment. Examples of parameters of the pretreatment include temperature, pressure, time, and pH.

[00131] In some embodiments, the parameters of the pretreatment are changed to vary the concentration of the components of the pretreated feedstock such that concentration of the components in the pretreated stock is optimal for fermentation with a microbe such as *C. phytofermentans*.

[00132] In some embodiments, the parameters of the pretreatment are changed such that concentration of accessible cellulose in the pretreated feedstock is 1%, 5%, 10%, 12%, 13%, 14%, 15%, 16%, 17%, 19%, 20%, 30%, 40% or 50%. In some embodiments, the parameters of the pretreatment are changed such that concentration of accessible cellulose in the pretreated feedstock is 5% to 30%. In some embodiments, the parameters of the pretreatment are changed such that concentration of accessible cellulose in the pretreated feedstock is 10% to 20%.

[00133] In some embodiments, the parameters of the pretreatment are changed such that concentration of hemicellulose in the pretreated feedstock is 1%, 5%, 10%, 12%, 13%, 14%, 15%, 16%, 17%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 40% or 50%. In some embodiments, the parameters of the pretreatment are changed such that concentration of hemicellulose in the

pretreated feedstock is 5% to 40%. In some embodiments, the parameters of the pretreatment are changed such that concentration of hemicellulose in the pretreated feedstock is 10% to 30%.

[00134] In some embodiments, the parameters of the pretreatment are changed such that concentration of soluble oligomers in the pretreated feedstock is 1%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%. Examples of soluble oligomers include, but are not limited to, cellobiose and xylobiose. In some embodiments, the parameters of the pretreatment are changed such that concentration of soluble oligomers in the pretreated feedstock is 30% to 90%. In some embodiments, the parameters of the pretreatment are changed such that concentration of soluble oligomers in the pretreated feedstock is 45% to 80%. In some embodiments, the parameters of the pretreatment are changed such that concentration of soluble oligomers in the pretreated feedstock is 45% to 80% and the soluble oligomers are primarily cellobiose and xylobiose.

[00135] In some embodiments, the parameters of the pretreatment are changed such that concentration of simple sugars in the pretreated feedstock is 1%, 5%, 10%, 12%, 13%, 14%, 15%, 16%, 17%, 19%, 20%, 30%, 40% or 50%. In some embodiments, the parameters of the pretreatment are changed such that concentration of simple sugars in the pretreated feedstock is 0% to 20%. In some embodiments, the parameters of the pretreatment are changed such that concentration of simple sugars in the pretreated feedstock is 0% to 5%. Examples of simple sugars include, but are not limited to, C5 and C6 monomers and dimers.

[00136] In some embodiments, the parameters of the pretreatment are changed such that concentration of lignins in the pretreated feedstock is 1%, 5%, 10%, 12%, 13%, 14%, 15%, 16%, 17%, 19%, 20%, 30%, 40% or 50%. In some embodiments, the parameters of the pretreatment are changed such that concentration of lignins in the pretreated feedstock is 0% to 20%. In some embodiments, the parameters of the pretreatment are changed such that concentration of lignins in the pretreated feedstock is 0% to 5%. In some embodiments, the parameters of the pretreatment are changed such that concentration of lignins in the pretreated feedstock is less than 1% to 2%. In some embodiments, the parameters of the pretreatment are changed such that the concentration of phenolics is minimized.

[00137] In some embodiments, the parameters of the pretreatment are changed such that concentration of furfural and low molecular weight lignins in the pretreated feedstock is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%. In some embodiments, the parameters of the pretreatment are changed such that concentration of furfural and low molecular weight lignins in the pretreated feedstock is less than 1% to 2%.

[00138] In some embodiments, the parameters of the pretreatment are changed such that concentration of accessible cellulose is 10% to 20%, the concentration of hemicellulose is 10% to 30%, the concentration of soluble oligomers is 45% to 80%, the concentration of simple sugars is 0% to 5%, and the concentration of lignins is 0% to 5% and the concentration of furfural and low molecular weight lignins in the pretreated feedstock is less than 1% to 2%.

[00139] In some embodiments, the parameters of the pretreatment are changed to obtain a high concentration of hemicellulose (*e.g.*, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70% or higher) and a low concentration of lignins (*e.g.*, 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, or 30%). In some embodiments, the parameters of the pretreatment are changed to obtain a high concentration of hemicellulose and a low concentration of lignins such that concentration of the components in the pretreated stock is optimal for fermentation with a microbe such as *C. phytofermentans*.

[00140] Certain conditions of pretreatment can be modified prior to, or concurrently with, introduction of a fermentative microorganism into the feedstock. For example, pretreated feedstock may be cooled to a temperature which allows for growth of the microorganism(s). As another example, pH may be altered prior to, or concurrently with, addition of one or more microorganisms.

[00141] Alteration of the pH of a pretreated feedstock may be accomplished by washing the feedstock (*e.g.*, with water) one or more times to remove an alkaline or acidic substance, or other substance used or produced during pretreatment. Washing may comprise exposing the pretreated feedstock to an equal volume of water 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more times. In another embodiment, a pH modifier can be added. For example, an acid, a buffer, or a material that reacts with other materials present can be added to modulate the pH of the feedstock. In some embodiments, more than one pH modifier can be used, such as one or more bases, one or more bases with one or more buffers, one or more acids, one or more acids with one or more buffers, or one or more buffers. When more than one pH modifiers are utilized, they can be added at the same time or at different times. Other non-limiting exemplary methods for neutralizing feedstocks treated with alkaline substances have been described, for example in U.S. Patent Nos. 4,048,341; 4,182,780; and 5,693,296.

[00142] In some embodiments, one or more acids can be combined, resulting in a buffer. Suitable acids and buffers that can be used as pH modifiers include any liquid or gaseous acid that is compatible with the microorganism. Non-limiting examples include peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, and hydrochloric acid. In some instances, the pH can be lowered to neutral pH or acidic pH, for example a pH of 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, or lower. In some embodiments, the pH is lowered and/or maintained within a range of about pH 4.5 to about 7.1, or about 4.5 to about 6.9, or about pH 5.0 to about 6.3, or about pH 5.5 to about 6.3, or about pH 6.0 to about 6.5, or about pH 5.5 to about 6.9 or about pH 6.2 to about 6.7.

### **Fermentive mixture**

[00143] In one embodiment, a fermentative mixture is provided which comprises a pretreated lignocellulosic feedstock comprising less than about 50% of a lignin component present in the feedstock prior to pretreatment and comprising more than about 60% of a hemicellulose component present in the feedstock prior to pretreatment; and a microorganism capable of fermenting a five-carbon sugar, such as xylose, arabinose or a combination thereof, and a six-carbon sugar, such as glucose, galactose, mannose or a combination thereof. In some instances, pretreatment of the lignocellulosic

feedstock comprises adding an alkaline substance which raises the pH to an alkaline level, for example NaOH. In some embodiments, NaOH is added at a concentration of about 0.5% to about 2% by weight of the feedstock. In other embodiments, pretreatment also comprises addition of a chelating agent. In some embodiments, the microorganism is a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans* or *Clostridium* sp. Q.D.

[00144] The present disclosure also provides a fermentative mixture comprising: a cellulosic feedstock pre-treated with an alkaline substance which maintains an alkaline pH, and at a temperature of from about 80°C to about 120°C; and a microorganism capable of fermenting a five-carbon sugar and a six-carbon sugar. In some instances, the five-carbon sugar is xylose, arabinose, or a combination thereof. In other instances, the six-carbon sugar is glucose, galactose, mannose, or a combination thereof. In some embodiments, the alkaline substance is NaOH. In some embodiments, NaOH is added at a concentration of about 0.5% to about 2% by weight of the feedstock. In some embodiments, the microorganism is a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans* or *Clostridium* sp. Q.D. In still other embodiments, the microorganism is genetically modified to enhance activity of one or more hydrolytic enzymes.

[00145] Further provided herein is a fermentative mixture comprising a cellulosic feedstock pre-treated with an alkaline substance which increases the pH to an alkaline level, at a temperature of from about 80°C to about 120°C; and a microorganism capable of uptake and fermentation of an oligosaccharide. In some embodiments the alkaline substance is NaOH. In some embodiments, NaOH is added at a concentration of about 0.5% to about 2% by weight of the feedstock. In some embodiments, the microorganism is a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans* or *Clostridium* sp. Q.D. In other embodiments, the microorganism is genetically modified to express or increase expression of an enzyme capable of hydrolyzing said oligosaccharide, a transporter capable of transporting the oligosaccharide, or a combination thereof.

[00146] Another aspect of the present disclosure provides a fermentative mixture comprising a cellulosic feedstock comprising cellulosic material from at least two sources, wherein said feedstock is pre-treated with a substance which increases the pH to an alkaline level, at a temperature of from about 80°C to about 120°C; and a microorganism capable of fermenting said cellulosic material from at least two different sources to produce a fermentive product at substantially a same yield coefficient. In some instances, the sources of cellulosic material are corn stover, bagasse, switchgrass or poplar. In some embodiments the alkaline substance is NaOH. In some embodiments, NaOH is added at a concentration of about 0.5% to about 2% by weight of the feedstock. In some embodiments, the microorganism is a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans*.

### **Fermentation**

[00147] In one embodiment methods are provided herein for the fermentation of biomass and the subsequent production of a useful end-product including, but not limiting to, an alcohol, ethanol, an organic acid, acetic acid, lactic acid, methane, or hydrogen or other chemical. In some embodiments,

biomass (*e.g.* corn stover) is processed or pretreated prior to fermentation. In one embodiment a method of pre-treatment includes but is not limited to, biomass particle size reduction, such as for example shredding, milling, chipping, crushing, grinding, or pulverizing. In some embodiments, biomass particle size reduction can include size separation methods such as sieving, or other suitable methods known in the art to separate materials based on size. In one embodiment size separation can provide for enhanced yields. In some embodiments, separation of finely shredded biomass (*e.g.* particles smaller than about 8 mm in diameter, such as, 8, 7.9, 7.7, 7.5, 7.3, 7, 6.9, 6.7, 6.5, 6.3, 6, 5.9, 5.7, 5.5, 5.3, 5, 4.9, 4.7, 4.5, 4.3, 4, 3.9, 3.7, 3.5, 3.3, 3, 2.9, 2.7, 2.5, 2.3, 2, 1.9, 1.7, 1.5, 1.3, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 mm) from larger particles allows the recycling of the larger particles back into the size reduction process, thereby increasing the final yield of processed biomass.

**[00148]** In some embodiments pretreatment methods can include treatment under conditions of high or low pH. High or low pH treatment includes, but is not limited to, treatment using concentrated acids or concentrated alkali, or treatment using dilute acids or dilute alkali. Alkaline compositions useful for treatment of biomass in methods described herein include, but are not limited to, caustic, such as caustic lime, caustic soda, caustic potash, sodium, potassium, ammonium hydroxide, calcium hydroxide, or calcium oxide. In some embodiments suitable amounts of alkaline useful for the treatment of biomass ranges from 0.01g to 3g of alkaline (*e.g.* caustic) for every gram of biomass to be treated. In some embodiments suitable amounts of alkaline useful for the treatment of biomass include, but are not limited to, about 0.01g of alkaline (*e.g.* caustic), 0.02g, 0.03g, 0.04g, 0.05g, 0.075g, 0.1g, 0.2g, 0.3g, 0.4g, 0.5g, 0.75g, 1g, 2g, or about 3g of alkaline (*e.g.* caustic) for every gram of biomass to be treated.

**[00149]** In another embodiment, biomass can be pre-treated at an elevated temperature and/or pressure. In one embodiment biomass is pre treated at a temperature range of 20°C to 400°C. In another embodiment biomass is pretreated at a temperature of about 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 80°C, 90°C, 100°C, 120°C, 150°C, 200°C, 250°C, 300°C, 350°C, 400°C or higher. In another embodiment, elevated temperatures are provided by the use of steam, hot water, or hot gases. In one embodiment steam can be injected into a biomass containing vessel. In another embodiment the steam, hot water, or hot gas can be injected into a vessel jacket such that it heats, but does not directly contact the biomass.

**[00150]** In another embodiment, a biomass can be treated at an elevated pressure. In one embodiment biomass is pre treated at a pressure range of about 1psi to about 30psi. In another embodiment biomass is pre treated at a pressure or about 1psi, 2psi, 3psi, 4psi, 5psi, 6psi, 7psi, 8psi, 9psi, 10psi, 12psi, 15psi, 18psi, 20psi, 22psi, 24psi, 26psi, 28psi, 30psi or more. In some embodiments, biomass can be treated with elevated pressures by the injection of steam into a biomass containing vessel. In other embodiments, the biomass can be treated to vacuum conditions prior or subsequent to alkaline or acid treatment or any other treatment methods provided herein.

**[00151]** In one embodiment alkaline or acid pretreated biomass is washed (*e.g.* with water (hot or cold) or other solvent such as alcohol (*e.g.* ethanol)), pH neutralized with an acid, base, or buffering agent

(*e.g.* phosphate, citrate, borate, or carbonate salt) or dried prior to fermentation. In one embodiment, the drying step can be performed under vacuum to increase the rate of evaporation of water or other solvents. Alternatively, or additionally, the drying step can be performed at elevated temperatures such as about 20<sup>0</sup>C, 25<sup>0</sup>C, 30<sup>0</sup>C, 35<sup>0</sup>C, 40<sup>0</sup>C, 45<sup>0</sup>C, 50<sup>0</sup>C, 55<sup>0</sup>C, 60<sup>0</sup>C, 65<sup>0</sup>C, 80<sup>0</sup>C, 90<sup>0</sup>C, 100<sup>0</sup>C, 120<sup>0</sup>C, 150<sup>0</sup>C, 200<sup>0</sup>C, 250<sup>0</sup>C, 300<sup>0</sup>C or more.

**[00152]** In some embodiments of compositions and methods described herein, the pretreatment step includes a step of solids recovery. The solids recovery step can be during or after pretreatment (*e.g.*, acid or alkali pretreatment), or before the drying step. In some embodiments, the solids recovery step provided by compositions and methods described herein includes the use of a sieve, filter, screen, or a membrane for separating the liquid and solids fractions. In one embodiment a suitable sieve pore diameter size ranges from about 0.001 microns to 8mm, such as about 0.005microns to 3mm or about 0.01 microns to 1mm. In one embodiment a sieve pore size has a pore diameter of about 0.01microns, 0.02 microns, 0.05 microns, 0.1 microns, 0.5 microns, 1 micron, 2 microns, 4 microns, 5 microns, 10 microns, 20 microns, 25 microns, 50 microns, 75 microns, 100 microns, 125 microns, 150 microns, 200 microns, 250 microns, 300 microns, 400 microns, 500 microns, 750 microns, 1mm or more.

**[00153]** Fed-batch culture is a kind of microbial process in which medium components, such as carbon substrate, nitrogen substrate, vitamins, minerals, growth factors, cofactors, etc. or biocatalysts (including, for example, fresh organisms, enzymes prepared by the Q microbe in a separate fermentation, enzymes prepared by other organisms, or a combination of these) are supplied to the fermentor during cultivation, but culture broth is not harvested at the same time and volume.

**[00154]** To improve bioconversion from soluble and insoluble substrates, such as those that can be used in biofuels production, various feeding strategies can be utilized to improve yields and/or productivity. This technique can be used to achieve a high cell density within a given time. It can also be used to maintain a good supply of nutrients and substrates for the bioconversion process. It can also be used to achieve higher titer and productivity of desirable products that might otherwise be achieved more slowly or not at all.

**[00155]** In another embodiment, the feeding strategy balances the cell production rate and the rate of hydrolysis of the biomass feedstock with the production of ethanol. Sufficient medium components are added in quantities to achieved sustained cell production and hydrolysis of the biomass feedstock with production of ethanol. In some embodiments, sufficient carbon and nitrogen substrate are added in quantities to achieve sustained production of fresh cells and hydrolytic enzymes for conversion of polysaccharides into lower sugars as well as sustained conversion of the lower sugars into fresh cells and ethanol.

**[00156]** In another embodiment, the level of a medium component is maintained at a desired level by adding additional medium component as the component is consumed or taken up by the organism. Examples of medium components included, but are not limited to, carbon substrate, nitrogen substrate, vitamins, minerals, growth factors, cofactors, and biocatalysts. The medium component can be added

continuously or at regular or irregular intervals. In some embodiments, additional medium component is added prior to the complete depletion of the medium component in the medium. In some embodiments, complete depletion can effectively be used, for example to initiate different metabolic pathways, to simplify downstream operations, or for other reasons as well. In some embodiments, the medium component level is allowed to vary by about 10% around a midpoint, in some embodiments, it is allowed to vary by about 30% around a midpoint, and in some embodiments, it is allowed to vary by 60% or more around a midpoint. Operation in some embodiments will maintain the medium component level by allowing the medium component to be depleted to an appropriate level, followed by increasing the medium component level to another appropriate level. In one embodiment, a medium component, such as vitamin, is added at two different time points during fermentation process. For example, one-half of a total amount of vitamin is added at the beginning of fermentation and the other half is added at midpoint of fermentation.

[00157] In another embodiment, the nitrogen level is maintained at a desired level by adding additional nitrogen-containing material as nitrogen is consumed or taken up by the organism. The nitrogen-containing material can be added continuously or at regular or irregular intervals. In some embodiments, additional nitrogen-containing material is added prior to the complete depletion of the nitrogen available in the medium. In some embodiments, complete depletion can effectively be used, for example to initiate different metabolic pathways, to simplify downstream operations, or for other reasons as well. In some embodiments, the nitrogen level (as measured by the grams of actual nitrogen in the nitrogen-containing material per liter of broth) is allowed to vary by about 10% around a midpoint, in some embodiments, it is allowed to vary by about 30% around a midpoint, and in some embodiments, it is allowed to vary by 60% or more around a midpoint. Operation in some embodiments will maintain the nitrogen level by allowing the nitrogen to be depleted to an appropriate level, followed by increasing the nitrogen level to another appropriate level. Useful nitrogen levels include levels of about 5 to about 10 g/L. In one embodiment levels of about 1 to about 12 g/L can also be usefully employed. In another embodiment levels, such as about 0.5, 0.1 g/L or even lower, and higher levels, such as about 20, 30 g/L or even higher are used. In another embodiment a useful nitrogen level is about 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 g/L. Such nitrogen levels can facilitate the production of fresh cells and of hydrolytic enzymes. Increasing the level of nitrogen can lead to higher levels of enzymes and/or greater production of cells, and result in higher productivity of desired products. Nitrogen can be supplied as a simple nitrogen-containing material, such as an ammonium compounds (*e.g.* ammonium sulfate, ammonium hydroxide, ammonia, ammonium nitrate, or any other compound or mixture containing an ammonium moiety), nitrate or nitrite compounds (*e.g.* potassium, sodium, ammonium, calcium, or other compound or mixture containing a nitrate or nitrite moiety), or as a more complex nitrogen-containing material, such as amino acids, proteins, hydrolyzed protein, hydrolyzed yeast, yeast extract, dried brewer's yeast, yeast hydrolysates, soy protein, hydrolyzed soy

protein, fermentation products, and processed or corn steep powder or unprocessed protein-rich vegetable or animal matter, including those derived from bean, seeds, soy, legumes, nuts, milk, pig, cattle, mammal, fish, as well as other parts of plants and other types of animals. Nitrogen-containing materials useful in various embodiments also include materials that contain a nitrogen-containing material including but not limited to mixtures of a simple or more complex nitrogen-containing material mixed with a carbon source, another nitrogen-containing material, or other nutrients or non-nutrients, and AFEX treated plant matter.

[00158] In another embodiment, the carbon level is maintained at a desired level by adding sugar compounds or material containing sugar compounds ("Sugar-containing material") as sugar is consumed or taken up by the organism. The sugar-containing material can be added continuously or at regular or irregular intervals. In some embodiments, additional sugar-containing material is added prior to the complete depletion of the sugar compounds available in the medium. In some embodiments, complete depletion can effectively be used, for example to initiate different metabolic pathways, to simplify downstream operations, or for other reasons as well. In some embodiments, the carbon level (as measured by the grams of sugar present in the sugar-containing material per liter of broth) is allowed to vary by about 10% around a midpoint, in some embodiments, it is allowed to vary by about 30% around a midpoint, and in some embodiments, it is allowed to vary by 60% or more around a midpoint. Operation in some embodiments will maintain the carbon level by allowing the carbon to be depleted to an appropriate level, followed by increasing the carbon level to another appropriate level. In some embodiments, the carbon level can be maintained at a level of about 5 to about 120 g/L. However, levels of about 30 to about 100 g/L can also be usefully employed as well as levels of about 60 to about 80 g/L. In one embodiment, the carbon level is maintained at greater than 25 g/L for a portion of the culturing. In another embodiment, the carbon level is maintained at about 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L, 16 g/L, 17 g/L, 18 g/L, 19 g/L, 20 g/L, 21 g/L, 22 g/L, 23 g/L, 24 g/L, 25 g/L, 26 g/L, 27 g/L, 28 g/L, 29 g/L, 30 g/L, 31 g/L, 32 g/L, 33 g/L, 34 g/L, 35 g/L, 36 g/L, 37 g/L, 38 g/L, 39 g/L, 40 g/L, 41 g/L, 42 g/L, 43 g/L, 44 g/L, 45 g/L, 46 g/L, 47 g/L, 48 g/L, 49 g/L, 50 g/L, 51 g/L, 52 g/L, 53 g/L, 54 g/L, 55 g/L, 56 g/L, 57 g/L, 58 g/L, 59 g/L, 60 g/L, 61 g/L, 62 g/L, 63 g/L, 64 g/L, 65 g/L, 66 g/L, 67 g/L, 68 g/L, 69 g/L, 70 g/L, 71 g/L, 72 g/L, 73 g/L, 74 g/L, 75 g/L, 76 g/L, 77 g/L, 78 g/L, 79 g/L, 80 g/L, 81 g/L, 82 g/L, 83 g/L, 84 g/L, 85 g/L, 86 g/L, 87 g/L, 88 g/L, 89 g/L, 90 g/L, 91 g/L, 92 g/L, 93 g/L, 94 g/L, 95 g/L, 96 g/L, 97 g/L, 98 g/L, 99 g/L, 100 g/L, 101 g/L, 102 g/L, 103 g/L, 104 g/L, 105 g/L, 106 g/L, 107 g/L, 108 g/L, 109 g/L, 110 g/L, 111 g/L, 112 g/L, 113 g/L, 114 g/L, 115 g/L, 116 g/L, 117 g/L, 118 g/L, 119 g/L, 120 g/L, 121 g/L, 122 g/L, 123 g/L, 124 g/L, 125 g/L, 126 g/L, 127 g/L, 128 g/L, 129 g/L, 130 g/L, 131 g/L, 132 g/L, 133 g/L, 134 g/L, 135 g/L, 136 g/L, 137 g/L, 138 g/L, 139 g/L, 140 g/L, 141 g/L, 142 g/L, 143 g/L, 144 g/L, 145 g/L, 146 g/L, 147 g/L, 148 g/L, 149 g/L, or 150 g/L.

[00159] The carbon substrate, like the nitrogen substrate, is necessary for cell production and enzyme production, but unlike the nitrogen substrate, it serves as the raw material for ethanol. Frequently, more carbon substrate can lead to greater production of ethanol.

[00160] In another embodiment, it can be advantageous to operate with the carbon level and nitrogen level related to each other for at least a portion of the fermentation time. In one embodiment, the ratio of carbon to nitrogen is maintained within a range of about 30:1 to about 10:1. In another embodiment, the ratio of carbon nitrogen is maintained from about 20:1 to about 10:1 or more preferably from about 15:1 to about 10:1. In another embodiment the ratio of carbon nitrogen is about 30:1, 29:1, 28:1, 27:1, 26:1, 25:1, 24:1, 23:1, 22:1, 21:1, 20:1, 19:1, 18:1, 17:1, 16:1, 15:1, 14:1, 13:1, 12:1, 11:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, or 1:1.

[00161] Maintaining the ratio of carbon and nitrogen ratio within particular ranges can result in benefits to the operation such as the rate of hydrolysis of carbon substrate, which depends on the amount of carbon substrate and the amount and activity of enzymes present, being balanced to the rate of ethanol production. Such balancing can be important, for example, due to the possibility of inhibition of cellular activity due to the presence of a high concentration of low molecular weight saccharides, and the need to maintain enzymatic hydrolytic activity throughout the period where longer chain saccharides are present and available for hydrolysis. Balancing the carbon to nitrogen ratio can, for example, facilitate the sustained production of these enzymes such as to replace those which have lost activity.

[00162] In another embodiment, the amount and/or timing of carbon, nitrogen, or other medium component addition can be related to measurements taken during the fermentation. For example, the amount of monosaccharides present, the amount of insoluble polysaccharide present, the polysaccharase activity, the amount of ethanol present, the amount of cellular material (for example, packed cell volume, dry cell weight, etc.) and/or the amount of nitrogen (for example, nitrate, nitrite, ammonia, urea, proteins, amino acids, etc.) present can be measured. The concentration of the particular species, the total amount of the species present in the fermentor, the number of hours the fermentation has been running, and the volume of the fermentor can be considered. In various embodiments, these measurements can be compared to each other and/or they can be compared to previous measurements of the same parameter previously taken from the same fermentation or another fermentation.

Adjustments to the amount of a medium component can be accomplished such as by changing the flow rate of a stream containing that component or by changing the frequency of the additions for that component. In one embodiment, the amount of polysaccharide can be reduced when the monosaccharides level increases faster than the ethanol level increases. In another embodiment, the amount of polysaccharide can be increased when the amount or level of monosaccharides decreases while the ethanol production approximately remains steady. In another embodiment, the amount of nitrogen can be increased when the monosaccharides level increases faster than the viable cell level. The amount of polysaccharide can also be increased when the cell production increases faster than the

ethanol production. In another embodiment the amount of nitrogen can be increased when the enzyme activity level decreases.

[00163] In another embodiment, different levels or complete depletion of a medium component can effectively be used, for example to initiate different metabolic pathways or to change the yield of the different products of the fermentation process. For instance, different levels or complete depletion of a medium component can effectively be used to increase the ethanol yield and productivity, to improve carbon utilization (*e.g.*, g ethanol/g sugar fermented) and reduced acid production (*e.g.*, g acid/g ethanol and g acid/g sugar fermented). In some embodiments, different levels or complete depletion of nitrogen can effectively be used to increase the ethanol yield and productivity, to improve carbon utilization (*e.g.*, g ethanol/g sugar fermented) and reduced acid production (*e.g.*, g acid/g ethanol and g acid/g sugar fermented). In some embodiments, different levels or complete depletion of carbon can effectively be used to increase the ethanol yield and productivity, to improve carbon utilization (*e.g.*, g ethanol/g sugar fermented) and reduced acid production (*e.g.*, g acid/g ethanol and g acid/g sugar fermented). In some embodiments, the ratio of carbon level to nitrogen level for at least a portion of the fermentation time can effectively be used to increase the ethanol yield and productivity, to improve carbon utilization (*e.g.*, g ethanol/g sugar fermented) and reduced acid production (*e.g.*, g acid/g ethanol and g acid/g sugar fermented).

[00164] In another embodiment, a fed batch operation can be employed, wherein medium components and/or fresh cells are added during the fermentation without removal of a portion of the broth for harvest prior to the end of the fermentation. In one embodiment a fed-batch process is based on feeding a growth limiting nutrient medium to a culture of microorganisms. In one embodiment the feed medium is highly concentrated to avoid dilution of the bioreactor. In another embodiment the controlled addition of the nutrient directly affects the growth rate of the culture and avoids overflow metabolism such as the formation of side metabolites. In one embodiment the growth limiting nutrient is a nitrogen source or a saccharide source.

[00165] In another embodiment, a modified fed batch operation can be employed wherein a portion of the broth is harvested at discrete times. Such a modified fed batch operation can be advantageously employed when, for example, very long fermentation cycles are employed. Under very long fermentation conditions, the volume of liquid inside the fermentor increases. In order to operate for very long periods, it can be advantageous to partially empty the fermentor, for example, when the volume is nearly full. A partial harvest of broth followed by supplementation with fresh medium ingredients, such as with a fed batch operation, can improve fermentor utilization and can facilitate higher plant throughputs due to a reduction in the time for tasks such as cleaning and sterilization of equipment. When the "partial harvest" type of operation is employed, the fermentation can be seeded with the broth that remains in the fermentor, or with fresh inoculum, or with a mixture of the two. In addition, broth can be recycled for use as fresh inoculum either alone or in combination with other fresh inoculum.

[00166] In some embodiments, a fed batch operation can be employed, wherein medium components and/or fresh cells are added during the fermentation when the hydrolytic activity of the broth has decreased. In some embodiments, medium components and/or fresh cells are added during the fermentation when the hydrolytic activity of the broth has decreased about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 75%, 80%, 85%, 90%, 95%, or 100%.

[00167] While the Q microbe can be used in long or short fermentation cycles, it is particularly well-suited for long fermentation cycles and for use in fermentations with partial harvest, self-seeding, and broth recycle operations due to the anaerobic conditions of the fermentation, the presence of alcohol, the fast growth rate of the organism, and, in some embodiments, the use of a solid carbon substrate, whether or not resulting in low sugar concentrations in the broth.

[00168] In one embodiment, fed-batch fermentation is performed on the treated biomass to produce a fermentation end-product, such as alcohol, ethanol, organic acid, acetic acid, lactic acid, methane, or hydrogen. In one embodiment, the fermentation process comprises simultaneous hydrolysis and fermentation of the biomass using one or more microorganisms such as a *Clostridium strain*, a *Trichoderma strain*, a *Saccharomyces strain*, a *Zymomonas strain*, or another microorganism suitable for fermentation of biomass. In another embodiment, the fermentation process comprises simultaneous hydrolysis and fermentation of the biomass using a microorganism that is *Clostridium phytofermentans*, *Clostridium algidixylanolyticum*, *Clostridium xylanolyticum*, *Clostridium cellulovorans*, *Clostridium cellulolyticum*, *Clostridium thermocellum*, *Clostridium josui*, *Clostridium papyrosolvans*, *Clostridium cellobioparum*, *Clostridium hungatei*, *Clostridium cellulosi*, *Clostridium stercorarium*, *Clostridium termitidis*, *Clostridium thermocopriae*, *Clostridium celerecrescens*, *Clostridium polysaccharolyticum*, *Clostridium populeti*, *Clostridium lentocellum*, *Clostridium chartatabidum*, *Clostridium aldrichii*, *Clostridium herbivorans*, *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvans*, *Caldicellulosiruptor saccharolyticum*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Eubacterium cellulosolvans*, *Butyrivibrio fibrisolvens*, *Anaerocellum thermophilum*, *Halocella cellulolytica*, *Thermoanaerobacterium thermosaccharolyticum*, *Sacharophagus degradans*, or *Thermoanaerobacterium saccharolyticum*.

[00169] In another embodiment, a fermentation to produce ethanol is performed by culturing a strain of the Q microbe in a medium having a high concentration of one or more carbon sources, and/or augmenting the culture with addition of fresh cells of Q microbe during the course of the fermentation. The resulting production of ethanol can be up to 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, and in some cases up to 10-fold and higher in volumetric productivity than a batch process and achieve a carbon conversion efficiency approaching the theoretical maximum. The theoretical maximum can vary with the substrate and product. For example, the generally accepted maximum efficiency for conversion of glucose to ethanol is 0.51 g ethanol/g glucose. In one embodiment the Q microbe can produce about 40-100% of a theoretical maximum yield of ethanol. In another embodiment, the Q microbe can produce up to about 40% of the theoretical maximum yield of ethanol.

In another embodiment, the Q microbe can produce up to about 50% of the theoretical maximum yield of ethanol. In another embodiment, the Q microbe can produce about 70% of the theoretical maximum yield of ethanol. In another embodiment, the Q microbe can produce about 90% of the theoretical maximum yield of ethanol. In another embodiment, the Q microbe can produce about 95% of the theoretical maximum yield of ethanol. In another embodiment, the Q microbe can produce about 95% of the theoretical maximum yield of ethanol. In another embodiment, the Q microbe can produce about 99% of the theoretical maximum yield of ethanol. In another embodiment, the Q microbe can produce about 100% of the theoretical maximum yield of ethanol. In one embodiment a Q microbe can produce up to about 1 %, 2 %, 3 %, 4 %, 5 %, 6 %, 7 %, 8 %, 9 %, 10 %, 11 %, 12 %, 13 %, 14 %, 15 %, 16 %, 17 %, 18 %, 19 %, 20 %, 21 %, 22 %, 23 %, 24 %, 25 %, 26 %, 27 %, 28 %, 29 %, 30 %, 31 %, 32 %, 33 %, 34 %, 35 %, 36 %, 37 %, 38 %, 39 %, 40 %, 41 %, 42 %, 43 %, 44 %, 45 %, 46 %, 47 %, 48 %, 49 %, 50 %, 51 %, 52 %, 53 %, 54 %, 55 %, 56 %, 57 %, 58 %, 59 %, 60 %, 61 %, 62 %, 63 %, 64 %, 65 %, 66 %, 67 %, 68 %, 69 %, 70 %, 71 %, 72 %, 73 %, 74 %, 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, 99.99 %, or 100 % of a theoretical maximum yield of ethanol.

**[00170]** In some embodiments, fermentation can be performed in an apparatus such as bioreactor, a fermentation vessel, a stirred tank reactor, or a fluidized bed reactor. In one embodiment the treated biomass can be supplemented with suitable chemicals to facilitate robust growth of the one or more fermenting organisms. In one embodiment a useful supplement includes but is not limited to, a source of nitrogen and/or amino acids such as yeast extract, cysteine, or ammonium salts (*e.g.* nitrate, sulfate, phosphate etc.); a source of simple carbohydrates such as corn steep liquor, and malt syrup; a source of vitamins such as yeast extract; buffering agents such as salts (including but not limited to citrate salts, phosphate salts, or carbonate salts); or mineral nutrients such as salts of magnesium, calcium, or iron. In some embodiments redox modifiers are added to the fermentation mixture including but not limited to cysteine or mercaptoethanol.

**[00171]** Chemicals used in the methods of compositions and methods described herein are readily available and can be purchased from a commercial supplier, such as Sigma-Aldrich. Additionally, commercial enzyme cocktails (*e.g.* accellerase™ 1000, CelluSeb-TL, CelluSeb-TS, Cellic™ CTec, STARGEN™, Maxaliq™, Spezyme®, Distillase®, G-Zyme®, Fermentzyme®, Fermgen™, GC 212, or Optimash™) or any other commercial enzyme cocktail can be purchased from vendors such as Specialty Enzymes & Biochemicals Co., Genencor, or Novozymes. Alternatively, enzyme cocktails can be prepared by growing one or more organisms such as for example a fungi (*e.g.* a *Trichoderma*, a *Saccharomyces*, a *Pichia*, a White Rot Fungus etc.), a bacteria (*e.g.* a *Clostridium* (*e.g.* *Clostridium phytofermentans*), or a coliform bacterium, a *Zymomonas* bacterium, *Sacharophagus degradans* etc.) in a suitable medium and harvesting enzymes produced therefrom. In some embodiments, the harvesting can include one or more steps of purification of enzymes.

[00172] In one embodiment the titer and/or productivity of fermentation end-product production by a microorganism (such as *Clostridium phytofermentans*) is improved by culturing the microorganism in a medium comprising one or more compounds comprising hexose and/or pentose sugars. In one embodiment, a process comprises conversion of a starting material (such as a biomass) to a biofuel, such as one or more alcohols. In one embodiment, methods of the invention comprise contacting substrate comprising both hexose (*e.g.* glucose, cellobiose) and pentose (*e.g.* xylose, arabinose) saccharides with a microorganism that can hydrolyse C5 and C6 saccharides to produce ethanol. In another embodiment, methods of the invention comprise contacting substrate comprising both hexose (*e.g.* glucose, cellobiose) and pentose (*e.g.* xylose, arabinose) saccharides with *C. phytofermentans* to produce ethanol.

[00173] In some embodiments of compositions and methods described herein, batch fermentation with a microorganism (such as *Clostridium phytofermentans*) of a mixture of hexose and pentose saccharides using the methods of compositions and methods described herein provides uptake rates of about 0.1-8 g/L/h or more of hexose (*e.g.* glucose, cellulose, cellobiose etc.), and about 0.1-8g/L/h or more of pentose (xylose, xylan, hemicellulose etc.). In some embodiments of compositions and methods described herein, batch fermentation with a microorganism (such as *Clostridium phytofermentans*) of a mixture of hexose and pentose saccharides using the methods of compositions and methods described herein provides uptake rates of about 0.1, 0.2, 0.4, 0.5, 0.6 0.7, 0.8, 1, 2, 3, 4, 5, or 6 g/L/h or more of hexose (*e.g.* glucose, cellulose, cellobiose etc.), and about 0.1, 0.2, 0.4, 0.5, 0.6 0.7, 0.8, 1, 2, 3, 4, 5, or 6 g/L/h or more of pentose (xylose, xylan, hemicellulose etc.).

[00174] In one embodiment a method for production of ethanol produces about 10 g/l to 120 g/l in 40 hours or less. In another embodiment a method for production of ethanol produces about 10 g/l, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L, 16 g/L, 17 g/L, 18 g/L, 19 g/L, 20g/L, 21 g/L, 22 g/L, 23 g/L, 24 g/L, 25g/L, 26 g/L, 27 g/L, 28 g/L, 29 g/L, 30 g/L, 31 g/L, 32 g/L, 33 g/L, 34 g/L, 35 g/L, 36 g/L, 37 g/L, 38 g/L, 39 g/L, 40 g/L, 41 g/L, 42 g/L, 43 g/L, 44 g/L, 45 g/L, 46 g/L, 47 g/L, 48 g/L, 49 g/L, 50 g/L, 51 g/L, 52 g/L, 53 g/L, 54 g/L, 55 g/L, 56 g/L, 57 g/L, 58 g/L, 59 g/L, 60 g/L, 61 g/L, 62 g/L, 63 g/L, 64 g/L, 65 g/L, 66 g/L, 67 g/L, 68 g/L, 69 g/L, 70 g/L, 71 g/L, 72 g/L, 73 g/L, 74 g/L, 75 g/L, 76 g/L, 77 g/L, 78 g/L, 79 g/L, 80 g/L, 81 g/L, 82 g/L, 83 g/L, 84 g/L, 85 g/L, 86 g/L, 87 g/L, 88 g/L, 89 g/L, 90 g/L, 91 g/L, 92 g/L, 93 g/L, 94 g/L, 95 g/L, 96 g/L, 97 g/L, 98 g/L, 99 g/L, 100 g/L, 110 g/l, 120 g/l, or more ethanol in 40 hours by the fermentation of biomass. In another embodiment, ethanol produced by a method comprising simultaneous fermentation of hexose and pentose saccharides. In another embodiment, ethanol is produced to by a microorganism comprising simultaneous fermentation of hexose and pentose saccharides.

[00175] In another embodiment a microorganism that produces a fermentative end-product tolerates in the presence of high alcohol (*e.g.* ethanol or butanol) concentrations. In one embodiment *Clostridium phytofermentans* tolerates in the presence of high alcohol (*e.g.* ethanol or butanol) concentrations. In one embodiment the microorganism can grow and function in alcohol (*e.g.* ethanol or butanol)

concentrations up to 15% v/v. In another embodiment the microorganism can grow and function in alcohol (e.g. ethanol or butanol) concentrations of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15% v/v. In one embodiment functioning in high alcohol concentrations includes the ability to continue to produce alcohol without undue inhibition or suppression by alcohol and/or other components present. In another embodiment functioning in high alcohol concentrations includes the ability to efficiently convert hexose and pentose carbon sources in a biomass feedstock to a fermentation end-product such as an alcohol. In one embodiment *Clostridium phytofermentans* tolerates the presence of high alcohol (e.g. ethanol or butanol) concentrations.

[00176] It has been observed that an ethanol concentration in a fermentation medium comprising *Clostridium phytofermentans* attains a plateau of about 15 g/L after about 36 - 48 hours of batch fermentation, with carbon substrate remaining in the medium. In another embodiment lowering the fermentation pH to about 6.5 and/or adding unsaturated fatty acids to the fermentation medium resulted in a significant increase in the amount of ethanol produced by the organism, with between about 20 g/L to about 30, 40, 50, 60, or 70 g/L or more of ethanol observed in the medium following a 48 - 96 hrs or longer fermentation. In addition, it has also been observed that the productivity of the organism was higher (to about 10 g/L-d) when the ethanol titer was low and lower (to about 2 g/L-d) than when the ethanol concentration was higher. Fermentation at reduced pH and/or with the addition of a lipid (e.g., fatty acids) can result in about a two to ten fold (such as a 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, or 10x increase) or higher increase in the ethanol production rate as compared to the unadjusted fermentation medium. In some embodiments of compositions and methods described herein, simultaneous fermentation of both hexose and pentose saccharides can also enable increases in ethanol productivity and/or yield. In some cases, the simultaneous fermentation of hexose and pentose carbohydrate substrates can be utilized in combination with fermentation at reduced pH and/or with the addition of a lipid (e.g., fatty acids) to further increase productivity, and/or yield. In one embodiment a lipid is a fat or oil, including without limitation the glyceride esters of fatty acids along with associated phosphatides, sterols, alcohols, hydrocarbons, ketones, and related compounds. In another embodiment a lipid is a phospholipid. In one embodiment a fatty acid is an aliphatic or aromatic monocarboxylic acid. In another embodiment a fatty acid is an unsaturated fatty acid. In one embodiment an unsaturated fatty acid is a fatty acid with 1 to 3 double bonds and a "highly unsaturated fatty acid" means a fatty acid with 4 or more double bonds. In another embodiment an unsaturated fatty acid is an omega-3 highly unsaturated fatty acid, such as eicosapentaenoic acid, docosapentaenoic acid, alpha linolenic acid, docosahexaenoic acid, and conjugates thereof. In another embodiment a fatty acid is a saturated fatty acid. In another embodiment a fatty acid is a vegetable oil, such as partially hydrogenated, include palm oil, cottonseed oil, corn oil, peanut oil, palm kernel oil, babassu oil, sunflower oil, safflower oil, or mixtures thereof. In another embodiment a composition comprising a fatty acid further comprises a wax, such as beeswax, petroleum wax, rice bran wax, castor wax, microcrystalline wax, or mixtures thereof.

[00177] In another embodiment the fermentation medium comprises a chelating agent (such as the dihydrate of trisodium citrate, or EDTA). In one embodiment a chelating agent is a chemical that forms soluble, complex molecules with certain metal ions, inactivating the ions so that they do not react with other elements or ions. In one embodiment, the concentration of a chelating agent in the fermentation medium is greater than about 0.2 g/L, greater than about 0.5 g/L, or greater than about 1 g/L. In another embodiment, the concentration of a chelating agent in the fermentation medium is less than about 10 g/L, less than about 5 g/L, or less than about 2 g/L. In one embodiment a biologically acceptable chelating agent is 5-Sulfosalicylic acid dihydrate, Ammonium citrate dibasic, Ammonium oxalate monohydrate, Citric acid, Ethylenediaminetetraacetic acid, Ethylenediaminetetraacetic acid disodium salt dihydrate, L-(+)-Tartaric acid, Potassium oxalate monohydrate, Potassium sodium tartrate tetrahydrate, Sodium citrate tribasic dihydrate, Sodium L-tartrate dibasic dihydrate, Sodium oxalate, Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, Magnesium citrate tribasic nonahydrate, Ethylenediaminetetraacetic acid diammonium salt, Ethylenediaminetetraacetic acid dipotassium salt dihydrate, Potassium tetraoxalate dihydrate, Sodium tartrate dibasic dihydrate, Ethylenediaminetetraacetic acid tripotassium salt dihydrate, Ethylenediaminetetraacetic acid trisodium salt dihydrate, Ammonium tartrate dibasic, Lithium citrate tribasic tetrahydrate, Potassium citrate monobasic, Sodium bitartrate monohydrate, Sodium citrate monobasic, Ethylenediaminetetraacetic acid tetrasodium salt hydrate, *N,N*-Dimethyldecylamine *N*-oxide, *N,N*-Dimethyldodecylamine *N*-oxide, Nitrilotriacetic acid, Potassium citrate tribasic, Potassium D-tartrate monobasic, Potassium peroxydisulfate, Potassium sodium tartrate, Pyromellitic acid hydrate, Sodium tartrate dibasic solution, Citrate Concentrated Solution, Ethylenediaminetetraacetic acid disodium salt, Edetate disodium, Sodium citrate, Ethylenediaminetetra(methylenephosphonic acid), dicarboxymethylglutamic acid, ethylenediaminedisuccinic acid (EDDS), methylamine, pyocyanin, pyoverdine, enterobactin, methionine *e.g.*, phytochelatin, malic acid, nitrilotriacetic acid, oxalic acid, or desferoxamine B. In one embodiment a chelating agent is chosen based on the specificity of the metal(s) targeted for chelation and/or by the ability of the chelating agent to function in the pretreatment environment. In one embodiment, where an alkaline pH is maintained by the addition of an alkaline agent to the feedstock, the chelating agent chosen would be capable of functioning at alkaline pH. In another embodiment, where an acid pH is maintained by the addition of an acid agent to the feedstock, the chelating agent chosen would be capable of functioning at acid pH. In another embodiment, where high temperature is utilized during pretreatment, the chelating agent chosen would be capable of functioning at high temperature. In another embodiment, a fermentation medium comprises more than one chelating agent. In one embodiment one or more chelating agent is added to a fermentation medium during fermentation of a biomass with a microorganism, such as *Clostridium phytofermentans*.

[00178] In another embodiment, a fermentation to produce ethanol is performed by culturing a strain of the Q microorganism and adding fresh medium components and fresh Q microbe cells while the cells in the fermentor are growing. Medium components, such as carbon, nitrogen, and combinations of these,

can be added as disclosed herein, as well as other nutrients, including vitamins, factors, cofactors, enzymes, minerals, salts, and such, sufficient to maintain an effective level of these nutrients in the medium. The medium and Q microbe cells can be added simultaneously, or one at a time. In another embodiment, fresh Q microbe cells can be added when hydrolytic enzyme activity decreases, especially when the activity of those hydrolytic enzymes that are more sensitive to the presence of alcohol decreases. After the addition of fresh Q microbe cells, a nitrogen feed or a combination of nitrogen and carbon feed and/or other medium components can be fed, prolonging the enzymatic production or other activity of the cells. In another embodiment, the cells can be added with sufficient carbon and nitrogen to prolong the enzymatic production or other activity of the cells sufficiently until the next addition of fresh cells. In another embodiment, fresh Q microbe cells can be added when both the nitrogen level and carbon level present in the fermentor increase. In another embodiment, fresh Q microbe cells can be added when the viable cell count decreases, especially when the nitrogen level is relatively stable or increasing. In another embodiment, fresh cells can be added when a significant portion of the viable cells are in the process of sporulation, or have sporulated. Fresh Q microbe cells can be when the portion of cells in the process of sporulation or have sporulated: *e.g.*, about 2% to about 100%, about 10% to about 75%, about 20% to about 50%, or about 25% to about 30% of the cells are in the process of sporulation or have sporulated.

[00179] In other embodiments, a fermentation to produce ethanol is performed by culturing recycled cells as inoculum. A higher population density can be used to increase the production of ethanol. Appropriate levels of inoculum include utilizing less than about 0.01% (v/v) or about 0.01% to about 0.1% (v/v), about 0.1% to about 1% (v/v), about 1% to about 3% (v/v), about 3% to about 5% (v/v) or even as high as 10% (v/v) or even higher. Cell content of the inoculum can be measured in various ways, such as by optical density, microscopic analysis, packed cell volume, dry cell weight, DNA analysis, etc. Suitable levels of cells in the inoculum can be about 0.01 g/mL to about 0.05 g/mL dry cell weight (DCW), about 0.05 g/mL to about 0.1 g/mL DCW, or about 0.1 g/mL to about 0.3 g/mL DCW. The total amount of cells inoculated into a fermentation medium can be determined by relating the level of cells, such as determined by dry cell weight or other appropriate means, and the level of inoculum. In some embodiments total amounts of cells include utilizing about 0.0001 to about 0.001 g dry cells per ml broth, about 0.001 to about 0.01 g dry cells per ml broth, or about 0.01 to about 0.03 g dry cells per ml broth. In some cases total amounts higher or lower than described herein can be used. Higher ethanol titers can be achieved by such techniques as varying the amount of recycled cells; varying the number of times cells are recycled; varying a medium component level (*e.g.* carbon and nitrogen levels, separately or in a coordinated fashion), such as by the means described herein; and varying a medium component source (*e.g.* carbon and/or nitrogen source), such as is described herein. Through techniques including these, high ethanol concentrations can be achieved. In one embodiment an ethanol concentration that can be achieved by methods described herein that is about 20 g/L, 21 g/L, 22 g/L, 23 g/L, 24 g/L, 25 g/L, 26 g/L, 27 g/L, 28 g/L, 29 g/L, 30 g/L, 31 g/L, 32 g/L, 33 g/L, 34 g/L,

35 g/L, 36 g/L, 37 g/L, 38 g/L, 39 g/L, 40 g/L, 41 g/L, 42 g/L, 43 g/L, 44 g/L, 45 g/L, 46 g/L, 47 g/L, 48 g/L, 49 g/L, 50 g/L, 51 g/L, 52 g/L, 53 g/L, 54 g/L, 55 g/L, 56 g/L, 57 g/L, 58 g/L, 59 g/L, 60 g/L, 61 g/L, 62 g/L, 63 g/L, 64 g/L, 65 g/L, 66 g/L, 67 g/L, 68 g/L, 69 g/L, 70 g/L, 71 g/L, 72 g/L, 73 g/L, 74 g/L, 75 g/L, 76 g/L, 77 g/L, 78 g/L, 79 g/L, 80 g/L, 81 g/L, 82 g/L, 83 g/L, 84 g/L, 85 g/L, 86 g/L, 87 g/L, 88 g/L, 89 g/L, 90 g/L, 91 g/L, 92 g/L, 93 g/L, 94 g/L, 95 g/L, 96 g/L, 97 g/L, 98 g/L, 99 g/L, 100 g/L, 101 g/L, 102 g/L, 103 g/L, 104 g/L, 105 g/L, 106 g/L, 107 g/L, 108 g/L, 109 g/L, 110 g/L, 111 g/L, 112 g/L, 113 g/L, 114 g/L, 115 g/L, 116 g/L, 117 g/L, 118 g/L, 119 g/L, 120 g/L, 121 g/L, 122 g/L, 123 g/L, 124 g/L, 125 g/L, 126 g/L, 127 g/L, 128 g/L, 129 g/L, 130 g/L, 131 g/L, 132 g/L, 133 g/L, 134 g/L, 135 g/L, 136 g/L, 137 g/L, 138 g/L, 139 g/L, 140 g/L, 141 g/L, 142 g/L, 143 g/L, 144 g/L, 145 g/L, 146 g/L, 147 g/L, 148 g/L, 149 g/L, 150 g/L, 151 g/L, 152 g/L, 153 g/L, 154 g/L, 155 g/L, 156 g/L, 157 g/L, 158 g/L, 159 g/L, 160 g/L, 161 g/L, 162 g/L, 163 g/L, 164 g/L, 165 g/L, 166 g/L, 167 g/L, 168 g/L, 169 g/L, 170 g/L, 171 g/L, 172 g/L, 173 g/L, 174 g/L, 175 g/L, 176 g/L, 177 g/L, 178 g/L, 179 g/L, 180 g/L, or 181 g/L.

[00180] In another embodiment, a fermentation to produce ethanol is performed by culturing a strain of the Q microorganism and adding recycled Q microbe cells while the cells in the fermentor are cell expansion stage (*e.g.* seed stage) and/or the final fermentation stage of a fermentation. Without intending to be limited to any theory the results described herein indicate that the recycled cells have a tolerance of higher ethanol concentrations and the ability to grow in such an environment. Thus, such a tolerance and ability can be useful for situations such as the cell expansion stage (*e.g.* seed stage) and the final fermentation stage of a fermentation where these concentrations of ethanol are present, including ethanol production fermentations, or for the production of other products in the presence of these concentrations of ethanol.

### **Plant process**

[00181] Generally, techniques such as cell recycle and partial harvest fermentation are not frequently used in production scale operations due to various problems inherent with these techniques. For example, "culture exhaustion," where the cells simply do not provide subsequent fermentations with adequate or similar yields and/or productivity as the original or earlier fermentation is not unusual. In addition, operation with the single culture for extended times, especially when broth is being harvested and there is a risk of breaking sterility, can lead to significant problems with contamination of the culture and fermentations that it is used for. As a result, the suitability of an organism for cell recycle and/or partial harvest fermentation is not generally expected.

[00182] In some instances, a process for converting biomass material into ethanol includes pretreating the biomass material (*e.g.*, "feedstock"), hydrolyzing the pretreated feedstock to convert polysaccharides to oligosaccharides, further hydrolyzing the oligosaccharides to monosaccharides, and converting the monosaccharides to ethanol. In some instances, the feedstock can be hydrolyzed directly to monosaccharides or other saccharides that can be utilized by the fermentation organism to produce ethanol or other products. If a different final product is desired, such as hydrocarbons, hydrogen,

methane, hydroxy compounds such as alcohols (*e.g.* butanol, propanol, methanol, etc.), carbonyl compounds such as aldehydes and ketones (*e.g.* acetone, formaldehyde, 1-propanal, etc.), organic acids, derivatives of organic acids such as esters (*e.g.* wax esters, glycerides, etc.) and other functional compounds including, but not limited to, 1, 2-propanediol, 1, 3-propanediol, lactic acid, formic acid, acetic acid, succinic acid, pyruvic acid, enzymes such as cellulases, polysaccharases, lipases, proteases, ligninases, and hemicellulases, the monosaccharides can be used in the biosynthesis of that particular compound. Feedstock material that can be utilized includes, for example, woody plant matter, *e.g.* materials from trees such as poplar, non-woody plant matter, cellulosic material, lignocellulosic material, hemicellulosic material, carbohydrates, pectin, starch, inulin, fructans, glucans, corn, corn stover, sugar cane, bagasse, grasses, switchgrass, bamboo, and material derived from these. The final product can then be separated and/or purified, as indicated by the properties for the desired final product. In some instances, compounds related to sugars such as sugar alcohols or sugar acids can be utilized as well.

**[00183]** More than one of these steps can occur at any given time. For example, hydrolysis of the pretreated feedstock and hydrolysis of the oligosaccharides can occur simultaneously in a single fermentation reaction vessel. As another example, hydrolysis of a pretreated feedstock, hydrolysis of oligosaccharides and conversion of monosaccharides to ethanol can occur simultaneously in a single reaction vessel. Alternately and one or more of these can occur simultaneously to the conversion of monosaccharides to ethanol.

**[00184]** Some enzymes can directly convert polysaccharides to monosaccharides, while others can hydrolyze polysaccharides to oligosaccharides. Still other enzymes can hydrolyze oligosaccharides to monosaccharides.

**[00185]** For some conditions, enzymes present in the fermentation can be obtained separately and then added to the fermentation or they can be produced by microorganisms present in the fermentation. In other conditions, the microorganisms present in the fermentation can produce some enzymes and some enzymes can be produced separately and added to the fermentation.

**[00186]** For the overall conversion of pretreated biomass to final product to occur at high rates, generally each of the enzymes for each conversion step is present with sufficiently high activity. If one of these enzymes is missing or is present in insufficient quantities, the production rate of ethanol, or other desired product will be reduced. The production rate can also be reduced if the microorganisms responsible for the conversion of monosaccharides to product only slowly take up monosaccharides and/or have only limited capability for translocation of the monosaccharides and intermediates produced during the conversion to ethanol.

**[00187]** In one embodiment, the enzymes of the method are produced by *C. phytofermentans* itself, including a range of hydrolytic enzymes suitable for the biomass materials used in the fermentation methods. In one embodiment, *C. phytofermentans* is grown under conditions appropriate to induce and/or promote production of the enzymes needed for the saccharification of the polysaccharide

present. The production of these enzymes can occur in a separate vessel, such as a seed fermentation vessel or other fermentation vessel, or in the production fermentation vessel where ethanol production occurs. When the enzymes are produced in a separate vessel, they can, for example, be transferred to the production fermentation vessel along with the cells, or as a relatively cell free solution liquid containing the intercellular medium with the enzymes. When the enzymes are produced in a separate vessel, they can also be dried and/or purified prior to adding them to the production fermentation vessel. The conditions appropriate for production of the enzymes are frequently managed by growing the cells in a medium that includes the biomass that the cells will be expected to hydrolyze in subsequent fermentation steps. Additional medium components, such as salt supplements, growth factors, and cofactors including, but not limited to phytate, amino acids, and peptides can also assist in the production of the enzymes utilized by the microorganism in the production of the desired products. [00188] Figure 12 illustrates an example of a method for producing chemical products from biomass by first treating biomass with an acid at elevated temperature and pressure in a hydrolysis unit. The biomass can first be heated by addition of hot water or steam. The biomass can be acidified by bubbling gaseous sulfur dioxide through the biomass that is suspended in water, or by adding a strong acid, *e.g.*, sulfuric, hydrochloric, or nitric acid with or without preheating/presteaming/water addition. During the acidification, the pH is maintained at a low level, *e.g.*, below about 5. The temperature and pressure can be elevated after acid addition. In addition to the acid already in the acidification unit, optionally, a metal salt such as ferrous sulfate, ferric sulfate, ferric chloride, aluminum sulfate, aluminum chloride, magnesium sulfate, or mixtures of these can be added to aid in the hydrolysis of the biomass. The acid-impregnated biomass is fed into the hydrolysis section of the pretreatment unit. Steam is injected into the hydrolysis portion of the pretreatment unit to directly contact and heat the biomass to the desired temperature. The temperature of the biomass after steam addition is, *e.g.*, between about 130° C and 220° C. The hydrolysate is then discharged into the flash tank portion of the pretreatment unit, and is held in the tank for a period of time to further hydrolyze the biomass, *e.g.*, into oligosaccharides and monomeric sugars. Steam explosion can also be used to further break down biomass. Alternatively, the biomass can be subject to discharge through a pressure lock for any high-pressure pretreatment process. Hydrolysate is then discharged from the pretreatment reactor, with or without the addition of water, *e.g.*, at solids concentrations between about 15% and 60%.

[00189] After pretreatment, the biomass can be dewatered and/or washed with a quantity of water, *e.g.* by squeezing or by centrifugation, or by filtration using, *e.g.* a countercurrent extractor, wash press, filter press, pressure filter, a screw conveyor extractor, or a vacuum belt extractor to remove acidified fluid. The acidified fluid, with or without further treatment, *e.g.* addition of alkali (*e.g.* lime) and or ammonia (*e.g.* ammonium phosphate), can be re-used, *e.g.*, in the acidification portion of the pretreatment unit, or added to the fermentation, or collected for other use/treatment. Products can be derived from treatment of the acidified fluid, *e.g.*, gypsum or ammonium phosphate. Enzymes or a mixture of enzymes can be added during pretreatment to assist, *e.g.* endoglucanases, exoglucanases,

cellobiohydrolases (CBH), beta-glucosidases, glycoside hydrolases, glycosyltransferases, lyases, and esterases active against components of cellulose, hemicelluloses, pectin, and starch, in the hydrolysis of high molecular weight components.

[00190] The fermentor is fed with hydrolyzed biomass, any liquid fraction from biomass pretreatment, an active seed culture of *Clostridium phytofermentans* cells, if desired a co-fermenting microbe, *e.g.*, yeast or *E. coli*, and, if required, nutrients to promote growth of *Clostridium phytofermentans* or other microbes. Alternatively, the pretreated biomass or liquid fraction can be split into multiple fermentors, each containing a different strain of *Clostridium phytofermentans* and/or other microbes, and each operating under specific physical conditions. Fermentation is allowed to proceed for a period of time, *e.g.*, between about 15 and 150 hours, while maintaining a temperature of, *e.g.*, between about 25° C and 50° C. Gas produced during the fermentation is swept from fermentor and is discharged, collected, or flared with or without additional processing, *e.g.* hydrogen gas can be collected and used as a power source or purified as a co-product.

[00191] After fermentation, the contents of the fermentor are transferred to product recovery. Products are extracted, *e.g.*, ethanol is recovered through distilled and rectification.

[00192] Figure 13 depicts a method for producing chemicals from biomass by charging biomass to a consolidated bioprocessing (CBP) unit. The biomass can be allowed to soak for a period of time, with or without addition of heat, water, enzymes, or acid/alkali. The pressure in the processing vessel can be maintained at or above atmospheric pressure. Acid or alkali can be added at the end of the pretreatment period for neutralization. At the end of the pretreatment period, or at the same time as pretreatment begins, an active seed culture of a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*) and, if desired, a co-fermenting microbe, *e.g.*, yeast or *E. coli*, and, if required, nutrients to promote growth of a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*) are added. Fermentation is allowed to proceed as described above. After fermentation, the contents of the fermentor are transferred to product recovery as described above.

[00193] Any combination of the chemical production methods and/or features can be utilized to make a hybrid production method. In any of the methods described herein, products can be removed, added, or combined at any step. A C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*) can be used alone or synergistically in combination with one or more other microbes (*e.g.* yeasts, fungi, or other bacteria). In some embodiments different methods can be used within a single plant to produce different end-products.

[00194] In another aspect, the invention features a fuel plant that includes a hydrolysis unit configured to hydrolyze a biomass material that includes a high molecular weight carbohydrate, and a fermentor configured to house a medium and contains a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*) dispersed therein.

[00195] In another aspect, the invention features methods of making a fuel or fuels that include combining a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*) and a

lignocellulosic material (and/or other biomass material) in a medium, and fermenting the lignocellulosic material under conditions and for a time sufficient to produce a fuel or fuels, *e.g.*, ethanol, propanol and/or hydrogen or another chemical compound.

[00196] In some embodiments, compositions and methods described herein provides a process for producing ethanol and hydrogen from biomass using acid hydrolysis pretreatment. In some embodiments, compositions and methods described herein provides a process for producing ethanol and hydrogen from biomass using enzymatic hydrolysis pretreatment. Other embodiments provide a process for producing ethanol and hydrogen from biomass using biomass that has not been enzymatically pretreated. Still other embodiments disclose a process for producing ethanol and hydrogen from biomass using biomass that has not been chemically or enzymatically pretreated, but is optionally steam treated.

[00197] Figure 14 discloses pretreatments that produce hexose or pentose saccharides or oligomers that are then unprocessed or processed further and either, fermented separately or together. Figure 14A depicts a process (*e.g.*, acid pretreatment) that produces a solids phase and a liquid phase which are then fermented separately. Figure 14B depicts a similar pretreatment that produces a solids phase and liquids phase. The liquids phase is separated from the solids and elements that are toxic to the fermenting microorganism are removed prior to fermentation. At initiation of fermentation, the two phases are recombined and cofermented together. This is a more cost-effective process than fermenting the phases separately. The third process (Figure 14C) is the least costly. The pretreatment results in a slurry of liquids or solids that are then cofermented. There is little loss of saccharides component and minimal equipment required.

### **Recovery of Ethanol or Other Fermentive End Products**

[00198] Further provided herein is a method of producing ethanol, comprising the steps of treating a feedstock with a substance which increases the pH to an alkaline level, contacting the feedstock with a microorganism; growing the microorganism under conditions which allow for uptake of a five-carbon sugar and a six-carbon sugar by the microorganism; and fermenting the five-carbon sugar and the six-carbon sugar, thereby producing the ethanol. In some instances, the five-carbon sugar is xylose, arabinose, or a combination thereof. In other instances, the six-carbon sugar is glucose, galactose, mannose, or a combination thereof. In some embodiments the alkaline substance is NaOH. In some embodiments, NaOH is added at a concentration of about 0.5% to about 2% by weight of the feedstock. Treating of the feedstock can be performed at a temperature of from about 80<sup>0</sup>C to about 120<sup>0</sup>C. In some embodiments, the microorganism is a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans*. In some embodiments, the microorganism is genetically modified to express or increase expression of an enzyme capable of fermenting the five-carbon sugar or the six-carbon sugar, a transporter capable of transporting the five-carbon sugar or said six-carbon sugar, or a combination thereof.

[00199] Also provided herein is a method of producing ethanol, comprising the steps of treating a feedstock with a substance which increases the pH to an alkaline level; contacting said feedstock with a microorganism, wherein said microorganism is capable of both hydrolyzing and fermenting said feedstock; and growing said microorganism under conditions which results in the production of one or more enzymes for hydrolyzing and fermenting said feedstock, thereby producing said ethanol. In some instances, the enzyme for hydrolyzing the feedstock is cellulase, hemicellulase, amylase, protease, chitinase, pectinase, keratinase or a combination thereof. In other instances, the feedstock comprises cellulose, starch, xylan, pectin, chitin, keratin, or a combination thereof. In some embodiments the alkaline substance is NaOH. In some embodiments, NaOH is added at a concentration of about 0.5% to about 2% by weight of the feedstock. Treating of the feedstock can be performed at a temperature of from about 80<sup>0</sup>C to about 120<sup>0</sup>C. In some embodiments, the microorganism is a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans*. In some embodiments, the microorganism is genetically modified to express or increase expression of an enzyme capable of hydrolyzing or fermenting the feedstock, a transporter capable of transporting a substrate for hydrolysis or fermentation, or a combination thereof.

[00200] Another embodiment provided by the present disclosure is a method of producing ethanol, comprising the steps of treating a feedstock with a substance which increases the pH to an alkaline level; contacting the feedstock with a microorganism, wherein the microorganism is capable of uptaking and processing oligosaccharides; and growing the microorganism under conditions which result in the production of one or more enzymes for hydrolyzing and fermenting the oligosaccharides, thereby producing said ethanol. In some instances, the oligosaccharide comprises glucose monomers, xylose monomers, or a combination thereof. In some embodiments the alkaline substance is NaOH. In some embodiments, NaOH is added at a concentration of about 0.5% to about 2% by weight of the feedstock. Treating of the feedstock can be performed at a temperature of from about 80<sup>0</sup>C to about 120<sup>0</sup>C. In some embodiments, the microorganism is a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans*.

[00201] A method of producing ethanol at a rate of from about 4 g/L/day to about 12 g/L/day in a fermentation reaction comprising: contacting a feedstock treated with a substance which increases the pH to an alkaline level, with a microorganism capable of both hydrolyzing and fermenting said feedstock, thereby producing said ethanol at a rate of from about 4 g/L/day to about 12 g/L/day. In some embodiments, the feedstock comprises cellulose, starch, xylan, pectin, chitin, keratin, or a combination thereof. In other embodiments, the feedstock is a combination of two or more of corn stover, bagasse, poplar, or switchgrass. In some embodiments the alkaline substance is NaOH. In some embodiments, NaOH is added at a concentration of about 0.5% to about 2% by weight of the feedstock. Treating of the feedstock can be performed at a temperature of from about 80<sup>0</sup>C to about 120<sup>0</sup>C. In some embodiments, the microorganism is a bacterium, such as a member of the genus *Clostridium*, for example *Clostridium phytofermentans*.

[00202] In another aspect of the invention, methods are provided for the recovery of the biofuels, *e.g.*, ethanol, from the fermentation. In one embodiment, broth will be harvested at some point during of the fermentation, and the final desired product or products will be recovered. The broth with ethanol to be recovered will include both ethanol and impurities. The impurities include materials such as water, cell bodies, cellular debris, excess carbon substrate, excess nitrogen substrate, other remaining nutrients, non-ethanol metabolites, and other medium components or digested medium components. During the course of processing the broth, the broth can be heated and/or reacted with various reagents, resulting in additional impurities in the broth.

[00203] In one embodiment, the processing steps to recover ethanol include several separation steps, including, for example, distillation of a high concentration ethanol material from a less pure ethanol-containing material. In other embodiments, the high concentration ethanol material can be further concentrated to achieve very high concentration ethanol, such as 98% or 99% or 99.5% (wt.) or even higher. Other separation steps, such as filtration, centrifugation, extraction, adsorption, etc. can also be a part of some recovery processes for ethanol or other biofuels.

[00204] Embodiments described herein can be scaled to commercially useful biofuels operations. Several of the embodiments described herein describe use of microorganisms (*e.g.*, *C. phytofermentans*) for biofuels production such as alcohols, *e.g.*, ethanol, butanol, propanol, methanol, and fuels such as hydrocarbons, hydrogen, methane, and hydroxy compounds. Other embodiments relate to the use of microorganisms (*e.g.*, *C. phytofermentans*) for the production of other products such as carbonyl compounds such as aldehydes and ketones (*e.g.* acetone, formaldehyde, 1-propanal, etc.), organic acids, derivatives of organic acids such as esters (*e.g.* wax esters, glycerides, etc.) and other functional compounds including, but not limited to, 1, 2-propanediol, 1, 3-propanediol, lactic acid, formic acid, acetic acid, succinic acid, pyruvic acid, enzymes such as cellulases, polysaccharases, lipases, proteases, ligninases, and hemicellulases.

[00205] Additional methods and compositions for treatment of biomass, pretreatment of biomass, enzymatic treatment of biomass, or preparation of biomass for fermentation or conversion to useful end-products are provided by US Patent Application Nos. 20090053770, 2007003 1918, 2007003 1953, 20090053777, 20090042259, 20090042266, 20090004698, 20090004692, 20090004706, 20090011474, 2009001 1484, 20080227161, 20080227162, 20080044877, 20080182323, 20070148751, 20060246563, and US Patent Nos. 5865898, 5628830, 5693296, 5837506, and 6090595 each of which are herein incorporated by reference in their entirety. Enzyme treatment of biomass results in degradation of high molecular weight carbohydrate polymers into smaller oligosaccharides and in some cases, eventually, monomeric hexose and pentose sugars. In the second step these sugars are fermented to an end-product (*e.g.* alcohol, ethanol, organic acid, acetic acid, lactic acid, methane, or hydrogen) using, for example, yeast or bacterial strains.

[00206] In other embodiments, the hydrolysis and fermentation process can be combined into a single step. In some cases, this can provide a process that is more economical than a two step process by

reducing capital and operational costs, for example, by minimizing the need for external enzyme treatment. In still other embodiments, conversion of both hexose and pentose sugars to end-products can provide enhanced yields of end-products per gram of biomass as compared to conversion of only hexoses or only pentoses, or as compared to processes which mainly convert pentoses but do not substantially convert hexoses or mainly convert hexoses but do not substantially convert pentoses. Commonly used species of yeast (*Saccharomyces cerevisiae*), fungi and bacteria have been reported to be able to readily convert hexose sugar (glucose) to ethanol. However, fermentation of pentose sugars (xylose and arabinose) is still a technological bottleneck for ethanol production from biomass. Some of the researchers have used genetic tools to obtain recombinants of *Zymomonas*, *E. coli*, *Saccharomyces* and other yeasts.

[00207] In one embodiment *Clostridium phytofermentans* or another *Clostridium* species (such as *Clostridium* sp. Q.D.) preferentially ferments oligomers instead of monosaccharides. This metabolic trait can be utilized to reduce the time and severity of pretreatment of biomass. For example, a less severe acid treatment resulting in the release of oligomers rather than monosaccharides reduces the time and cost of chemicals of the pretreatment process. This can result in lower overall cost of producing a fermentation end-product. Fewer sugars are degraded during such a process thus adding to the higher saccharide content of the biomass and an increased yield of ethanol or other chemical product.

[00208] In one embodiment a biomass feedstock is pretreated under conditions of low severity to produce a feedstock enriched in oligosaccharides as compared to monomer saccharides. In another embodiment a biomass feedstock is pretreated under conditions of low severity to produce a pretreated feedstock that comprises more oligosaccharides as compared to monomer saccharides. In one embodiment the pretreated feedstock comprises more available five carbon sugars than six carbon sugars.

[00209] In one embodiment low severity pretreatment comprises treating a biomass feedstock with an acid and heat for a first period of time. In one embodiment the acid is dilute. In one embodiment the low severity conditions comprises dilute acid hydrolysis. In one embodiment the acid is at a concentration of 0.01 to 5% by volume (e.g. 1 ml of 100% glacial acetic acid in 100 ml of a diluent is a 1% acetic acid solution). In another embodiment the dilute acid is at 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, or 6% by volume. In one embodiment the pH of the acid is from about 4.5 to 6.9, or about 5 to 6.5 or about 5.5 to 6, or about 6 to 6.9. In another embodiment the pH of the acid is about 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, or 6.9. In one embodiment the first period of time is for 1 minute to 4 hrs or 10 minutes to 6 hours or 30 minutes to 12 hours. In another embodiment the first period of time is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,

24, 25, 26, 27, 28, 29 30, 31, 32 33, 34 35, 36 37 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 minutes. In another embodiment the first period of time is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 hrs. In another embodiment the biomass feedstock is heated at about 80 to 200<sup>0</sup>C, or 100<sup>0</sup>C to 180<sup>0</sup>C, or 120<sup>0</sup>C to 170<sup>0</sup>C or 130<sup>0</sup>C to 150<sup>0</sup>C or 140<sup>0</sup>C. In another embodiment the biomass feedstock is heated at about 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, or 200<sup>0</sup>C.

[00210] In another embodiment low severity pretreatment comprises treating a biomass feedstock with an acid and heat for a first period of time and treating the biomass feedstock with heat for a second period of time. In another embodiment the pretreated feedstock comprises more oligosaccharides as compared to monomer saccharides. In one embodiment the pretreated feedstock comprises more available five carbon sugars than six carbon sugars. In one embodiment the second period of time is for 1 minute to 4 hrs or 10 minutes to 6 hours or 30 minutes to 12 hours. In another embodiment the second period oftime is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 30, 31, 32 33, 34 35, 36 37 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 minutes. In another embodiment the second period oftime is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 hrs. In another embodiment the biomass feedstock is heated for a second period oftime at about 80 to 200<sup>0</sup>C, or 100<sup>0</sup>C to 180<sup>0</sup>C, or 120<sup>0</sup>C to 170<sup>0</sup>C or 130<sup>0</sup>C to 150<sup>0</sup>C or 140<sup>0</sup>C. In another embodiment the biomass feedstock is heated for a second period oftime at about 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, or 200<sup>0</sup>C.

[00211] In another embodiment a biomass feedstock is pretreated with a dilute sulfuric acid solution (e.g. 0.5% by volume) for about thirty minutes at about 140C. Further, the biomass feedstock is heated at 190C for about an hour and a half. The biomass feedstock is then neutralized with NaOH and used as a carbon source for a microorganism to ferment and saccharify to produce a fermentation end-product such as ethanol. In one embodiment the pretreated feedstock that comprises more oligosaccharides than monomer saccharides. In another embodiment the pretreated feedstock comprises more available five carbon sugars than six carbon sugars.

[00212] In another embodiment a feedstock that is pretreated under low severity conditions has an acidic pH. In one embodiment the feedstock has its pH neutralized by the addition of a base. In one embodiment the base is sodium hydroxide or ammonium hydroxide. In another embodiment the feedstock is not detoxified prior to incubation with a microorganism that produces a fermentation end-product. In another embodiment the feedstock is detoxified prior to incubation with a microorganism that produces a fermentation end-product. In another embodiment the feedstock is neutralized but not detoxified prior to incubation with a microorganism that produces a fermentation end-product.

[00213] In another embodiment a pretreated biomass feedstock that comprises more oligosaccharides than monosaccharides is further pre-treated to convert the oligosaccharides to monosaccharides. In one embodiment the further pre-treatment comprises incubation with acid or one or more enzymes for a period of time. In another embodiment the further pre-treatment comprises heat. In one embodiment the further pre-treatment converts substantially all of the oligosaccharides to monosaccharides.

[00214] In some embodiments, the methods of compositions and methods described herein provide for a fermentation process, such as for example a continuous fermentation process, a batch fermentation process, or a fed-batch fermentation process (*e.g.* constant or variable volume). In some embodiments, the methods provide for the fermentation of biomass with a microorganism, such as *Clostridium phytofermentans* or another *Clostridium* species. In some cases, a fed-batch fermentation process is provided for ethanol production from biomass (*e.g.* corn stover or any biomass provided herein) using a microorganism, such as *Clostridium phytofermentans* or another *Clostridium* species. In one embodiment a method provides for titers of 5 to 200 g/L of ethanol with a production rate of about 0.5 to 20 g/L/d. In another embodiment a method provides for an ethanol yield of about 0.1- 1 grams ethanol per gram of biomass loaded in the fermentor. In some embodiments, a method provides for yields of about 45% -99.5%, or more of the theoretical maximum possible yield of fermentation end product (*e.g.* alcohol, ethanol, organic acid, acetic acid, lactic acid, methane, or hydrogen).

[00215] In one embodiment a method provides for titers of at least about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 4, 142, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 160, 170, 180, 190, or 200 g/L or more of ethanol with a production rate of about 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 g/L/d or more. In another embodiment a method provides for an ethanol yield of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1 g or more ethanol per g of biomass loaded in the fermentor. In some embodiments, a method provides for yields of at least about 50%, 60%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or more of the theoretical maximum possible yield of fermentation end product (*e.g.* alcohol, ethanol, organic acid, acetic acid, lactic acid, methane, or hydrogen).

[00216] In another embodiment a biomass is pre-treated with a surfactant prior to fermentation with a microorganism. In another embodiment a biomass is contacted with a surfactant during fermentation with a microorganism. In one embodiment the surfactant is a Tween series of surfactant (*e.g.*, Tween 20 or Tween 80) or a Triton series of surfactant (*e.g.* Triton X-100). In another embodiment the surfactant is polysorbate 60, polysorbate 80, propylene glycol, sodium dioctylsulfo succinate, sodium lauryl sulfate, lactic esters of fatty acids, polyglycerol esters of fatty acids, or mixtures thereof. In another embodiment a biomass is pre-treated with a surfactant and a lipid prior to fermentation with a microorganism. In another embodiment a biomass is contacted with a surfactant and a lipid during fermentation with a microorganism.

[00217] In some embodiments aerobic/anaerobic cycling is used for the bioconversion of cellulosic or lignocellulosic material to fuels and chemicals. In some embodiments, the anaerobic microorganism can ferment biomass directly without the need of a pretreatment. In some embodiments, feedstocks are contacted with biocatalysts capable of breaking down plant-derived polymeric material into lower molecular weight products that can subsequently be transformed by biocatalysts to fuels and/or other desirable chemicals.

[00218] In one embodiment hydrolysis of the pretreated feedstock and hydrolysis of the oligosaccharides by a microorganism occurs simultaneously in a single fermentation reaction vessel. In one embodiment the microorganism is *Clostridium phytofermentans*. In another embodiment, hydrolysis of a pretreated feedstock, hydrolysis of oligosaccharides and conversion of monosaccharides to ethanol can occur simultaneously in a single reaction vessel. In one embodiment a single microorganism performs both of the hydrolysis and the conversion. In one embodiment the microorganism is *Clostridium phytofermentans*. In another embodiment a first and second species of microorganisms perform the hydrolysis and the conversion steps.

[00219] In one embodiment the process comprises treating the biomass in a closed container with a microorganism that can saccharify C5/C6 saccharides. In another embodiment the process comprises treating the biomass in a closed container with *Clostridium phytofermentans* bacterium or another *Clostridium* species under conditions wherein the *Clostridium phytofermentans* or other microorganism produces saccharolytic enzymes sufficient to substantially convert the biomass into monosaccharides and disaccharides. In another embodiment, the process comprises treating the biomass in a container with a microorganism that can saccharify C5/C6 saccharides and adding one or more enzymes to aid in the breakdown or detoxification of carbohydrates or lignocellulosic material. In another embodiment, the process comprises treating the biomass in a container with a *Clostridium phytofermentans* or another similar C5/C6 *Clostridium* species and adding one or more enzymes to aid in the breakdown or detoxification of carbohydrates or lignocellulosic material. In some embodiments, the culture can then be contacted after fermentation with a first microorganism (such as *Clostridium phytofermentans*) with a second microorganism where the second organism is capable of substantially converting the monosaccharides and disaccharides into a desired fermentation end-product, such as a fuel (*e.g.* ethanol

or butanol). In one embodiment the second microorganisms is a fungi. In another embodiment the second microorganism is yeast. In another embodiment the second microorganism is *Saccharomyces bayanus*, *Saccharomyces boulardii*, *Saccharomyces bulderi*, *Saccharomyces cariocanus*, *Saccharomyces cariocus*, *Saccharomyces cerevisiae*, *Saccharomyces chevalieri*, *Saccharomyces dairenensis*, *Saccharomyces ellipsoideus*, *Saccharomyces martiniae*, *Saccharomyces monacensis*, *Saccharomyces norbensis*, *Saccharomyces paradoxus*, *Saccharomyces pastorianus*, *Saccharomyces spencerorum*, *Saccharomyces turicensis*, *Saccharomyces unisporus*, *Saccharomyces uvarum*, *Saccharomyces zonatus*. In another embodiment the second microorganism is *Saccharomyces* or *Candidia*. In another embodiment the second microorganism is a *Clostridia* species such as *C. thermocellum*, *C. acetobutylicum*, and *C. cellovorans*, or *Zymomonas mobilis*.

**[00220]** In some embodiments, a process is provided for producing a biofuel or other chemical from a lignin-containing biomass. The process comprises: 1) contacting the lignin-containing biomass with an aqueous alkaline solution at a concentration sufficient to hydrolyze at least a portion of the lignin-containing biomass; 2) neutralizing the treated biomass to a pH between 5 to 9 (e.g. 5.5, 6, 6.5, 7, 7.5, 8, 8.5, or 9); 3) treating the biomass in a closed container with a *Clostridium phytofermentans* or another similar C5/C6 *Clostridium* species bacterium under conditions wherein the *Clostridium phytofermentans*, optionally with the addition of one or more enzymes to the container, substantially converts the treated biomass into monosaccharides and disaccharides, and/or biofuel or other fermentation end-product; and 4) optionally, introducing a culture of a second microorganism wherein the second organism is capable of substantially converting the monosaccharides and disaccharides into a fermentation end-product, such as a biofuel.

**[00221]** In one aspect, provided herein is a fuel plant that includes a hydrolysis unit configured to hydrolyze a biomass material that includes a high molecular weight carbohydrate, and a fermentor configured to house a medium and contains microorganisms dispersed therein. In one embodiment the microorganism is *Clostridium phytofermentans*.

**[00222]** In another aspect, provided herein are methods of making a fuel or chemical end product that includes combining a microorganism (such as *Clostridium phytofermentans* cells or a similar C5/C6 *Clostridium* species) and a lignocellulosic material (and/or other biomass material) in a medium, and fermenting the lignocellulosic material under conditions and for a time sufficient to produce a fermentation end-product, such as a fuel (e.g., ethanol, propanol, methane or hydrogen).

**[00223]** In some embodiments, a process is provided for producing a fermentation end-product (such as ethanol or hydrogen) from biomass using acid hydrolysis pretreatment. In some embodiments, a process is provided for producing a fermentation end-product (such as ethanol or hydrogen) from biomass using enzymatic hydrolysis pretreatment. In another embodiment a process is provided for producing a fermentation end-product (such as ethanol or hydrogen) from biomass using biomass that has not been enzymatically pretreated. In another embodiment a process is provided for producing a fermentation end-product (such as ethanol or hydrogen) from biomass using biomass that has not been chemically or

enzymatically pretreated, but is optionally steam treated. In another aspect, provided herein are end-products made by any of the processes described herein.

[00224] Those skilled in the art will appreciate that a number of modifications can be made to the methods exemplified herein. For example, a variety of promoters can be utilized to drive expression of the heterologous genes in a recombinant microorganism (such as *Clostridium phytofermentans*). The skilled artisan, having the benefit of the instant disclosure, will be able to readily choose and utilize any one of the various promoters available for this purpose. Similarly, skilled artisans, as a matter of routine preference, can utilize a higher copy number plasmid. In another embodiment, constructs can be prepared for chromosomal integration of the desired genes. Chromosomal integration of foreign genes can offer several advantages over plasmid-based constructions, the latter having certain limitations for commercial processes. Ethanogenic genes have been integrated chromosomally in *E. coli* B; see Ohta et al. (1991) Appl. Environ. Microbiol. 57:893-900. In general, this is accomplished by purification of a DNA fragment containing (1) the desired genes upstream from an antibiotic resistance gene and (2) a fragment of homologous DNA from the target organism. This DNA can be ligated to form circles without replicons and used for transformation. Thus, the gene of interest can be introduced in a heterologous host such as *E. coli*, and short, random fragments can be isolated and ligated in *Clostridium phytofermentans* to promote homologous recombination.

[00225] Large Scale Fermentation End-Product Production from Biomass

[00226] In one aspect a fermentation end-product (*e.g.*, ethanol) from biomass is produced on a large scale utilizing a microorganism, such as *C. phytofermentans*. In one embodiment, one first hydrolyzes a biomass material that includes high molecular weight carbohydrates to lower molecular weight carbohydrates, and then ferments the lower molecular weight carbohydrates utilizing of microbial cells to produce ethanol. In another embodiment, one ferments the biomass material itself without chemical and/or enzymatic pretreatment. In the first method, hydrolysis can be accomplished using acids, *e.g.*, Bronsted acids (*e.g.*, sulfuric or hydrochloric acid), bases, *e.g.*, sodium hydroxide, hydrothermal processes, steam explosion, ammonia fiber explosion processes ("AFEX"), lime processes, enzymes, or combination of these. Hydrogen, and other products of the fermentation can be captured and purified if desired, or disposed of, *e.g.*, by burning. For example, the hydrogen gas can be flared, or used as an energy source in the process, *e.g.*, to drive a steam boiler, *e.g.*, by burning. Hydrolysis and/or steam treatment of the biomass can, *e.g.*, increase porosity and/or surface area of the biomass, often leaving the cellulosic materials more exposed to the microbial cells, which can increase fermentation rate and yield. Removal of lignin can, *e.g.*, provide a combustible fuel for driving a boiler, and can also, *e.g.*, increase porosity and/or surface area of the biomass, often increasing fermentation rate and yield. In some embodiments, the initial concentration of the carbohydrates in the medium is greater than 20 mM, *e.g.*, greater than 30 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, or even greater than 500 mM.

[00227] In one aspect, the invention features a fuel plant that includes a hydrolysis unit configured to hydrolyze a biomass material that includes a high molecular weight carbohydrate, a fermentor

configured to house a medium with a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*) dispersed therein, and one or more product recovery system(s) to isolate an end-product or end-products and associated by-products and co-products.

[00228] In another aspect, the invention features methods of making an end-product or end-products that include combining a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*) and a biomass feed in a medium, and fermenting the biomass material under conditions and for a time sufficient to produce a biofuel, chemical product or fermentation end-products (*e.g.* ethanol, propanol, hydrogen, lignin, terpenoids, and the like).

[00229] Generally, there are two basic approaches to producing one or more fermentation end-products from biomass on a large scale utilizing a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*). In all methods, depending on the type of biomass and its physical manifestation, one of the processes can comprise a milling of the carbonaceous material, via wet or dry milling, to reduce the material in size and increase the surface to volume ratio (physical modification).

[00230] In one embodiment, a biomass material comprising includes high molecular weight carbohydrates is hydrolyzed to delignify it or to separate the carbohydrate compounds from noncarbohydrate compounds. Using a combination of heat, chemical, and/or enzymatic treatment, the hydrolyzed material can be separated to form liquid and dewatered streams, which can be separately treated and kept separate or recombined, and then ferments the lower molecular weight carbohydrates utilizing a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*) to produce one or more chemical products. In the second method, one ferments the biomass material itself without heat, chemical, and/or enzymatic pretreatment. In the first method, hydrolysis can be accomplished using acids (*e.g.* sulfuric or hydrochloric acids), bases (*e.g.* sodium hydroxide), hydrothermal processes, AFEX, lime processes, enzymes, or combination of these. Hydrolysis and/or steam treatment of the biomass can, *e.g.*, increase porosity and/or surface area of the biomass, often leaving the cellulosic materials more exposed to a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans*), which can increase fermentation rate and yield. Hydrolysis and/or steam treatment of the biomass can, *e.g.*, produce by-products or co-products which can be separated or treated to improve fermentation rate and yield, or used to produce power to run the process, or used as products with or without further processing. Removal of lignin can, *e.g.*, provide a combustible fuel for driving a boiler. Gaseous (*e.g.*, methane, hydrogen or CO<sub>2</sub>), liquid (*e.g.* ethanol and organic acids), or solid (*e.g.* lignin), products of the fermentation can be captured and purified if desired, or disposed of, *e.g.*, by burning. For example, the hydrogen gas can be flared, or used as an energy source in the process, *e.g.*, to drive a steam boiler, *e.g.*, by burning. Products exiting the fermentor can be further processed, *e.g.* ethanol can be transferred to distillation and rectification, producing a concentrated ethanol mixture or solids can be separated for use to provide energy or as chemical products.

[00231] In some embodiments, the treatment includes a step of treatment with acid. In some embodiments, the acid is dilute. In some embodiments, the acid treatment is carried out at elevated

temperatures of between about 85 and 140<sup>0</sup>C. In some embodiments, the method further comprises the recovery of the acid treated biomass solids, for example by use of a sieve. In some embodiments, the sieve comprises openings of approximately 150-250 microns in diameter. In some embodiments, the method further comprises washing the acid treated biomass with water or other solvents. In some embodiments, the method further comprises neutralizing the acid with alkali. In some embodiments, the method further comprises drying the acid treated biomass. In some embodiments, the drying step is carried out at elevated temperatures between about 15-45<sup>0</sup>C. In some embodiments, the liquid portion of the separated material is further treated to remove toxic materials. In some embodiments, the liquid portion is separated from the solid and then fermented separately. In some embodiments, a slurry of solids and liquids are formed from acid treatment and then fermented together.

[00232] Any combination of the chemical production methods and/or features can be utilized to make a hybrid production method. In any of the methods described herein, products can be removed, added, or combined at any step. A C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans* or *Clostridium* sp. Q.D.) can be used alone or synergistically in combination with one or more other microbes (*e.g.* yeasts, fungi, or other bacteria). In some embodiments different methods can be used within a single plant to produce different end-products.

[00233] In another aspect, the invention features a fuel plant that includes a hydrolysis unit configured to hydrolyze a biomass material that includes a high molecular weight carbohydrate, and a fermentor configured to house a medium and contains a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans* or *Clostridium* sp. Q.D) dispersed therein.

[00234] In another aspect, the invention features methods of making a fuel or fuels that include combining a C5/C6 hydrolyzing microorganism (*e.g.*, *Clostridium phytofermentans* or *Clostridium* sp. Q.D) and a lignocellulosic material (and/or other biomass material) in a medium, and fermenting the lignocellulosic material under conditions and for a time sufficient to produce a fuel or fuels, *e.g.*, ethanol, propanol and/or hydrogen or another chemical compound.

[00235] In some embodiments, compositions and methods described herein provides a process for producing ethanol and hydrogen from biomass using acid hydrolysis pretreatment. In some embodiments, compositions and methods described herein provides a process for producing ethanol and hydrogen from biomass using enzymatic hydrolysis pretreatment. Other embodiments provide a process for producing ethanol and hydrogen from biomass using biomass that has not been enzymatically pretreated. Still other embodiments disclose a process for producing ethanol and hydrogen from biomass using biomass that has not been chemically or enzymatically pretreated, but is optionally steam treated.

[00236] In another aspect of the invention, methods are provided for the recovery of the fermentive end products, such as an alcohol (*e.g.* ethanol, propanol, methanol, butanol, etc.) another biofuel or chemical product.

[00237] In one embodiment a process can be scaled to produce commercially useful biofuels. In another embodiment the Q microbe is used to produce an alcohol, *e.g.*, ethanol, butanol, propanol, methanol, or a fuel such as hydrocarbons hydrogen, methane, and hydroxy compounds. In another embodiment the Q microbe is used to produce a carbonyl compound such as an aldehyde or ketone (*e.g.* acetone, formaldehyde, 1-propanal, etc.), an organic acid, a derivative of an organic acid such as an ester (*e.g.* wax ester, glyceride, etc.), 1, 2-propanediol, 1, 3-propanediol, lactic acid, formic acid, acetic acid, succinic acid, pyruvic acid, or an enzyme such as a cellulase, polysaccharase, lipases, protease, ligninase, and hemicellulase.

[00238] While preferred embodiments of compositions and methods described herein have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

## EXAMPLES

### **Example 1. Ethanol Production by *Clostridium phytofermentans* at Reduced pH.**

[00239] Three bioreactors were filled with 300 mL media containing 20 g/L cellobiose, 1.5 g/L  $\text{KH}_2\text{PO}_4$ , 2.9 g/L  $\text{K}_2\text{HPO}_4$ , 4.6 g/L ammonium sulfate, 2 g/L cysteine-HCl, 3 g/L sodium citrate, 1 g/L  $\text{MgCl}_2 \cdot \text{OH}_2\text{O}$ , 0.15 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.00125 g/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 6 g/L yeast extract (Bacto). The fermentors were operated under fed-batch mode by continuously feeding concentrated media containing 200 g/L cellobiose at 1.4 mL/h. The bioreactor 1, 2 and 3 were operated at controlled pH of 7.5, 7 and 6.5, respectively.

[00240] The fermenters were monitored for ethanol concentration throughout the fermentation. The results are shown in Table 3 and Figure 1. These results show that fermentation at pH less than 7.5 results in an increase in the concentration of ethanol and an increase in the productivity of ethanol.

[00241] Table 3. Ethanol Concentration for Fermentation at Different pHs.

|         | BR1    | BR2  | BR3    |
|---------|--------|------|--------|
| time, h | pH 7.5 | pH 7 | pH 6.5 |
| 0       | 0.04   | 0.00 | 0.17   |
| 20.5    | 2.68   | 4.19 | 4.22   |
| 48.5    | 6.15   | 9.80 | 10.7   |
| 68.5    | 9.00   | 13.0 | 13.5   |

|       |      |      |      |
|-------|------|------|------|
| 92.5  | 11.9 | 15.3 | 15.3 |
| 116.5 | 11.6 | 15.4 | 15.3 |
| 144.5 | 11.5 | 13.5 | 16.1 |
| 175.5 | 11.8 | 15.6 | 16.4 |

**Example 2. Genetic modification of *Clostridium phytofermentans* to produce increased biofuels, including ethanol.**

[00242] Plasmids suitable for use in *C. phytofermentans* were constructed using portions of plasmids obtained from bacterial culture collections. Plasmid pIMPI is a non-conjugal plasmid that can replicate in *E. coli* as well as a range of gram positive bacterial species and it encodes for resistance to erythromycin. *C. phytofermentans* is highly sensitive to erythromycin being unable to grow at concentrations of 0.5 micrograms of erythromycin per ml of microbial growth media. The broad host range conjugal plasmid RK2 contains all of the genes needed for a bacterial conjugation system which include: an origin of replication specific to the DNA polymerase of the conjugation system, conjugal DNA replication genes, and genes encoding for the synthesis of pili to enable the recognition of potential recipient bacterial cells and to serve as the conduit through which single stranded plasmid DNA is transferred by cell-to-cell contact from donor to recipient cells. We obtained the origin of transfer for the RK2 conjugal system from plasmid pRK290 which was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) as DSM 3928, and the other conjugation functions of RK2 were obtained from pRK2013 which was obtained from DSMZ as DSM 5599. The polymerase chain reaction was used to amplify the 112 basepair origin of transfer region (oriT) from pRK290 using primers that added Cla I restriction sites flanking the oriT region. This DNA fragment was inserted into the Cla I site of pIMPI to yield plasmid pIMPT. pIMPT was shown to be able to be transferred from one strain of *E. coli* to another when pRK2013 was also present to supply other conjugation functions. However, pIMPT could not be demonstrated to be conjugally transferred - from *E. coli* to *C. phytofermentans*. We reasoned that the promoter driving the expression of the erythromycin resistance gene in pIMPT may not function in *C. phytofermentans* so we used PCR to amplify the promoter of the alcohol dehydrogenase gene *C. phytofermentans* 1029 from the *C. phytofermentans* chromosome and it was used to replace the promoter of the erythromycin gene in pIMPT to create pIMPT1029. When pRK2013 is also present to supply other conjugation functions we demonstrated that pIMPT1029 could be conjugally transferred from *E. coli* to *C. phytofermentans*. Successful transfer of plasmid DNA into *C. phytofermentans* was demonstrated by virtue of the ability of the *C. phytofermentans* derivative containing pIMPT1029 to grow on media containing up to 10 micrograms per ml erythromycin and by use of PCR primers to specifically amplify two genetic regions specific to pIMPT1029 from the *C. phytofermentans* derivative but not from a control *C. phytofermentans* culture that does not contain the plasmid.

[00243] The method of accomplishing conjugal transfer of pIMPT1029 from *E. coli* to *C. phytofermentans* consists of first constructing an *E. coli* strain (DH5alpha) that contains both pIMPT1029 and pRK2013. Then fresh cells of this *E. coli* culture and fresh cells of the *C. phytofermentans* recipient culture are obtained by growth to mid-log phase using appropriate growth media (L broth and QMI media respectively). The two bacterial cultures are then centrifuged to yield cell pellets and the pellets are resuspended in the same media to obtain cell suspensions that are concentrated about ten-fold and having cell densities of about  $10^{10}$  cells per ml. These concentrated cell suspensions are then mixed to achieve a donor-to-recipient ratio of five-to-one then this cell suspension was spotted onto QMI agar plates and incubated anaerobically at 30 degrees Centigrade for 24 hours. Then the cell mixture was removed from the QMI plate and placed on solid or in liquid QMI media containing antibiotics chosen to allow the survival of only *C. phytofermentans* recipient cells that express erythromycin resistance. This was accomplished by using a combination of antibiotics that consisted of trimethoprim at 20 micrograms per ml, cycloserine at 250 micrograms per ml, and erythromycin at 10 micrograms per ml. The *E. coli* donor was unable to survive exposure to these concentrations of trimethoprim and cycloserine, while the *C. phytofermentans* recipient was unable to survive exposure to this concentration of erythromycin (but could tolerate trimethoprim and cycloserine at these concentrations). Accordingly, after incubation of these antibiotic-containing plates or liquid media for 5-to-7 days at 30 degrees Centigrade under anaerobic conditions derivatives of *C. phytofermentans* were obtained that were erythromycin resistant and these *C. phytofermentans* derivatives were subsequently shown to contain pIMPT1029 as demonstrated by PCR analyses.

[00244] The surprising result was that the only a specially constructed derivative of the erythromycin resistance gene that contained the *C. phytofermentans* promoter from the alcohol dehydrogenase gene could be functionally expressed in *C. phytofermentans*.

[00245] Other genes of interest, either from *C. phytofermentans* or from heterologous sources would be introduced into the pIMPT construct and would be used to transform *C. phytofermentans* to introduce the genes and, hence, the gene products that would be used for increasing the environmental tolerance of *C. phytofermentans* to ethanol, acidic pH, or other toxic intermediates encountered during the production of biofuels.

[00246] Supporting Data: A map of the plasmid pIMPT1029 is produced in Figure 5, along with the DNA sequence of this plasmid, provided as SEQ ID NO: 1

[00247] SEQ ID NO: 1:

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cacgagggagcttccagggggaaacgcctgtatctttatagctcctgtcgggttcgccacctgacttgagcgtcgattttgtgatgct  
cgtcagggggggcggagcctatggaaaaacgccagcaacgcggccttttacggcttctggccttttctggccttttctcacatgttctt  
cctgcgttatcccctgattctgtggataaccgtattaccgctttgagtgagctgataccgctcggcagccgaacgccgagcgcagc  
gagtcagtgagcggaggaagcggaga .

### **Example 3. Pretreatment of feedstocks.**

[00248] In this example, one method of pretreating a feedstock is described.

[00249] To digest a feedstock (corn stover, bagasse, corn cobs, etc.) with sodium hydroxide (NaOH), digestion is performed depending on the need for treated biomass (corn stover, bagasse, corn cobs, etc). In this example, corn stover is used. Corn stover is weighed, chopped and screened through 2 mm sieve into the digestion vessel. NaOH solution prepared in tap water is added to each vessel to achieve a 10% solids slurry in regards to corn stover in a 1% w/v solution of NaOH. The vessels are closed and sealed and valves are drained. Heating capacity and ramp profile is set to achieve a 10 minute heating to the desired treatment temperature (*e.g.*, 80-120<sup>0</sup>C). Agitation and cooking are begun and the digestion is run for the desired time (*e.g.*, 20 to 60 minutes). After digestion is done cool down the vessels to room temperature. Transfer the digested material for washing and solids recovery.

[00250] To neutralize (adjust pH) the alkaline digested biomass, the digested slurry is cooled to room temperature and transferred through a 150 micron sieve/screen to separate solids from the alkaline liquor. Gentle pressure is applied to the slurry to remove liquid from the sample. The collected alkaline liquor can be recycled over the filter bed suspended by the 150 micron screen. This will allow for retention of finer particulates smaller than 150 micron. The feedstock is washed with 5 to 7 volumes of water or until the pH has returned to near neutral (as determined by use of a pH meter or pH).

[00251] To recover solids after alkaline digested and neutralization of biomass slurry, once the digested slurry has been neutralized, the slurry is transferred through a centrifuge or through a 150 micron

sieve/screen to separate solids from the neutralized liquor. Gentle pressure is applied to remove as much water from the retained solids as possible and the solid fraction is collected for drying.

[00252] To dry digested and neutralized biomass solids, spread the neutralized pretreated biomass evenly over a drying pan and transfer the pan to the drying oven. The drying oven is set to a desired temperature (*e.g.*, 35 °C) and drying continues until the moisture content reaches the desired level (*e.g.*, less than 5%). Occasional stirring can be used to promote drying.

#### **Example 4. Fermentation of NaOH-pretreated corn stover.**

[00253] In this example, the ability of *C. phytofermentans* to ferment alkaline pretreated corn stover was analyzed.

[00254] A Fermentation media recipe: (FM media): Base media: 50g/l NaOH pretreated corn stover, yeast extract 10 g/L, corn steep powder 5 g/L,  $K_2HPO_4$  3 g/L,  $KH_2PO_4$  1.6 g/L, TriSodium citrate.2H<sub>2</sub>O 2 g/L, Citric acid. H<sub>2</sub>O 1.2 g/L,  $(NH_4)_2SO_4$  0.5 g/L, NaCl 1 g/L, Cysteine.HCl 1 g/L, are dissolved in deionized water to achieve final volume, the pH is adjusted to 6.5 and the medium is degassed with nitrogen and autoclaved at 121°C for 30 min. IOOX Salt Stock:  $MgCl_2 \cdot 6H_2O$  80 g/L,  $CaCl_2 \cdot 2H_2O$  10 g/L,  $FeSO_4 \cdot H_2O$  0.125 g/L, TriSodium citrate.2H<sub>2</sub>O 3.0 g/L.

[00255] Culturing procedure. The fermentation media is prepared according to the recipe above. Components of the Base media are combined into a single vessel and degassed with nitrogen prior to sterilization. A 100X salts stock is prepared and sterilized separately. Sterilization is achieved by autoclave treatment at 121°C for 30 minutes. After sterilization, base media is supplemented with a 1% v/v dose of IOOX salts and to achieve the final concentrations. All additions are prepared anaerobically and aseptically.

[00256] An inoculum of *Clostridium phytofermentans* sp is prepared by propagation in QM media 24 hrs to an active cell density of  $2 \times 10^9$  cells per ml. the cells were concentrated by centrifugation and then transferred into the FM media bottles to achieve an initial cell density of  $2 \times 10^9$  cells per ml for the start of fermentation. Cultures are then incubated at pH 6.5 and at 35°C for 120 hrs or until fermentations were complete.

[00257] Product formation was determined by HPLC analysis using refractive index detection. Compositional analysis for the NaOH treated corn stover is obtained via NREL standard methods using two-stage acid hydrolysis procedures.

#### **Example 5. Effect of severity factor on oligomeric sugar contents**

Corn stover was pre-treated prior to fermentation under various severity factor. The severity factor is a combination of reaction time, temperature and acid or base concentration. The calculation of severity factor is indicated in table 4. The severity factors for various fermentations are indicated in table 4 and figure 15. Oligomeric sugar contents from each run was quantitated and illustrated in figure 16. As illustrated in figure 16, sample batches fermented under low severity condition, such as sample batch

"a", batch "b", contain mainly oligomeric sugars. As severity factor is increased, percentage of oligomeric sugars was decreased.

[00258] Table 4. Severity factors

| Run ID | Catalysts   | Stage 1 (removing hemicellulose) |          | Stage 2 (Steam explosion) |          | Total Severity Factor (SF) |
|--------|-------------|----------------------------------|----------|---------------------------|----------|----------------------------|
|        |             | Temp(T)                          | Time (t) | Temp(T)                   | Time (t) |                            |
| a      | None        | 170.00                           | 35.00    | 190.00                    | 1.50     | 2.41                       |
| b      | H2SO4, 0.5% | 140.00                           | 30.00    | 190.00                    | 1.50     | 2.53                       |
| 1      | H2SO4, 0.5% | 150.00                           | 25.00    | 190.00                    | 1.50     | 3.22                       |
| 2      | H2SO4, 0.5% | 160.00                           | 20.00    | 190.00                    | 1.50     | 3.71                       |
| 3      | H2SO4, 0.5% | 160.00                           | 25.00    | 190.00                    | 1.50     | 3.95                       |
| 4      | H2SO4, 0.5% | 170.00                           | 15.00    | 190.00                    | 1.50     | 4.05                       |
| 5      | H2SO4, 0.5% | 170.00                           | 20.00    | 190.00                    | 1.50     | 4.19                       |

Formulation for the calculation of severity factor

$$SF = \ln \left[ t \cdot \exp \left( \frac{T - 100}{14.75} \right) \right] - pH$$

**Example 6. Efficient ethanol production via direct fermentation of oligomeric sugars.**

[00259] Figure 17 illustrates a plant process in which sample "b" was processed. Corn stover was pretreated under conditions with a severity factor of 2.53. The resulting mixture of solid and liquid was neutralized with NaOH. Subsequently, Sample "b" was inoculated with a culture of *Clostridium phytofermentans* and a small amount of cellulase enzyme (5- 10 fpu/g TS) was added to the fermentation to assist hydrolysis of the cellulose. The entire process was performed in a single bioreactor without a separation step, such as the separation of solid from liquid. Further, no detoxification step was performed.

[00260] To identify oligomers in sample "b", reflective index response was measured. As illustrated in figure 18, sample "b" was highly oligomeric. Continued fermentation of sample "b" with *Clostridium phytofermentans* resulted in direct production of ethanol (figure 20). A portion of sample "b" was further treated with acid and enzymes, which resulted in the production of a sample comprising predominantly monomelic sugars (figure 19). The monomeric sample will be fermented with a strain of microorganism as indicated in figure 17 to produce ethanol.

## CLAIMS

WHAT IS CLAIMED IS:

1. A product for biofuel production comprising:
  - a) a biomass feedstock pretreated under low severity conditions wherein said pretreated feedstock comprises a higher amount of oligosaccharides after said pretreatment than before; and
  - b) a microorganism that is capable of directly fermenting and scarifying said oligosaccharides to produce a fermentation end-product.
2. The product of claim 1, wherein said pretreated feedstock comprises a greater percentage of oligosaccharides than monomer saccharides.
3. The product of claim 1, wherein said microorganism produces an enzyme which saccharifys a portion of said feedstock, and wherein said enzyme is selected from the group consisting of a cellulase, a hemicellulase, an amylase, a protease, a chitinase, a pectinase, a keratinase, or a combination thereof.
4. The product of claim 1, wherein said feedstock comprises a greater amount of five carbon monomer saccharides than six carbon monomer saccharides.
5. The product of claim 4, wherein said five carbon monomer sugars comprise xylose, arabinose, or a combination thereof.
6. The product of claim 4, wherein said six-carbon sugars are glucose, galactose, mannose, or a combination thereof.
7. The product of claim 1, wherein said low severity conditions comprise treating said feedstock with a dilute acid.
8. The product of claim 7, wherein said dilute acid hydrolysis comprises treating said feedstock with peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, or hydrochloric acid.
9. The product of claim 1, wherein said microorganism is ferments five-carbon saccharides and six-carbon saccharides.
10. The product of claim 1, wherein said microorganism is a *Clostridium*.
11. The product of claim 1, wherein said microorganism is *Clostridium phytofermentans* .
12. The product of claim 1, wherein said microorganism is *Clostridium* sp. Q.D.
13. The product of claim 1, wherein said microorganism is genetically modified.
14. The product of claim 1, wherein said low severity conditions comprises incubating said feedstock at a temperature of from about 80<sup>0</sup>C to about 170<sup>0</sup>C.
15. The product of claim 1, wherein said low severity conditions comprises incubating said feedstock at a temperature of 140<sup>0</sup>C.
16. The product of claim 7, wherein said product further comprises a second substance that neutralizes an acidic pH of said feedstock.
17. The product of claim 16, wherein said second substance is ammonium hydroxide.

18. The product of claim 15, wherein said neutralized feedstock comprises substantially more oligosaccharides than monomer five-carbon or six-carbon sugars.
19. The product of claim 8, wherein said feedstock is treated with sulfuric acid to decrease its pH to between 6.1 to 6.9.
20. A method of producing a fermentation end-product, comprising:
  - a) treating a feedstock with an acid for a first period of time at about 120<sup>0</sup>C to 180<sup>0</sup>C;
  - b) treating said feedstock for a second period of time at about 180<sup>0</sup>C to 200<sup>0</sup>C;
  - c) neutralizing the pH of said feedstock with a base; and
  - d) incubating said neutralized feedstock with a microorganism that directly ferments and saccharifys said feedstock to produce a fermentation end-product.
21. The method of claim 20, wherein said acid is peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, or hydrochloric acid.
22. The method of claim 21, wherein said acid is sulfuric acid.
23. The method of claim 22, wherein said sulfuric acid is provided in an aqueous solution at a concentration of about 0.5% to about 2% by volume.
24. The method of claim 20, wherein said bases is ammonium hydroxide.
25. The method of claim 20, wherein said ammonium hydroxide is added at a concentration of about 0.5% to about 2% by weight of said feedstock.
26. The method of claim 20, wherein the pH of said feedstock in step a) is decreased to between 6.1 to 6.9.
27. The method of claim 20, wherein said fermentation end-product is an alcohol.
28. The method of claim 20, wherein said fermentation end-product is ethanol.
29. The method of claim 20, wherein said microorganism is a *Clostridium*.
30. The method of claim 20, wherein said microorganism is *Clostridium phytofermentans*.
31. The method of claim 20, wherein said microorganism is *Clostridium* sp. Q.D.
32. The method of claim 20, wherein said microorganism is genetically modified.
33. The method of claim 20, wherein after step a) and b) said neutralized feedstock comprises a greater percentage of oligosaccharides than monomer saccharides.
34. The method of claim 33, wherein said neutralized feedstock comprises a greater amount of five carbon monomer saccharides than six carbon monomer saccharides.
35. The method of claim 20, wherein said five-carbon sugars are xylose, arabinose, or a combination thereof.
36. The method of claim 20, wherein said six-carbon sugars are glucose, galactose, mannose, or a combination thereof.
37. The method of claim 20, wherein said step a) is performed at a temperature of from about 120<sup>0</sup>C to about 140<sup>0</sup>C.
38. The product of claim 37, wherein said temperature is at 140<sup>0</sup>C.

39. The method of claim 20, wherein said neutralized feedstock comprises more oligosaccharides than five-carbon or six-carbon monomer saccharides.
40. The method of claim 20, wherein said second period of time is greater than said first period of time.
41. The method of claim 20, wherein said first period of time is thirty minutes.
42. The method of claim 20, wherein the pretreated feedstock is not detoxified.
43. The method of claim 20, wherein said second period of time is an hour and a half.
44. A method of producing a fermentation end-product, comprising:
  - a) Pretreating a biomass feedstock comprising:
    - i) treating a feedstock with sulfuric acid for a first period of time at about 140<sup>0</sup>C;
    - ii) treating said feedstock for a second period of time at about 190<sup>0</sup>C, wherein said second period of time is greater than said first period of time; and
    - iii) neutralizing the pH of said feedstock with ammonium hydroxide, wherein said feedstock is not is not detoxified; and
  - b) incubating said pretreated feedstock with *Clostridium phytofermentans* to produce ethanol.
45. A method of producing a fermentation end-product, comprising:
  - a) Pretreating a biomass feedstock comprising:
    - i) treating a feedstock with a first acid for a first period of time at about 130<sup>0</sup>C to 180<sup>0</sup>C;
    - ii) treating said feedstock for a second period of time at about 180<sup>0</sup>C to 200<sup>0</sup>C;
    - iii) treating said feedstock for a third period of time with a second acid and one or more enzymes; and
    - iv) neutralizing the pH of said feedstock with a base, wherein said neutralized feedstock comprises substantially more monomer saccharides than oligosaccharides; and
  - b) incubating said neutralized feedstock with a microorganism that directly ferments and saccarifys said feedstock to produce a fermentation end-product.
46. The method of claim 45, wherein said first or second acid is peroxyacetic acid, sulfuric acid, lactic acid, citric acid, phosphoric acid, or hydrochloric acid.
47. The method of claim 45, wherein said first or second acid is sulfuric acid.
48. The method of claim 47, wherein said sulfuric acid is provided in an aqueous solution at a concentration of about 0.5% to about 2% by volume.
49. The method of claim 45, wherein said base is ammonium hydroxide.
50. The method of claim 49, wherein said ammonium hydroxide is added at a concentration of about 0.5% to about 2% by weight of said feedstock.
51. The method of claim 45, wherein the pH of said feedstock in step a) is decreased to between 6.1 to 6.9.
52. The method of claim 45, wherein said fermentation end-product is an alcohol.
53. The method of claim 45, wherein said fermentation end-product is ethanol.
54. The method of claim 45, wherein said microorganism is a *Clostridium*.

55. The method of claim 45, wherein said microorganism is *Clostridium phytofermentans* .
56. The method of claim 45, wherein said microorganism is *Clostridium* sp. Q.D.
57. The method of claim 45, wherein said microorganism is a yeast.
58. The method of claim 45, wherein said microorganism is *E. coli*
59. The method of claim 45, wherein said microorganism is *Z. mobilis*.
60. The method of claim 45, wherein said microorganism is genetically modified.
61. The method of claim 45, wherein after step a) and b) said neutralized feedstock comprises a greater percentage of monomer saccharides than oligosaccharides.
62. A method of producing a fermentation end-product, comprising:
  - a) Pretreating a biomass feedstock comprising:
    - i) treating a feedstock with an acid for a first period of time at about 130<sup>0</sup>C to 180<sup>0</sup>C;
    - ii) treating said feedstock for a second period of time at about 180<sup>0</sup>C to 200<sup>0</sup>C, wherein said steps i) and ii) produce a pretreated feedstock with substantially more oligosaccharides than monomer saccharides; and
  - b) incubating said feedstock with a microorganism that directly ferments and saccharifys said feedstock to produce a fermentation end-product.
63. A method of producing a pretreated biomass feedstock comprising:
  - a) treating a feedstock with sulfuric acid for a first period of time at about 140<sup>0</sup>C;
  - b) treating said feedstock for a second period of time at about 190<sup>0</sup>C, wherein said second period of time is greater than said first period of time; and
  - c) neutralizing the pH of said feedstock with ammonium hydroxide, wherein said feedstock is not detoxified.

FIGURE 1

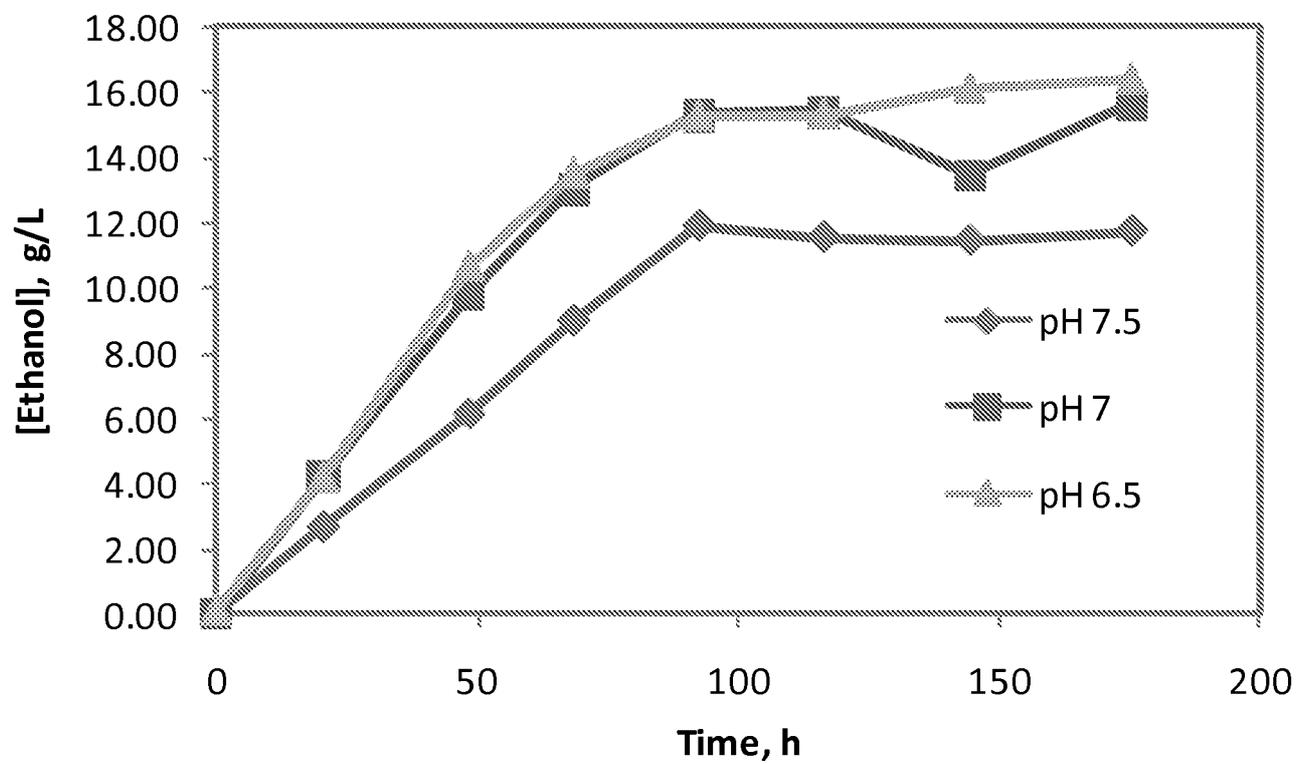


FIGURE 2

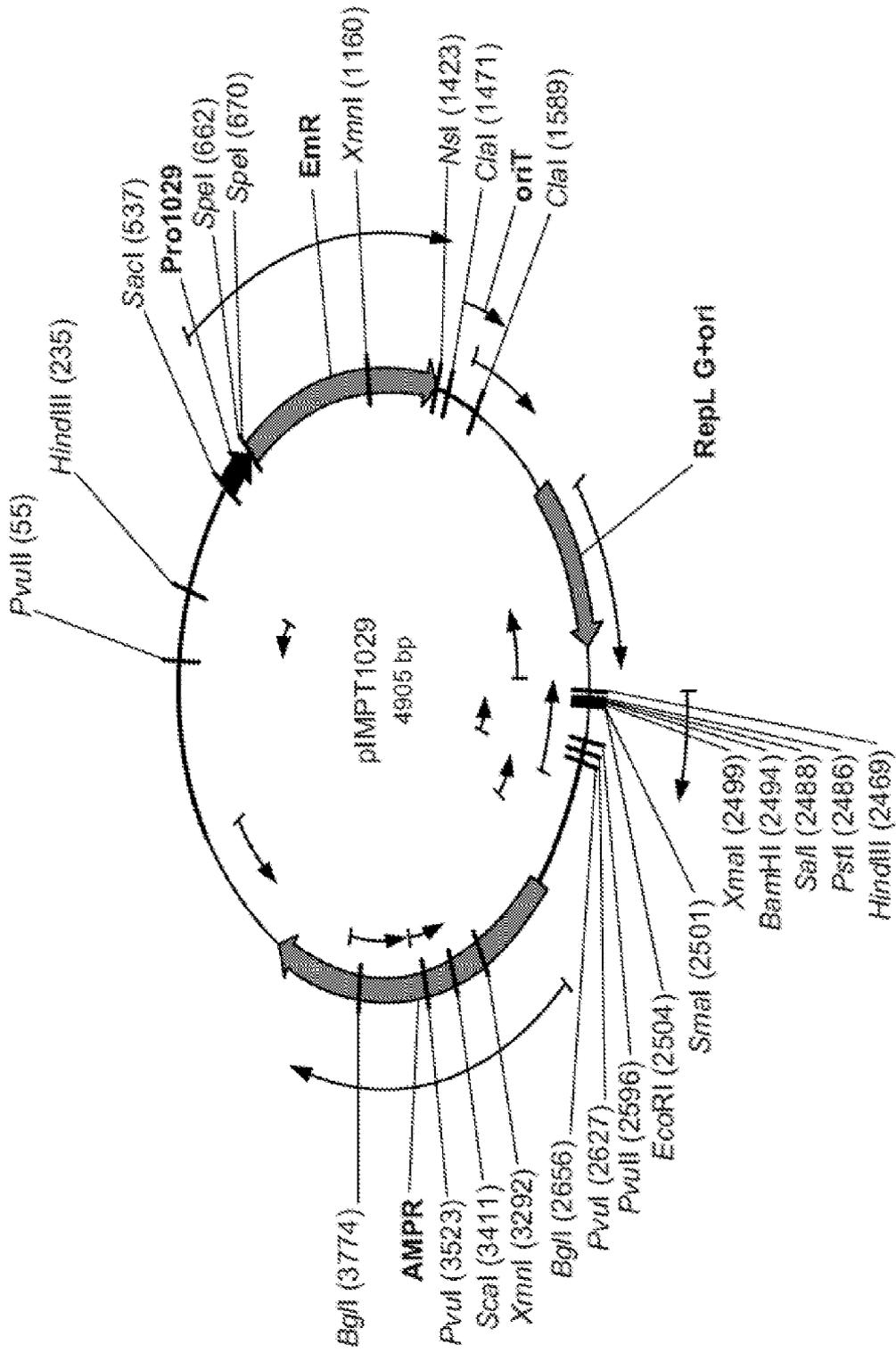


FIGURE 3

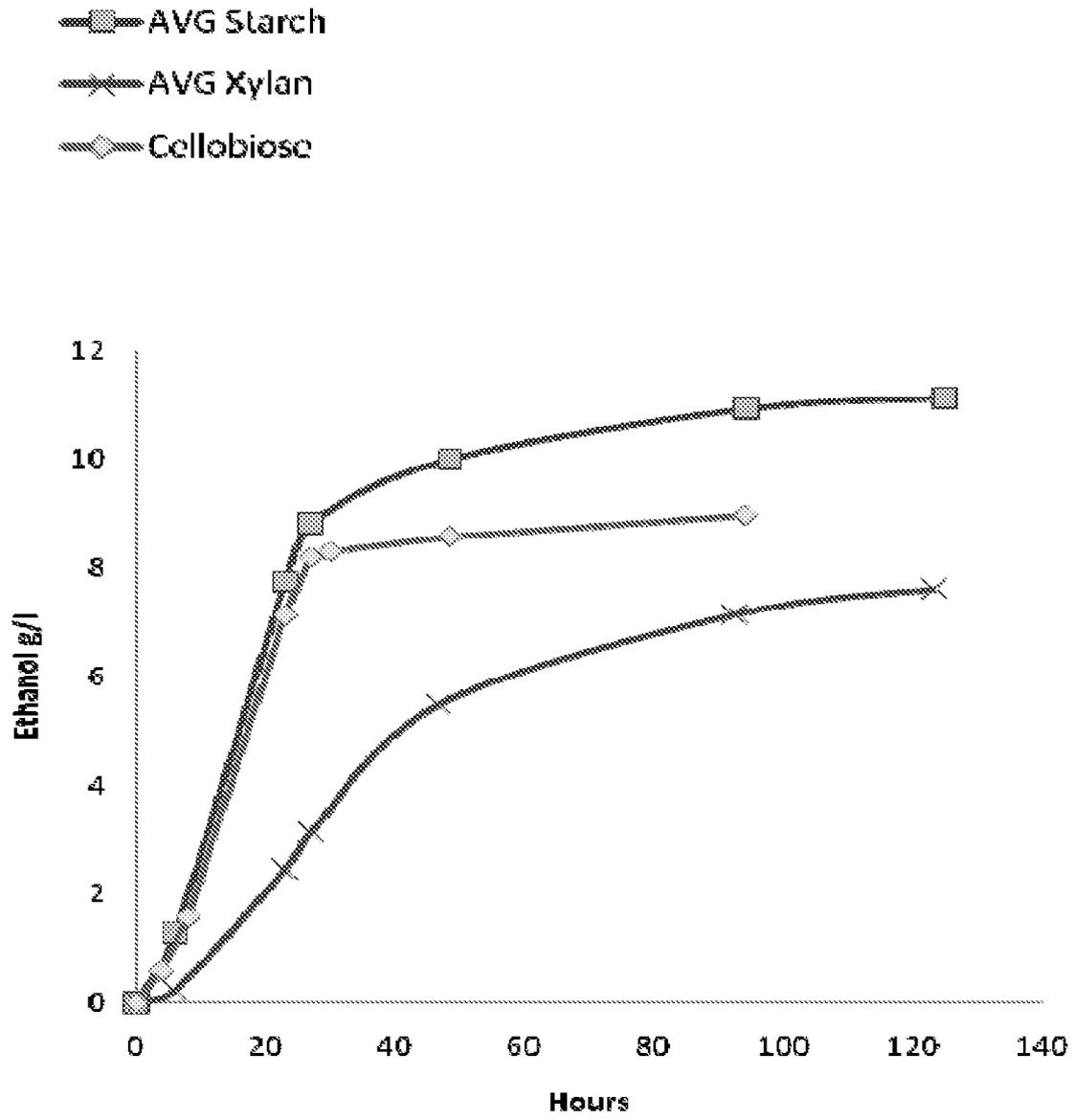
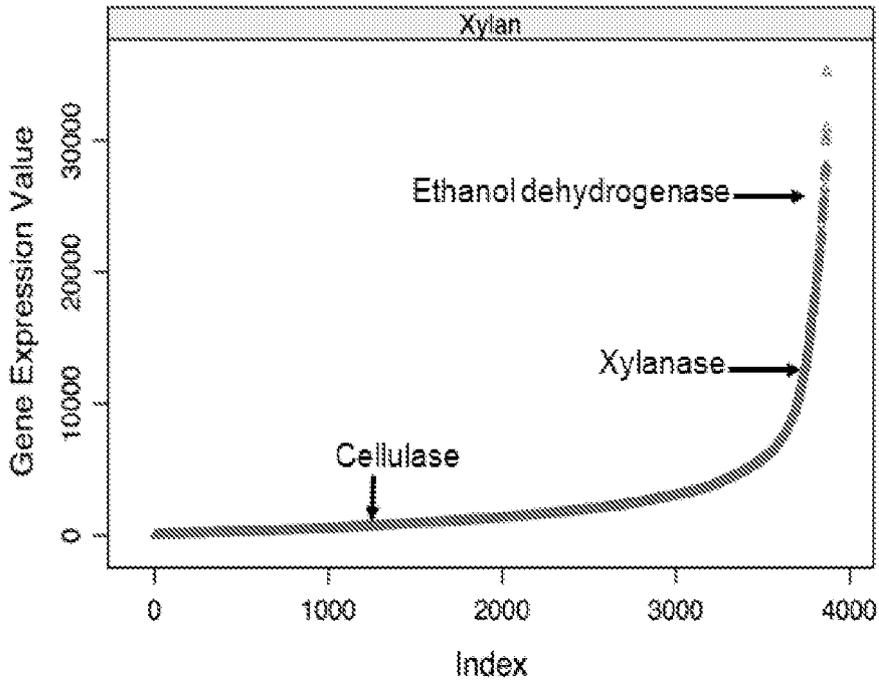


FIGURE 4

A



B

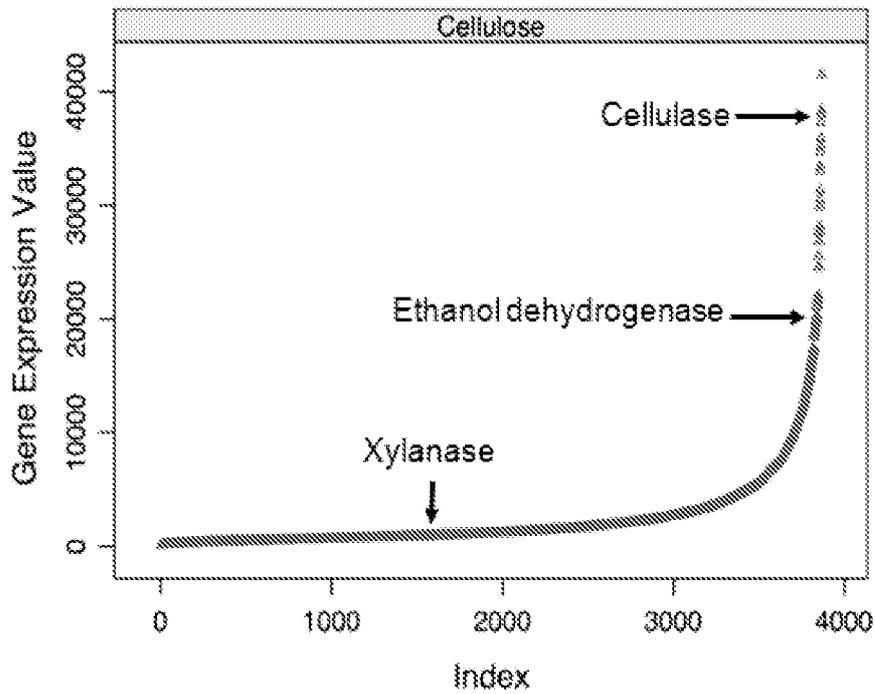
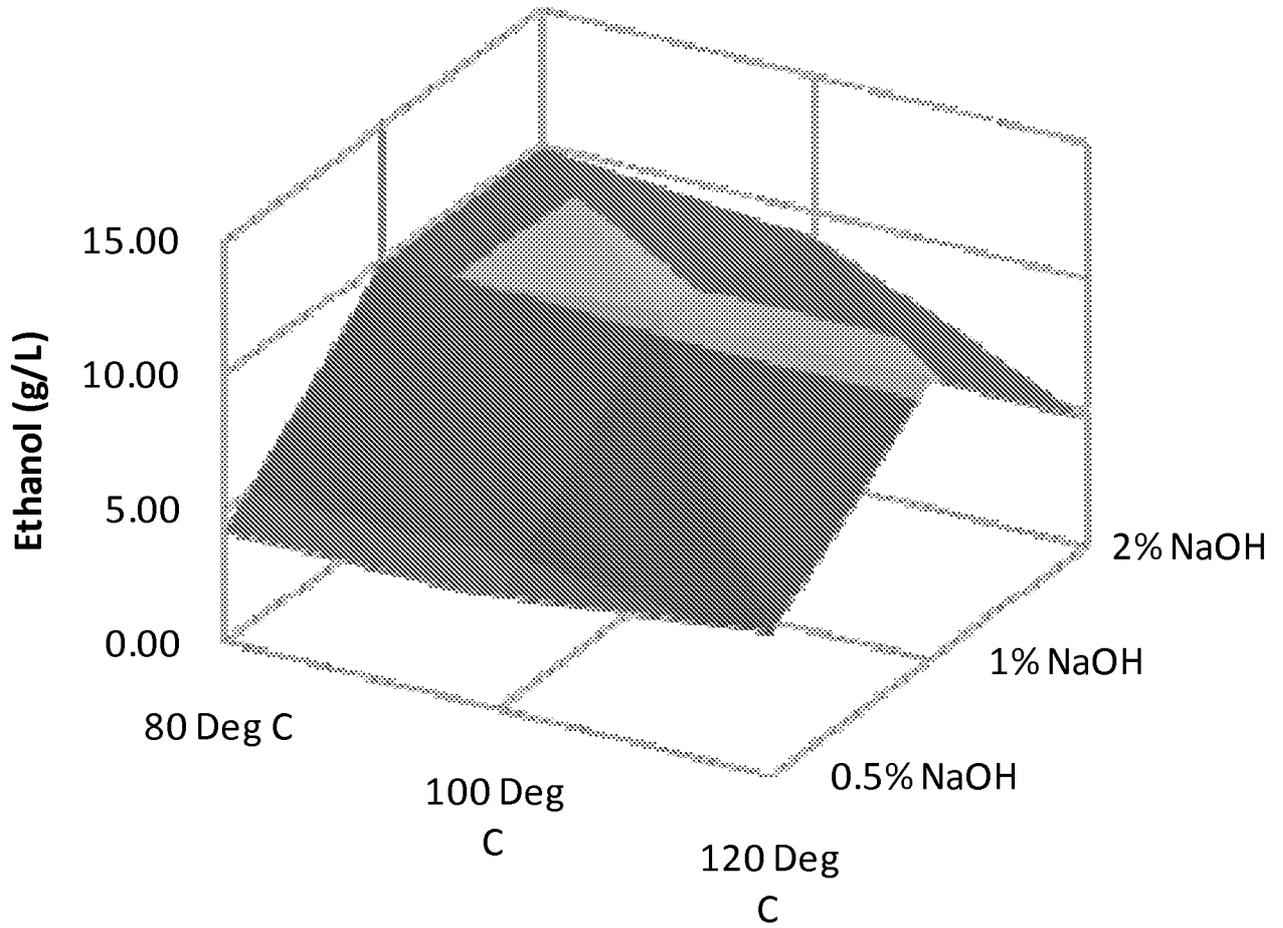


FIGURE 5



- 10.00-15.00
- 5.00-10.00
- 0.00-5.00

FIGURE 6

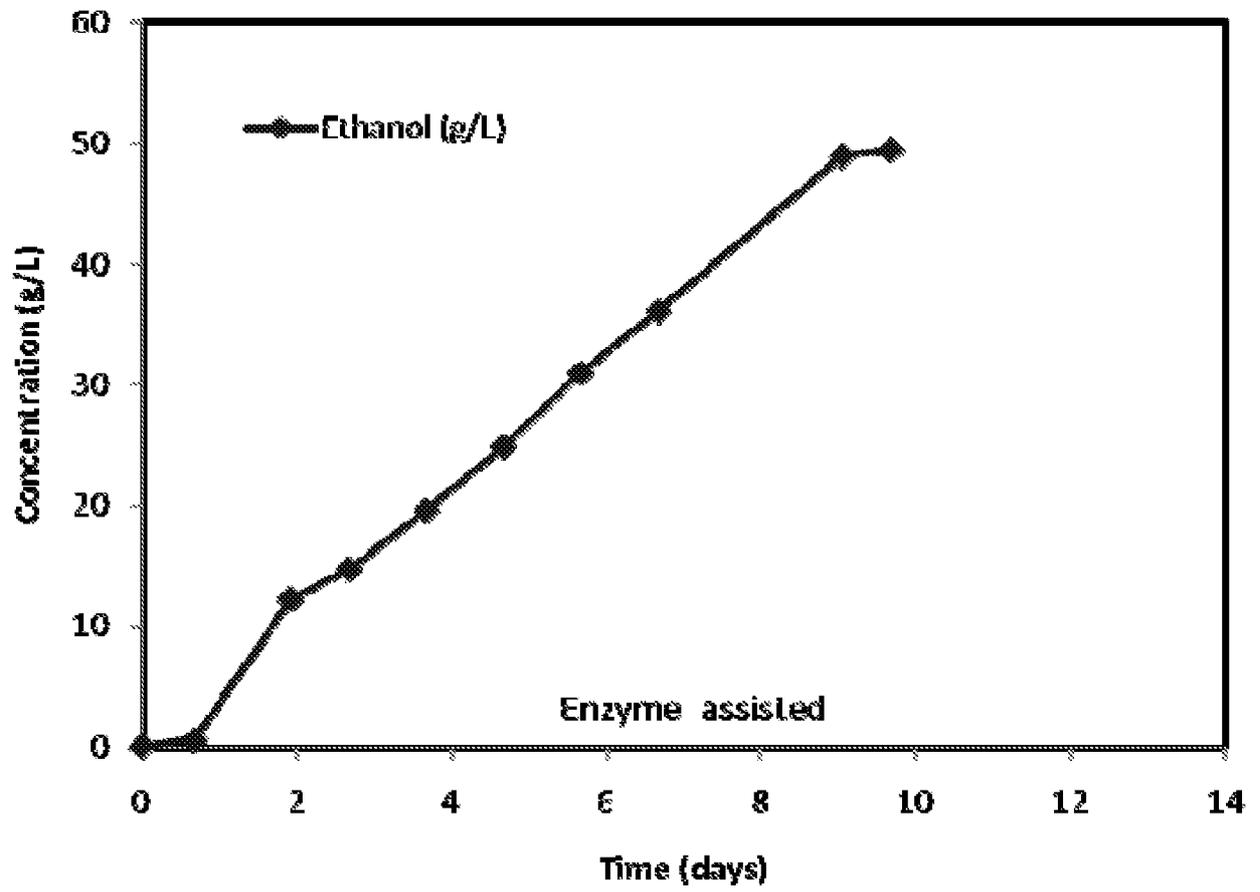


FIGURE 7

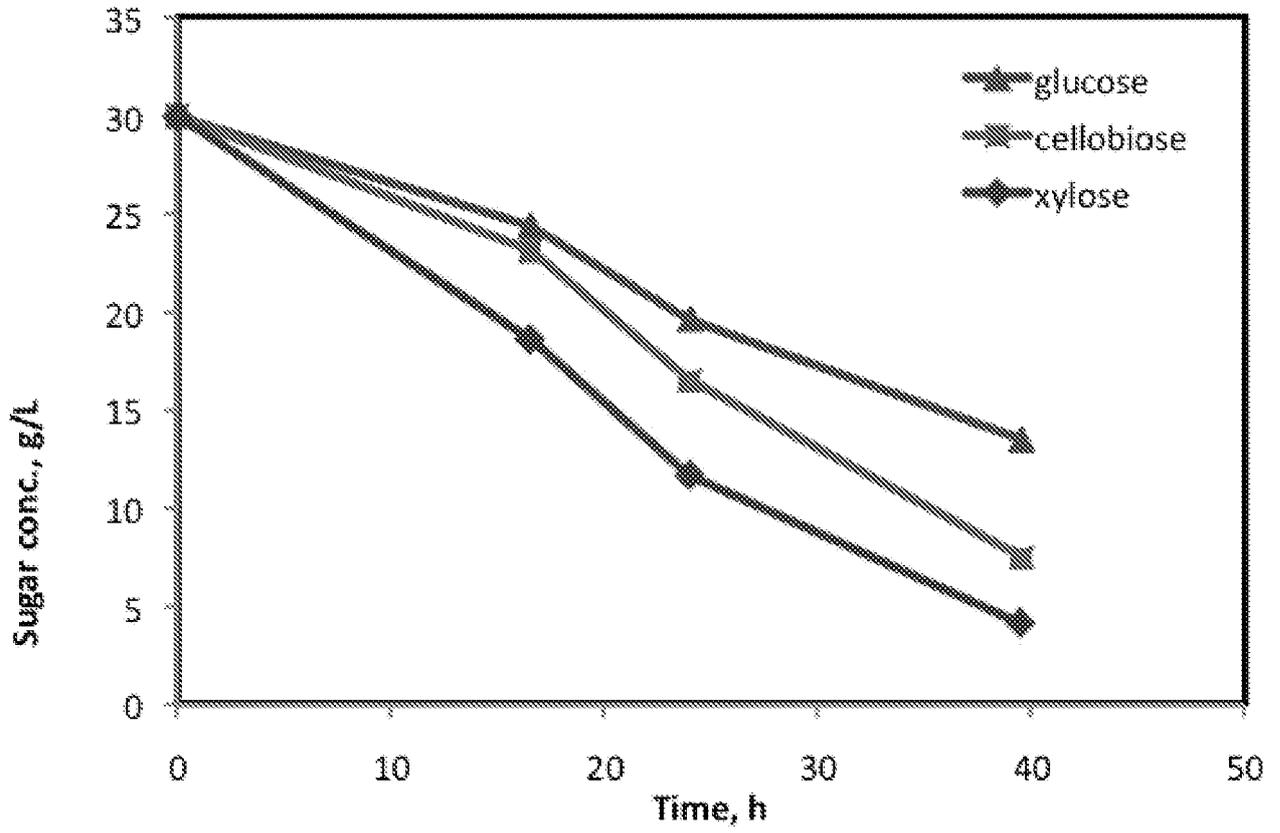


FIGURE 8

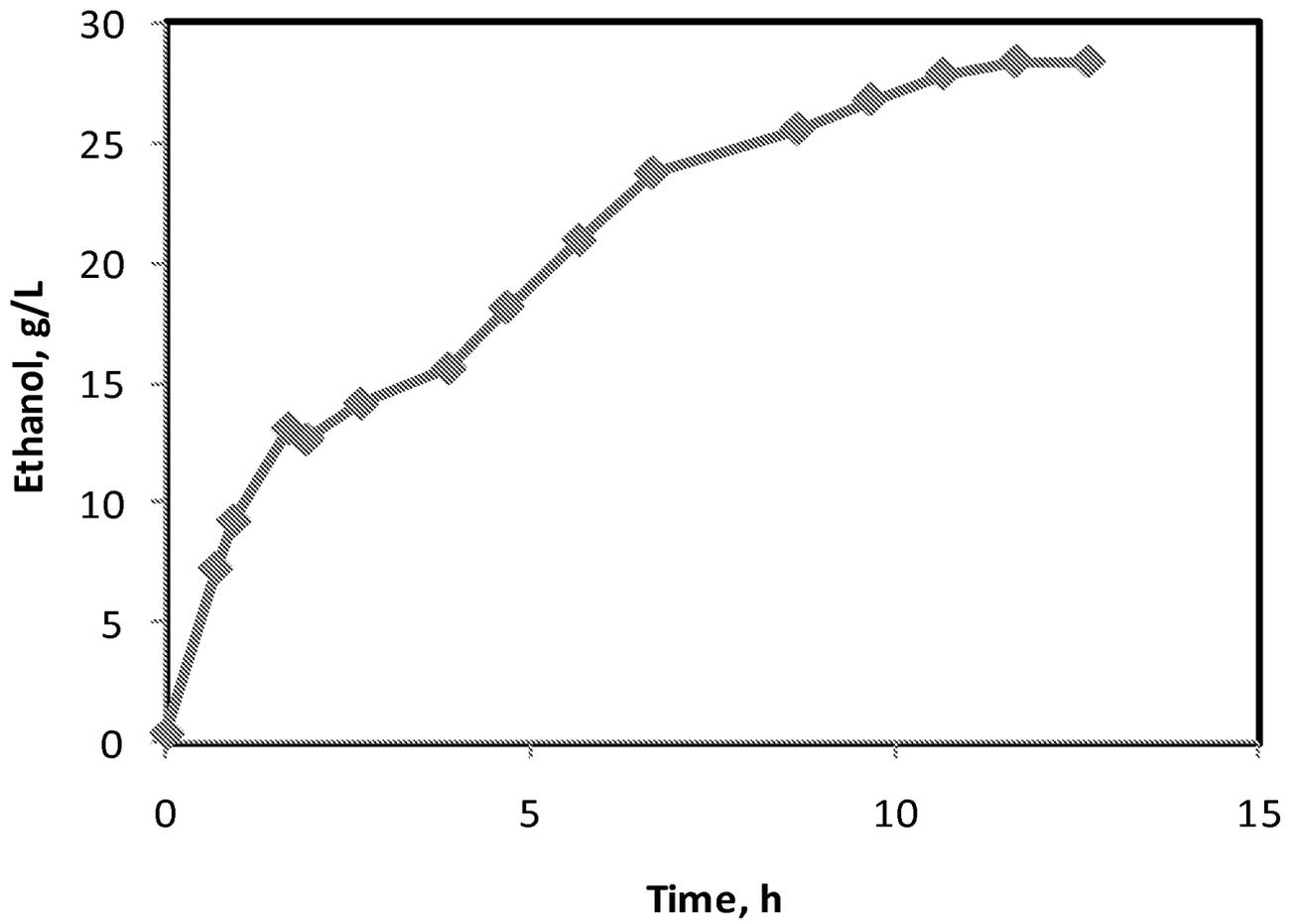
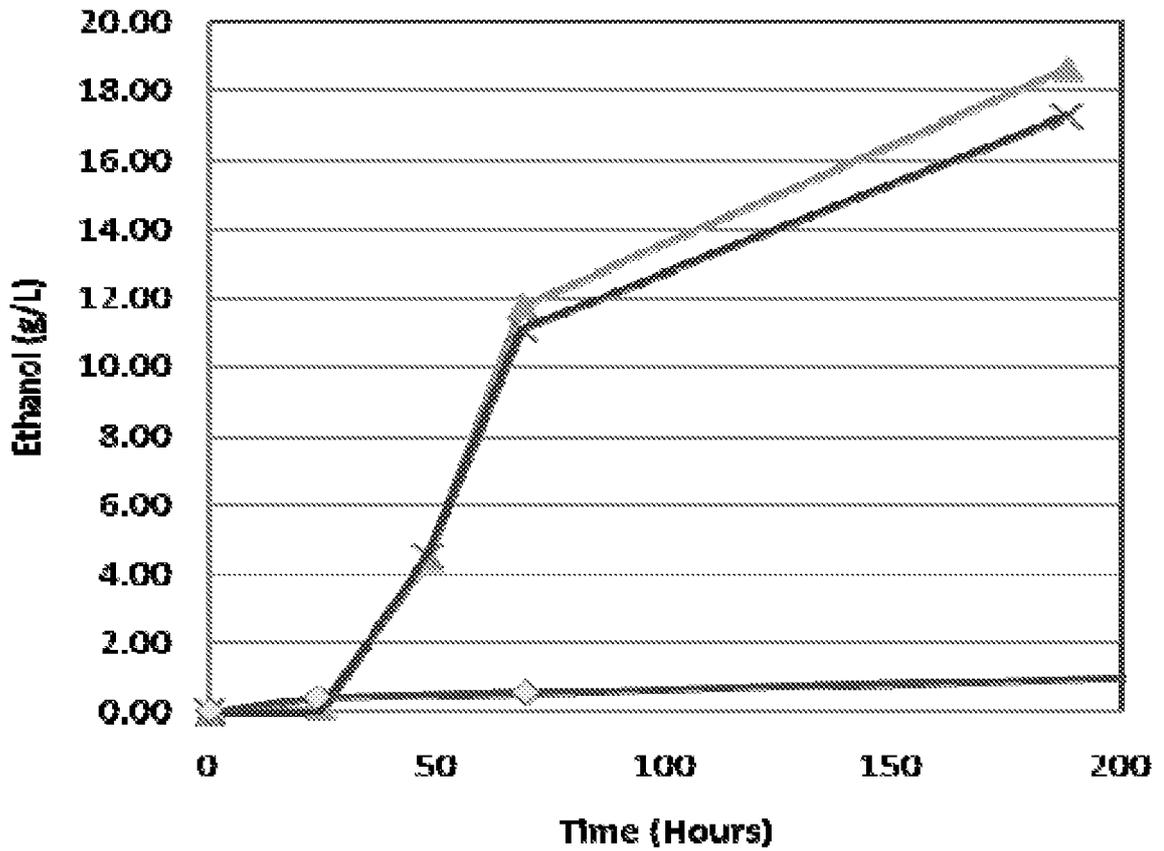


FIGURE 9

# AVG Ethanol of Treated MLW



5% ACT 1 + Cellulose B

5% ACT 2 + Cellulose B

5% Untreated ACT, Sample 2  
(03/04)

FIGURE 10

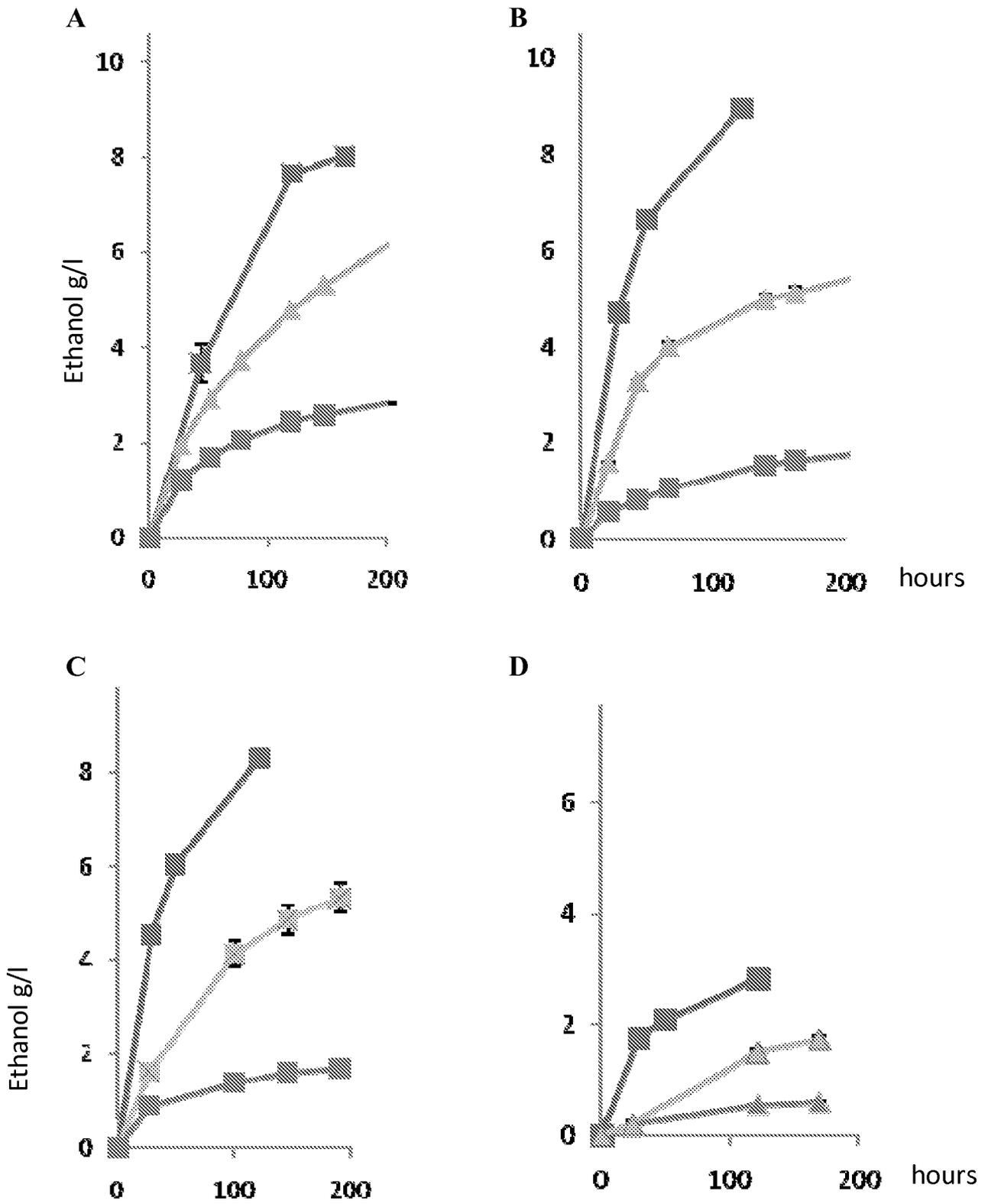


FIGURE 11

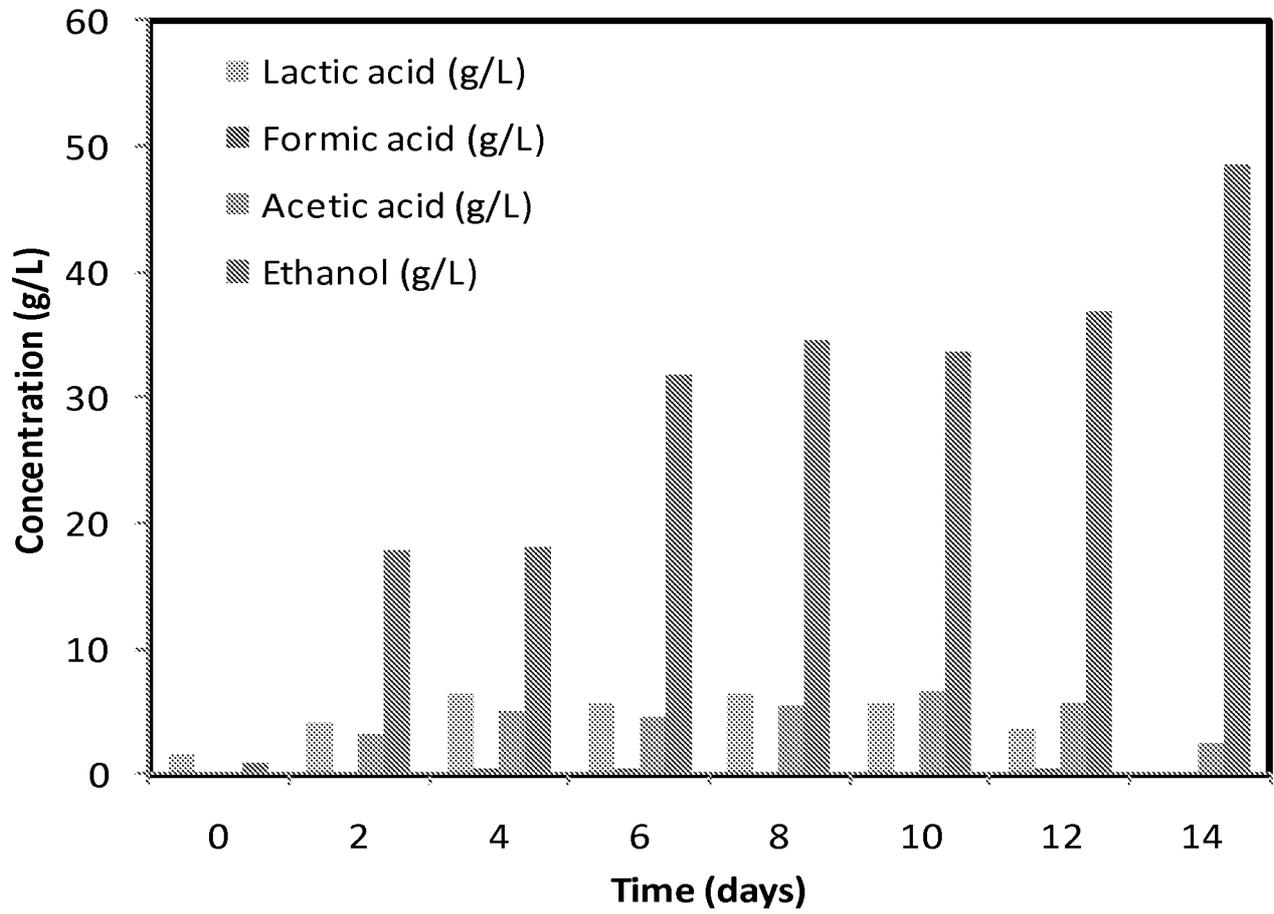


FIGURE 12

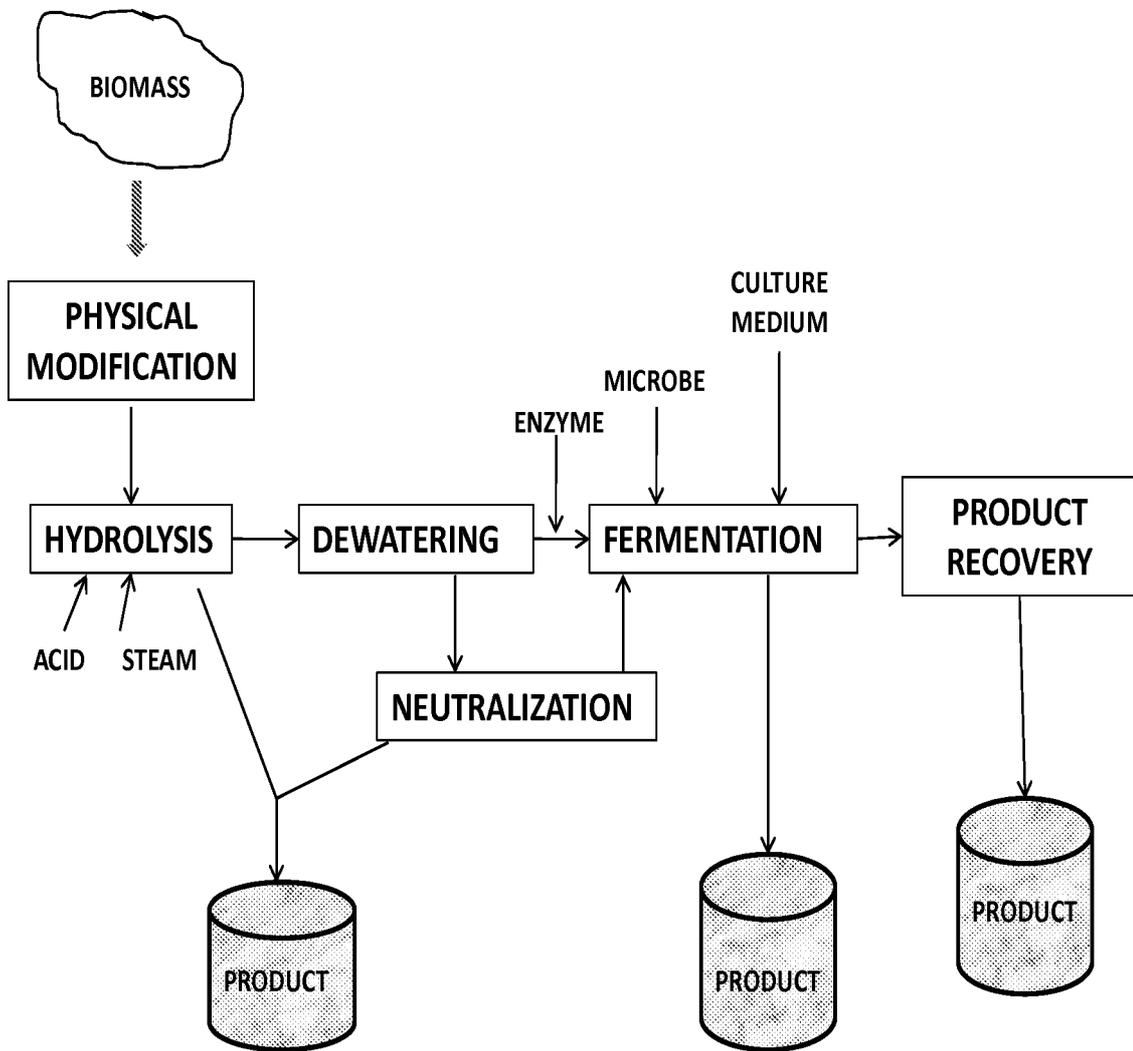


FIGURE 13

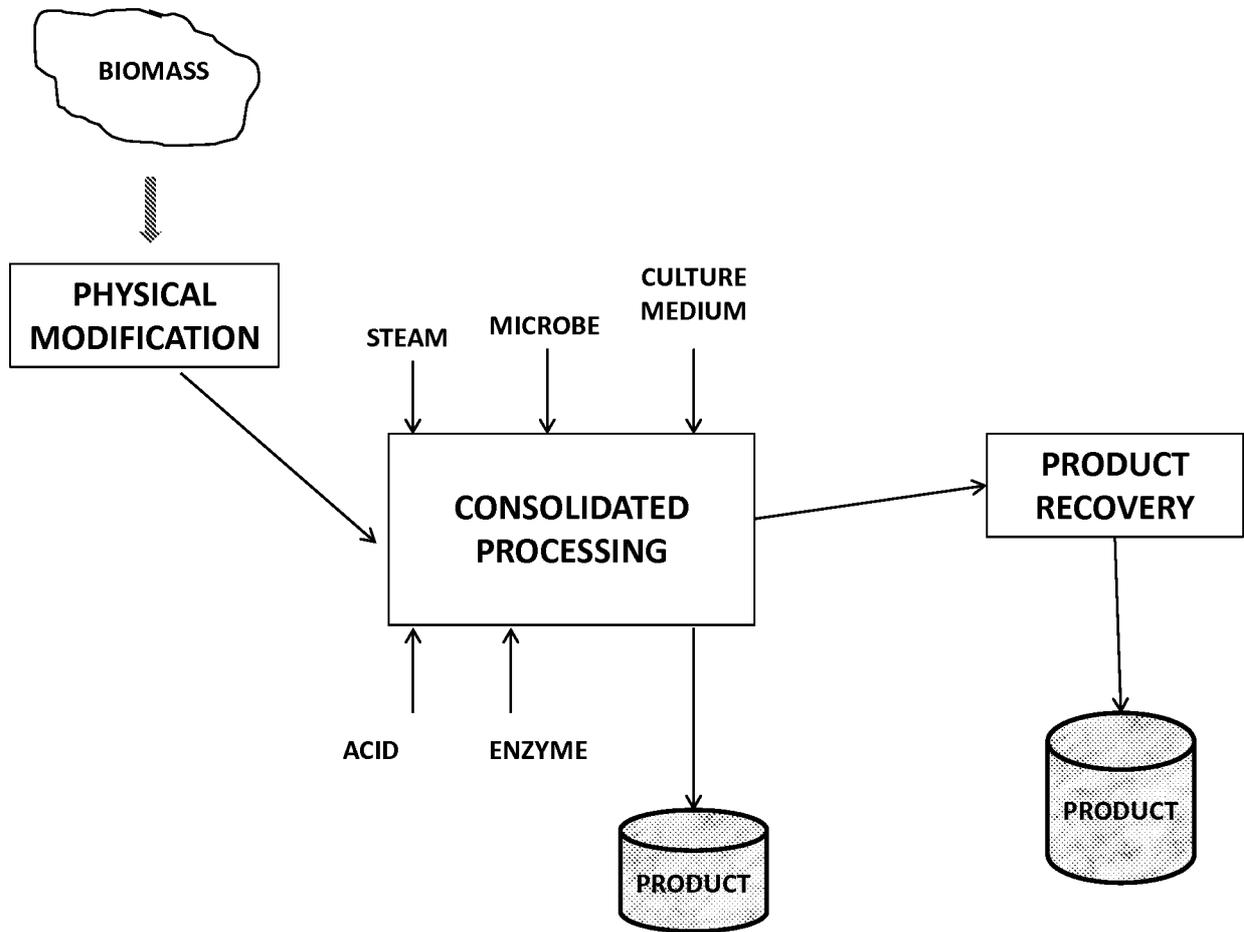


FIGURE 14

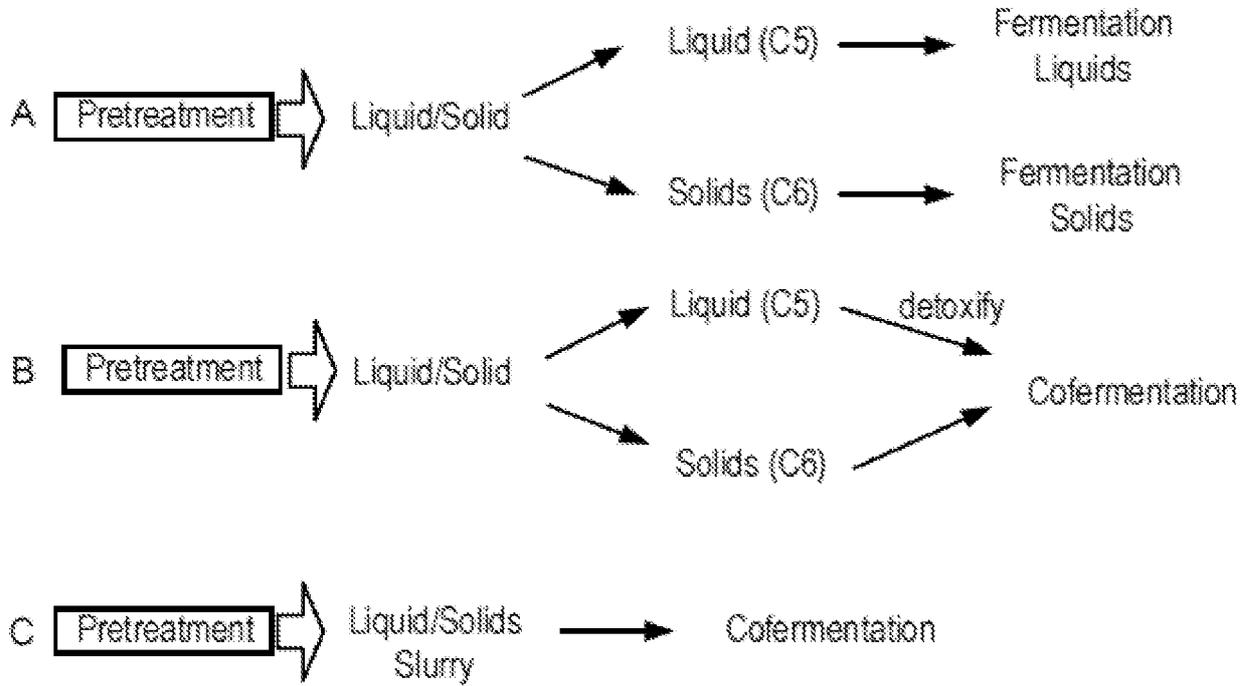


FIGURE 15

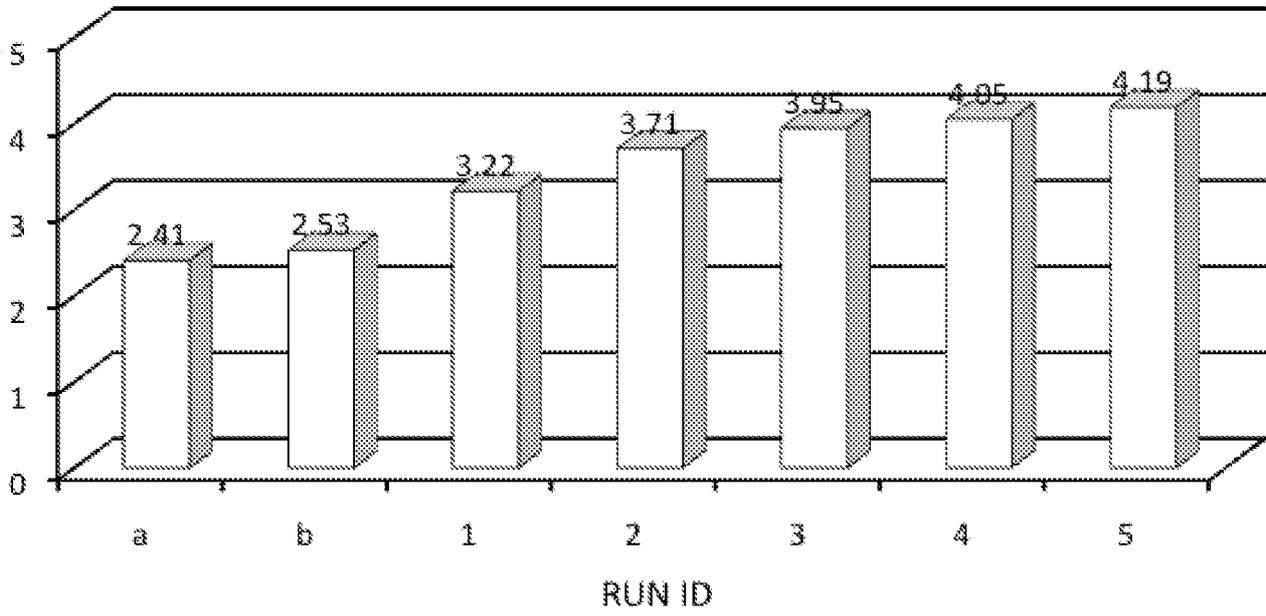


FIGURE 16

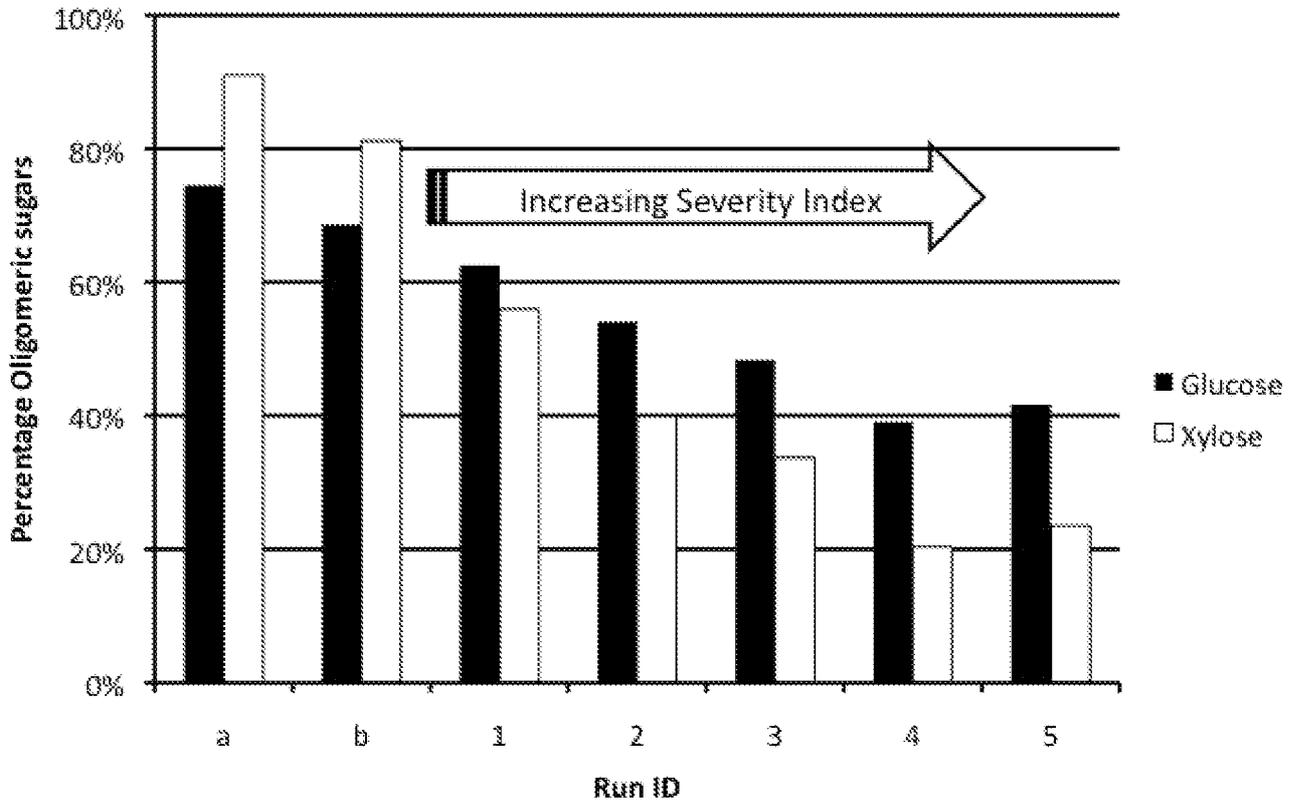


FIGURE 17

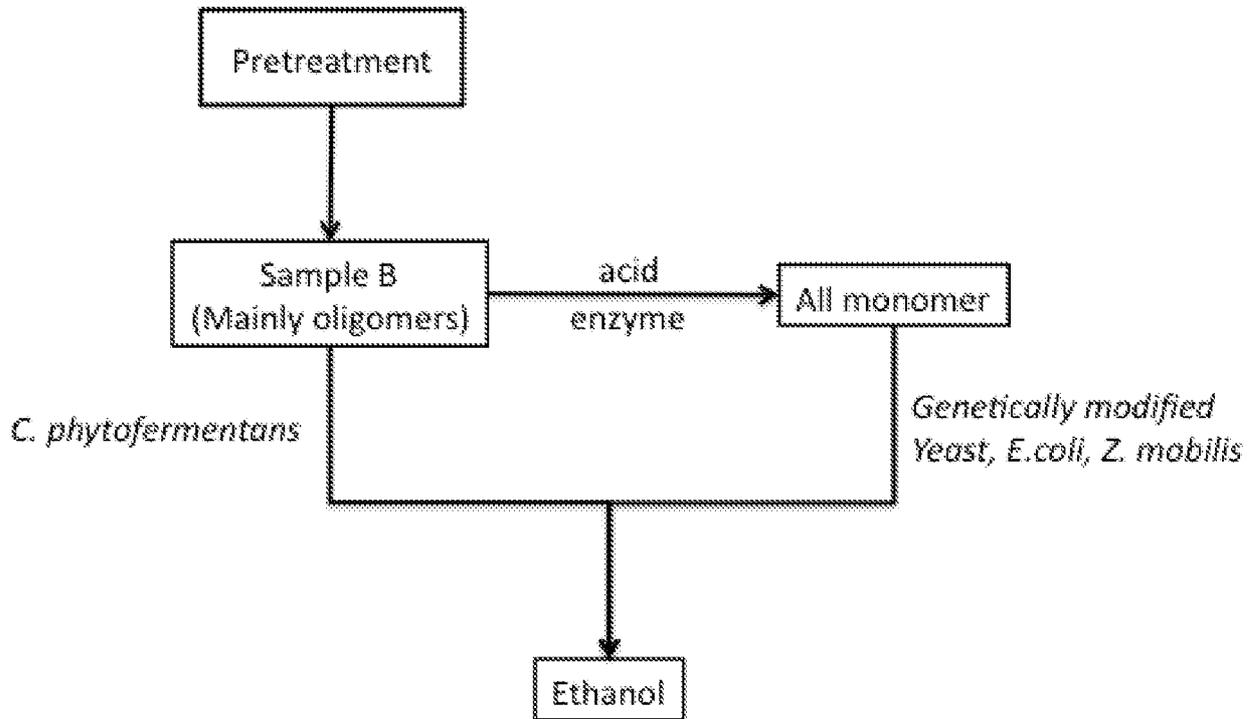


FIGURE 18

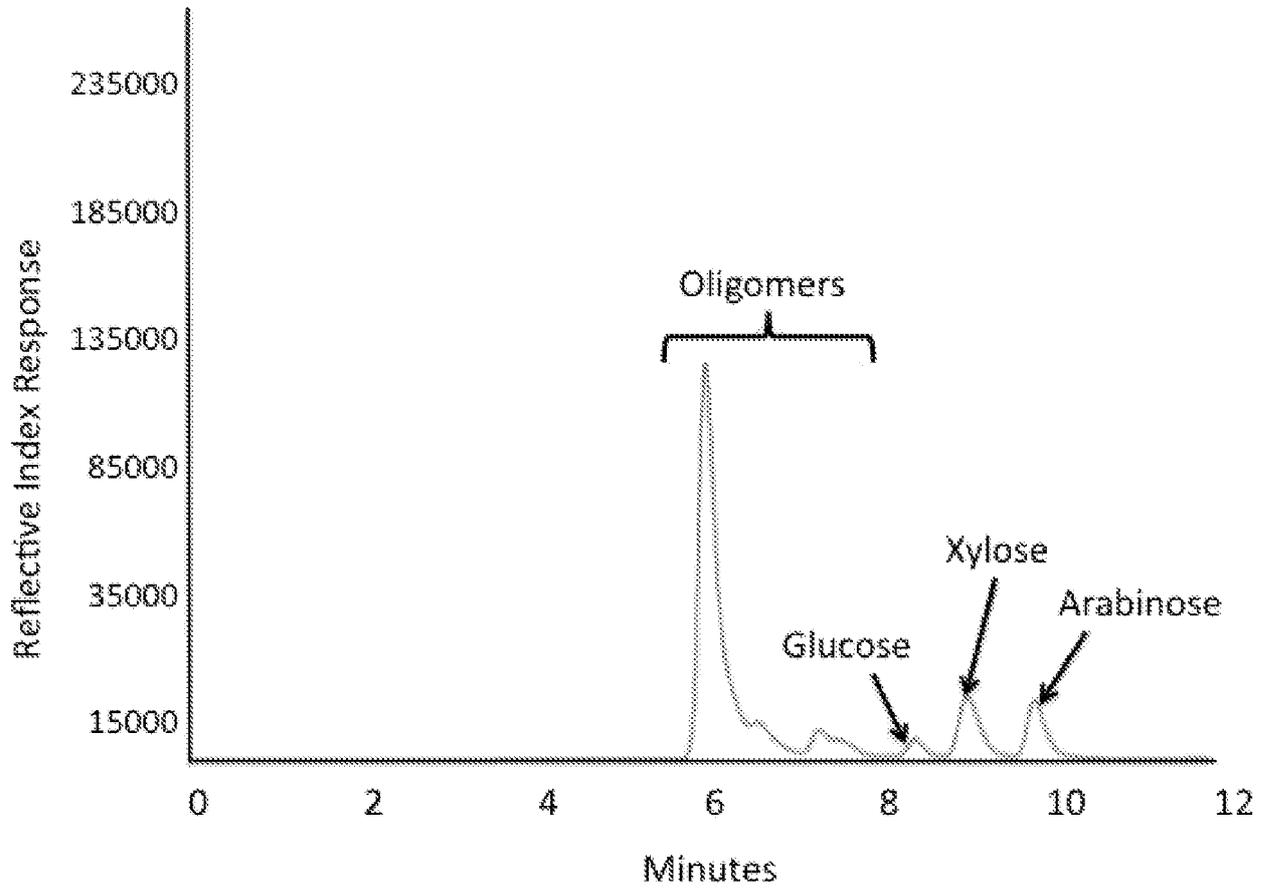


FIGURE 19

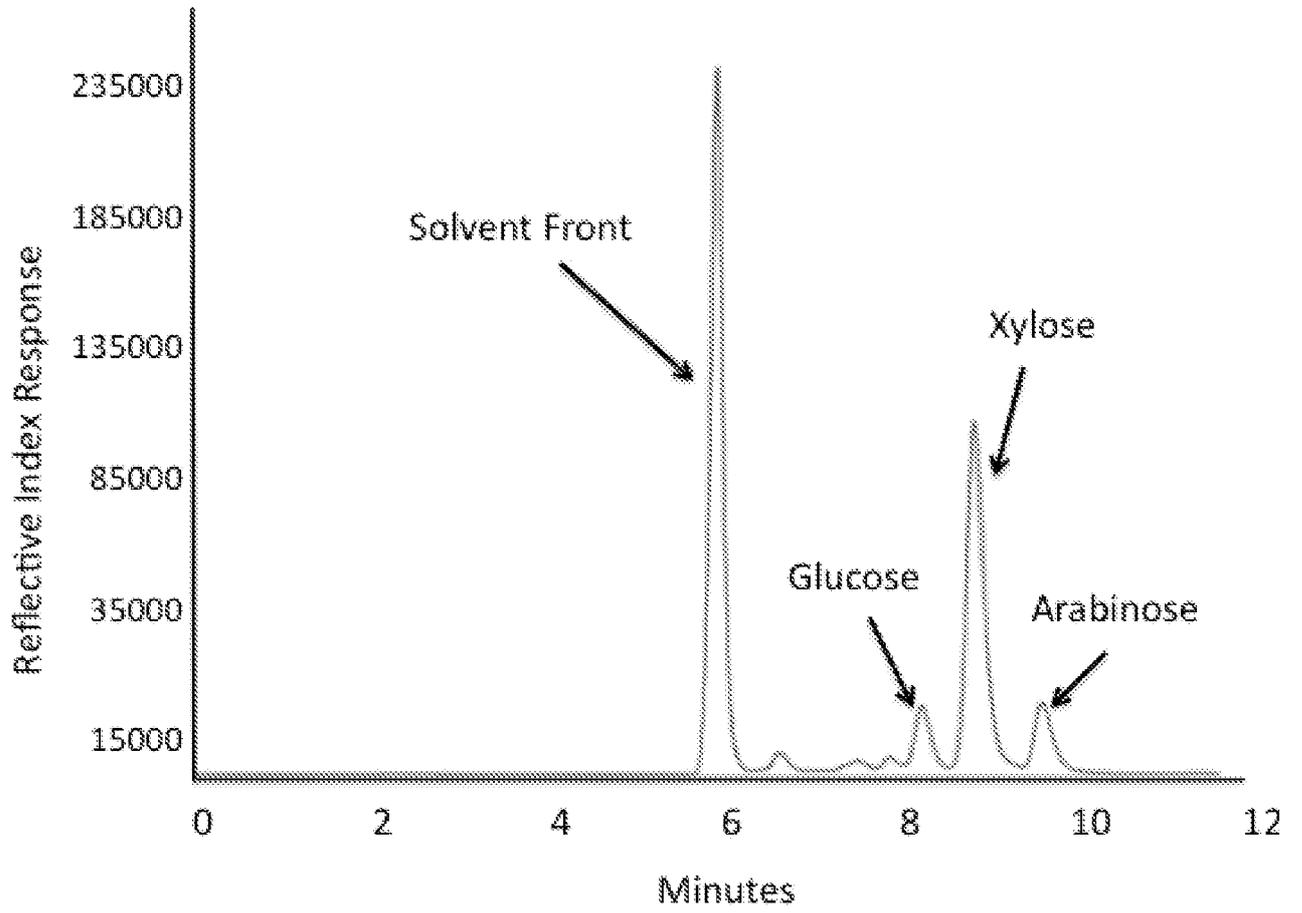
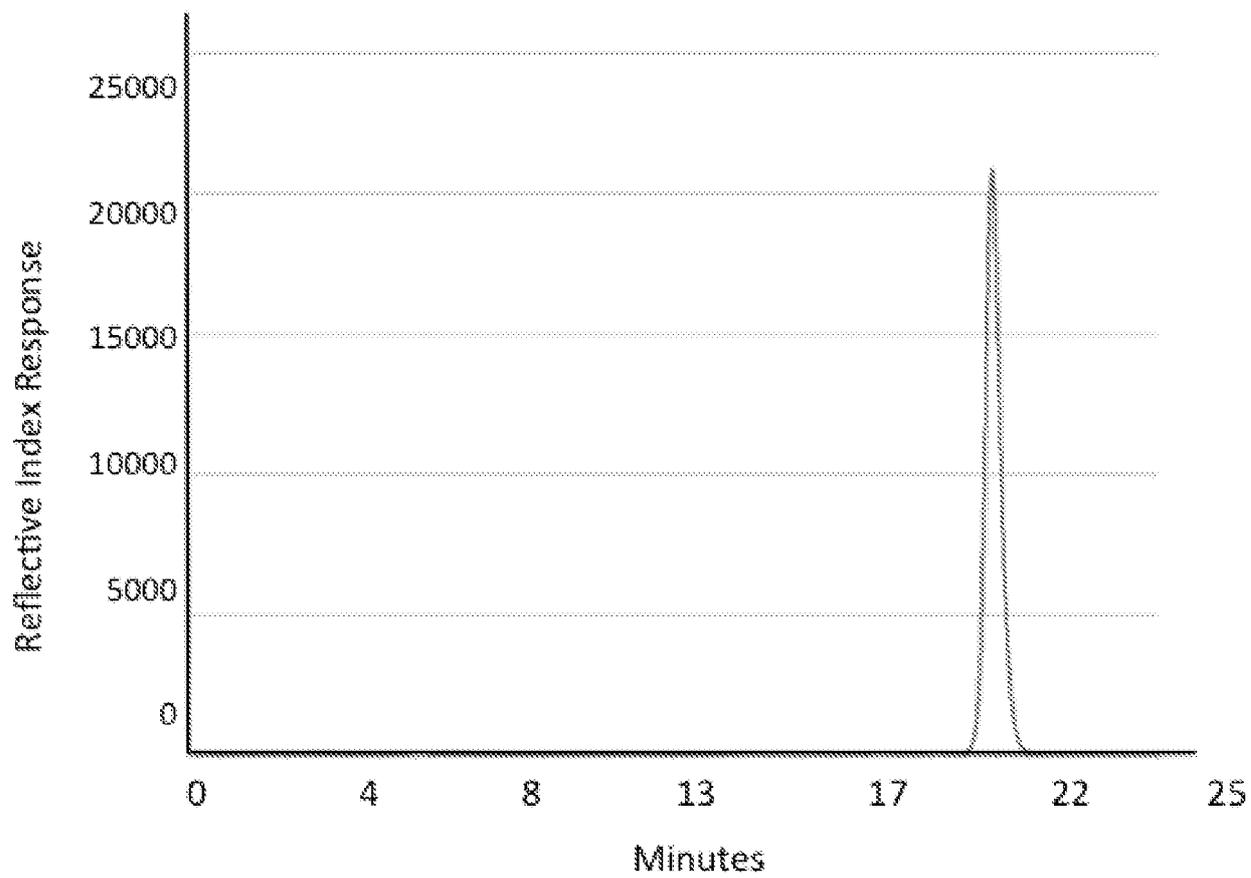


FIGURE 20



**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/JS 10/40502

**A CLASSIFICATION OF SUBJECT MATTER**

**IPC(8) - C12N 1/00; C12N 1/20 (201 0.01 )**  
**USPC - 435/243, 435/252.1 , 435/252.7**

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)  
 IPC(8) - C12N 1/00; C12N 1/20 (2010.01)  
 USPC - 435/243, 435/252.1, 435/252.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 WEST - DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=YES; OP=ADJ; Google Scholar  
 search terms: biomass, LignocellulosS, cellulosS, hemicellulos\$, carbon, hydrocarbS, feedstock, conversS, fermenS, reactor, treat, treats, proces\$, hydrolyS, saccharifS, oligosacchS, polysacchS, oligomer, phytofermentaS, clostπdS, microorganism, microbe, bacterS, yeast,

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category*     | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No   |
|---------------|---|--|
| X<br>---<br>Y | US 2009/001 1484 A 1 (BERLIN et al.) 08 January 2009 (08.01.2009) para [0005]-[0010]; [0015]; [0031]; [0035H0037]; [0040]-[0042]; abstract.   | 1-10, 13, 16, 19<br>---<br>11, 12, 14, 15, 17, 18, 20-44, 62, 63 |
| Y             | US 2009/0068714 A 1 (LESCHINE et al.) 12 March 2009 (12.03.2009) para [0017]; abstract  | 11, 30, 44, 55   |
| Y             | LESCHINE S. A novel biocatalyst for Cellulosic Ethanol Production. UMass Amherst [online], September 19, 2008 [retrieved on 2010-09-07]. Retrieved from the Internet: <URL: http://scholarworks.umass.edu/cgi/i/viewcontent.cgi?article=1005&context=timbr> | 12, 31, 56   |
| Y             | US 2003/0199049 A 1 (NGUYEN et al.) 23 October 2003 (23.10.2003) para [0021]; [0024]; [0036]; [0046]-[0050]; [0052]; [0053]; [0055]; abstract.  | 14, 15, 17, 18, 20-63  |
| Y             | US 2009/00533777 A 1 (HENNESSEY et al.) 26 February 2009 (26 02.2009) para [0008]-[0016]; [0023]; [0039]; [0046]; [0048]-[0050]; [0059]-[0061]; [0067]; [0104]; [01 18]; [0127]; [0142]; Table 1.   | 45-61  |

**D** Further documents are listed in the continuation of Box C

|   |  |
|---|--|
| <ul style="list-style-type: none"> <li>• Special categories of cited documents</li> </ul>   | <ul style="list-style-type: none"> <li>"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</li> </ul>  |
| <ul style="list-style-type: none"> <li>"A" document defining the general state of the art which is not considered to be of particular relevance</li> </ul>  | <ul style="list-style-type: none"> <li>"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</li> </ul>   |
| <ul style="list-style-type: none"> <li>"E" earlier application or patent but published on or after the international filing date</li> </ul>   | <ul style="list-style-type: none"> <li>"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</li> </ul> |
| <ul style="list-style-type: none"> <li>"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)</li> </ul> | <ul style="list-style-type: none"> <li>"&amp;" document member of the same patent family</li> </ul>  |
| <ul style="list-style-type: none"> <li>"O" document referring to an oral disclosure, use, exhibition or other means</li> </ul>  |  |
| <ul style="list-style-type: none"> <li>"P" document published prior to the international filing date but later than the priority date claimed</li> </ul>  |  |

Date of the actual completion of the international search  
 07 September 2010 (07.09.2010)

Date of mailing of the international search report  
**20 SEP 2010**

Name and mailing address of the ISA/US  
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