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(54) Title: COMPOSITIONS AND METHODS FOR IMPROVED SACCHARIFICATION OF BIOMASS

(57) Abstract: Compositions and methods are provided for enhancing saccharification of biomass with one or more enzymes to enhance availability of substrates for fermentation by a microorganism. Microorganisms are also modified to enhance activity of one or more hydrolytic enzymes that are present endogenously in or are introduced heterologously into a host microorganism.

COMPOSITIONS AND METHODS FOR IMPROVED SACCHARIFICATION OF BIOMASS

CLAIM OF PRIORITY

[0001] This application claims the benefit of priority from U.S. Provisional Patent Application Serial No. 61/221,523, filed on June 29, 2009, and U.S. Non-Provisional Patent Application Serial No. 12/630,784, filed on December 3, 2009, which are incorporated herein by reference in their entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 30, 2009, is named 37836717.txt, and is 81,443 bytes in size.

BACKGROUND OF THE INVENTION

[0003] 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). Biomass includes agricultural residues (corn stalks, grass, straw, grain hulls, bagasse, etc.), animal waste (manure from cattle, poultry, and hogs), woody materials (wood or bark, sawdust, timber slash, and mill scrap), municipal waste (waste paper, recycled toilet papers, yard clippings, etc.), and energy crops (poplars, willows, switch grass, alfalfa, prairie bluestem, algae etc.). Lignocellulosic biomass has cellulose and hemicellulose as two major components. To obtain a high fermentation efficiency of lignocellulosic biomass to end-product (yield) it is important to provide an appropriate fermentation environment to enhance end-product yield. More complete saccharification of biomass and fermentation of the saccharification products results in higher fuel yields.

[0004] Unfortunately, many organisms used for fermentation of carbonaceous substrates cannot saccharify polysaccharides into monosaccharides. Progress in bioproduct fermentation has been hampered by lack of suitable microorganisms that can effectively hydrolyze and metabolize all of the sugars present in a biomass. This increases the cost of fermentation because hydrolysis must be accomplished by the addition of expensive mixtures of enzymes that can hydrolyze five and six carbon polysaccharides. There is considerable need for organisms that can efficiently utilize polysaccharides such as cellulose and hemicellulose with little or no enzyme addition during fermentation.

SUMMARY OF THE INVENTION

[0005] In one aspect of the invention, a process is provided for producing a fermentive end-product comprising contacting a carbonaceous biomass with (a) a microorganism that hydrolyses and ferments said biomass; and (b) an external source of one or more enzymes that are capable of enhancing said hydrolysis, and allowing sufficient time for said hydrolysis and fermentation to produce a fermentive end-product wherein the one or more enzymes do not include a xylanase, a hemicellulase, a glucanase or glucosidase, and wherein said external source is not the microorganism.

[0006] In another aspect of the invention, a process is provided for producing a fermentive end-product comprising contacting a carbonaceous biomass with (a) a microorganism that hydrolyses and ferments said biomass; and (b) an external source of a single enzyme that is capable of enhancing said hydrolysis, and allowing sufficient time for the hydrolysis and fermentation to produce a fermentive end-product wherein the enzyme is a cellulase and wherein the external source is not the microorganism.

[0007] In a further aspect of the invention, a process is provided for producing a fermentive end-product comprising contacting a carbonaceous biomass with (a) a microorganism that hydrolyses and ferments the biomass; and (b) an external source of one or more enzymes that are capable of enhancing said hydrolysis, and allowing sufficient time for the hydrolysis and fermentation to produce a fermentive end-product wherein the one or more enzymes comprise cellulase, and wherein the cellulase and the microorganism act synergistically to enhance hydrolysis.

[0008] In another aspect of the invention, a process is provided for producing a fermentive end-product comprising contacting a carbonaceous biomass with (a) a recombinant microorganism that hydrolyses and ferments the biomass; and (b) an external source of one or more enzymes that are capable of enhancing the hydrolysis, and allowing sufficient time for the hydrolysis and fermentation to produce a fermentive end-product.

[0009] Any of the above processes can be carried out where contact is in a large-scale fermentation vessel, wherein the fermentation vessel is adapted to provide suitable conditions for fermentation of one or more carbohydrate into fermentive end-products. Further, any of the above processes can be carried out where the microorganism is capable of direct fermentation of C5 and C6 carbohydrates. Useful microorganisms include a bacterium, a species of Clostridia, *Clostridium phytofermentans* or *Clostridium. sp. Q.D.*

[0010] . Further the microorganism can be non-recombinant or recombinant. If recombinant, the microorganism can comprise one or more heterologous polynucleotides that enhance the

activity of one or more cellulases, or one or more polynucleotides that encode one or more copies of an endogenous cellulase.

[0011] In a further embodiment, in any of the above processes, the biomass can comprise one or more of corn steep solids, corn steep liquor, malt syrup, xylan, cellulose, hemicellulose, fructose, glucose, mannose, rhamnose, or xylose. In another embodiment, in any of the above processes, the biomass can comprise municipal waste, wood, plant material, plant material extract, a natural or synthetic polymer, or a combination thereof. Further, the plant material can be switchgrass, bagasse, corn stover or poplar.

[0012] In another embodiment, the fermentive end-product of any of the above processes is an alcohol. Further, the fermentive end-product of any of the above processes can be ethanol, lactic acid, acetic acid or formic acid.

[0013] Any of the above processes can also be carried out where the microorganism is capable of uptake of one or more complex carbohydrates. These processes also include biomass that comprises a higher concentration of oligomeric carbohydrates relative to monomeric carbohydrates.

[0014] In a further aspect of this invention, the polynucleotides used in the processes of this invention comprise all or a portion of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 14, SEQ ID NO: 18, or SEQ ID NO: 32, or one or more polynucleotides that encodes one or more enzymes selected from the group consisting of Cphy_3202, Cphy_2058, Cphy_1163, Cphy_3367, Cphy_1100, Cphy_1510, Cphy_3368, and Cphy_2128.

[0015] The processes of this invention also include embodiments wherein the external source of one or more enzymes is in an amount from about 0.4 to about 15 filter paper unit (FPU)/gram cellulose, or wherein cellulase is in an amount from about 0.4 to about 15 filter paper unit (FPU)/gram cellulose, or wherein the enhanced hydrolytic activity is equivalent to addition of cellulases in an amount sufficient to provide activity of about 0.4 to about 15 filter paper unit (FPU)/gram cellulose.

[0016] In a further embodiment, the processes of this invention provide hydrolysis that result in a greater concentration of cellobiose and/or larger oligomers, relative to monomeric carbohydrates. The monomeric carbohydrates can comprise xylose and arabinose.

[0017] In a further embodiment, the processes of this invention can be carried out with a biomass that is pre-conditioned with alkali treatment.

[0018] In another aspect of this invention, the recombinant microorganism useful for hydrolysis and fermentation during the processes of this invention produces one or more

hydrolytic enzyme encoded by a variant having a polynucleotide sequence with an identity of 70% or more compared to a sequence selected from SEQ ID NO 1, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 14, or a combination thereof.

[0019] In a further embodiment of this invention, a product for production of a fermentive end-product can comprise (a) a carbonaceous biomass, (b) a microorganism that hydrolyses and ferments the biomass; and (c) an external source of one or more enzymes that are capable of enhancing the hydrolysis, wherein the one or more enzymes do not include a xylanase, a hemicellulase, a glucanase or glucosidase, and wherein the external source is not said microorganism.

[0020] In another aspect of the invention, a product is provided for production of a fermentive end-product, the product comprising (a) a carbonaceous biomass, (b) a microorganism that hydrolyses and ferments the biomass; and (c) an external source of a single enzyme that is capable of enhancing the hydrolysis, wherein one or more enzymes is a cellulase.

[0021] In another aspect of the invention, a product is provided for production of a fermentive end-product, the product comprising (a) a carbonaceous biomass, (b) a microorganism that hydrolyses and ferments the biomass; and (c) an external source of one or more enzymes that are capable of enhancing the hydrolysis, wherein the one or more enzymes comprise cellulase, and wherein the cellulase and the microorganism act synergistically to produce an end product.

[0022] In another aspect of the invention, a product is provided for production of a fermentive end-product, the product comprising (a) a carbonaceous biomass, (b) a microorganism that hydrolyses and ferments the biomass; and (c) an external source of one or more enzymes that are capable of enhancing the hydrolysis.

[0023] This invention further comprises any of the above products wherein the microorganism is capable of direct fermentation of C5 and C6 carbohydrates. A useful microorganism to produce the products of this invention is a bacterium, a species of Clostridia, or *Clostridium phytofermentans*. The microorganism can be non-recombinant. Further the microorganism can comprise one or more heterologous polynucleotides that enhance the activity of one or more cellulases or can be modified to provide enhanced activity of one or more cellulase.

[0024] This invention further includes any of the above products wherein the biomass comprises one or more of corn steep solids, corn steep liquor, malt syrup, xylan, cellulose, hemicellulose, fructose, glucose, mannose, rhamnose, or xylose. The biomass of this invention can also comprise municipal waste, wood, plant material, plant material extract, a natural or synthetic polymer, or a combination thereof. Useful plant material is switchgrass, bagasse, corn stover or poplar.

[0025] This invention further comprises any of the above products wherein the fermentive end-product is an alcohol or comprises ethanol, lactic acid, acetic acid and formic acid.

[0026] In another embodiment of this invention, the products include a microorganism capable of uptake of one or more complex carbohydrates.

[0027] In another embodiment of this invention, the products include a biomass comprising a higher concentration of oligomeric carbohydrates relative to monomeric carbohydrates.

[0028] In another embodiment of this invention, the products for production of a fermentive end-product include an enzyme identified in Table 2. In a further embodiment, the products include one or more polynucleotides that are SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 14, SEQ ID NO: 17, or SEQ ID NO: 32 or one or more polynucleotides encoding Cphy_3202, Cphy_2058, Cphy_1163, Cphy_3367, Cphy_1100, Cphy_1510, Cphy_3368, or Cphy_2128.

[0029] One aspect of this invention includes a vector comprising: (a) one or more polynucleotides encoding a biomass degrading enzyme; and (b) a sequence encoding an erythromycin resistance gene. In another embodiment, this invention includes a vector comprising: (a) one or more polynucleotides encoding a biomass degrading enzyme; and (b) a SacII restriction site. Either of these vectors can further comprise a constitutively active promoter. In a further embodiment, the constitutively active promoter is a Cphy_3510 promoter.

[0030] The vectors of this invention can further comprise a polynucleotide that encodes Cphy_3289 or Cphy_3290. The vectors of this invention can also include one or more polynucleotides such as SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 14, SEQ ID NO: 17, or SEQ ID NO: 32 or one or more polynucleotides that hybridizes to SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 14, SEQ ID NO: 18 or SEQ ID NO: 32 under conditions of medium stringency.

[0031] Vectors of this invention can encode biomass degrading enzymes such as Cphy_3202, Cphy_2058, Cphy_1163, Cphy_3367, Cphy_1100, Cphy_1510, Cphy_3368, or Cphy_2128.

[0032] In another embodiment, vectors of this invention can comprise polynucleotides encoding a polypeptide of SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, or SEQ ID NO: 29.

[0033] In another aspect of this invention, vectors can comprise a polynucleotide that encodes a polypeptide that is expressed in a species of gram$^+$ bacteria, gram$^-$ bacteria, a yeast, or other microbe. The polynucleotide can also encode a cellulase.

[0034] In another embodiment, vectors of this invention can encode a biomass degrading enzyme that is a cellulolytic, hemicellulolytic, or ligninolytic enzyme.

[0035] A further embodiment of this invention includes a microorganism comprising any of the vectors of the invention. The recombinant microorganism can have increased expression of one or more biomass degrading enzymes as compared to a non-recombinant microorganism, wherein the one or more biomass degrading enzymes are cellulolytic, hemicellulolytic, or ligninolytic enzymes.

[0036] Such useful recombinant microorganisms with enhanced hydrolytic activity will express one or more biomass degrading enzymes encoded by SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, or SEQ ID NO: 14, or the chaperonin proteins encoded by SEQ ID NO: 17. Other useful recombinant microorganisms with enhanced hydrolytic activity will express one or more biomass degrading enzymes wherein the one or more biomass degrading enzymes are SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, or express the chaperonin proteins SEQ ID NO: 26 and SEQ ID NO: 27. In a further embodiment, the recombinant microorganisms will express one or more biomass degrading enzymes wherein the one or more biomass degrading enzymes are Cphy_3202, Cphy_2058, Cphy_1163, Cphy_3367, Cphy_1100, Cphy_1510, Cphy_3368, or Cphy_2128. The recombinant microorganism can further comprise one or both of the chaperonin proteins encoded by all or a portion of SEQ ID NO: 17.

[0037] The recombinant microorganisms of this invention can comprise a Cphy_3510 promoter.

[0038] The recombinant microorganisms of this invention can be species of *Clostridium*, including but not limited to *C. phytofermentans*, but they can also be a bacterium or yeast or other fungal cell. The plasmids and promoters of this invention are designed to work in any microbe.

[0039] In a further embodiment, the microorganism of this invention can demonstrate enhanced hydrolytic activity that is equivalent to the addition of cellulases in an amount sufficient to provide activity of about 0.4 to about 15 filter paper unit (FPU)/gram cellulose. These microorganisms can comprise one or more heterologous polynucleotides that enhance the activity of one or more cellulases or any biomass-degrading enzyme.

[0040] In another aspect of this invention the microorganisms can hydrolyze and ferment biomass that comprises one or more of corn steep solids, corn steep liquor, Distillers Dried Solubles (DDS), Distillers Dried Grains (DDG), Condensed Distillers Solubles (CDS), Distillers Wet Grains (DWG), Distillers Dried Grains with Solubles (DDGS), malt syrup, xylan, cellulose, hemicellulose, fructose, glucose, mannose, rhamnose, or xylose. Further, the biomass can comprise municipal waste, wood, plant material, plant material extract, a natural or synthetic polymer, or a combination thereof.

[0041] In another embodiment, the microorganisms of this invention are capable of uptake of one or more complex carbohydrates and can utilize biomass comprising a higher concentration of oligomeric carbohydrates relative to monomeric carbohydrates. In some of these microorganisms, the biomass degrading enzyme is Cphy_3202, Cphy_2058, Cphy_1163, Cphy_3367, Cphy_1100, Cphy_1510, Cphy_3368, or Cphy_2128.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are used, and the accompanying drawings of which:

[0043] Figures 1A-1B illustrates ethanol production from corn stover utilizing a cocktail mix (A) and individual enzyme(s) components of the cocktail mix (B).

[0044] Figure 2 illustrates saccharification yield.

[0045] Figure 3 illustrates ethanol production from corn stover utilizing a cocktail mix (A) and individual enzyme(s) components of the cocktail mix (B).

[0046] Figure 4 illustrates a hydrolysis plot showing *C. phytofermentans* (Q) saccharification.

[0047] Figure 5 illustrates enzyme-assisted fermentation of corn stover.

[0048] Figure 6 illustrates a pathway map for cellulose hydrolysis and fermentation.

[0049] Figure 7 illustrates a plasmid map for pIMP1.

[0050] Figure 8 illustrates a plasmid map for pIMCphy.

[0051] Figure 9 illustrates a plasmid map for pCphyP3510.

[0052] Figure 10 illustrates CMC-congo red plate and Cellazyme Y assays.

[0053] Figure 11 illustrates a plasmid map for pCphyP3510-1163.

[0054] Figure 12 illustrates the nucleic acid sequence of Cphy_1163 and relevant primers.

[0055] Figure 13 illustrates the nucleic acid sequence of Cphy_3367 and relevant primers.

[0056] Figure 14 illustrates the nucleic acid sequence of Cphy_3368 and relevant primers.

[0057] Figure 15 illustrates the nucleic acid sequence of Cphy_3202 and relevant primers.

[0058] Figure 16 depicts the nucleic acid sequence of Cphy_2058 and relevant primers.

[0059] Figure 17 depicts the nucleic acid sequences of Cphy_3289 and Cphy_3290 and relevant primers.

[0060] Figure 18 depicts the amino acid sequence of Cphy_1163.

[0061] Figure 19 illustrates the amino acid sequence of Cphy_3367.

[0062] Figure 20 illustrates the amino acid sequence of Cphy_3368.

[0063] Figure 21 illustrates the amino acid sequence of Cphy_3202.

[0064] Figure 22 illustrates the amino acid sequence of Cphy_2058.

[0065] Figure 23 illustrates the amino acid sequence of Cphy_1100.

[0066] Figure 24 illustrates the amino acid sequence of Cphy_1510.

[0067] Figure 25 illustrates the amino acid sequence of Cphy_2128.

[0068] Figure 26 illustrates the amino acid sequence of Cphy_3289 chaperonin GroEL.

[0069] Figure 27 illustrates the amino acid sequence of Cphy_3290 chaperonin GroES.

[0070] Figure 28 illustrates the nucleic acid sequence of Cphy_3510.

[0071] Figure 29 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.

[0072] Figure 30 depicts a method for producing fermentation end products from biomass by charging biomass to a fermentation vessel.

[0073] Figure 31 discloses pretreatments that produce hexose or pentose saccharides or oligomers that are then unprocessed or processed further and either, fermented separately or together.

INCORPORATION BY REFERENCE

[0074] 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

[0075] The following description and examples illustrate embodiments of the present invention in detail. Generally, methods and compositions directed to saccharification and fermentation of various biomass substrates to one or more fermentive products are disclosed.

[0076] Unless characterized differently, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

[0077] Definitions

[0078] The term "enzyme reactive conditions" as used herein, refers to an environmental condition (*i.e.*, such factors as temperature, pH, lack of inhibiting substances) which will permit the enzyme to function. Enzyme reactive conditions can be either *in vitro*, such as in a test tube, or *in vivo*, such as within a cell.

[0079] The term "about" as used herein, refers to a range that is 15% plus or minus from a stated numerical value within the context of the particular usage. For example, about 10 would include a range from 8.5 to 11.5.

[0080] The terms "function" and "functional" as used herein refer to biological or enzymatic function.

[0081] The term "gene" as used herein, refers to a unit of inheritance that occupies a specific locus on a chromosome and consists of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (*i.e.*, introns, 5' and 3' untranslated sequences). The term "host cell" includes an individual cell or cell culture which can be or has been a recipient of any recombinant vector(s) or isolated polynucleotide. Host cells include progeny of a single host cell, and the progeny can not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected, transformed, or infected *in vivo* or *in vitro* with a recombinant vector or a polynucleotide. A host cell which comprises a recombinant vector is a recombinant host cell, recombinant cell, or recombinant microorganism.

[0082] The term "isolated" as used herein, refers to material that is substantially or essentially free from components that normally accompany it in its native state. For example, an "isolated polynucleotide", as used herein, refers to a polynucleotide, which has been purified from the sequences which flank it in a naturally-occurring state, *e.g.*, a DNA fragment which has been removed from the sequences that are normally adjacent to the fragment. Alternatively, an "isolated peptide" or an "isolated polypeptide" and the like, as used herein, refer to *in vitro* isolation and/or purification of a peptide or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, *i.e.*, it is not associated with *in vivo* substances.

[0083] The term "increased" or "increasing" as used herein, refers to the ability of one or more recombinant microorganisms to produce a greater amount of a given product or molecule (*e.g.*,

commodity chemical, biofuel, or intermediate product thereof) as compared to a control microorganism, such as an unmodified microorganism or a differently-modified microorganism. An "increased" amount is typically a "statistically significant" amount, and can include an increase that is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (including all integers and decimal points in between, *e.g.*, 1.5, 1.6, 1.7. 1.8, etc.) the amount produced by an unmodified microorganism or a differently modified microorganism.

[0084] The term "operably linked" as used herein means placing a gene under the regulatory control of a promoter, which then controls the transcription and optionally the translation of the gene. In one example for the construction of promoter/structural gene combinations, the genetic sequence or promoter is positioned at a distance from the gene transcription start site that is approximately the same as the distance between that genetic sequence or promoter and the gene it controls in its natural setting; *i.e.* the gene from which the genetic sequence or promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of function. Similarly, a regulatory sequence element can be positioned with respect to a gene to be placed under its control in the same position as the element is situated in its in its natural setting with respect to the native gene it controls.

[0085] The term "Constitutive promoter" refers to a polynucleotide sequence that induces transcription or is typically active, (*i.e.*, promotes transcription), under most conditions, such as those that occur in a host cell. A constitutive promoter is generally active in a host cell through a variety of different environmental conditions.

[0086] The term "Inducible promoter" refers to a polynucleotide sequence that induces transcription or is typically active only under certain conditions, such as in the presence of a specific transcription factor or transcription factor complex, a given molecule factor (*e.g.*, IPTG) or a given environmental condition (*e.g.*, CO2concentration, nutrient levels, light, heat). In the absence of that condition, inducible promoters typically do not allow significant or measurable levels of transcriptional activity.

[0087] The terms "polynucleotide" or "nucleic acid" as used herein designates mRNA, RNA, cRNA, rRNA, cDNA or DNA. The term typically refers to polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

[0088] As will be understood by those skilled in the art, a polynucleotide sequence can include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or can be adapted to express, proteins, polypeptides, peptides and the like. Such segments can be naturally isolated, or modified synthetically by the hand of man.

[0089] Polynucleotides can be single-stranded (coding or antisense) or double-stranded, and can be DNA (genomic, cDNA or synthetic) or RNA molecules. In one embodiment additional coding or non-coding sequences can, be present within a polynucleotide. In another embodiment a polynucleotide can be linked to other molecules and/or support materials.

[0090] Polynucleotides can comprise a native sequence (*i.e.*, an endogenous sequence) or can comprise a variant, or a biological functional equivalent of such a sequence. Polynucleotide variants can contain one or more base substitutions, additions, deletions and/or insertions, as further described below. In one embodiment a polynucleotide variant encodes a polypeptide with the same sequence as the native protein. In another embodiment a polynucleotide variant encodes a polypeptide with substantially similar enzymatic activity as the native protein. In another embodiment a polynucleotide variant encodes a protein with increased enzymatic activity relative to the native polypeptide. The effect on the enzymatic activity of the encoded polypeptide can generally be assessed as described herein.

[0091] A polynucleotide encoding a polypeptide can be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length can vary considerably. In one embodiment the maximum length of a polynucleotide sequence which can be used to transform a microorganism is governed only by the nature of the recombinant protocol employed.

[0092] The terms "polynucleotide variant" and "variant" and the like refer to polynucleotides that display substantial sequence identity with any of the reference polynucleotide sequences or genes described herein, and to polynucleotides that hybridize with any polynucleotide reference sequence described herein, or any polynucleotide coding sequence of any gene or protein referred to herein, under low stringency, medium stringency, high stringency, or very high stringency conditions that are defined hereinafter and known in the art. These terms also encompass polynucleotides that are distinguished from a reference polynucleotide by the addition, deletion or substitution of at least one nucleotide. Accordingly, the terms "polynucleotide variant" and "variant" include polynucleotides in which one or more nucleotides have been added or deleted, or replaced with different nucleotides. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference polynucleotide whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide, or has increased activity in relation to the reference polynucleotide (*i.e.*, optimized). Polynucleotide variants include, for example, polynucleotides having at least 50% (and at least 51% to at least 99% and

all integer percentages in between) sequence identity with a reference polynucleotide described herein.

[0093] The terms "polynucleotide variant" and "variant" also include naturally-occurring allelic variants that encode these enzymes. Examples of naturally-occurring variants include allelic variants (same locus), homologs (different locus), and orthologs (different microorganism). Naturally occurring variants such as these can be identified and isolated using well-known molecular biology techniques including, for example, various polymerase chain reaction (PCR) and hybridization-based techniques as known in the art. Naturally-occurring variants can be isolated from any microorganism that encodes one or more genes having a suitable enzymatic activity described herein (*e.g.*, C--C ligase, diol dehyodrogenase, pectate lyase, alginate lyase, diol dehydratase, transporter, etc.).

[0094] Non-naturally occurring variants can be made by mutagenesis techniques, including those applied to polynucleotides, cells, or microorganisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. In certain aspects, non-naturally occurring variants can have been optimized for use in a given microorganism (*e.g.*, *E. coli*), such as by engineering and screening the enzymes for increased activity, stability, or any other desirable feature. The variations can produce both conservative and non-conservative amino acid substitutions (as compared to the originally encoded product). For polynucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of a reference polypeptide. Variant polynucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a biologically active polypeptide. Generally, variants of a reference polynucleotide sequence will have at least about 30%, 40% 50%, 55%, 60%, 65%, 70%, generally at least about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity with the reference polynucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters. In one embodiment a variant polynucleotide sequence encodes a protein with substantially similar activity compared to a protein encoded by the respective reference polynucleotide sequence. Substantially similar activity means variant protein activity that is within +/- 15% of the activity of a protein encoded by the respective reference polynucleotide sequence. In another embodiment a variant polynucleotide sequence encodes a protein with

greater activity compared to a protein encoded by the respective reference polynucleotide sequence.

[0095] The terms "hybridizes under low stringency, hybridizes medium stringency, hybridizes high stringency, or hybridizes very high stringency conditions" as used herein, refers to conditions for hybridization and washing. Guidance for performing hybridization reactions can be found in Ausubel et al., "Current Protocols in Molecular Biology", John Wiley & Sons Inc, 1994-1998, Sections 6.3.1-6.3.6. Aqueous and non-aqueous methods are described in that reference and either can be used.

[0096] The term "low stringency" as used herein, refers to conditions that include and encompass from at least about 1% v/v to at least about 15% v/v formamide and from at least about 1 M to at least about 2 M salt for hybridization at 42° C., and at least about 1 M to at least about 2 M salt for washing at 42° C. Low stringency conditions also can include 1% Bovine Serum Albumin (BSA), 1 mM EDTA, 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS for hybridization at 65° C., and (i) 2X SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$ (pH 7.2), 5% SDS for washing at room temperature. One embodiment of low stringency conditions includes hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45° C., followed by two washes in 0.2X SSC, 0.1% SDS at least at 50° C. (the temperature of the washes can be increased to 55° C. for low stringency conditions).

[0097] The term "Medium stringency" as used herein, refers to conditions that include and encompass from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization at 42° C., and at least about 0.1 M to at least about 0.2 M salt for washing at 55° C. Medium stringency conditions also can include 1% Bovine Serum Albumin (BSA), 1 mM EDTA, 0.5 M NaHPO4 (pH 7.2), 7% SDS for hybridization at 65° C., and (i) 2X SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$ (pH 7.2), 5% SDS for washing at 60-65° C. One embodiment of medium stringency conditions includes hybridizing in 6X SSC at about 45° C., followed by one or more washes in 0.2X SSC, 0.1% SDS at 60° C.

[0098] The term "High stringency" as used herein, refers to conditions that include and encompass from at least about 31% v/v to at least about 50% v/v formamide and from about 0.01 M to about 0.15 M salt for hybridization at 42° C., and about 0.01 M to about 0.02 M salt for washing at 55° C. High stringency conditions also can include 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS for hybridization at 65° C., and (i) 0.2X SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$ (pH 7.2), 1% SDS for washing at a temperature in

excess of 65° C. One embodiment of high stringency conditions includes hybridizing in 6X SSC at about 45° C., followed by one or more washes in 0.2X SSC, 0.1% SDS at 65° C.

[0099] Due to the degeneracy of the genetic code, amino acids can be substituted for other amino acids in a protein sequence without appreciable loss of the desired activity (see Table 1 below). It is thus contemplated that various changes can be made in the peptide sequences of the disclosed protein sequences, or their corresponding nucleic acid sequences without appreciable loss of the biological activity.

[00100] In making such changes, the hydropathic index of amino acids can be considered. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle, J. Mol. Biol., 157: 105-132, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

[00101] Amino acids have been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics. These are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cysteine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate/glutamine/aspartate/asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

[00102] It is known in the art that certain amino acids can be substituted by other amino acids having a similar hydropathic index or score and result in a protein with similar biological activity, *i.e.*, still obtain a biologically-functional protein. In one embodiment, the substitution of amino acids whose hydropathic indices are within +/-0.2 is preferred, those within +/-0.1 are more preferred, and those within +/-.0.5 are most preferred.

[00103] It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101 (Hopp, which is herein incorporated by reference in its entirety) states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0.+-.1); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5.+-.1); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4).

[00104] It is understood that an amino acid can be substituted by another amino acid having a similar hydrophilicity score and still result in a protein with similar biological activity, *i.e.*, still obtain a biologically functional protein. In one embodiment the substitution of amino acids whose hydropathic indices are within +/-0.2 is preferred, those within +/-0.1 are more preferred, and those within +/-.0.5 are most preferred.

[00105] As outlined above, amino acid substitutions can be based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take any of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine. Changes which are not expected to be advantageous can also be used if these resulting proteins have the same or improved characteristics, relative to the unmodified polypeptide from which they are engineered.

[00106] In one embodiment a polynucleotide comprises codons in its protein coding sequence that are optimized to increase the thermostability of an mRNA transcribed from the polynucleotide. In one embodiment this optimization does not change the amino acid sequence encoded by the polynucleotide. In another embodiment a polynucleotide comprises codons in its protein coding sequence that are optimized to increase translation efficiency of an mRNA from the polynucleotide in a host cell. In one embodiment this optimization does not change the amino acid sequence encoded by the polynucleotide.

[00107] The RNA codon table below (Table 1) shows the 64 codons and the amino acid for each. The direction of the mRNA is 5' to 3'.

Table 1

		2nd base			
		U	**C**	**A**	**G**
1st base	**U**	UUU (Phe/F) Phenylalanine	UCU (Ser/S) Serine	UAU (Tyr/Y) Tyrosine	UGU (Cys/C) Cysteine
		UUC (Phe/F) Phenylalanine	UCC (Ser/S) Serine	UAC (Tyr/Y) Tyrosine	UGC (Cys/C) Cysteine
		UUA (Leu/L) Leucine	UCA (Ser/S)	UAA Ochre (*Stop*)	UGA Opal (*Stop*)

			Serine		
		UUG (Leu/L) Leucine	UCG (Ser/S) Serine	UAG Amber (*Stop*)	UGG (Trp/W) Tryptophan
	C	CUU (Leu/L) Leucine	CCU (Pro/P) Proline	CAU (His/H) Histidine	CGU (Arg/R) Arginine
		CUC (Leu/L) Leucine	CCC (Pro/P) Proline	CAC (His/H) Histidine	CGC (Arg/R) Arginine
		CUA (Leu/L) Leucine	CCA (Pro/P) Proline	CAA (Gln/Q) Glutamine	CGA (Arg/R) Arginine
		CUG (Leu/L) Leucine	CCG (Pro/P) Proline	CAG (Gln/Q) Glutamine	CGG (Arg/R) Arginine
	A	AUU (Ile/I) Isoleucine	ACU (Thr/T) Threonine	AAU (Asn/N) Asparagine	AGU (Ser/S) Serine
		AUC (Ile/I) Isoleucine	ACC (Thr/T) Threonine	AAC (Asn/N) Asparagine	AGC (Ser/S) Serine
		AUA (Ile/I) Isoleucine	ACA (Thr/T) Threonine	AAA (Lys/K) Lysine	AGA (Arg/R) Arginine
		AUG[A] (Met/M) Methionine	ACG (Thr/T) Threonine	AAG (Lys/K) Lysine	AGG (Arg/R) Arginine
	G	GUU (Val/V) Valine	GCU (Ala/A) Alanine	GAU (Asp/D) Aspartic acid	GGU (Gly/G) Glycine
		GUC (Val/V) Valine	GCC (Ala/A) Alanine	GAC (Asp/D) Aspartic acid	GGC (Gly/G) Glycine
		GUA (Val/V) Valine	GCA (Ala/A) Alanine	GAA (Glu/E) Glutamic acid	GGA (Gly/G) Glycine
		GUG (Val/V) Valine	GCG (Ala/A) Alanine	GAG (Glu/E) Glutamic acid	GGG (Gly/G) Glycine

[A] The codon AUG both codes for methionine and serves as an initiation site: the first AUG in an mRNA's coding region is where translation into protein begins.

[00108] In one embodiment a method disclosed which uses variants of full-length polypeptides having any of the enzymatic activities described herein, truncated fragments of these full-length polypeptides, variants of truncated fragments, as well as their related biologically active fragments. Typically, biologically active fragments of a polypeptide can participate in an interaction, for example, an intra-molecular or an inter-molecular interaction. An inter-molecular interaction can be a specific binding interaction or an enzymatic interaction (*e.g.*, the interaction can be transient and a covalent bond is formed or broken). Biologically active fragments of a polypeptide/enzyme an enzymatic activity described herein include peptides comprising amino acid sequences sufficiently similar to, or derived from, the amino acid sequences of a (putative) full-length reference polypeptide sequence. Typically, biologically active fragments comprise a domain or motif with at least one enzymatic activity, and can include one or more (and in some cases all) of the various active domains. A biologically active fragment of a an enzyme can be a polypeptide fragment which is, for example, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400, 450, 500, 600 or more contiguous amino acids, including all integers in between, of a reference polypeptide sequence. In certain embodiments, a biologically active fragment comprises a conserved enzymatic sequence, domain, or motif, as described elsewhere herein and known in the art. Suitably, the biologically-active fragment has no less than about 1%, 10%, 25%, or 50% of an activity of the wild-type polypeptide from which it is derived.

[00109] The term "exogenous" as used herein, refers to a polynucleotide sequence or polypeptide that does not naturally occur in a given wild-type cell or microorganism, but is typically introduced into the cell by a molecular biological technique, *i.e.*, engineering to produce a recombinant microorganism. Examples of "exogenous" polynucleotides include vectors, plasmids, and/or man-made nucleic acid constructs encoding a desired protein or enzyme.

[00110] The term "endogenous" as used herein, refers to naturally-occurring polynucleotide sequences or polypeptides that can be found in a given wild-type cell or microorganism. For example, certain naturally-occurring bacterial or yeast species do not typically contain a benzaldehyde lyase gene, and, therefore, do not comprise an "endogenous" polynucleotide sequence that encodes a benzaldehyde lyase. In this regard, it is also noted that even though a microorganism can comprise an endogenous copy of a given polynucleotide sequence or gene, the introduction of a plasmid or vector encoding that sequence, such as to over-express or otherwise regulate the expression of the encoded protein, represents an

"exogenous" copy of that gene or polynucleotide sequence. Any of the of pathways, genes, or enzymes described herein can utilize or rely on an "endogenous" sequence, or can be provided as one or more "exogenous" polynucleotide sequences, and/or can be used according to the endogenous sequences already contained within a given microorganism.

[00111] The term "sequence identity" for example, comprising a "sequence 50% identical to," as used herein, refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" can be calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, I) or the identical amino acid residue (*e.g.*, Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

[00112] The terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides can each comprise (1) a sequence (*i.e.*, only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150 in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window can comprise additions or deletions (*i.e.*, gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window can be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, Wis., USA) or by inspection and the best alignment (*i.e.*, resulting in the highest percentage homology over the comparison

window) generated by any of the various methods selected. Reference also can be made to the BLAST family of programs as for example disclosed by Altschul et al., 1997, Nucl. Acids Res. 25:3389, which is herein incorporated by reference in its entirety. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel et al., "Current Protocols in Molecular Biology", John Wiley & Sons Inc, 1994-1998, Chapter 15, which is herein incorporated by reference in its entirety.

[00113] The term "transformation" as used herein, refers to the permanent, heritable alteration in a cell resulting from the uptake and incorporation of foreign DNA into the host-cell genome. This includes the transfer of an exogenous gene from one microorganism into the genome of another microorganism as well as the addition of additional copies of an endogenous gene into a microorganism.

[00114] The term "vector" as used herein, refers to a polynucleotide molecule, such as a DNA molecule. It can be derived, from a plasmid, bacteriophage, yeast or virus, into which a polynucleotide can be inserted or cloned. A vector can contain one or more unique restriction sites and can be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integrable with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector can be an autonomously replicating vector, *i.e.*, a vector that exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a linear or closed circular plasmid, an extra-chromosomal element, a mini-chromosome, or an artificial chromosome. The vector can contain any means for assuring self-replication. Alternatively, the vector can be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Such a vector can comprise specific sequences that allow recombination into a particular, desired site of the host chromosome. A vector system can comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. A vector can be one which is operably functional in a bacterial cell, such as a cyanobacterial cell. The vector can include a reporter gene, such as a green fluorescent protein (GFP), which can be either fused in frame to one or more of the encoded polypeptides, or expressed separately. The vector can also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable transformants.

[00115] The terms "wild-type" and "naturally-occurring" as used herein are used interchangeably to refer to a gene or gene product that has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild type gene or gene product (*e.g.*, a polypeptide) is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene.

[00116] The term "fuel" or "biofuel" 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 and includes, but is not limited to, hydrocarbons, hydrogen, methane, hydroxy compounds such as alcohols (*e.g.* ethanol, butanol, propanol, methanol, etc.), and carbonyl compounds such as aldehydes and ketones (*e.g.* acetone, formaldehyde, 1-propanal, etc.).

[00117] The terms "fermentation end-product" or "end-product" as used herein has its ordinary meaning as known to those skilled in the art and can include one or more biofuels, chemical additives, processing aids, food additives, organic acids (*e.g.* acetic, lactic, formic, citric acid etc.), derivatives of organic acids such as esters (*e.g.* wax esters, glycerides, etc.) or other functional compounds. These end-products include, but are not limited to, an alcohol, ethanol, butanol, methanol, 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 can be present as a pure compound, a mixture, or an impure or diluted form.

[00118] Various end-products can be produced through saccharification and fermentation using enzyme-enhancing products and processes. Examples of end-products include but are not limited to 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-butanonedione, indolylethane, indolylethene, 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-hexanone, 4-methyl-1-(4-hydroxyphenyl)-3-hexanone, 5-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-decadecene, 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-

hydroxy propanal, 1,3-propanediol, 4-hydroxybutanal, 1,4-butanediol, 3-hydroxy-2-butanone, 2,3-butandiol, 1,5-pentane diol, homocitrate, homoisocitorate, b-hydroxy adipate, glutarate, glutarsemialdehyde, glutaraldehyde, 2-hydroxy-1-cyclopentanone, 1,2-cyclopentanediol, 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, phenylacetoaldehyde, 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-hydeoxyphenyl)-4-phenylbutane, 1-(4-hydeoxyphenyl)-4-phenyl-1-butene, 1-(4-hydeoxyphenyl)-4-phenyl-2-butene, 1-(4-hydeoxyphenyl)-4-phenyl-2-butanol, 1-(4-hydeoxyphenyl)-4-phenyl-2-butanone, 1-(4-hydeoxyphenyl)-4-phenyl-2,3-butanediol, 1-(4-hydeoxyphenyl)-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-hydroxyphenylacetoaldehyde, 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-acetoaldehyde, 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 or formic acid.

[00119] 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 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, death, dormant, vegetative, sporulating, etc.

[00120] The term "external source" as it relates to a quantity of an enzyme or enzymes provided to a product or a process means that the quantity of the enzyme or enzymes is not produced by a microorganism in the product or process. An external source of an enzyme can include, but is not limited to an enzyme provided in purified form, cell extracts, culture medium or an enzyme obtained from a commercially available source.

[00121] The term "plant polysaccharide" as used herein has its ordinary meaning as known to those skilled in the art and can comprise one or more carbohydrate 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 can repeat in a regular pattern, or non-regular pattern. 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.

[00122] The term "fermentable sugars" as used herein has its ordinary meaning as known to those skilled in the art and can include one or more sugars and/or sugar derivatives that can be used as a carbon source by the microorganism, including monomers, dimers, and polymers of these compounds including two or more of these compounds. In some cases, the microorganism can 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.

[00123] 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 used by the microorganism at hand. For some microorganisms, this would include 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 microorganisms, the

allowable chain-length can be longer (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 monomer units or more) and for some microorganisms the allowable chain-length can be shorter (*e.g.* 1, 2, 3, 4, 5, 6, or 7 monomer units).

[00124] The term "carbonaceous biomass" as used herein has its ordinary meaning as known to those skilled in the art and can include one or more biological material that can be converted into a biofuel, chemical or other product. Carbonaceous biomass can comprise municipal waste, wood, plant material, plant extract, a natural or synthetic polymer, or a combination thereof.

[00125] Plant matter can include, but is not limited to, woody plant matter, non-woody plant matter, cellulosic material, lignocellulosic material, hemicellulosic material, carbohydrates, pectin, starch, inulin, fructans, glucans, corn, corn stover, sugar cane, grasses, switch grass, bamboo, algae and material derived from these.

[00126] "Biomass" can include, but is not limited to, woody or non-woody plant matter, aquatic or marine biomass, fruit-based biomass such as fruit waste, and vegetable-based biomass such as vegetable waste, and animal based biomass among others. Examples of aquatic or marine biomass 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* sp. and the like. In one embodiment, biomass does not include fossilized sources of carbon, such as hydrocarbons that are typically found within the top layer of the Earth's crust (*e.g.*, natural gas, nonvolatile materials composed of almost pure carbon, like anthracite coal, etc.).

[00127] Examples of fruit and/or vegetable biomass include, but are not limited to, any source of pectin such as plant peel and pomace including citrus, orange, grapefruit, potato, tomato, grape, mango, gooseberry, carrot, sugar-beet, and apple, among others. In one embodiment plant matter is characterized by the chemical species present, such as proteins, polysaccharides and oils. In one embodiment plant matter includes agricultural waste byproducts or side streams such as pomace, corn steep liquor, corn steep solids, distillers grains, Distillers Dried Solubles (DDS), Distillers Dried Grains (DDG), Condensed Distillers Solubles (CDS), Distillers Wet Grains (DWG), Distillers Dried Grains with Solubles (DDGS), peels, pits, fermentation waste, straw, lumber, sewage, garbage or food leftovers. These materials can come from farms, forestry, industrial sources, households, etc. In another embodiment biomass comprises animal matter, including, for example milk, meat, fat, animal processing waste, and animal waste. The term "feedstock" is frequently used to refer to biomass being used for a process, such as those described herein. Examples of polysaccharides, oligosaccharides, monosaccharides or other sugar components of biomass include, but are not

limited to, alginate, agar, carrageenan, fucoidan, floridean starch, pectin, gluronate, mannuronate, mannitol, lyxose, cellulose, hemicellulose, glycerol, xylitol, glucose, mannose, galactose, xylose, xylan, mannan, arabinan, arabinose, glucuronate, galacturonate (including di- and tri-galacturonates), rhamnose, and the like.

[00128] 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, such as for example the entire contents of a fermentation reaction can be referred to as a fermentation broth.

[00129] 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 (*e.g.* g/L/d) is different from "titer" (*e.g.* g/L) in that productivity includes a time term, and titer is analogous to concentration.

[00130] The term "biocatalyst" as used herein has its ordinary meaning as known to those skilled in the art and can include one or more enzymes and/or microorganisms, including solutions, suspensions, and mixtures of enzymes and microorganisms. In some contexts this word will refer to the possible use of either enzymes or microorganisms to serve a particular function, in other contexts the word will refer to the combined use of the two, and in other contexts the word will refer to only one of the two. The context of the phrase will indicate the meaning intended to one of skill in the art.

[00131] 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:

[00132] $C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$

[00133] 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 yield. In one embodiment, 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 embodiment, 10g of cellulose can provide 7.5g of glucose which can provide a maximum theoretical conversion efficiency of about 7.5g*51% or 3.8g of ethanol. In other cases, the efficiency of the saccharification step can be calculated or determined, *i.e.*, saccharification yield. Saccharification yields can include between about 10-100%, about 20-90%, about 30-80%, about 40-70% or about 50-60%, such as about 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%, 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% or about 100% for any carbohydrate carbon sources larger than a single monosaccharide subunit.

[00134] The saccharification yield takes into account the amount of ethanol, and acidic products produced plus the amount of residual monomeric sugars detected in the media. The ethanol figures resulting from media components are not adjusted in this experiment. These can account for up to 3 g/l ethanol production or equivalent of up to 6g/l sugar as much as +/-10%-15% saccharification yield (or saccharification efficiency). For this reason the saccharification yield % can be greater than 100% for some plots.

[00135] The terms "pretreatment" or "pretreated" as used herein refer 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 a 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 is 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. For example, other chemicals can be added to neutralize or detoxify the biomass or saccharide streams resulting from earlier pretreatment.

[00136] The terms "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 microorganisms, 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, although it can also 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 skill in the art the techniques being considered.

[00137] The term "SSF" as used herein, refers to simultaneous saccharification fermentation. The term "SHF" means sequential hydrolysis followed by subsequent fermentation.

[00138] A term "phytate" as used herein has its ordinary meaning as known to those skilled in the art can be include phytic acid, its salts, and its combined forms as well as combinations of these.

[00139] The term "recombinant" as used herein, refers to a microorganism is genetically modified to comprise one or more heterologous or endogenous nucleic acid molecules. Such nucleic acid molecules can be comprised extrachromosomally or integrated into the chromosome of a microorganism. The term "non-recombinant" means a microorganism is not genetically modified. For example, a recombinant microorganism can be modified to

overexpress an endogenous gene encoding an enzyme through modification of promoter elements (*e.g.*, replacing an endogenous promoter element with a constitutive or highly active promoter). Alternatively, a recombinant microorganism can be modified by introducing a heterologous or another copy of an endogenous nucleic acid molecule encoding a protein that is not otherwise expressed in the host microorganism.

[00140] The term "sugar compounds" as used herein has its ordinary meaning as known to those skilled in the art and can include monosaccharide sugars, including but not limited to 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. Included within this description are disaccharides; trisaccharides; oligosaccharides; polysaccharides; and sugar chains, branched and/or linear, of any length.

[00141] Generally, compositions and methods are provided for enzyme conditioning of feedstock or biomass to allow saccharification and fermentation to one or more industrially useful fermentive end-products.

[00142] In one aspect one or more products are provided for production of a biofuel from biomass.

[00143] Enzyme-assisted fermentation involves mixtures of enzymes, derived from several microorganisms, which are added during saccharification steps in order to improve end-product yield during fermentation by increasing hydrolysis. This can be an expensive proposition since mixes of enzymes are expensive and affect the final cost of a biofuel produced by a fermentation processes.

[00144] As demonstrated in the disclosure herein, it was observed that for certain enzyme-assisted cocktails tested at various concentrations, certain enzymes did not substantially affect saccharification and fermentation of the biomass tested. Furthermore, the enzyme cocktail (Novozymes A/S, Krogshoejvej, 36, 2880, Bagsvaerd, Denmark) is recommended for use at 1X. However, by increasing dosing of enzymes to double that of recommended dosage (Fig. 1A demonstrates that various concentrations of a cocktail mix, *i.e.*, 0.25X to 2X) no significant difference in ethanol production is observed in a simultaneous saccharification and fermentation. At 25% the recommended dosage more than 80% fermentation (as compared to full dosing) is observed. In addition, the non-enzyme assisted fermentation achieved only about 20% of theoretical yield.

[00145] Fig. 1B illustrates the performance of the individual enzyme components of the cocktail mix used in Fig. 1A, and were supplied at 1X. The results demonstrate that none of the individual components performed as well as the cocktail mixture. However, the results

also demonstrate that individually, β-glucosidase, xylanase and hemicellulase did not enhance ethanol production. Cellulase alone (NS50013, Novozymes, *supra*) resulted in greater than 63% yield relative to theoretical yield. Furthermore, a β-glucanase/xylanase mix that contains cellulase and hemicellulase activity also enhanced ethanol production.

[00146] In one embodiment feedstock is saccharified by a microorganism to produce a fermentation end-product such as ethanol. In one embodiment the microorganism is genetically modified so that it comprises one or more polynucleotides that encodes one ore more cellulases. In one embodiment the one or more polynucleotides are heterologous. In another embodiment the microorganism is genetically modified so that it comprises one or more additional copies of a polynucleotide that encodes an endogenous cellulase as compared to the wild-type microorganism.

[00147] In one embodiment, feedstock is saccharified by a microorganism that is a *Clostridium strain,* a *Trichoderma strain*, a *Saccharomyces strain,* a *Zymomonas strain*, or another microorganism suitable for fermentation of biomass. In another embodiment, feedstock is saccharified by a microorganism that is *Clostridium phytofermentans, Clostridium* sp. Q.D, *Clostridium algidixylanolyticum, Clostridium xylanolyticum, Clostridium cellulovorans, Clostridium cellulolyticum, Clostridium thermocellum, Clostridium josui, Clostridium papyrosolvens, 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 cellulosolvens, Caldicellulosiruptor saccharolyticum, Ruminococcus albus, Ruminococcus flavefaciens, Fibrobacter succinogenes, Eubacterium cellulosolvens, Butyrivibrio fibrisolvens, Anaerocellum thermophilum, Halocella cellulolytica, Thermoanaerobacterium thermosaccharolyticum, Sacharophagus degradans, or Thermoanaerobacterium saccharolyticum.*

[00148] In another embodiment feedstock is saccharified by a microorganism that is a *Clostridium* strain. In another embodiment feedstock is saccharified by a microorganism that is *Clostridium phytofermentans* or *Clostridium sp. Q.D.*

[00149] *Clostridium sp. Q.D* is described in U.S. serial No. 61/327,051, which is herein incorporated by reference in its entirety. *Clostridium* sp. Q.D forms moist, shiny, beige, opaque, irregular or undulate colonies. The cells are entire, small, short rods, diplo or chains, motile, and form subterminal endospores. Q.D is able to utilize crystalline cellulose as a carbon source, and can form ethanol and acetic acid as major end products. *Clostridium* sp.

Q.D is a gram-positive bacterium, deposited under NRRL Accession No. NRRL B-50361 at the Agricultural Research Service Culture Collection, an International Depositary Authority, (National Center for Agricultural Utilization Research, U.S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604 U.S.A.) , wherein the bacterium is an anaerobic, obligate mesophile that produces colonies that are beige pigmented, wherein the bacterium can use polysaccharides as a sole carbon source and can reduce acetaldehyde into ethanol. The 16S rRNA gene sequence from *Clostridium* sp. Q.D shares 90% similarity to *Clostridium phytofermentans*, but is closer to *Clostridium algidixylanolyticum* starin SPL73 (99%), *Clostridium sp. U201* (99%), and swine fecal bacterium strain RF3G-Cel2 (99%). *Clostridium* sp. Q.D can hydrolyze polysaccharides and higher saccharides that contain hexose sugar units, pentose sugar units, or that contain both, into lower saccharides and in some cases monosaccharides.

[00150] In one embodiment a synergistic effect is observed with respect to saccharification yield when utilizing an organism that is capable of direct saccharification, *e.g.*, *C. phytofermentans* or *Clostridium sp. Q.D* and an external source of a cellulase to achieve complete saccharification and fermentation (Fig. 2). In other words, the presence of cellulase enhances the effects of *C. phytofermentans* saccharification so that the resulting hydrolysis is better than expected from the addition of the two.

[00151] Saccharification yield of feedstock contacted with *C. phytofermentans* is generally not affected by the addition of an external source of β-glucosidase. The non-microbe innoculated reactions exhibited poor saccharification yield. The pH of the reactions was at about pH 6.5 and the temperature was about 35°C. Saccharification yield can be enhanced by decreasing pH to about 5.4 and increasing temperature to about 65°C. This result indicates that β-glucosidase can be excluded when adding an external source of enzymes to enhance saccharification and fermentation of feedstock.

[00152] Furthermore, various dosages of a hydrolytic enzyme cocktail (from 0.25 to 2X) result in theoretical ethanol conversions (Fig. 3A) that are similar. In addition, the curves for individual enzyme augmentations illustrate the impact of cellulase additions and also clearly demonstrate the ineffectiveness of B-glucosidase, xylanase and hemicelluloses additions (Fig. 3B). Figure 3A and 3B correspond to the experiments illustrated in Figures 1A and 1B, but are different in providing FPUs (Filter Paper Units) for hydrolytic enzymes or cellulases that are present in the cocktail mix.

[00153] When examining saccharification with and without a microbe, the extent of hydrolysis using enzyme alone resulted in a reduced saccharification yield, *i.e.*, 16.8% (Fig. 4).

[00154] Furthermore, reducing dosages from 1.68 FPU/gram of glucan to lower than about 0.4 FPU resulted in proportional reduction to 0.168 FPU and 0.084 FPU loadings (Fig. 5).

[00155] Utilization of cellulases as the external source for enhancing saccharification and the fermentation yield, provides a substantial improvement in rate and yield of cellulose utilization by supplementation of additional endo-glucanase activity. The addition of a cellulase enzyme alone obviates the requirement of other enzymes for the saccharification of polysaccharides in a C5/C6 fermenting microorganism. In fact, small amounts of a cellulase synergistically enhance the rate of hydrolysis of C6 sugars so that biofuel production is more rapid and more efficient. This discovery will significantly reduce the cost of producing biofuels such as ethanol, hydrogen, methane and the like.

[00156] *Clostridium phytofermentans* is one microorganism that can simultaneously hydrolyze and ferment hexose (C6) and pentose (C5) polysaccharides. This microorganism has a complement of enzymes to adapt to any biomass substrate. However, the hydrolysis of cellulose in the naturally-occurring microorganism is initially slower than desirable for cost-effective production of biofuels. Unlike other microorganisms, β-glucosidase does not enhance the hydrolysis of cellulose in this microorganism. In one embodiment there is substantial improvement in the rate and yield of cellulose utilization for a microrganism by upregulation or supplementation of additional endo-glucanase activity. The addition of small amounts of a cellulase enzyme alone synergistically enhance the rate of hydrolysis of C6 sugars in *C. phytofermentans* and increases the yield of fermentation end-products. *In a similar manner, small amounts of a cellulase enzyme synergistically enhance the rate of saccharification in Clostridium. sp. Q.D.*

[00157] In one embodiment, a product for production of a biofuel comprises: a carbonaceous biomass, a microorganism that is capable of direct hydrolysis and fermentation of said biomass, and an external source of one or more enzymes that are capable of enhancing said hydrolysis, wherein said one or more enzymes do not include a xylanase, a hemicellulase, a glucanase or glucosidase, and wherein said external source is not said microorganism.

[00158] In another embodiment a product for production of a biofuel is provided comprising: a carbonaceous biomass, a microorganism that is capable of direct hydrolysis and fermentation of said biomass, wherein said microorganism is modified to provide enhanced activity of one or more cellulases.

[00159] In another embodiment a product for production of fermentive end-products comprises: (a) a fermentation vessel comprising a carbonaceous biomass; (b) a microorganism

that is capable of direct hydrolysis and fermentation of said biomass; and (c) a source of one or more enzymes that is external to said microorganism, wherein said one or more enzymes do not include a xylanase, a hemicellulase, a glucanase or glucosidase; wherein the fermentation vessel is adapted to provide suitable conditions for fermentation of one or more carbohydrates into fermentive end-products.

[00160] In one embodiment a microorganism is capable of direct fermentation of C5 (five carbon chain polysaccharide) and/or C6 (six carbon chain polysaccharide) carbohydrates. In one embodiment, such a capability is achieved through modifying the microorganism to express one or more genes encoding proteins associated with C5 and C6 carbohydrate metabolization.

[00161] Microorganisms that can be used in acomposition or method disclosed herein include but are not limited to bacteria, yeast or fungi. In some embodiments, two or more different microorganisms can be used during saccharification and/or fermentation processes to produce an end-product. Microorganisms used can be recombinant, non-recombinant or wild type.

[00162] In one embodiment, a microorganism used in a composition or method disclosed herein is a strain of Clostridia. The strain can be *C. acetobutylicum, C. bejeirinckii, C. saccharoperbutylacetonicum, C. butylicum, C. butyricum, C. perfringens, C. tetani, C. sporogenes, C. thermocellum, C. saccarolyticum* (now *Thermoanaerobacter saccarolyticum*), *C. thermosulfurogenes* (now *Thermoanaerobacter thermosulfurigenes*), *C. thermohydrosulfuricum* (now *Thermoanaerobacter ethanolicus*), *C.* sp. Q.D, and *C. phytofermentans.* In one embodiment, the microorganism is *Clostridium phytofermentans*. In another embodiment, the microorganism is *C.* sp. Q.D.

[00163] In one embodiment a microorganism can be modified to comprise one or more heterologous polynucleotides that enhance enzyme function. In one embodiment, enzymatic function is increased for one or more cellulase enzymes or other hydrolases.

[00164] In another embodiment a microrganism can be modified to comprise one or more additional copies of an endogenous polynucleotide that encodes a protein. In one embodiment the protein is a cellulase enzyme. In another embodiment the protein is a hydrolase enzyme. In another embodiment a microrganism can be modified to comprises more than one additional copies of an endogenous polynucleotide that encodes a protein.

[00165] In another embodiment a microorganism can be capable of uptake of one or more complex carbohydrates from a biomass (*e.g.*, biomass comprises a higher concentration of oligomeric carbohydrates relative to monomeric carbohydrates).

[00166] In one embodiment, one or more enzymes from an external source (*e.g.*, enzymes provided in purified form, cell extracts, culture medium or commercially available source) is added to a product or process disclosed herein.

[00167] In one embodiment a product or a process is disclosed for producing an end-product from biomass, a carbonaceous biomass is contacted with: (1) a microorganism that is capable of direct hydrolysis and fermentation of said biomass, and/or (2) an external source of one or more enzymes that are capable of enhancing said hydrolysis, wherein said one or more enzymes do not include a xylanase, a hemicellulase, a glucanase or glucosidase, and wherein said external source is not said microorganism; thereby producing a fermentive medium; and allowing sufficient time for said hydrolysis and fermentation to produce a biofuel.

[00168] Furthermore, a microorganism that is used with or without an external source of one or more enzymes, can itself be modified to enhance enzyme function of one or more enzymes associated with hydrolyzation of biomass, fermentation of a polysaccharide or monosaccharide, or both.

[00169] Enzyme activity can also be enhanced by modifying conditions in a reaction vessel, including but not limited to time, pH of a culture medium, temperature, concentration of nutrients and/or catalyst, or a combination thereof.

[00170] A reaction vessel can be configured to separate one or more desired end-products.

[00171] Enzymes added externally can be in an amount from about 0.5 FPU/gram cellulose to about 20 FPU/gram cellulose, about 0.5 FPU/gram cellulose to about 40 FPU/gram cellulose, about 10 to about 30 FPU/gram cellulose, about 15 to about 25 FPU/gram cellulose, or about 20 to about 40 FPU/gram cellulose. In various embodiments, one or more cellulase enzymes can be added to a product or process disclosed herein to enhance saccharification and increase substrates available for fermentation.

[00172] In one embodiment a modified microorganism can have enhanced activity of an enzyme that is equivalent to addition of said cellulases in an amount sufficient to provide activity of about 0.5 FPU/gram cellulose to about 20 FPU/gram cellulose, about 0.5 FPU to about 40 FPU/gram cellulose, about 10 to about 30 FPU/gram cellulose, about 15 to about 25 FPU/gram cellulose, or about 20 to about 40 FPU/gram cellulose.

[00173] In one embodiment a product or process can provide hydrolysis of a biomass resulting in a greater concentration of cellobiose relative to monomeric carboyhdrates. Such monomeric carbohydrates can comprise glucose, xylose and arabinose.

[00174] In one embodiment, batch fermentation with a microorganism and of a mixture of hexose and pentose saccharides can provide uptake rates of about 0.1, 0.2, 0.4, 0.5, 0.6 0.7, 0.8, 1, 2, 3, 4, 5, or about 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 about 6 g/L/h or more of pentose (xylose, xylan, hemicellulose etc.). For example, either *C. phytofermentans* or *C.* sp. Q.D is capable of direct fermentation of C5 and C6 sugars.

[00175] In another embodiment a product or process disclosed herein can produce about 15 g/L, about 20g/L, about 25g/L, about 30 g/L, about 35 g/L, about 40 g/L, about 45 g/L, about 50 g/L, about 60 g/L, about 70 g/L, about 80 g/L, or about 100 g/L production of ethanol. Such levels of ethanol can be observed in 10, 20, 30, 40, 50 or 60 hours of fermentation. In some embodiments the ethanol productivities provided by a process of the disclosed herein is due to the simultaneous fermentation of hexose and pentose saccharides.

[00176] Production of high levels of alcohol from biomass requires the ability for the microorganism to thrive generally in the presence of elevated alcohol levels, the ability to continue to produce alcohol without undue inhibition or suppression by the alcohol and/or other components present, and the ability to efficiently convert the multitude of different hexose and pentose carbon sources found in a biomass feedstock.

[00177] Fermentation at reduced pH and/or with the addition of fatty acids can result in about a three to five to 10 fold or higher increase in the ethanol production rate. In some embodiments, 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 used in combination with fermentation at reduced pH and/or with the addition of fatty acids to further increase productivity, and/or yield.

[00178] Biomass

[00179] 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. 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.

[00180] In some embodiments aerobic/anaerobic cycling is employed for the bioconversion of cellulosic/lignocellulosic material to fuels and chemicals. In some embodiments, the anaerobic microorganism can ferment biomass directly without the need of a pretreatment. In certain 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.

[00181] In some embodiments, a process for simultaneous saccharification and fermentation of cellulosic solids from biomass into biofuel or another end-product is provided. The process comprises treating the biomass in a closed container with a microorganism under conditions where the microorganism produces saccharolytic enzymes sufficient to substantially convert the biomass into oligomers, monosaccharides and disaccharides. The organism subsequently converts the oligomers, monosaccharides and disaccharides into ethanol and/or another biofuel or product.

[00182] In an alternative embodiment, a process for saccharification and fermentation comprises treating the biomass in a container with the microorganism and adding one or more enzymes before, concurrent or after contacting the biomass with the microorganism, wherein the enzymes added aid in the breakdown or detoxification of carbohydrates or lignocellulosic material.

[00183] In some instances, enzymes added do not include a xylanase, a hemicellulase, a glucanase or glucosidase. In other embodiments, the amount of exogenous cellulase is greatly reduced, one-quarter or less of the amount normally added to a fermentation wherein the organism cannot saccharify the biomass.

[00184] Examples of second cultures include but are not limited to *Saccharomyces cerevisiae*, Clostridia species such as *C. thermocellum, C. acetobutylicum*, and *C. cellovorans*, and *Zymomonas mobilis.*

[00185] In one embodiment, a process of producing a biofuel from a lignin-containing biomass is provided. In one embodiment 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 a *Clostridium* sp. Q.D bacterium under conditions wherein the *Clostridium phytofermentans* or the *Clostridium* sp. Q.D, optionally with the addition of one or more enzymes to the container, substantially converts the treated biomass into oligomers, monosaccharides and disaccharides, and/or biofuel or other fermentation end-product; and 4) optionally, introducing a culture of a second microorganism wherein the second microorganism is capable of substantially converting the oligomers, monosaccharides and disaccharides into biofuel.

[00186] Biofuel plant and process of producing biofuel

[00187] 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*. In another embodiment, it is *Clostridium* sp. Q.D.

[00188] 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, *Clostridium* sp. Q.D 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).

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.

[00189] In another aspect, provided herein are end-products made by any of the processes described herein.

[00190] 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* or *Clostridium* sp. Q.D). 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* or *Clostridium* sp. Q.D to promote homologous recombination.

[00191] Large Scale Fermentation End-Product Production from Biomass

[00192] 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* or *Clostridium* sp. Q.D . 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.

[00193] Biomass processing plant and process of producing products from biomass

[00194] 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* or *Clostridium* sp. Q.D) 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.

[00195] 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* or *Clostridium* sp. Q.D) 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).

[00196] In another aspect, the invention features end-products made by any of the processes described herein.

[00197] Large Scale Production of Fermentation End- Products From Biomass

[00198] 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* or *Clostridium* sp. Q.D). 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).

[00199] 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* or *Clostridium* sp. Q.D) 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, ammonia fiber explosion 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* or *Clostridium* sp. Q.D), 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_2), 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.

[00200] 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°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°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.

[00201] Fig. 29 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%.

[00202] 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.

[00203] The fermentor is fed with hydrolyzed biomass, any liquid fraction from biomass pretreatment, an active seed culture of *Clostridium phytofermentans* or *Clostridium* sp. Q.D 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* or *Clostridium* sp. Q.D 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.

[00204] After fermentation, the contents of the fermentor are transferred to product recovery. Products are extracted, *e.g.*, ethanol is recovered through distilled and rectification.

[00205] Chemical Production From Biomass Without Pretreatment

[00206] Fig. 30 depicts a method for producing chemicals from biomass by charging biomass to a fermentation vessel. 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* or *Clostridium* sp. Q.D) 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* or *Clostridium* sp. Q.D) 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. 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.

[00207] 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.

[00208] 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.

[00209] In some embodiments, the present invention provides a process for producing ethanol and hydrogen from biomass using acid hydrolysis pretreatment. In some embodiments, the present invention 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.

[00210] Figure 31 discloses pretreatments that produce hexose or pentose saccharides or oligomers that are then unprocessed or processed further and either, fermented separately or together. Figure 31A depicts a process (*e.g.*, acid pretreatment) that produces a solids phase and a liquid phase which are then fermented separately. Figure 31B 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 31C) 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.

[00211] Modification to Enhance Enzyme Activity

[00212] In one embodiment one or more modifications hydrolysis and/or fermentation conditions can be implemented to enhance end-product production. Examples of such modifications include genetic modification to enhance enzyme activity in a microorganism that already comprises genes for encoding one or more target enzymes, introducing one or more heterogeneous nucleic acid molecules into a host microorganism to express and enhance activity of an enzyme not otherwise expressed in the host, modifying physical and chemical conditions to enhance enzyme function (*e.g.*, modifying and/or maintaining a certain temperature, pH, nutrient concentration, temporal), or a combination of one or more such modifications.

[00213] Genetic Modification

[00214] In one embodiment, a microorganism can be genetically modified to enhance enzyme activity of one or more enzymes, including but not limited to cellulase(s). Examples of such modifications include modifying endogenous nucleic acid regulatory elements to increase expression of one or more enzymes (*e.g.*, operably linking a gene encoding a target enzyme to a strong promoter), introducing into a microorganism additional copies of endogenous nucleic acid molecules to provide enhanced activity of an enzyme by increasing its production, and operably linking genes encoding one or more enzymes to an inducible promoter or a combination thereof.

[00215] In another embodiment a microorganism can be modified to enhance an activity of one or more cellulases, or enzymes associated with cellulose processing (*e.g.*, Fig. 6). The classification of cellulases is usually based on grouping enzymes together that forms a family with similar or identical activity, but not necessary the same substrate specificity. One of these classifications is the CAZy system (CAZy stands for Carbohydrate-Active enZymes), for example, where there are 115 different Glycoside Hydrolases (GH) listed, named GH1 to GH155. Each of the different protein families usually has a corresponding enzyme activity. This database includes both cellulose and hemicellulase active enzymes. Furthermore, the entire annotated genome of *C. phytofermentans* is available on the worldwideweb at www.ncbi.nlm.nih.gov/sites/entrez.

[00216] Several examples of cellulase enzymes whose function can be enhanced for expression endogenously or for expression heterologously in a microorganism include one or more of the genes disclosed in Table 2.

Table 2

Cellulase Protein ID	**Description (on www.ncbi.nlm.nih.gov/sites/entrez)**
ABX43556	Cellulase [*Clostridium phytofermentans* ISDg] gi|160429993|gb|ABX43556.1|[160429993] Cphy_3302
ABX42426	Cellulase [*Clostridium phytofermentans* ISDg] gi|160428863|gb|ABX42426.1|[160428863] Cphy_2058
ABX41541	Cellulase [*Clostridium phytofermentans* ISDg] gi|160427978|gb|ABX41541.1|[160427978] Cphy_1163
ABX43720	Cellulose 1,4-beta-cellobiosidase [*Clostridium phytofermentans* ISDg] gi|160430157|gb|ABX43720.1|[160430157] Cphy_3367
ABX41478	Cellulase M Cphy_1100
ABX41884	Endo-1,4-beta-xylanase Cphy_1510

ABX43721	Cellulase 1,4-beta-cellobiosidase Cphy_3368
ABX42494	Mannan endo-1,4-beta-mannosidase, Cellulase 1,4-beta-cellobiosidase Cphy_2128

[00217] The Glycosyl hydrolase family 9 (GH9): O-Glycosyl hydrolases are a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. A classification system for glycosyl hydrolases, based on sequence similarity, has led to the definition of 85 different families PUBMED:7624375, PUBMED:8535779, PUBMED:. This classification is available on the CAZy (CArbohydrate-Active EnZymes) web site PUBMED. Because the fold of proteins is better conserved than their sequences, some of the families can be grouped in 'clans'. The Glycoside hydrolase family 9 comprises enzymes with several known activities, such as endoglucanase and cellobiohydrolase. In *C. phytofermentans*, a GH9 cellulase is ABX43720 (Table 2).

[00218] Cellulase enzyme activity can be enhanced in a microorganism. In one embodiment a cellulase disclosed in Table 2 is enhanced in a microorganism.

[00219] In one embodiment a hydrolytic enzyme is selected from the annotated genome of *C. phytofermentans* for utilization in a product or process disclosed herein. In one embodiment the hydrolytic enzyme is an endoglucanase, chitinase, cellobiohydrolase or endo-processive cellulases (either on reducing or non-reducing end).

[00220] In one embodiment a microorganism, such as *C. phytofermentans* can be modified to enhance production of one or more cellulase or hydrolase enzymes. In another embodiment one or more enzymes can be heterologous expressed in a host (*e.g.*, a bacteria or yeast). For heterologous expression bacteria or yeast can be modified through recombinant technology. (*e.g.*, Brat et al. Appl. Env. Microbio. 2009; 75(8):2304-2311, disclosing expression of xylose isomerase in *S. cerevisiae* and which is herein incorporated by reference in its entirety).

[00221] In another embodiment other modifications can be made to enhance end-product (*e.g.*, ethanol) production in a recombinant microorganism. For example, the host 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.

[00222] A variety of promoters (*e.g.*, constitutive promoters, inducible promoters) can be used to drive expression of the heterologous genes in a recombinant host microorganism.

[00223] Promoter elements can be selected and mobilized in a vector (*e.g.*, pIMPCphy). For example, a transcription regulatory sequence is operably linked to gene(s) of interest (*e.g.*, in a expression construct). The promoter can be any array of DNA sequences that interact specifically with cellular transcription factors to regulate transcription of the downstream gene. The selection of a particular promoter depends on what cell type is to be used to express the protein of interest. In one embodiment a transcription regulatory sequences can be derived from the host microorganism. In various embodiments, constitutive or inducible promoters are selected for use in a host cell. Depending on the host cell, there are potentially hundreds of constitutive and inducible promoters which are known and that can be engineered to function in the host cell.

[00224] Promoters typically used in recombinant technology, such as *E. coli* lac and trp operons, the tac promoter, the bacteriophage pL promoter, bacteriophage T7 and SP6 promoters, beta-actin promoter, insulin promoter, baculoviral polyhedrin and p10 promoter, can be used to initiate transcription..

[00225] In one embodiment a constitutive promoter can be used including, but not limited to the int promoter of bacteriophage lamda, the bla promoter of the beta-lactamase gene sequence of pBR322, hydA or thlA in *Clostridium*, *S. coelicolor* hrdB, or whiE, the CAT promoter of the chloramphenicol acetyl transferase gene sequence of pPR325, Staphylococcal constitutive promoter blaZ and the like.

[00226] In another embodiment an inducible promoter can be used that regulates the expression of downstream gene in a controlled manner, such as under a specific condition of a cell culture. Examples of inducible prokaryotic promoters include, but are not limited to, the major right and left promoters of bacteriophage, the trp, reca, lacZ, AraC and gal promoters of *E. coli*, the alpha-amylase (Ulmanen Ett at., J. Bacteriol. 162:176-182, 1985, which is herein incorporated by reference in its entirety) and the sigma-28-specific promoters of *B. subtilis* (Gilman et al., Gene sequence 32:11-20 (1984) , which is herein incorporated by reference in its entirety), the promoters of the bacteriophages of Bacillus (Gryczan, In: The Molecular Biology of the Bacilli, Academic Press, Inc., NY (1982) , which is herein incorporated by reference in its entirety), Streptomyces promoters (Ward et at., Mol. Gen. Genet. 203:468-478, 1986, which is herein incorporated by reference in its entirety), and the like. Exemplary prokaryotic promoters are reviewed by Glick (J. Ind. Microtiot. 1:277-282, 1987, which is herein incorporated by reference in its entirety); Cenatiempo (Biochimie 68:505-516, 1986,

which is herein incorporated by reference in its entirety); and Gottesrnan (Ann. Rev. Genet. 18:415-442, 1984, which is herein incorporated by reference in its entirety).

[00227] A promoter that is constitutively active under certain culture conditions, can be inactive in other conditions. For example, the promoter of the hydA gene from *Clostridium acetobutylicum*, wherein expression is known to be regulated by the environmental pH. Furthermore, temperature-regulated promoters are also known and can be used. In some embodiments, depending on the desired host cell, a pH-regulated or temperature-regulated promoter can be used with an expression constructs to initiate transcrription. Other pH-regulatable promoters are known, such as P170 functioning in lactic acid bacteria, as disclosed in US Patent Application No. 20020137140, which is herein incorporated by reference in its entirety.

[00228] In general, to express the desired gene/nucleotide sequence efficiently, various promoters can be used; *e.g.*, the original promoter of the gene, promoters of antibiotic resistance genes such as for instance kanamycin resistant gene of Tn5, ampicillin resistant gene of pBR322, and promoters of lambda phage and any promoters which can be functional in the host cell. For expression, other regulatory elements, such as for instance a Shine-Dalgarno (SD) sequence (*e.g.*, AGGAGG and so on including natural and synthetic sequences operable in a host cell) and a transcriptional terminator (inverted repeat structure including any natural and synthetic sequence) which are operable in a host cell (into which a coding sequence is introduced to provide a recombinant cell) can be used with the above described promoters.

[00229] Examples of promoters that can be used with a product or process disclosed herein include those disclosed in the following patent documents: US20040171824, US 6410317, WO 2005/024019 , which are herein incorporated by reference in their entirety. Several promoter-operator systems, such as lac, (D. V. Goeddel et al., "Expression in Escherichia coli of Chemically Synthesized Genes for Human Insulin", Proc. Nat. Acad. Sci. U.S.A., 76:106-110 (1979) , which is herein incorporated by reference in its entirety); trp (J. D. Windass et al. "The Construction of a Synthetic *Escherichia coli* Trp Promoter and Its Use in the Expression of a Synthetic Interferon Gene", Nucl. Acids. Res., 10:6639-57 (1982), which is herein incorporated by reference in its entirety) and λ PL operons (R. Crowl et al., "Versatile Expression Vectors for High-Level Synthesis of Cloned Gene Products in *Escherichia coli*", Gene, 38:31-38 (1985), which is herein incorporated by reference in its entirety) in *E. coli* and have been used for the regulation of gene expression in recombinant cells. The corresponding repressors are the lac repressor, trpR and cI, respectively.

[00230] Repressors are protein molecules that bind specifically to particular operators. For example, the lac repressor molecule binds to the operator of the lac promoter-operator system, while the cro repressor binds to the operator of the lambda pR promoter. Other combinations of repressor and operator are known in the art. See, *e.g.*, J. D. Watson et al., Molecular Biology Of The Gene, p. 373 (4th ed. 1987), which is herein incorporated by reference in its entirety. The structure formed by the repressor and operator blocks the productive interaction of the associated promoter with RNA polymerase, thereby preventing transcription. Other molecules, termed inducers, bind to repressors, thereby preventing the repressor from binding to its operator. Thus, the suppression of protein expression by repressor molecules can be reversed by reducing the concentration of repressor (depression) or by neutralizing the repressor with an inducer.

[00231] Analogous promoter-operator systems and inducers are known in other microorganisms. In yeast, the GAL10 and GAL1 promoters are repressed by extracellular glucose, and activated by addition of galactose, an inducer. Protein GAL80 is a repressor for the system, and GAL4 is a transcriptional activator. Binding of GAL80 to galactose prevents GAL80 from binding GAL4. Then, GAL4 can bind to an upstream activation sequence (UAS) activating transcription. See Y. Oshima, "Regulatory Circuits For Gene Expression: The Metabolisms Of Galactose And Phosphate" in The Molecular Biology Of The Yeast Sacharomyces, Metabolism And Gene Expression, J. N. Strathern et al. eds. (1982), which are herein incorporated by reference in their entirety.

[00232] Transcription under the control of the PHO5 promoter is repressed by extracellular inorganic phosphate, and induced to a high level when phosphate is depleted. R. A. Kramer and N. Andersen, "Isolation of Yeast Genes With mRNA Levels Controlled By Phosphate Concentration", Proc. Nat. Acad. Sci. U.S.A., 77:6451-6545 (1980), which is herein incorporated by reference in its entirety. A number of regulatory genes for PHO5 expression have been identified, including some involved in phosphate regulation.

[00233] Matα2 is a temperature-regulated promoter system in yeast. A repressor protein, operator and promoter sites have been identified in this system. A. Z. Sledziewski *et al.*, "Construction Of Temperature-Regulated Yeast Promoters Using The Matα2 Repression System", Bio/Technology, 6:411-16 (1988), which is herein incorporated by reference in its entirety.

[00234] Another example of a repressor system in yeast is the CUP1 promoter, which can be induced by $Cu^{+}2$ ions. The CUP1 promoter is regulated by a metallothionine protein. J.

A. Gorman et al., "Regulation Of The Yeast Metallothionine Gene", Gene, 48:13-22 (1986), which is herein incorporated by reference in its entirety.

[00235] Similarly, to obtain a desired expression level of one or more cellulases, a higher copy number plasmid canbe used. Constructs can be prepared for chromosomal integration of the desired genes. Chromosomal integration of foreign genes can offer several advantages over plasmid-based constructions. Ethanologenic genes have been integrated chromosomally in *E. coli* B; see Ohta et al. (1991) Appl. Environ. Microbiol. 57:893-900, which is herein incorporated by reference in its entirety. 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 microorganism. 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 operably linked to target genes (*e.g.*, genes encoding cellulase enzymes) to promote homologous recombination.

[00236] In some embodiments, a microorganism 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 microorganisms on substrates that comprise carbon sources that will be used during the generation of end-products by fermentation. Screening can also include measuring the production of end-products during growth of the microorganism, 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.

[00237] In some embodiments host cells (e.g., microorganisms) can be transformed with multiple genes encoding one or more enzymes. For example, a single transformed cell can contain exogenous nucleic acids encoding an entire biodegradation pathway. One example of a pathway can include genes encoding an exo-β-glucanase, and endo-β-glucanase, and an endoxylanase. Such cells transformed with entire pathways and/or enzymes extracted from them, can saccharify certain components of biomass more rapidly than the naturally-occurring organism. Constructs can contain multiple copies of the same gene, and/or multiple genes encoding the same enzyme from different organisms, and/or multiple genes with mutations in

one or more parts of the coding sequences. For example, multiple copies of Cphy_3367 or Cphy_3368 (SEQ ID NO:5 or SEQ ID NO:8, respectively) can increase saccharification, thus increasing the rate and yield of fermentation products. In some embodiments, the nucleic acid sequences encoding the genes can be similar or identical to the endogenous gene. There can be a percent similarity of 70% or more in comparing the base pairs of the sequences.

[00238] In another embodiment, more effective biomass degradation pathways can be created by transforming host cells with multiple copies of enzymes of the pathway and then combining the cells producing the individual enzymes. This approach allows for the combination of enzymes to more particularly match the biomass of interest by altering the relative ratios of the multiple-transformed strains. In one embodiment two times as many cells expressing the first enzyme of a pathway can be added to a mix where the first step of the reaction pathway is a limiting step of the overall reaction pathway.

[00239] In one embodiment biomass-degrading enzymes are made by transforming host cells (*e.g.*, microbial cells such as bacteria, especially Clostridial cells, algae, and fungi) and/or organisms comprising host cells with nucleic acids encoding one or more different biomass degrading enzymes (*e.g.*, cellulolytic enzymes, hemicellulolytic enzymes, xylanases, lignases and cellulases). In some embodiments, a single enzyme can be produced. For example, a cellulase which breaks down pretreated cellulose fragments into cellodextrins or double glucose molecules (cellobiose) or a cellulase which splits cellobiose into glucose, can be produced. In other embodiments, multiple copies of an enzyme can be transformed into an organism to overcome a rate-limiting step of a reaction pathway.

EXAMPLES

Example 1. Cellulase Enzyme Addition

[00240] To study the effects of exogenous enzyme supplementation, hydrolytic enzyme mixtures and individual hydrolytic enzymes were added during the fermentation of a corn stover biomass.

[00241] The following operating conditions and process parameters for *C. phytofermentans* were followed for fermentation of NaOH-pretreated corn stover with enzyme augmentation in 250 ml shake flasks with 100 ml of culture medium (Table 3).

[00242] **Table 3**

Operating conditions	
pH	6.5; range of from about 6.0 to about 7.0
Temperature	35° C.....Shake flasks were incubated in temperature controlled cabinets
Agitation	175 rpm
Degassing	Sparging with N_2 to achieve redox potential less than -300 mV
Base for pH control	4N NaOH
Mode of operation	Batch
Inoculation size	0.5 g/L on dry wt. ($2x10^9$ CFU) use dfo reach flask

[00243] Seed propagation media (QM1) recipe:

QM Base Media:	g/L:
KH_2PO_4	1.92
K_2HPO_4	10.60
Ammonium sulfate	4.60
Sodium citrate tribasic * $2H_2O$	3.00
Bacto yeast extract	6.00
Cysteine	2.00

20x Substrate Stock	g/L:
Maltose	400.00

100X QM Salts solution:	g/L
$MgCl_2 \cdot 6H_2O$	100
$CaCl_2 \cdot 2H_2O$	15
$FeSO_4 \cdot 7H_2O$	0.125

[00244] The seed propagation media was prepared according to the recipe above. Base media, salts and substrates were degassed with nitrogen prior to autoclave sterilization. Following sterilization, 94 ml of base media was combined with 1ml of 100X salts and 5mls of 20X substrate to achieve a final concentrations. All additions were prepared anaerobically and aseptically.

[00245] Fermentation media: (FM media)

[00246] Base media (g/L) was prepared with: 50g/l NaOH pretreated corn stover, yeast extract 10, corn steep powder 5, K_2HPO_4 3, KH_2PO_4 1.6, TriSodium citrate$\cdot 2H_2O_2$ 2, Citric

acid·H_2O 1.2, $(NH_4)_2SO_4$ 0.5, NaCl 1, Cysteine.HCl 1, dissolved in deionized water to achieve final volume, adjusted to pH to 6.5, degassed with nitrogen and autoclaved 121°C for 30 min.

[00247] 100X Salt Stock (g/L):

[00248] $MgCl_2.6H_2O$ 80, $CaCl_2.2H_2O$ 10, $FeSO_4.7H_2O$ 0.125, TriSodium citrate.$2H_2O_2$ 3.0

[00249] Culturing procedure:

[00250] The fermentation media was prepared according to the protocol above. Components of the Base media were combined to a single vessel and degassed with nitrogen prior to sterilization. A 100X salts stock was prepared and sterilized separately. After sterilization base media was supplemented with a 1% v/v dose of 100X salts to achieve a final concentration. All additions were prepared anaerobically and aseptically.

[00251] Enzymes were obtained from Novozymes and mixtures (cocktails) were prepared separately and sterilized by sterile filtration using 0.2μm filters. The prepared enzymes were then added to the FM corn stover media immediately prior to time of inoculation. Other enzymes and mixtures of enzymes from several different manufacturers were also tested with similar results.

[00252] Inoculum of *Clostridium phytofermentans* was prepared by propagation in QM media 24 hrs to an active cell density of $2X10^9$ cells per ml. The cells were concentrated by centrifugation and then transferred into the FM media bottles to achieve an initial cell density of $2x\ 10^9$ cells per ml for the start of fermentation.

[00253] Cultures were then incubated at pH 6.5 and at 35°C for 120 hr or until fermentations were complete. Product formation was determined by HPLC analysis using refractive index detection. Compositional analysis for the NaOH-treated corn stover was obtained via NREL standard methods using two-stage acid hydrolysis procedures.

[00254] The addition of cellulase mixtures exhibiting as little as 0.4 FPU per gram of glucan supplemented with B-glucosidase, hemicellulase, pectinase and xylanase resulted in >95% theoretical yields of ethanol from fermentation of 50g/l NaOH-treated corn stover. The addition of a single endo-cellulase complex at 1.68FPU per gram of glucan resulted in greater than 90% theoretical saccharification and greater than 70% fermentation yield. (Table 4.) Addition of B-glucosidase, xylanase, or hemicellulase alone had little to no impact to the rate, titer or yield of Q fermentations. Based on the microorganism's metabolism of cellulose in the conditions studied, these enzymes were not observed to impact the fermentation process. For fermentation, addition of B-glucosidase does not significantly impact the fermentation process.

[00255] The table below shows the adjusted loadings in terms of FPU/gram of glucan as a standard enzyme unit. The activities were adjusted based on the reduction in the enzyme-reduced activity at the lowered temperature and higher pH ranges. Experiments were performed at pH 6.5 and temperature of 35°C, which resulted in more than 80% loss of cellulase activity. This factor was figured into the loading calculations - FPU: filter paper unit, CBU cellobiosidase unit, FBG fungal glucanase unit, FXU: fungal xylanase unit.

Table 4

Description of enzyme cocktail	***FPU/ gram glucan**	**CBU/ gram glucan**	***FBG/ gram biomass**	***FXU/ gram biomass**
2x mixture	3.36	15.00	2.36	19.3
1x mixture	1.68	7.50	1.18	9.65
.5x mixture	0.84	3.75	0.59	4.83
.25x mixture	0.42	1.88	0.30	2.41
NS50013 cellulase	1.68	NA	NA	NA
NS50010 B-glucosidase	NA	7.5	NA	NA
NS50012 hemicellulase	NA	NA	0.28	NA
NS50030 xylanase	NA	NA	0.25	NA
NS22002 glucanase/xylanase	NA	NA	0.9	9.4
No exogenous enzyme	NA	NA	NA	NA
Compositional analysis of 1% NaOH treated corn stover				
* activity adjusted based on % activity retained at 35°C				

[00256] Carbohydrate analysis for NaOH-treated corn stover; Glucan: 53.37%; Xylan: 27.5 %; Arabinan: 3.6%. Total sugar equivalents: 95.4%.

[00257] The difference in the fermentation profiles for the cultures with enzyme mixture additions from 0.25 to 2X result in the theoretical ethanol conversions described in Fig. 1A. The curves for individual enzyme augmentations illustrate the impact of cellulase additions and also clearly demonstrate the ineffectiveness of B-glucosidase, xylanase and hemicelluloses additions described in Fig. 1B.

[00258] Conversion efficiency of SSF fermentations for Corn stover with various enzyme loadings and individual enzyme loadings is further provided in Table 5.

[00259] **Table 5**

Description of enzyme cocktail	Time (days)	Initial sugar equivalents g/l	Residual sugars g/l	Ethanol titer g/l	consumed sugars (assumes .45 g/g)	Ethanol yield (% theoretical)	Saccharification yield
2x mixture	7.00	48.66	3.90	23.66	52.58	95%	116%
1x mixture	7.00	48.66	2.32	22.90	50.89	92%	109%
.5x mixture	7.00	48.66	0.72	23.83	52.96	96%	110%
.25x mixture	7.00	48.66	1.12	22.53	50.07	91%	105%
NS50013 Cellulase	7.00	48.66	5.66	17.60	39.11	71%	92%
NS50010 B-glucosidase	7.00	48.66	2.65	6.88	15.29	28%	37%
NS50012 Hemicellulase	7.00	48.66	3.20	6.91	15.36	28%	38%
NS50030 xylanase	7.00	48.66	1.66	7.88	17.51	32%	39%
NS22002 glucanase/xylanase	7.00	48.66	2.73	10.35	23.00	42%	53%
No exogenous enzyme	7.00	48.66	2.40	6.13	13.62	25%	33%
Compositional analysis of 1% NaOH treated corn stover		glucan	xylan	arabinan	Total Sugar equivalents		
		53.3 %	27.4 %	3.6%	95.4 %		
* activity adjusted based on % activity retained at 35°C							

[00260] Fig. 2 and Fig. 4 show the synergistic effect of hydrolytic enzyme on *C. phytofermentans* saccharification efficiency. Fig. 3A demonstrates the reduced amount of enzyme mixture necessary for peak ethanol yield during *C. phytofermentans* saccharification and fermentation of corn stover. Fig. 3B shows that cellulase alone is the hydrolytic enzyme responsible for the higher ethanol yield during *C. phytofermentans* saccharification and fermentation of corn stover. During these fermentations, significantly lower amounts of hydrolytic enzymes than normally used during biofuel production with other organisms resulted in high rates and yield of ethanol with *C. phytofermentans* (Fig. 5).

Example 2. Microorganism Modification

[00261] Constitutive Expression of Cellulases I

[00262] pIMPCphy

[00263] Plasmids suitable for use in *Clostridium phytofermentans* were constructed using portions of plasmids obtained from bacterial culture collections (Deutsche Sammlung von

Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7 B, 38124 Braunschweig, Germany, hereinafter "DSMZ"). Plasmid pIMP1 is a non-conjugal shuttle vector that can replicate in *Escherichia coli* and *C. phytofermentans;* additionally, pIMP1 (Fig. 7) encodes for resistance to erythromycin ($Em^{R)}$. The origin of transfer for the RK2 conjugal system was obtained from plasmid pRK29O (DSMZ) as DSM 3928, and the other conjugation functions of RK2 were obtained from pRK2013 (DSMZ) as DSM 5599. The polymerase chain reaction (PCR) was used to amplify the 112 base pair origin of transfer region (oriT) from pRK29O using primers that added ClaI restriction sites flanking the oriT region. This DNA fragment was inserted into the ClaI site on pIMPl to yield plasmid pIMPT. pIMPT was shown to able to be transferred from one strain of *E. coli* to another when pRK2013 was also present to supply other conjugation functions. PCR was used to amplify the promoter of the alcohol dehydrogenase (Adh) gene Cphy_1029 from the *C. phytofermentans* chromosome and it was used to replace the promoter of the erythromycin gene in pIMPT to create pIMPTCphy (Fig. 8). The successful transfer of pIMPTCphy into *C. phytofermentans* via electroporation was demonstrated by the ability to grow in the presence of 10 μg/mL erythromycin. In addition to phenotypic proof of electroporation provided by the growth on erythromycin, successive plasmid isolations from *C. phytofermentans* confirmed that the same plasmid was isolated from *Clostridium phytofermentans* and transferred into *E.* coli and recovered.

[00264] The method of conjugal transfer of pIMPTCphy from *E. coli* to *C. phytofermentans* involved constructing an *E. coli* strain (DH5alpha) that contains both pIMPTCphy and pRK20l3. Fresh cells *E. coli* culture and fresh cells of the *C. phytofermentans* recipient culture were obtained by growth to mid-log phase using appropriate growth media (L broth and QM1 media respectively). The two bacterial cultures were then centrifuged to yield cell pellets and the pellets resuspended in the same media to obtain cell suspensions that were concentrated about ten-fold having cell densities of about 10^{10} cells per ml. These concentrated cell suspensions were then mixed to achieve a donor-to-recipient ratio of five-to-one, then the cell suspension was spotted onto QM1 agar plates and incubated anaerobically at 30° C for 24 hours. The cell mixture was removed from the QM1 plate and placed on solid or in liquid QM1 media containing antibiotics that allow the survival of *C. phytofermentans* recipient cells expressing erythromycin resistance. This was accomplished by using a combination of antibiotics consisting of trimethoprim (20 μg/ml), cycloserine (250 μg/ml), and erythromycin (10 μg/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 anaerobic incubation on antibiotic-containing plates or liquid media for 5 to 7 days at 30° C, derivatives of *C. phytofermentans* were obtained that were erythromycin resistant and these *C. phytofermentans* derivatives were subsequently shown to contain pIMPCphy as demonstrated by PCR analyses.

[00265] A map of the plasmid pIMPCphy is shown in Figure 8, and the DNA sequence of this plasmid is provided as SEQ ID NO:1.

[00266] SEQ ID NO: 1:

gcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgcagctggcacgacaggtttcccgactggaaagcgggc
agtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggctttacactttatgcttccggctcgtatgttgtgtggaat
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cacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgct
cgtcaggggggcggagcctatggaaaaacgccagcaacgcggcctttttacggttcctggccttttgctggccttttgctcacatgttcttt
cctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgccgagcgcagc
gagtcagtgagcgaggaagcggaaga.

[00267] The vector pIMPCphy was constructed as a shuttle vector for *C. phytofermentans*. It has an Ampicillin-resistance cassette and an Origin of Replication (ori) for selection and replication in *E.coli*. It contains a Gram-positive origin of replication that allows the replication of the plasmid in *C. phytofermentans*. In order to select for the presence of the plasmid, the pIMPCphy carries an erythromycin resistance gene under the control of the *C. phytofermentans* promoter of the gene Cphy1029. This plasmid can be transferred to *C.*

phytofermentans by electroporation or by transconjugation with an *E.coli* strain that has a mobilizing plasmid, for example pRK2030. A plasmid map of pIMPCphy is depicted in Fig. 8. pIMPCphy is an effective replicative vector system for all microbes, including all gram$^+$ and gram$^-$ bacteria, and fungi (including yeasts).

[00268] *Constitutive Promoter*

[00269] In a first step, several promoters from *C. phytofermentans* were chosen that show high expression of their corresponding genes in all growth stages as well as on different substrates. A promoter element can be selected by selecting key genes that would necessarily be involved in constitutive pathways (*e.g.*, ribosomal genes, or for ethanol production, alcohol dehydrogenase genes). Examples of promoters from such genes include but are not limited to:

[00270] Cphy_1029: iron-containing alcohol dehydrogenase

[00271] Cphy_3510: Ig domain-containing protein

[00272] Cphy_3925: bifunctional acetaldehyde-CoA/alcohol dehydrogenase

[00273] *Cloning of Promoter*

[00274] The different promoters in the upstream regions of the genes were amplified by PCR. The primers for this PCR reaction were chosen in a way that they include the promoter region but do not include the ribosome binding sites of the downstream gene. The primers were engineered to introduce restriction sites at the end of the promoter fragments that are present in the multiple cloning site of pIMPCphy but are otherwise not present in the promoter region itself, for example SalI, BamHI, XmaI, SmaI, EcoRI.

[00275] The PCR reaction was performed with a commercially available PCR Kit, *e.g.* GoTaq® Green Master Mix (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711 USA), according to the manufacturer's conditions. The reaction is run in a thermal cycler, *e.g.* Gene Amp System 2400 (PerkinElmer, 940 Winter St., Waltham MA 02451 USA). The PCR products were purified with the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich Corp., St. Louis, MO, USA). Both the purified PCR products as well as the plasmid pIMPCphy were then digested with the corresponding enzymes with the appropriate amounts according to the manufacturer's conditions (restriction enzymes from New England Biolabs, 240 County Road, Ipswich, MA 01938 USA and Promega). The PCR products and the plasmid were then analyzed and gel-purified on a Recovery FlashGel (Lonza Biologics, Inc., 101 International Drive, Portsmouth, NH 03801 USA). The PCR products were subsequently ligated to the plasmid with the Quick Ligation Kit (New England Biolabs) and competent cells of *E.coli* (DH5α) are transformed with the ligation mixtures and plated on LB plates with 100 µg/ml ampicillin. The plates are incubated overnight at 37°C.

[00276] Ampicillin resistant *E.coli* colonies were picked from the plates and restreaked on new selective plates. After growth at 37°C, liquid LB medium with 100 μg/ml ampicillin was inoculated with a single colony and grown overnight at 37°C. Plasmids were isolated from the liquid culture with the Gene Elute™ Plasmid isolation kit.

[00277] *Miniprep Kit (Sigma-Aldrich).*

[00278] Plasmids were checked for the right insert by PCR reaction and restriction digest with the appropriate primers and by restriction enzymes respectively. To ensure the sequence integrity, the insert is sequenced at this step.

[00279] *Cloning of Cellulase genes*

[00280] One or more genes disclosed in Table 2, which can include each gene's own ribosome binding sites, were amplified via PCR and subsequently digested with the appropriate enzymes as described previously under *Cloning of Promoter*. Resulting plasmids were also treated with the corresponding restriction enzymes and the amplified genes are mobilized into plasmids through standard ligation. *E.coli* were transformed with the plasmids and correct inserts were verified from transformants selected on selection plates.

[00281] *Transconjugation*

[00282] *E.coli* DH5α along with the helper plasmid pRK2030, were transformed with the different plasmids discussed above. *E.coli* colonies with both of the foregoing plasmids were selected on LB plates with 100 μg/ml ampicillin and 50 μg/ml kanamycin after growing overnight at 37°C. Single colonies were obtained after re-streaking on selective plates at 37°C. Growth media for *E.coli* (*e.g.* LB or LB supplemented with 1% glucose and 1% cellobiose) was inoculated with a single colony and either grown aerobically at 37°C or anaerobically at 35°C overnight. Fresh growth media was inoculated 1:100 with the overnight culture and grown until mid log phase. A *C. phytofermentans* strain was also grown in the same media until mid log.

[00283] The two different cultures, *C. phytofermentans* and *E.coli* with pRK2030 and one of the plasmids, were then mixed in different ratios, e. g. 1:1000, 1:100, 1:10, 1:1, 10:1, 100:1, 1000:1. The mating was performed in either liquid media, on plates or on 25 mm Nucleopore Track-Etch Membrane (Whatman, Inc., 800 Centennial Avenue, Piscataway, NJ 08854 USA) at 35°C. The time was varied between 2h and 24h, and the mating media was the same growth media in which the culture was grown prior to the mating. After the mating procedure, the bacteria mixture was either spread directly onto plates or first grown on liquid media for 6h to 18h and then plated. The plates contain 10μg/ml erythromycin as selective

agent for *C. phytofermentans* and 10 μg/ml Trimethoprim, 150 μg/ml Cyclosporin and 100 μg/ml Nalidixic acid as counter selectable media for *E .coli*.

[00284] After 3 to 5 days incubation at 35°C, erythromycin-resistant colonies were picked from the plates and restreaked on fresh selective plates. Single colonies were picked and the presence of the plasmid is confirmed by PCR reaction.

[00285] *Cellulase gene expression*

[00286] The expression of the cellulase genes on the different plasmids was then tested under conditions where there is little to no expression of the corresponding genes from the chromosomal locus. Positive candidates showed constitutive expression of the cloned cellulases.

[00287] Constitutive Expression of Cellulases I

[00288] pCphyP3510-1163

[00289] Two primers were chosen to amplify Cphy_1163 using *C. phytofermentans* genomic DNA as template. The two primers were: cphy_1163F: 5' -CCG CGG AGG AGG GTT TTG TAT GAG TAA AAT CAG AAG AAT AGT TTC-3 (SEQ ID NO: 3), which contained a SacII restriction enzyme site and ribosomal site; and cphy_1163R: CCC GGG TTA GTG GTG GTG GTG GTG GTG TTT TCC ATA ATA TTG CCC TAA TGA (SEQ ID NO: 4), which containing a XmaI site and His-tag (SEQ ID NO: 31). The amplified gene was cloned into Topo-TA first, then digested with SacII and XmaI, the cphy_1163 fragment was gel purified and ligated with pCPHY3510 digested with SacII and XmaI, respectively. The plasmid was transformed into *E.coli*, purified and then transformed into *C. phytofermentans* by electroporation. The plasmid map is shown in Figure 11.

[00290] The transformants from the QM plate, which contained 20 μg/ml of erythromycin, were transformed into QM liquid medium, which contained 2% cellobiose and 20 μg/ml of erythromycin. The enzyme activities from the supernatant of overnight culture were assayed by CMC-congo red plate assay and Cellazyme T assay kit (Megazyme International Ireland, Ltd., Bray Business Park, Bray, Co., Wicklow, Ireland). The CMC-congo plate and the Cellazyme T assays indicated the transformant of *C.phytofermentans* /pCphy3510_1163 showed increased activity than that of the control strain (Figure 10). The CEL-T assay showed the tranformant had an activity level of 54.5 mU/ml (left box "3") whereas the control activity was only 3.7 mU/ml (right box "2").

[00291] Using the methods above and the primers described in Figs. 13, 14, 15, and 16, respectively, genes encoding Cphy_3367, Cphy_3368, Cphy_3202 and Cphy_2058 were cloned into pCphy3510 to produce pCphy3510_3367, pCphy3510_3368, pCphy3510_3202,

and pCphy3510_2058 respectively. These vectors were transformed into *C. phytofermentans* via electroporation as described *supra*. In addition, genes encoding the heat shock chaperonin proteins, Cphy_3289 (GroES, Fig. 15) and Cphy_3290 (GroEL, Fig. 15) were incorporated into pCphy3510. In another embodiment, an endogenous or exogenous gene can be cloned into this vector and used to transform *C. phytofermentans*, another bacteria or fungal cell.

[00292] While preferred embodiments of the present invention 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 can 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.

CLAIMS

WHAT IS CLAIMED IS:

1. A process for producing a fermentive end-product comprising:

(a) contacting a carbonaceous biomass with:

i. a recombinant microorganism that hydrolyses and ferments said biomass, wherein said microorganism is capable of direct fermentation of C5 and C6 carbohydrates; and

ii. an external source of one or more enzymes that are capable of enhancing said hydrolysis, wherein said one or more enzymes comprise a cellulase, and wherein the cellulase and the microorganism act synergistically to enhance hydrolysis; and

(b) allowing sufficient time for said hydrolysis and fermentation to produce a fermentive end-product.

2. A process for producing a fermentive end-product comprising:

(a) contacting a carbonaceous biomass with:

i. a recombinant *Clostridia* microorganism that hydrolyses and ferments said biomass; and

ii. optionally, an external source of one or more enzymes that are capable of enhancing said hydrolysis, and

(b) allowing sufficient time for said hydrolysis and fermentation to produce a fermentive end-product.

3. A process for producing a fermentive end-product comprising:

(a) contacting a carbonaceous biomass with:

i. a microorganism that hydrolyses and ferments said biomass; and

ii. an external source of one or more enzymes that are capable of enhancing said hydrolysis; and

(b) allowing sufficient time for said hydrolysis and fermentation to produce a fermentive end-product, wherein said one or more enzymes do not include a xylanase, a hemicellulase, a glucanase or glucosidase, and wherein said external source is not said microorganism.

4. The process of claims 1-3, wherein said contact is in a large-scale fermentation vessel, wherein the fermentation vessel is adapted to provide suitable conditions for fermentation of one or more carbohydrate into fermentive end-products.
5. The process of claims 1 or 3, wherein said microorganism is capable of direct fermentation of C5 and C6 carbohydrates.
6. The process of claims 1 or 3, wherein said microorganism is a bacterium.
7. The process of claims 1 or 3, wherein said microorganism is a species of *Clostridia*.
8. The process of claims 1-3, wherein said microorganism is *Clostridium phytofermentans*.
9. The process of claims 1-3, wherein said microorganism is *Clostridium. sp. Q.D.*
10. The process of claims 3, wherein the microorganism is non-recombinant.
11. The process of claims 1 or 2, wherein said microorganism comprises one or more heterologous polynucleotides or one or more copies of an endogenous polynucleotide that enhance said activity of one or more cellulases.
12. The process of claims 1-3, wherein said biomass comprises one or more of corn steep solids, corn steep liquor, malt syrup, xylan, cellulose, hemicellulose, fructose, glucose, mannose, rhamnose, or xylose.
13. The process of claims 1-3, wherein the biomass comprises municipal waste, wood, plant material, plant material extract, a natural or synthetic polymer, or a combination thereof.
14. The process of claim 13, wherein said plant material is switchgrass, bagasse, corn stover, poplar, 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), or lactic acid.
15. The process of claims 1-3, wherein said fermentive end-product is an alcohol.
16. The process of claims 1-3, wherein said fermentive end-product is ethanol, lactic acid, acetic acid or formic acid.
17. The process of claims 1-3, wherein said microorganism is capable of uptake of one or more complex carbohydrates.
18. The process of claims 1-3, wherein said biomass comprises a higher concentration of oligomeric carbohydrates relative to monomeric carbohydrates.
19. The process of claim 11, wherein said one or more heterologous polynucleotides or said one or more copies of an endogenous polynucleotide comprises SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, or SEQ ID NO: 14.
20. The process of claim 11, said one or more heterologous polynucleotides or said one or more copies of an endogenous polynucleotide encodes one or more enzymes selected

from the group consisting of Cphy_3202, Cphy_2058, Cphy_1163, Cphy_3367, Cphy_1100, Cphy_1510, Cphy_3368, and Cphy_2128.

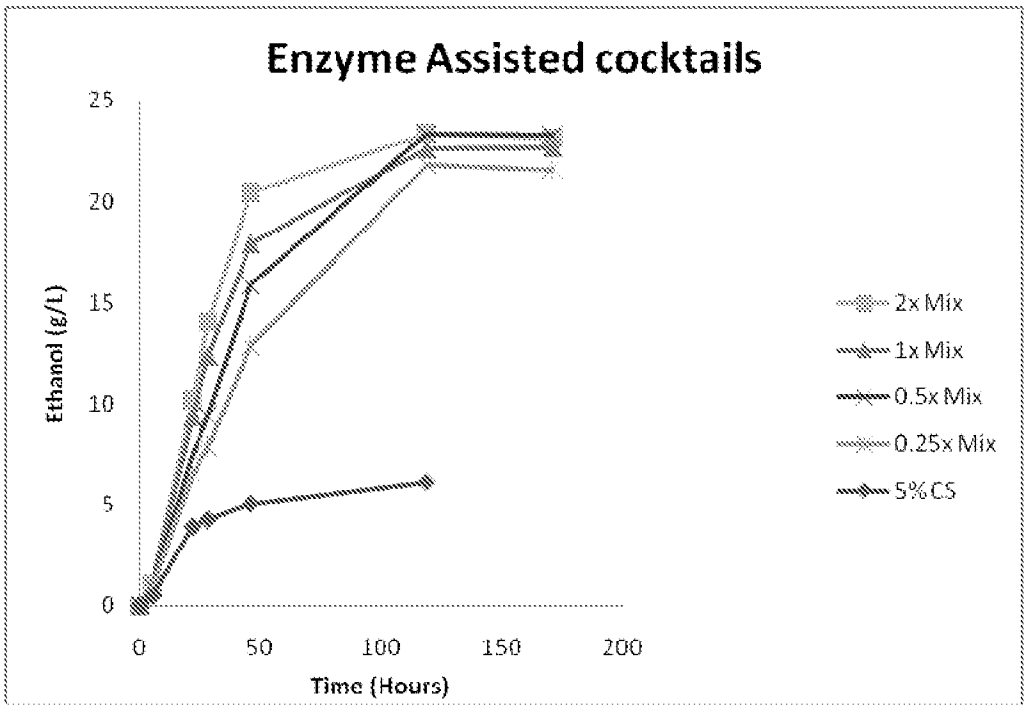
21. The process of claims 1-3, wherein said external source of one or more enzymes is in an amount from about 0.4 to about 15 filter paper unit (FPU)/gram cellulose.
22. The process of claim 1, wherein said cellulase is in an amount from about 0.4 to about 15 filter paper unit (FPU)/gram cellulose.
23. The process of claims 1-3, wherein said enhanced activity is sufficient to provide activity of about 0.4 to about 15 filter paper unit (FPU)/gram cellulose.
24. The process of claims 1-3, wherein said hydrolysis provides results in a greater concentration of cellobiose and/or larger oligomers, relative to monomeric carbohydrates.
25. The process of claim 24, wherein said monomeric carbohydrates comprise xylose and arabinose.
26. The process of claims 1-3, wherein said biomass is pre-conditioned with acid treatment or steam.
27. The process of claims 1-3, wherein said microorganism produces one or more hydrolytic enzyme encoded by a variant having a polynucleotide sequence with an identity of 70% or more compared to a sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, or SEQ ID NO: 14.
28. The process of claims 1-3, wherein said microorganism is a recombinant *Clostridium* microorganism comprising one or more additional copies of an endogenous polynucleotide, wherein said one or more additional copies of a polynucleotide encodes SEQ ID NO: 17, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 or SEQ ID NO: 27.
29. A recombinant *Clostridium* microorganism comprising one or more additional copies of an endogenous polynucleotide that encodes Cphy_3202, Cphy_2058, Cphy_1163, Cphy_3367, Cphy_1100, Cphy_1510, Cphy_3368, or Cphy_2128.
30. A recombinant *Clostridium* microorganism comprising one or more additional copies of an endogenous polynucleotide, wherein said one or more additional copies is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, or SEQ ID NO: 14.
31. A recombinant *Clostridium* microorganism comprising one or more additional copies of an endogenous polynucleotide, wherein said one or more additional copies of a polynucleotide encodes SEQ ID NO: 17, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO:

22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 or SEQ ID NO: 27.

32. A vector comprising: (a) one or more polynucleotides encoding a biomass degrading enzyme; and (b) a sequence encoding an erythromycin resistance gene.
33. The vector of claim 32, further comprising a SacII restriction site.
34. The vector of claim 32, further comprising a constitutively active promoter.
35. The vector of claim 32, wherein said constitutively active promoter is a Cphy_3510 promoter.
36. The vector of claim 32, wherein said biomass degrading enzyme is Cphy_3289 or Cphy_3290.
37. The vector of claim 32, wherein said biomass degrading enzyme is Cphy_3202, Cphy_2058, Cphy_1163, Cphy_3367, Cphy_1100, Cphy_1510, Cphy_3368, or Cphy_2128.
38. The vector of claim 32, wherein said one or more polynucleotides is SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 14, SEQ ID NO: 17, or SEQ ID NO: 32
39. The vector of claim 32, wherein said one or more polynucleotides hybridizes to SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 14, SEQ ID NO: 18 or SEQ ID NO: 32 under conditions of medium stringency.

FIGURE 1

A.


Enzyme Assisted cocktails
Ethanol (g/L)
25
20
15
10
5
0
0
50
100
150
200
Time (Hours)
2x Mix
1x Mix
0.5x Mix
0.25x Mix
5% CS

B.

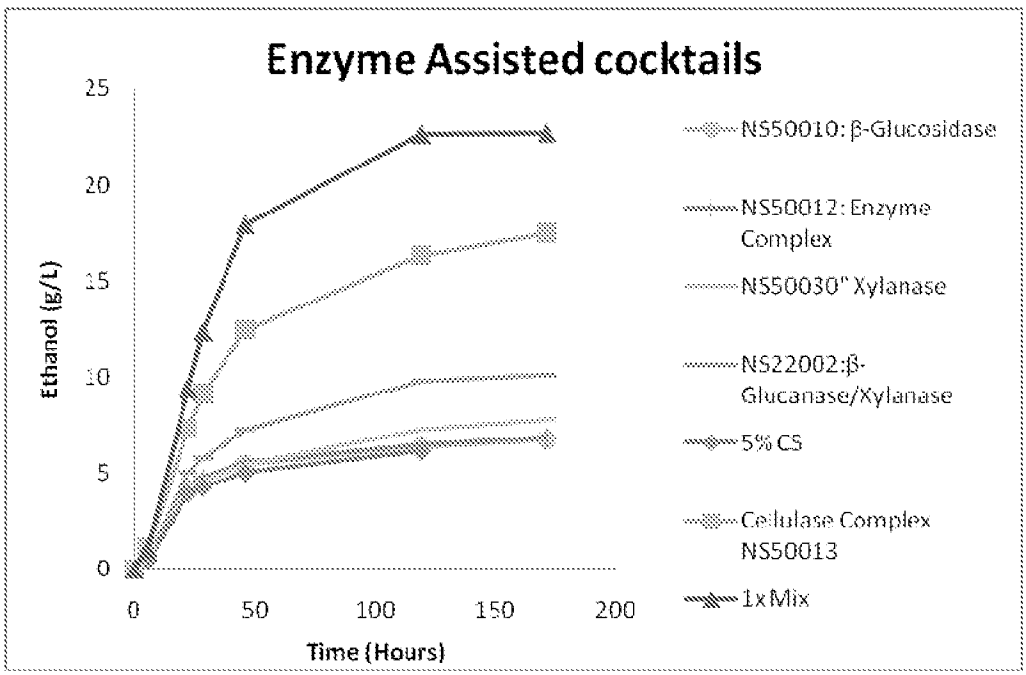

Enzyme Assisted cocktails
Ethanol (g/L)
25
20
15
10
5
0
0
50
100
150
200
Time (Hours)
NS50010: β-Glucosidase
NS50012: Enzyme Complex
NS50030" Xylanase
NS22002:β-Glucanase/Xylanase
5% CS
Cellulase Complex NS50013
1x Mix

FIGURE 2

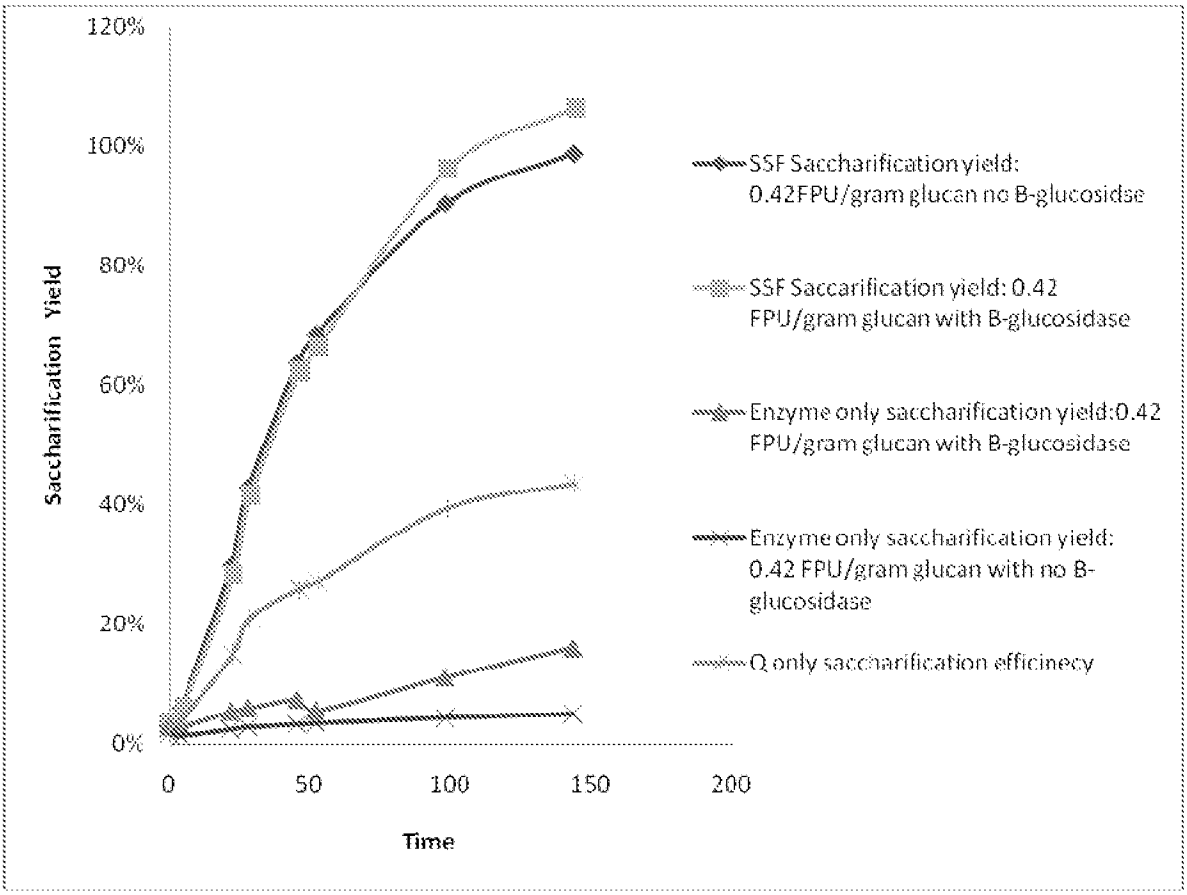
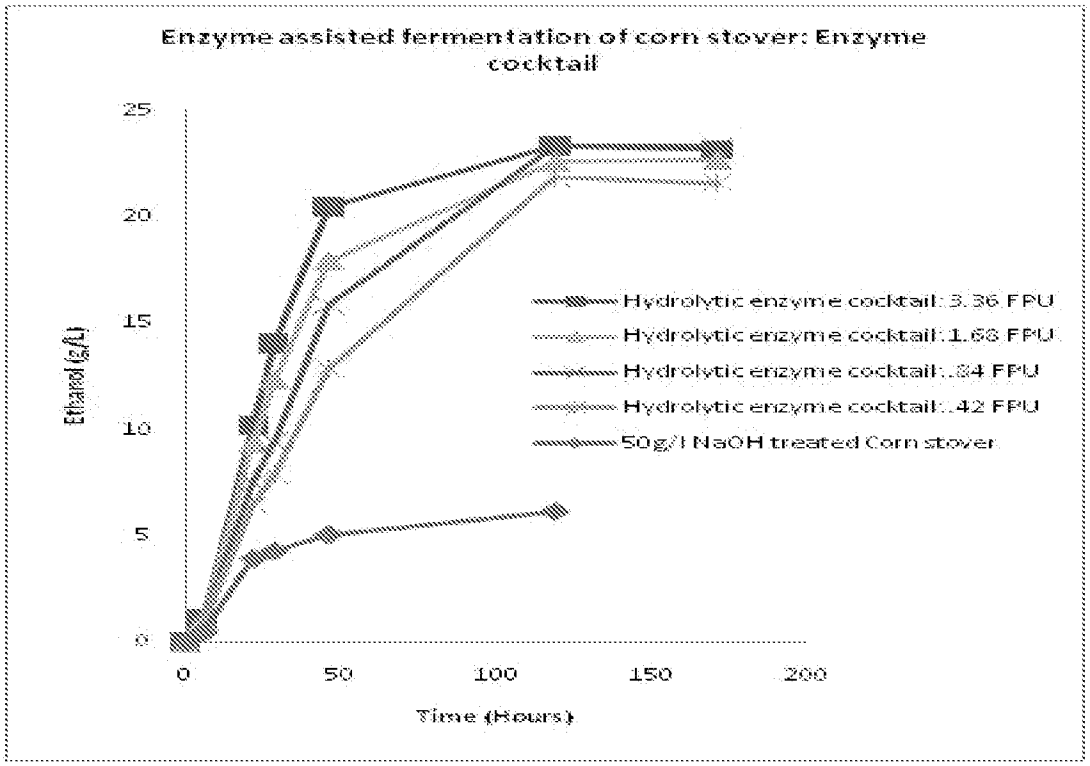

120%
100%
80%
60%
40%
20%
0%
Saccharification Yield
0
50
100
150
200
Time
SSF Saccharification yield: 0.42FPU/gram glucan no B-glucosidse
SSF Saccarification yield: 0.42 FPU/gram glucan with B-glucosidase
Enzyme only saccharification yield:0.42 FPU/gram glucan with B-glucosidase
Enzyme only saccharification yield: 0.42 FPU/gram glucan with no B-glucosidase
Q only saccharification efficinecy

FIGURE 3

A.


Enzyme assisted fermentation of corn stover: Enzyme cocktail
Ethanol (g/L)
Time (Hours)
Hydrolytic enzyme cocktail: 3.36 FPU
Hydrolytic enzyme cocktail: 1.68 FPU
Hydrolytic enzyme cocktail: .84 FPU
Hydrolytic enzyme cocktail: .42 FPU
50g/l NaOH treated Corn stover

B.

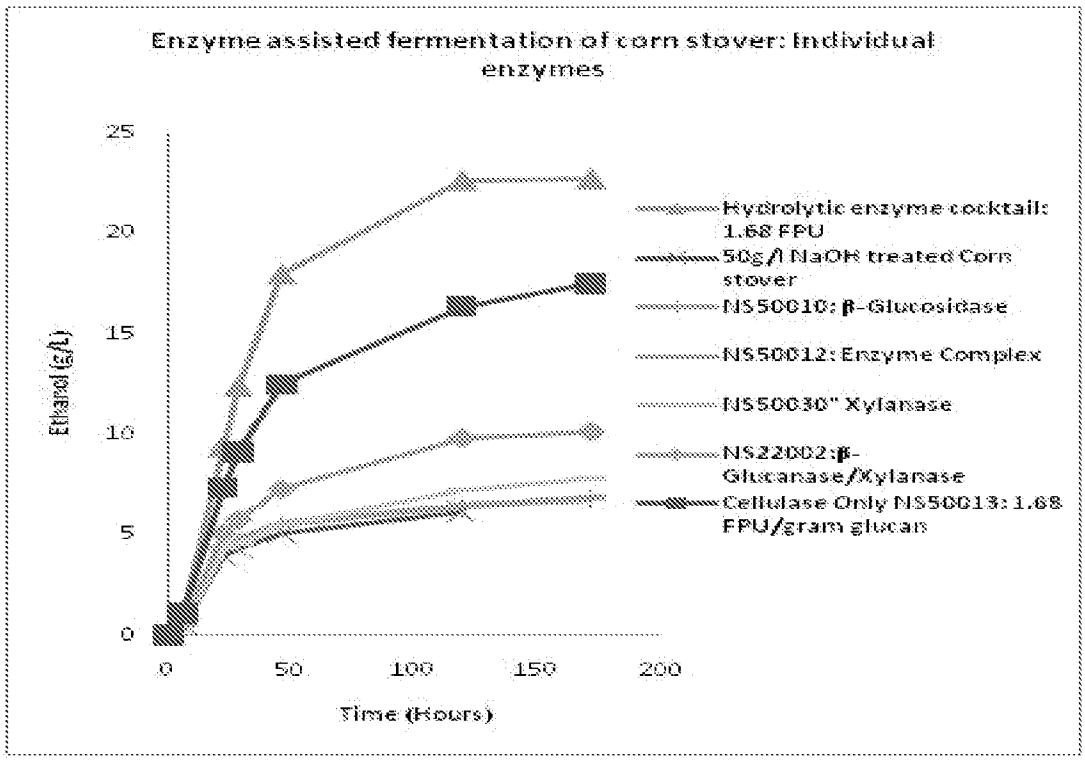

Enzyme assisted fermentation of corn stover: Individual enzymes
Ethanol (g/L)
Time (Hours)
Hydrolytic enzyme cocktail: 1.68 FPU
50g/l NaOH treated Corn stover
NS50010: β-Glucosidase
NS50012: Enzyme Complex
NS50030" Xylanase
NS22002: β-Glucanase/Xylanase
Cellulase Only NS50013: 1.68 FPU/gram glucan

FIGURE 4

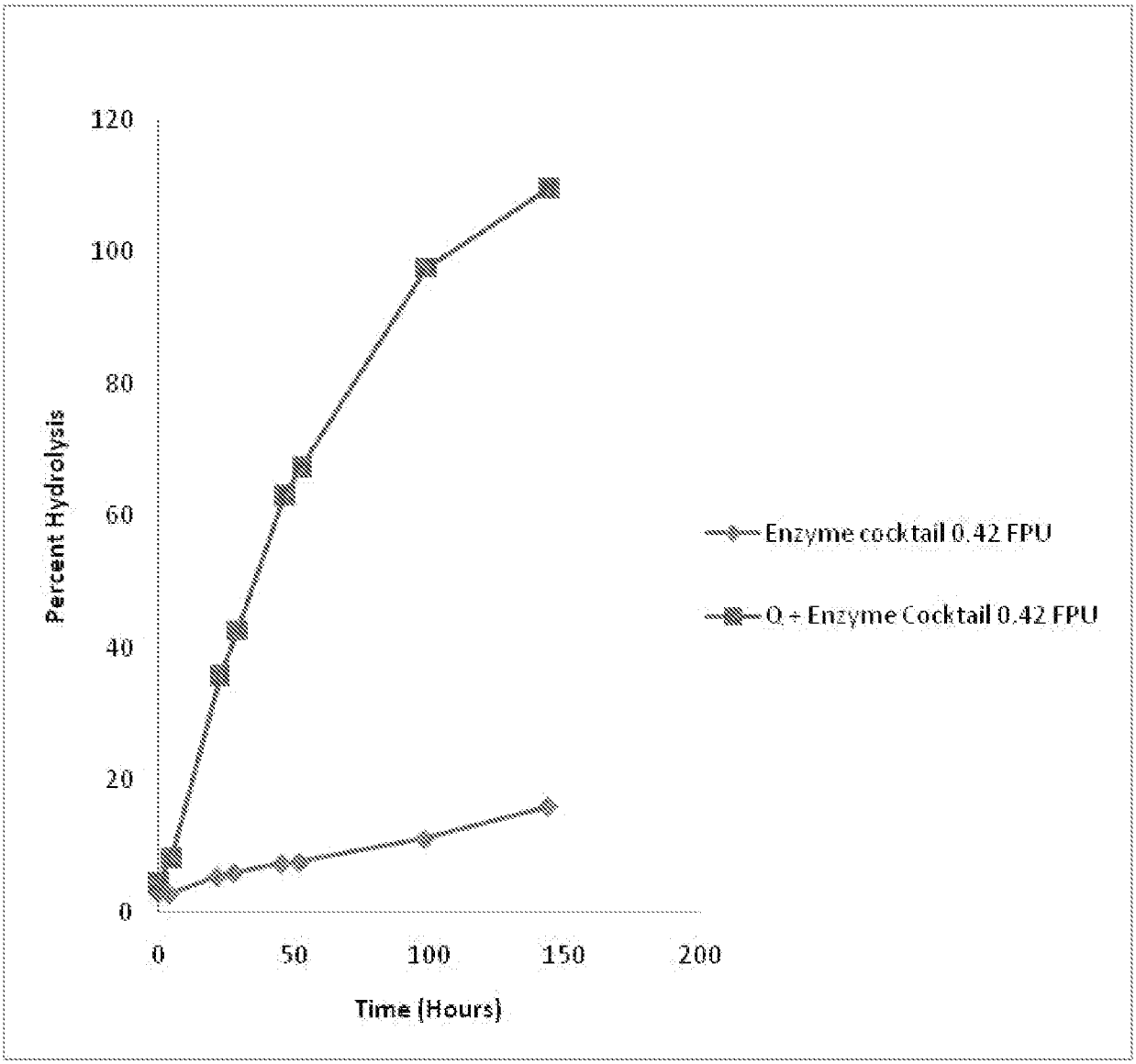

120
100
80
60
40
20
0
Percent Hydrolysis
0
50
100
150
200
Time (Hours)
Enzyme cocktail 0.42 FPU
Q + Enzyme Cocktail 0.42 FPU

FIGURE 5

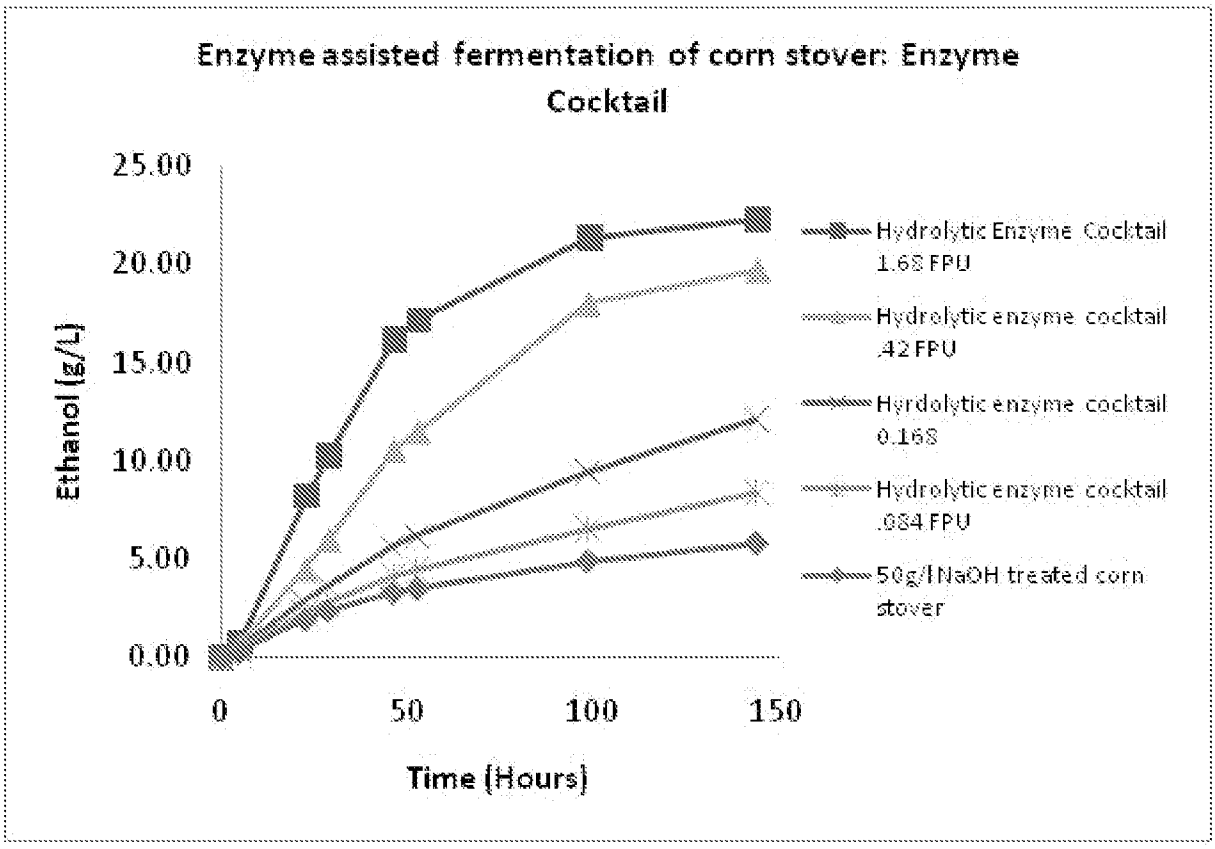

Enzyme assisted fermentation of corn stover: Enzyme Cocktail
25.00
20.00
15.00
10.00
5.00
0.00
Ethanol (g/L)
0
50
100
150
Time (Hours)
Hydrolytic Enzyme Cocktail 1.68 FPU
Hydrolytic enzyme cocktail .42 FPU
Hyrdolytic enzyme cocktail 0.168
Hydrolytic enzyme cocktail .084 FPU
50g/l NaOH treated corn stover

FIGURE 6

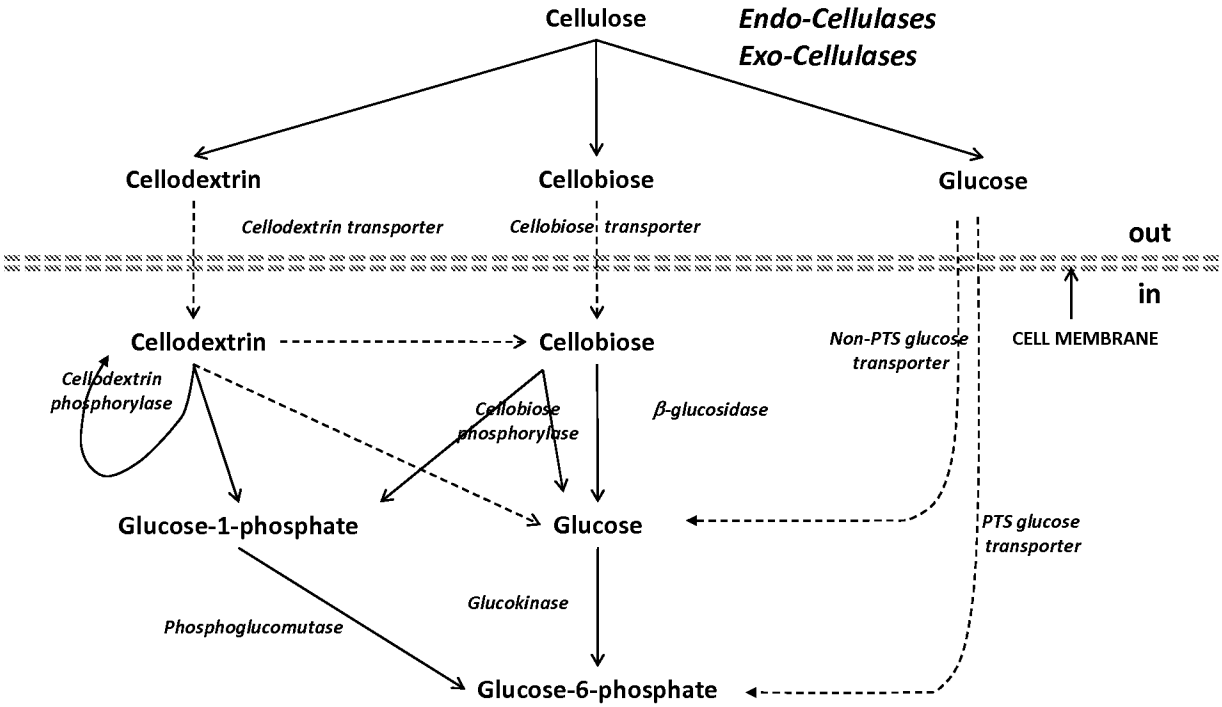

Cellulose
Endo-Cellulases
Exo-Cellulases
Cellodextrin
Cellobiose
Glucose
Cellodextrin transporter
Cellobiose transporter
out
in
Cellodextrin
Cellobiose
Non-PTS glucose transporter
CELL MEMBRANE
Cellodextrin phosphorylase
Cellobiose phosphorylase
β-glucosidase
Glucose-1-phosphate
Glucose
PTS glucose transporter
Glucokinase
Phosphoglucomutase
Glucose-6-phosphate

FIGURE 7

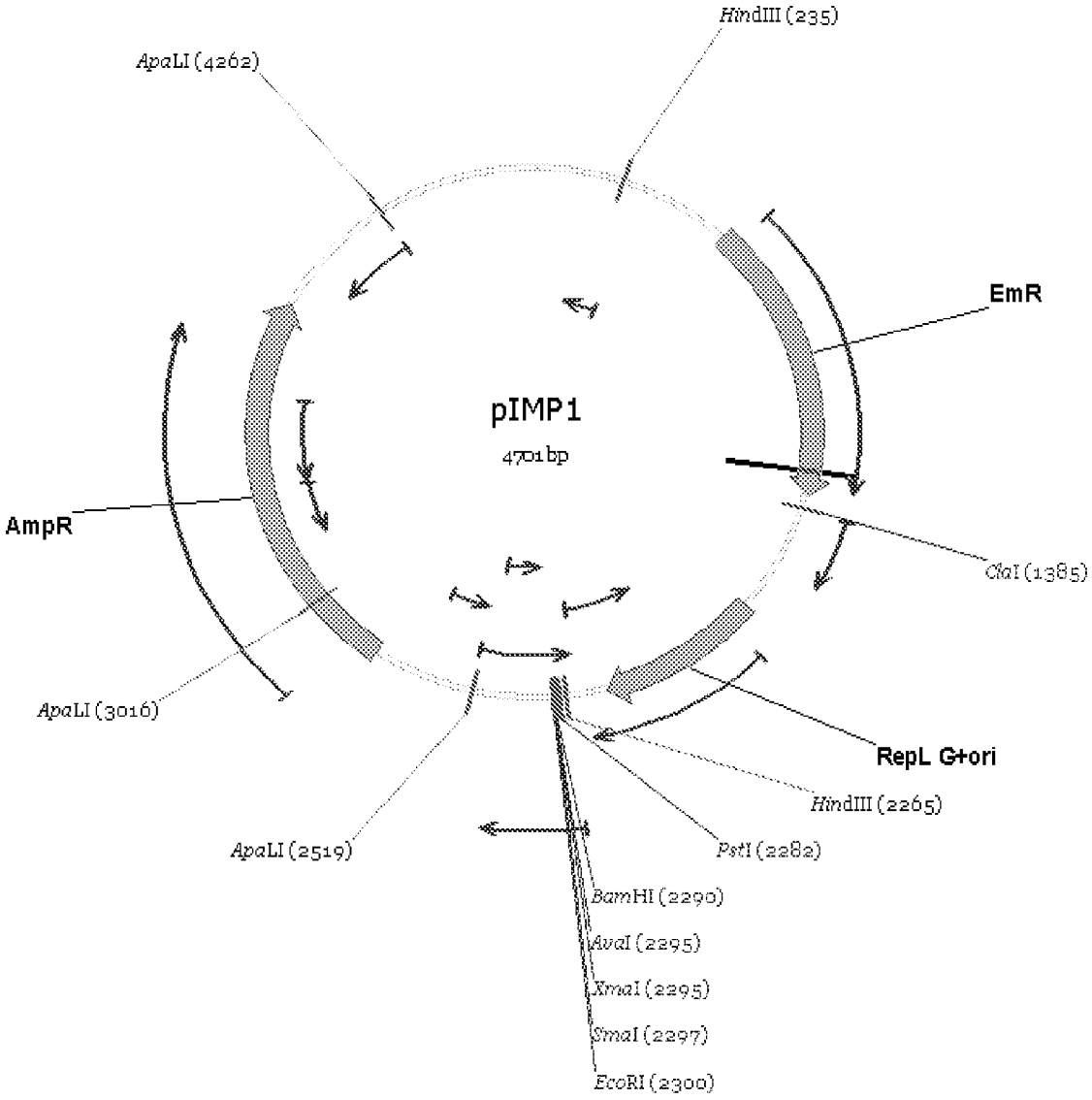
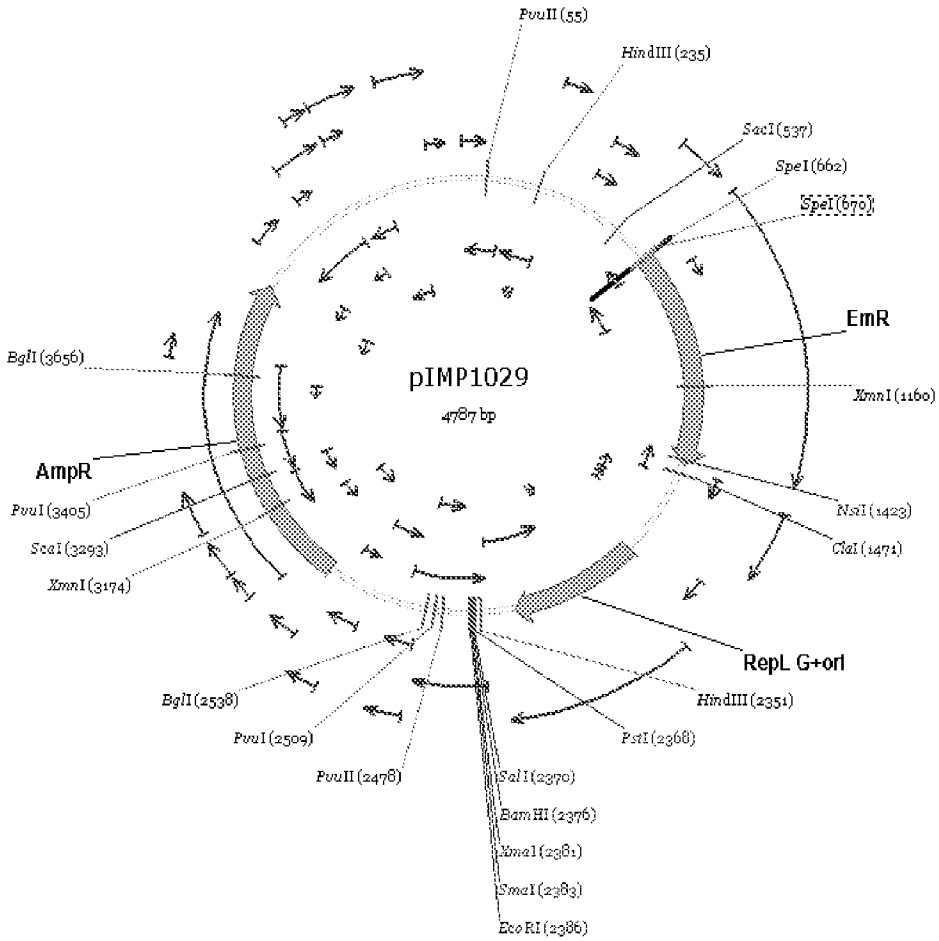

HindIII (235)
ApaLI (4262)
EmR
pIMP1
4701bp
AmpR
ClaI (1385)
ApaLI (3016)
RepL G+ori
HindIII (2265)
ApaLI (2519)
PstI (2282)
BamHI (2290)
AvaI (2295)
XmaI (2295)
SmaI (2297)
EcoRI (2300)

FIGURE 8



pIMPCphy

FIGURE 9

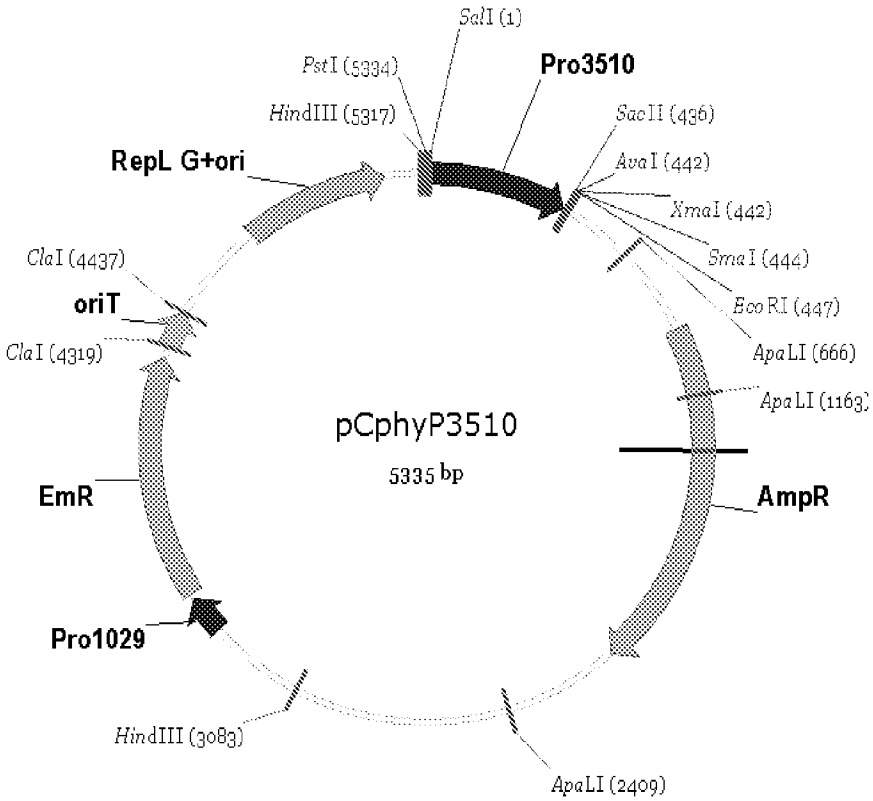

SalI (1)
Pro3510
PstI (5334)
HindIII (5317)
SacII (436)
AvaI (442)
XmaI (442)
SmaI (444)
EcoRI (447)
ApaLI (666)
ApaLI (1163)
RepL G+ori
ClaI (4437)
oriT
ClaI (4319)
pCphyP3510
5335 bp
EmR
AmpR
Pro1029
HindIII (3083)
ApaLI (2409)

FIGURE 10

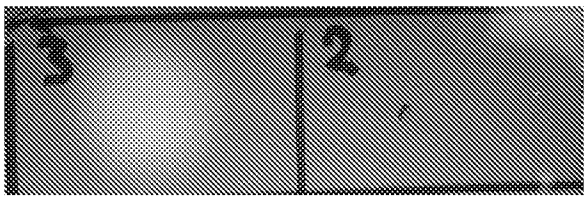


FIGURE 11

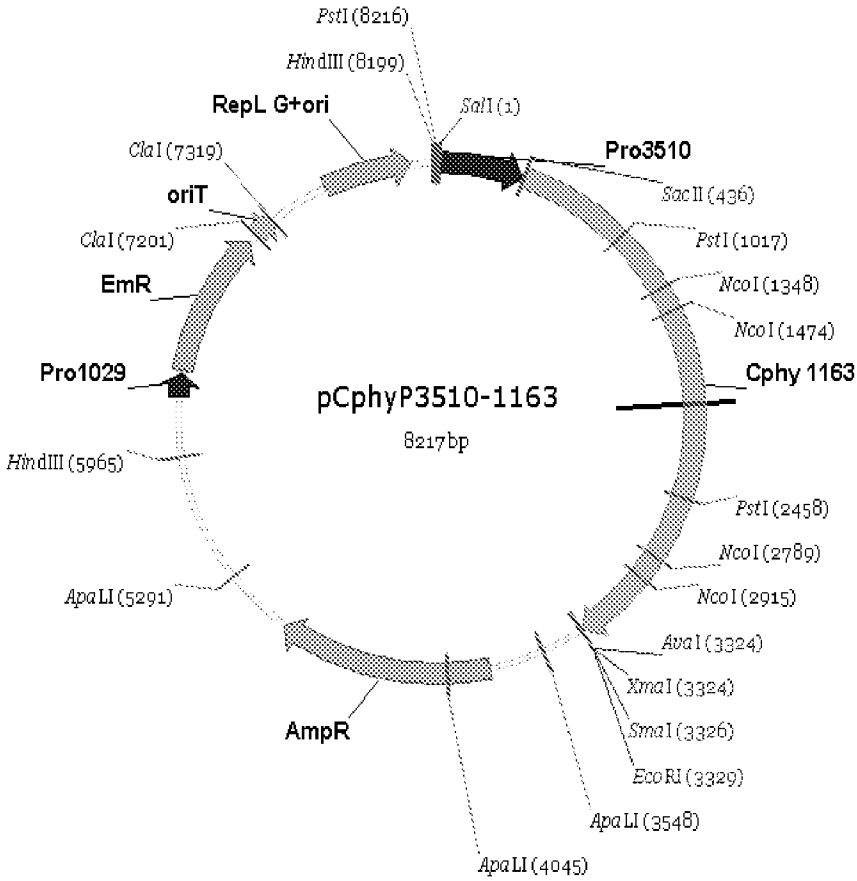

PstI (8216)
HindIII (8199)
SalI (1)
RepL G+ori
ClaI (7319)
Pro3510
SacII (436)
oriT
ClaI (7201)
PstI (1017)
EmR
NcoI (1348)
NcoI (1474)
Pro1029
Cphy 1163
pCphyP3510-1163
8217bp
HindIII (5965)
PstI (2458)
NcoI (2789)
ApaLI (5291)
NcoI (2915)
AvaI (3324)
XmaI (3324)
AmpR
SmaI (3326)
EcoRI (3329)
ApaLI (3548)
ApaLI (4045)

FIGURE 12

Cphy_1163 GH5 Cellulase

TTTTCTAGAGATCACTCATATAGGGAGCTATAAAAAATATTAGAAAAAGGGGAAATTTA
ATTATGAGTAAAATCAGAAGAATAGTTTCATTGTTTTTGATTGTAACAATCTTTATCACG
ACATGCTTTTTTAATGTTGGTCAAAAGGTATATGCTGCTGATACGAATAATGATGATTG
GCTACATTGTGTAGGCAATAAAATTTATGACATGAATGGCAATGAGGTTTGGCTGACCG
GTGCGAATTGGTTTGGTTTTAACTGTACTGAAAATGTATTTCATGGTGCATGGTACGATA
TTAAGGGGATGTTAACTAATATTGCAAACAGAGGAATAGGATTTTTAAGAGTTCCAATT
TCAACGGAACTTTTGTATAGTTGGATGATAGGCAAACCTAATAAAGTTTCAAGTGTGAC
CGCTGTCAATAATCCACCTTATTATGTATGCAACCCTGATTTTTATGATCCTACAACAAA
TAGTGTTAAAAATAGTATGGAAATATTTGATATCATTATGGGATACTGCAAACAATTGG
GGATCAAAGTAATGGTAGATGTTCATAGTCCGGATGCAAATAATTCAGGTCATAACTAT
CCGTTATGGTATGGGTTAACTACGACTACTGCAGGTGAAATAACGACAGATAAGTGGAT
CAATACTCAAGCATGGCTGGCTGATAAATACAAAAATGACGATACTATTCTGGCATTTG
ATATAAAAAATGAACCTCATGGACAGAGGGGATATAGTACTACAACACCTACTAATAT
AGCAAAATGGGATAATTCCACAGATGAGAATAACTGGAAGTATGCGGCGGAAAGATGT
GCGAAAGCTATACTTGCTAAAAACCCTAAATTATTAATTATGATTGAAGGTGTTGAACA
ATACCCTAAAACTGAAAAAGGTTATAACTATAATACACCGGATGTATGGGGAGCTACTG
GTGATCAGTCTCCATGGTATAGTGCTTGGTGGGGTGGAAATTTAAGAGGAGTAAAGGAT
TATCCAATTAATATAGGCACTCTCAATAGTCAGATCGTCTATTCCCCTCATGACTATGGT
CCTTCCGTATACAACCAACCATGGTTTGATAAGGATTTTACAACTCAGACCCTATTAGAT
GATTATTGGTATAATACTTGGGCATATATTAAAGATAAAGGTATTGCACCACTTTTGAT
AGGTGAGTGGGGAGGTTTTATGGATGGTGGAAAGAACCAGAAATGGATGACATTATTA
AGAGATTATATAGTAAATAATCGTATCCACCATACATTCTGGTGTATCAATCCGAACTC
AGGGGATACTGGAGGTTTACTAGGATATGATTGGCAAACTTGGGATGAAGCAAAATAC
GCTTTATTAAAACCTGCATTATGGCAGTCAAATGGTAAATTTATTGGTCTAGACCATCA
GACACCTCTTGGTGTAAATGGTATATCATTAGGGCAATATTATGGAAAATAAATGAACA
AAAAAATAAAATAATTTATAGAGAATGGGTTCAAATCGAGCCCATTCTTTTTTGTGCGA
AGCCGTAGCCTTAAGAAACTTTTTTGGT(SEQ ID NO: 2)

Forward Primer (SacII):
5'- CCG CGG AGG AGG GTT TTG TAT GAG TAA AAT CAG AAG AAT AGT TTC (SEQ ID NO: 3)

Reverse Primer (XmaI):
5'- CCC GGG TTA GTG GTG GTG GTG GTG GTG TTT TCC ATA ATA TTG CCC TAA TGA(SEQ ID NO: 4)

FIGURE 13

Cphy_3367 GH48 Cellulase

atgaaaaagataataagtcttttattagtgataacacttctgatatccatggcaccatcgaaagctgacgcagcggaaaccaattataattacggaga
agctcttcaaaaatcaatcatgttttatgagtttcaacgttctggtaaactgccaagtaccattcggaataattggagaggtgactctggtttaaccgat
ggagcagatgttggtttggatctaactggtggctggtatgatgctggtgatcatgtaaaatttaatcttcctttggcttatactgtaacaatgttagcatg
ggcagtatatgaagaagaggctactctttcaaaggcaggccaattaagttatttattagatgaaattaagtggtctagtgattacctaattaaatgtcat
ccacaagcaaatgtattttattatcaggttggtaatggaaatacagatcactcttggtggggacctgctgaagttatgcagatggctagaccgtcctat
aaggttgatttaaataacccaggttctactgtagtaggagaagcagcagcagctcttgcagcaacagcacttatatataagacaaaagaccctactt
attcagcaacttgccttcgtcatgcaaaagagcttttaattttgcagatacaacaaaaagcgatgctggatatacagcagcaagtgggttctatactt
cctatagtggattttatgatgaattatcctgggcagctacatggatttaccttgcaagtggagaagcgacctatttggataaggcagaatcttatgtag
ccaaatggggaacagaacctcaatcttccacattaagttataagtgggcacaaaactgggatgatgttcactatggtgcagctttattattagcaaga
attacaaataaagcaatttataagaacaatattgaaatgcatcttgactattggactacagggtataatggtagtcgtattacttatacaccaaaaggac
ttgcttggttagattcctgggggtgcattaagatatgcgacgacaacagcatttctagcaagtgtttatgctgattggagcggatgtagtgctggaaaa
gttagtacttacaatgcatttgcgaaacagcaggtagattatgcattaggaagtaccggaagaagttttgtggttggatatggtgtaaattctccaaca
agacctcatcatagaactgctcatagttcatgggcagacagtcagacggagccaaattaccatagacacaccatttatggtgctttagtaggtggac
ctggtaataatgatagttatgaggataacattaataattatgtaaacaatgaaatcgcttgtgactataatgcaggttttgttggcgcattggctaaagtt
tataaaacatatggcggaacaccaattgcaaactttaaggcaatcgaaacagtaacaaacgatgagttatttattcaagctggtattaatgcctctggt
ccatcttttatcgaagtaaaggcattggttttcaatgagacaggttggccagctcgtgttaccgataaattatcctttaagtattttattgatatctcggaa
tatgtagcaaagggatatacaaagaatgattttacggtatcgacaaattataacaatggagcaaccacatcggcattgcttccttgggatgctgcga
ataatatctattatgtgaatgtagacttctctggaactaagatttatcctggtggacagtctgcatataagaaagaagtacaatttagaattgctggtcca
caaaacgttaatatatgggacaattccaatgactactcctttacacaaattgctaatgttagttcaggaaataccgtaaagaccacatatataccattgt
atgataatggtaaattagtatttggtaatgagccaaagacgggtgttccttctgcaagtcttgataagactacagcaaactttgacaaaaacccagct
gtatccgcagatataccagtaaccattaactataatggtaatacattaacagcggttaagaatggaacaacggttttaacgaaaggtactgattatact
gtatctggtaatgtagtaacgttatctaagaatttatttcttagcacagagcgctagtacggttacttttaacatttgtatttagtggcggtaacgatgcaac
attaaaagtgactttagtagatacttctccaagtgcatccattaatccaaattctgctgtctttgataaggctagcggaaaacaggaaaatatagttatta
cgcttacaccaaatggcaatacctagctggacttaagaatgggtctaagagcctggtaactggaactgattataccgtttccggaacaacagtgac
gattctatcttcttatttaagtcaatttgcagtaggaagtcaatctattgtatttgaaatgaataaagggacaaatccagtcttagcagttaccattaagga
ttcttctgttgttactccaacaggaaatattaaacttcaaatgtttaatggaaattcttctgcaacaacgaatggcattgcaccaagaattaaattaattaa
caccggaactactgcaatcaacttatccgatgttaagattcgctattattatacaatcaatggcgaaaaggatcaggcattctggtgtgattattcgac
gattggtagttccaatgtaaatggtactttcgtaaagatgagtacaccaaaaacaaatgcagattactatctagaatttttcatttaagtccgctgccgga
actttaaacgcagggcaaagtattgaagttcaaggaagatttttctaaggtagactggacaaactatacacaaacagatgattattcgtttggtgatag
taactcaagttatgctgattggaataagacaacagtatatatctctgatgttttggtttgggggagtcgaaccataataggagaaaaaaatgtaataatttttt
agaggggtcataacttagtatacatgtctgtatatgaggtccgacacgtgccacacggcatgtgtcgggcctcatttttatacagcgtgtatgtgacc
ttattcatgacaagggatcgtccgcgaatagtgctacttcattcatgaaagaattgtttatgagggatgatgctttcttatattaataaatgctgtgattat
ccaaaaaaaaatttattaagtaatctatgataactatagttgatatttacaattacttacattataattgttaaaaaatggttta(SEQ ID NO: 5)

CACTTTCTATTGCCAGTATGGTAC

Primers Cphy_3367:
Forward (SacII): **CCGCGG**GCAGAAAGGATGAGAGATATGAAAAAG(SEQ ID NO: 6)
Reverse (XmaI): **CCCGGGCTCCTATTATGGTTCGACTCCCCA**(SEQ ID NO: 7)

FIGURE 14

Cphy_3368 GH48 Cellulase

gattagtggatttgtttttcatgaaaatgatgaaatatttttttatatttgcttattcataacaatgtgaagttctaaaaatacttttttgtgtttaaaaaaattaaata
tcttttatacgaaagtttcgcaccattttataaaagagaattaattgataactcatttataaaaattgacatttttttttatgcatgttgaacaaaaaaatacctt
ttacttgtaattgacagaaaatgtaaatagtgataatatacagagtgtacagacaagtttgatatataaatataaaatacagacgaaatatgagaaagg
aagatattgttaaataaatgacgtaattgtcttgaatatatattgaattataaatcaaattattatacataagcctacctaaagtgaaaagagggaaacgt
agaattttgtaaataaacctagtaaaatcgctaatcttatattatttagaaccttcagatcaattgatatataaatttgaaaatttatgcaaagaaaggaag
ggatgagaagtgcgttcgaatgccttatgtcattgacgtggagagttaattaataaggagggttttgtatgcaaaaacgtatctacaagagagtagc
agcagccattatgaccgctgcaatggtagttactctagttcctcagggagcaaagacatccgctttggctggtgaaactgagcaagctttagcggc
ggcagcaacaagggggaacctatgagcaacgttttatggacttatggtcggatattaaaaacccaaagaacggttatttagtcctcagggaattccg
tatcattctattgaaacaatgattgtagaagctcctgattatggtcatgtaactactagtgaggcaatgagttactatatgtggcttgaagctatgtacgg
caagtttacaggtgacttttctggatatgggaccgcttggaatgtagcagaaaaatatatgattccaacggatgcagatcaaccaccaaccagtatg
agtaagtatacaccgagtaaacctgcaacttatgcacctgagtatcaggatcctagtcagtacccagcgaagctcgattcgagtgctcctgttggta
gtgacccaatttggtcacagcttgttgcagcttatggacggaatacaatctatggtatgcactggttactagatgttgataactggtatggatttggttct
aggggagacggaacctcaaaaccatcctatatcaacacattccaacgtggagaacaggaatcaacatgggaaacaattcctcagccatgctggg
atacaatgaaatatggtggaacgaatggtttcctcgacttattcactggcgatagttcctatgcacagcaatttaagtatacggatgcaccagatgct
gatgcaagagcaattcaggctgcttattgggcaagtgaatgggcgaaagattatggtgtaaatgtcgatacttattcatcaaaagctacgatgatgg
gcgattatcttcgttattccatgtttgataaatattttagaaaaataggtaattctacagttgctggtacaggatatgatgcatcacattatctgttatcctg
gtattatgcttgggggcggtggaattacagctgattgggcatgggttattggatctagccataaccactttggttatcaaaatcctatggcagcttgggt
tttatctcaaaattcgaagtttaaaccaaaaactacaaatggacaggctgactgggcaacaagccttactagacagcttgaattctatcagtggttac
aatcttcagaaggtgggattgccggtggtgctagtaactcaaagaatggtcgttatgaaacttggccagctggaacagctacattctatggaatgg
gctatgaagcaaacccagtttataaagatccaggaagtaatacctggtttggtttccaggcatggtcaatgcaacgtgttgcagaatattattataag
acaaatgatgtaaaagcaaaacagatcctagataaatgggtagcttgggttaaatctgtggttgtattaaaagcagatggaacctttacgataccaa
gtacacttgactggagtggtcagccagatacatggacaggctcctatactggaaactctaagcttcatgtgacagtggttgattctggtaccgacctt
ggcgttacaggatctcttgcaaatgctttgttatattatagtaaagctgcaaacgatgtagctgcaaagaacttagcaaaagaattattagatcgcgta
tggaaattatatcgtgatgataagggcgttgctgctcctgaagcacgtgcagattataagagattctttgagcagaccgtttatgttccaagtacctta
atggtaagatgccaaatggtgatgtgattaagtctggtattaaattcttagatatccgttctaagtatttacaagacccatcttatccaaagttactggca
gcatatcagagtaataagtcaccagagtttatatatcatagattctgggctcagtgtgatgtagcgcttgcaaatggtgtatatgcactcctttatgaga
atggttctggcactactgattatgctaatattaatcctacaaatggttcctttgataaagctgtaggaaaacaagcagatcttagtacaacattatcaatg
caaggttatacctttgttaacttaagtaaaggtactacacctctgacttaaatacagactatactgttaatggtactacagtagtccttaagaaagaatt
cctatctacattaccacttggtgatactacaattacttttaatttagcaattcttatacaaaaccttttgtagtaaccgttgtggatacaacagtagttgtg
gtagtaggagatgttaaggttcagatgttcaatggaaatactagtgcaacaacgaatggaattgcacctcgttttttatcttgtaaatacaggctctaata
gtattaatctttctgatgtaaagcttcgttactactatacaattgatggtgagaagagccagagtttctggtgtgattggtcatcgattggaagcagtaat
gttaccggaacttttgtaaagatggcaactccaaagactggagcagattattatcttgagattggatttacaagtggtgctggttctttaaaggcagga
cagggaatcgaagttcaaggtagattctcaaaaactgactggtctaattataccccagactggggattattcatttaatagcagcggtaactcctatgtt
gattggaacaaggcaactgcttatataagtggaaaacttaattgggggtatcgaaccatgaaattttcttttaagtttggcaatagaaagtgttaagtcac
tttctattgccagtatggtacgcagaaaggatgagagat(SEQ ID NO: 8)

Primers Cphy_3368:
Forward (SacII): **CCGCGG**GTTAATTAATAAGGAGGGTTTTGTATGC(SEQ ID NO: 9)
Reverse (XmaI): **CCCGGG**CCAAACTTAAAAGAAAATTTTATG(SEQ ID NO: 10)

FIGURE 15

Cphy_3202 – Cellulase (2628 bp)

ggtacaaa**gacgaaaagagatagcgactat**gttatgtaaaagcatagtctttatctctttttgctcttagcaaaaaaatcgtttataaaaaacgtata
aaataatacaaaacaagtataatatcataggttgaaaattgtaaataaatggtatattatttttatataggacaaacagtcctatcaaataattataagga
ggaatagcaa**atg**aaaagaaaactgaaacaaagatgtgctgttttagtggcagttgcaacgatgatagcttcgttgcaatgggggagagtgccag
tacaagcagtaacagcagacggtcttacctctcaacagtatgttgaggcaatgggcgaaggctggaacttaggaaattccttgatggttttgattct
gatacttcaaaaccagatcaaggcgagaccgcttggggaaatcctaaggttacaaaagagctaatccatgcagtcaaacaaaaaggctatagta
gtatccgcataccaatgaccctatatcgtagatatacggagagcaatggtgtatgcactatcgatagcgcatggatagcacgttacaaagaagtag
tagattatgcagttgcagaaggtttatacgttatgataaacattcaccatgattcctggatatggttatcttcatgggatggaaataagagttctgtgcaa
tatgtaagatttactcagatgtgggatcaacttgcgaaggcatttaaagattatccgttacaagtatgttttgaaacgataaatgagccgaactttcaaa
actctggaaacgttactgcacagaataaattagatatgcttaaccaagcggcttacaatataattcgtgcctctggtggatcaaatgcaaagagaatg
attgttttaccatcactaaatacgaaccatgataatagtgtaccattagctgatttcataactaaattgaatgattctaatatcattgcaaccgttcattatta
tagtgaatgggtatttagtgctaaccttggtaagacaagctttgatgaagatttatggggaaatggtgattacactcctcgtgatgcggtaaataagg
cgtttgataccatttccaatgcatttacagcaaaaaaaatcggtgttgttatcggagaatttggtcttttaggttatgactctgattttgaaaataatcaac
caggcgaagaattaaaatattatgagtatatgaattatgtagctagacaaaagaaaatgtgccttatgttttggggataacggatctggaattaatcgta
acgactctaagtatagttggaaaaaacctatagttggaaagatgttagaagtatctatgacaggacgttcctcttatgcaacaggccttgataccattt
acctaaacggcagctcatttaatgatattaatatcccgcttactctaaacggtaacacctttgttggagttacaggattaaccagtggtaccgattttac
gtataaccaatccaatgcaacactaacattaaaatcatcctacgtgaagaaggtttatgatgcaatgggaagtaattatggtacggtagctgatttggt
acttaagttttcaagtggagctgattggcatgagtatttagtgaaatacaaagcaccagtatttcaaaatgcgaatggaactgtttccaatggaattaat
attccagttcaatttaacggaagtaaactccgtcgttctacagcttatataggttctaatcgagttggcccgaatcaaagctggtggatgtatttagagt
atggtgcaacttttgtggcgaactatacgaacaatattttaaccattaagcctgatttctttaaggatggttctgtttatgatggaaatatatcatttgagat
ggagttttatgatggacaaaagttaaaatataatcttaataaatcaaatggtaacataacaggaactgcagcagcagtaacccctacaccaacacca
acggcgacaccaacaccaacagcgacgccaacaccaaccgtaacaccaaaaccaacaataaccccaacagtaacgccgacaccaacagtaa
cgccaaaaccaacaataacaccgacagtaacaccaactcctactccaatcccaggaacaggtccagttacattaaaatacgaagtaacgaatact
tgggataagcatacacaggcgaatattacattaaccaatacctctaatacagcactaaagaatttgttgtatcatttacttataaagggtatatagacc
aaatgtggagtgcagatttggttagtcaaaattcgggtaccattacagtgaagggaccagcatgggctacgaatctagatccagggcaaagtataa
catttggttttattgcttcacatgatacaccgtctgttgatccaccatcaaatgttactttagttagttcaaat**taa**aattgtattcaaa**taatttttaagga**
tttacattattatttttacataaaacagattaattcaatctctggtatcgttaaaaatgaataggatattcagtatgagttaatgactatgatagaaatatca
aaaaagactaggttatcatttatgctttctatagtgtaggataacctagtttttttctaataat(SEQ ID NO: 11)

Forward
CCGCGGGACGAAAAGAGATAGCGACTAT(SEQ ID NO: 12)

Reverse
CCCGGGATGTAAATCCTTAAAAATTA(SEQ ID NO: 13)

FIGURE 16

Cphy_2058 – Cellulase (1594 bp)

cacaacgagttttttatatctatttttatttccagttcttaaaatagaaagatacttttcagttttct**tattgcataatcccataact**aaatgtaaaattata
ccatagttacgagcaagtagcgttttgttagtgtagctgaaatgcaaataacgagtgttgactaactagagagttataaatggtttagttatgtagaaat
tactatatcttttgaaggg**atg**tgtgttgaattgatgagaaagtttccaattatagtatcaggtattttagtatgtgcaacattattaagtggctgtagcca
aaatatgtcaaatgttgataaatccacagtgaatttgaattcgcagaatacagacaatcaaaatcaagatgcaaataataaagactcggataatcaa
ggcgcaaataatcaagagtcaaataatcaagaatcacaaaaccctgaattaaaaaatacaccaacacctcaaatcatagtactaccaggtgacag
cacaattgaagcaaacgaaattgtagccccaaccatcacatttgaacaaaaagaaataccatcgaatccagcaataacttttgtacataatatgaaa
ataggatggaaccttggaaatacatttgacgcagtgagtgattccaatctaatggatgaacttaattatgaaagctcatggtgtggtgtaaaaacaac
agaagagatgatgaaagcaattaaagatgctgggtttcagtcgattagaataccagtatcgtggcacaatcatgtttctggtgatgattttattataag
cgaagtatggcttaaccgagtacaagaagtggtcgattatgctatcaataatgatatgtatgtgatattaaatactcaccatgatgtaagtaaaaatttt
attatccaagtaatgaaaatttagaatcttctaaaaaatatatcaacgcagtatggacacaagtaagtgaacgattttcttcctatggagaaaagttatt
atttgaagggatgaacgaaccaaggcttgcaggttctaattacgaatggtggttagatttatcaaagcctgagtgtaaagaagcaatcgaatgtatta
atcaattaaatcaggaatttgttgataccgttcgcaaatcgggaggagagaatacttctaggtatcttctgataccagggtatgatgcatcgtctcaat
atgcacttattaatgattataagttaccaaaagataatataaatgatcgtttaattgtatcagtacatgcatatctaccatatgactttgccctaaaaagtc
caaaggaaagtggcagtatatcagaatggaattcaaaaatagctggatgtactaaggagatagattctttttaaatagtttatatatgaagtttataaa
aaatggagttcccgtaattatcggtgaatttggtgccagagataaagaaaataatttagaatctcgggtagagtatgctacttattatataggtgctgc
aaaagcgaatggaatcacatgcttctggtgggataatcatgcatttaaaggggacgggggaaaacttcggtcttttttgatagaaagagttgtactataa
aatatcctgagatattgcaaggtttaatgaaatacgcagaa**taa**tagatttatt**atagtagtagcatttcttac**ttaaatctttcatggctcttttg(SEQ ID NO: 14)

Forward (SacII)
CCGCGGTATTGCATAATCCCATAACT(SEQ ID NO: 15)

Reverse (XmaI)
CCCGGGGTAAGAAATGCTACTACTAT(SEQ ID NO: 16)

FIGURE 17

Cphy_3289 and Cphy_3290 GroEL and GroES, respectively.

ATTGAAGTAAAATTTACTAATATATACTCAACGATTAGCACTCGAATAGAGTGAGTGCT
AATAACTGATATGATACAGTATTAAAGGAGGAAATAAATATGAAATTAGTGCCATTAG
GAGACAAAGTTGTATTAAAGCAACTCGTAGCAGAAGAGACTACAAAGTCGGGTATTGT
ATTACCTGGTCAGGCAAAAGAAAAACCACAACAAGCAGAGGTTGTTGCAGTTGGCCCT
GGTGGAATGGTGGATGGAAAAGAAGTAACCATGCAGGTTAAAGTTGGCGACAAAGTTA
TTTATTCCAAATATGCTGGAACTGAAGTAAAATTAGATGAAGAAGAATTTATCGTTGTA
AAACAAAACGATATCGTAGCAATTGTAGCAGATTAATAATAATAAAGTGTGAATCATA
TTTTTATGATTCATATAAATAAAACGTGAAAATATAATCAGAATTATTATAAATTAAAA
CTTGGAGGAATACAATTATGGCTAAGGATATTAAATATAGTGCAGATGCCAGAGTAGC
GATGGAAGCTGGTGTAAACAAGTTAGCAAATACAGTAAGAGTAACTCTAGGACCAAAG
GGAAGAAATGTAGTTCTTGATAAGTCCTTCGGTGCACCATTAATTACAAATGATGGTGT
TACTATTGCAAAAGAAATTGAGTTAGAAGATTCCTTTGAGAATATGGGCGCTCAGCTTG
TAAAAGAAGTTGCTACAAAGACAAATGATGTAGCAGGTGATGGTACAACAACAGCTAC
TGTTCTTGCTCAAGCTATGATTAACGAAGGTATGAAAAATCTTGCAGCAGGTGCTAATC
CGATCATCTTAAGAAGAGGTATGAAGAAAGCTACTGATTGCGCTGTTGAAGCAATTAGC
AGTATGAGCTCAGCAATTAATGGTAAAGATCAGATTGCTAAGGTTGCTGCAATCTCTGC
TGGCGATGATTCCGTAGGTGAGATGGTTGCGGATGCTATGGATAAAGTTAGCAAAGATG
GTGTTATCACCATTGAAGAGTCTAAGACTATGCAGACTGAGCTTGACTTAGTAGAAGGT
ATGCAATTTGACCGTGGATATGTTTCCGCATATATGGCGACTGATATGGATAAGATGGA
AGCAAATCTAGATAATCCATATATTTTAATCACAGATAAGAAGATCAGCAACATTCAGG
AGATCCTTCCTGTTCTTGAGCAGATTGTTCAAAGTGGATCCAGATTATTAATCATCGCTG
AAGATATCGAAGGCGAAGCTTTAACAACATTAGTAATCAATAAGTTAAGAGGGACATT
CACTGTTGTTGGTGTTAAGGCGCCAGGTTATGGTGATAGAAGAAAGGCTATGTTACAAG
ATATCGCTATTTTAACTGGTGGTACTGTTATCTCTGATGAACTTGGCCTTGACTTAAAAG
AAGCTACATTAGATCAGCTTGGTCGTGCAAAATCCGTTAAAATTCAGAAAGAAAACACT
ATCATTGTTGATGGTGAAGGAAATAAAGCAGAAATCGAAGCTAGAATTTCTCAGATTAA
GGCTCAGATTGCTGAAACAACATCAGAATTTGATAAAGAAAAATTACAGGAGAGACTT
GCTAAACTTGCAGGTGGTGTAGCTGTAATTCGTGTTGGTGCTGCAACAGAGACTGAGAT
GAAAGAGAAGAAGCTTCGTATGGAAGATGCTTTAGCAGCTACAAGAGCAGCTGTGGAA
GAAGGTATTATCGCAGGTGGCGGTTCTGCTTACATCCATGCATCTAAGGAAGTTGCTAA
ACTTGCTGCTAAATTAGAAGGTGATGAGAGAACTGGTGCACAGATTATATTAAAAGCAT
TAGAAGCTCCATTATCATGCATCGCTCAAAACGCTGGTTTAGAAGGCGCTGTTATTGTT
AACAAGGTTAGAGAAAAGAAAACAGGTGTTGGTTTCAATGCCCTAACTGAGAAGTATG
TAGATATGGTAGAAGACGGAATTCTTGATCCTTCTAAGGTTACAAGAAGTGCTCTTCAG
AATGCAACCAGTGTTGCTTCTACATTCTTAACAACAGAAGCTGCAGTTGCATCCATTAA
AGAACCAGCTCCAGCTATGCCAGCAGGCGGCCCTGGCGGAATGGGTATGATGTAATTT
ACTGATATAAAATTAATATATCTTAAGACTTTAGAATCCATGTATAAGTATGAGTATAC
ATGGATTCTTTCAGCGTATAGGAAATATTGTTGTAATTACCTATAAACAAGAAAAAGTA
CGTTTTCGATATGAAGTATATTCGCATTATTCCTTAAAAGATAATAAAGTCCTTTTACTT
TTATAAATAATTTAGTATAATAAAGGTAGCAAGGAATCG(SEQ ID NO: 17)

Forward Primer (SacII):
CCGCGGTTAAAGGAGGAAATAAATATGAAAT(SEQ ID NO: 18)
Reverse Primer (XmaI):
CCCGGGATTAATTTTATATCAGTAAATTACAT(SEQ ID NO: 19)

FIGURE 18

Cphy_1163 cellulase (EC:3.2.1.4)

MSKIRRIVSLFLIVTIFITTCFFNVGQKVYAADTNNDDWLHCVGNKIYDMNGNEVWLTGAN
WFGFNCTENVFHGAWYDIKGMLTNIANRGIGFLRVPISTELLYSWMIGKPNKVSSVTAVNN
PPYYVCNPDFYDPTTNSVKNSMEIFDIIMGYCKQLGIKVMVDVHSPDANNSGHNYPLWYGL
TTTTAGEITTDKWINTQAWLADKYKNDDTILAFDIKNEPHGQRGYSTTTPTNIAKWDNSTD
ENNWKYAAERCAKAILAKNPKLLIMIEGVEQYPKTEKGYNYNTPDVWGATGDQSPWYSA
WWGGNLRGVKDYPINIGTLNSQIVYSPHDYGPSVYNQPWFDKDFTTQTLLDDYWYNTWA
YIKDKGIAPLLIGEWGGFMDGGKNQKWMTLLRDYIVNNRIHHTFWCINPNSGDTGGLLGY
DWQTWDEAKYALLKPALWQSNGKFIGLDHQTPLGVNGISLGQYYGK (SEQ ID NO: 20)

FIGURE 19

Cphy_3367 cellulose 1,4-beta-cellobiosidase (EC:3.2.1.4 3.2.1.91)

MKKIISLLLVITLLISMAPSKADAAETNYNYGEALQKSIMFYEFQRSGKLPSTIRNNWRGDSG
LTDGADVGLDLTGGWYDAGDHVKFNLPLAYTVTMLAWAVYEEEATLSKAGQLSYLLDEI
KWSSDYLIKCHPQANVFYYQVGNGNTDHSWWGPAEVMQMARPSYKVDLNNPGSTVVGE
AAAALAATALIYKTKDPTYSATCLRHAKELFNFADTTKSDAGYTAASGFYTSYSGFYDELS
WAATWIYLASGEATYLDKAESYVAKWGTEPQSSTLSYKWAQNWDDVHYGAALLLARITN
KAIYKNNIEMHLDYWTTGYNGSRITYTPKGLAWLDSWGALRYATTTAFLASVYADWSGCS
AGKVSTYNAFAKQQVDYALGSTGRSFVVGYGVNSPTRPHHRTAHSSWADSQTEPNYHRHT
IYGALVGGPGNNDSYEDNINNYVNNEIACDYNAGFVGALAKVYKTYGGTPIANFKAIETVT
NDELFIQAGINASGPSFIEVKALVFNETGWPARVTDKLSFKYFIDISEYVAKGYTKNDFTVST
NYNNGATTSALLPWDAANNIYYVNVDFSGTKIYPGGQSAYKKEVQFRIAGPQNVNIWDNS
NDYSFTQIANVSSGNTVKTTYIPLYDNGKLVFGNEPKTGVPSASLDKTTANFDKNPAVSADI
PVTINYNGNTLTAVKNGTTVLTKGTDYTVSGNVVTLSKNYFLAQSASTVTLTFVFSGGNDA
TLKVTLVDTSPSASINPNSAVFDKASGKQENIVITLTPNGNTLAGLKNGSKSLVTGTDYTVS
GTTVTILSSYLSQFAVGSQSIVFEMNKGTNPVLAVTIKDSSVVTPTGNIKLQMFNGNSSATTN
GIAPRIKLINTGTTAINLSDVKIRYYYTINGEKDQAFWCDYSTIGSSNVNGTFVKMSTPKTNA
DYYLEFSFKSAAGTLNAGQSIEVQGRFSKVDWTNYTQTDDYSFGDSNSSYADWNKTTVYIS
DVLVWGVEP (SEQ ID NO: 21)

FIGURE 20

Cphy_3368 cellulose 1,4-beta-cellobiosidase (EC:3.2.1.91)

MQKRIYKRVAAAIMTAAMVVTLVPQGAKTSALAGETEQALAAAATRGTYEQRFMDLWSD
IKNPKNGYFSPQGIPYHSIETMIVEAPDYGHVTTSEAMSYYMWLEAMYGKFTGDFSGYGTA
WNVAEKYMIPTDADQPPTSMSKYTPSKPATYAPEYQDPSQYPAKLDSSAPVGSDPIWSQLV
AAYGRNTIYGMHWLLDVDNWYGFGSRGDGTSKPSYINTFQRGEQESTWETIPQPCWDTMK
YGGTNGFLDLFTGDSSYAQQFKYTDAPDADARAIQAAYWASEWAKDYGVNVDTYSSKAT
MMGDYLRYSMFDKYFRKIGNSTVAGTGYDASHYLLSWYYAWGGGITADWAWVIGSSHN
HFGYQNPMAAWVLSQNSKFKPKTTNGQADWATSLTRQLEFYQWLQSSEGGIAGGASNSK
NGRYETWPAGTATFYGMGYEANPVYKDPGSNTWFGFQAWSMQRVAEYYYKTNDVKAK
QILDKWVAWVKSVVVLKADGTFTIPSTLDWSGQPDTWTGSYTGNSKLHVTVVDSGTDLGV
TGSLANALLYYSKAANDVAAKNLAKELLDRVWKLYRDDKGVAAPEARADYKRFFEQTVY
VPSTFNGKMPNGDVIKSGIKFLDIRSKYLQDPSYPKLLAAYQSNKSPEFIYHRFWAQCDVAL
ANGVYALLYENGSGTTDYANINPTNGSFDKAVGKQADLSTTLSMQGYTFVNLSKGTTPLTL
NTDYTVNGTTVVLKKEFLSTLPLGDTTITFNFSNSYTKPFVVTVVDTTVVVVVGDVKVQMF
NGNTSATTNGIAPRFYLVNTGSNSINLSDVKLRYYYTIDGEKSQSFWCDWSSIGSSNVTGTF
VKMATPKTGADYYLEIGFTSGAGSLKAGQGIEVQGRFSKTDWSNYTQTGDYSFNSSGNSY
VDWNKATAYISGKLNWGIEP (SEQ ID NO: 22)

FIGURE 21

Cphy_3202 cellulase (EC:3.2.1.4)

MKRKLKQRCAVLVAVATMIASLQWGRVPVQAVTADGLTSQQYVEAMGEGWNLGNSFDG
FDSDTSKPDQGETAWGNPKVTKELIHAVKQKGYSSIRIPMTLYRRYTESNGVCTIDSAWIAR
YKEVVDYAVAEGLYVMINIHHDSWIWLSSWDGNKSSVQYVRFTQMWDQLAKAFKDYPLQ
VCFETINEPNFQNSGNVTAQNKLDMLNQAAYNIIRASGGSNAKRMIVLPSLNTNHDNSVPL
ADFITKLNDSNIIATVHYYSEWVFSANLGKTSFDEDLWGNGDYTPRDAVNKAFDTISNAFT
AKKIGVVIGEFGLLGYDSDFENNQPGEELKYYEYMNYVARQKKMCLMFWDNGSGINRND
SKYSWKKPIVGKMLEVSMTGRSSYATGLDTIYLNGSSFNDINIPLTLNGNTFVGVTGLTSGT
DFTYNQSNATLTLKSSYVKKVYDAMGSNYGTVADLVLKFSSGADWHEYLVKYKAPVFQN
ANGTVSNGINIPVQFNGSKLRRSTAYIGSNRVGPNQSWWMYLEYGATFVANYTNNILTIKP
DFFKDGSVYDGNISFEMEFYDGQKLKYNLNKSNGNITGTAAAVTPTPTPTATPTPTATPTPT
VTPKPTITPTVTPTPTVTPKPTITPTVTPTPTPIPGTGPVTLKYEVTNTWDKHTQANITLTNTSN
TALKNFVVSFTYKGYIDQMWSADLVSQNSGTITVKGPAWATNLDPGQSITFGFIASHDTPSV
DPPSNVTLVSSN (SEQ ID NO: 23)

FIGURE 22

Cphy_2058 cellulase (EC:3.2.1.4)

MCVELMRKFPIIVSGILVCATLLSGCSQNMSNVDKSTVNLNSQNTDNQNQDANNKDSDNQ
GANNQESNNQESQNPELKNTPTPQIIVLPGDSTIEANEIVAPTITFEQKEIPSNPAITFVHNMKI
GWNLGNTFDAVSDSNLMDELNYESSWCGVKTTEEMMKAIKDAGFQSIRIPVSWHNHVSGD
DFIISEVWLNRVQEVVDYAINNDMYVILNTHHDVSKNFYYPSNENLESSKKYINAVWTQVS
ERFSSYGEKLLFEGMNEPRLAGSNYEWWLDLSKPECKEAIECINQLNQEFVDTVRKSGGEN
TSRYLLIPGYDASSQYALINDYKLPKDNINDRLIVSVHAYLPYDFALKSPKESGSISEWNSKI
AGCTKEIDSFLNSLYMKFIKNGVPVIIGEFGARDKENNLESRVEYATYYIGAAKANGITCFW
WDNHAFKGDGENFGLFDRKSCTIKYPEILQGLMKYAE (SEQ ID NO: 24)

FIGURE 23

Cphy_1100 peptidase M42 family protein

METKEYIIKTLETLVNIPSPSGFTKEVMEFVEREANKYHYSCEYIKRGGLIITVPGESNDCLGL
SAHVDTLGAMVRSIGGDGIIRFTCVGGYTMNSIEGEYCKIHTREGKVYTGTILSKSPSVHSY
DDARTLERKDRNMEIRIDEKVENKEDVLNLGINNGDYISFDARFEYTKSGFIKSRHLDDKAS
VAVLLGLLKDLSEQNVTPKRTLKILISNLEEIGMGASYIPSEISEFIAVDMGAIGDDLAGNEYS
VSICALDSSGPYDYELTTKLMNLAKDKQIPYVVDIFPHYGSDASAAMRGGNNIRAALIGQGI
HASHGMERTHETALIATLDLLKVYVG (SEQ ID NO: 25)

FIGURE 24

Cphy_1510 endo-1,4-beta-xylanase (EC:3.2.1.8)

(A)MLQKMNGKVKKILGISIAFLMLIMVIPTSIAKAATNKTYDFNSMTYQSTWGVTYSISNGS
GTFNFTGQYREIKFNLPETLDMSQCTSVTFNASSPNGQIAFKLYDTSGNQVAVVYNFNSNTS
DCTFAPNSTAKVNSIGIMAQGTNNYSAVVNRVTFTMTGGSSGTGSSTLLNTYGNILKNSGT
AVNLSQLQNSNTLSVIKTQYNSITLENEMKPDAVLGSSSTLMTVAQAKSNGYYIPSSYTEST
VPTLKFSTIDAVLQICYNNGLKLRGHTLVWHSQTPDWFFRTGYSSSGSYVSQAVMDARME
MFIRSYMSHIYNGSYGSVVYAWDVVNEYLHASTSGWSQVYGSNLGTTPSYVKKAFQYAY
DCLSSFGLTNSVKLFYNDYNTYEVTDQILSLVNFINSGTKLCAGVGMQSHLNTSYPSVSAYK
TAMQKFLNAGYEVQVTELDVTNTSASTQATYVYDLMTAILSLKKAGGNITGITWWGLYDS
VSWRASQNPLLFSNLTTPKESYNKALQAFTDAGY (SEQ ID NO: 26)

FIGURE 25

Cphy_2128 mannan endo-1,4-beta-mannosidase (EC:3.2.1.78 3.2.1.91)

MKGLVKKVSTFIIALSLLFSFITVPVSAGTALFVIESENTRLSSELQVVTAIYGQPKPGYSGAG
FVWMQNSGTLTFDVTVPETGMYTISTRYMQELSTDGRLQYLSVNGINKGSFMLPYTTVWS
DFNFGIYKLTKGSNTIQIKAGWGFAYFDTFTVDYAKLDSLNVAPTLTDSQATSETKLLMKY
LTEVYGKHIISGQQEIYGGGNNGNSELEFDWIHNLTGKYPAIRGFDFMNYNPLYGWEDGTT
NRIIDWVNNKNGIATGCWHITVPKDFNSYKLGDFVDWQKTTYKPTETNFKTANAVIPGTKE
YQYVMLTIDDLAKQLLILQQNNVPIILRPYHEAEGNGGANGEGAWFWWASAGAKVYKQL
WEMLYTRLTQTYGLHNIIWTYNSYVYSTSPAWYPGDNLVDIVGYDKYNTVYNRYDGLSG
VPNEDAITTIFYQLVDLTNGKKMVAMTENDTVPSLQNLTEEKSGWLYFCPWYGEYLMSSA
NNYTKTLKELYQSNYVITLDELPNLKVDDQTPSSTITPTSITFDKYTPNQSDKTISIVFNGNTL
NSLKLGTTTLKINVDYTLSGNNLILKKTFLSQLPTGESSIVFDFDKGKDPVLKVNVVDSTPSA
TITPVNATFDKAVDKQQNINVTVNLNGLSLVSVLNNSNKLLLNKDYTVSGNTIVLQKSYLST
LSLGESKLTFQFSAGNNPVLTLNVIDTTAPVITGNLVIQAFNGNTSASTNGISPKFKLINTGNT
DINLSDIKLRYYYTIDGEISQNFWCDWASVGSENVKGEFVKLAKPVSGADYYLEISFTASAG
TLKAGQNAEIQARFAKSNWSNYNQADDYSFNASSSQYVNSDKVTGYLSNKLVWGIEP
(SEQ ID NO: 27)

FIGURE 26

Cphy_3289 chaperonin GroEL; K04077 chaperonin GroEL

MAKDIKYSADARVAMEAGVNKLANTVRVTLGPKGRNVVLDKSFGAPLITNDGVTIAKEIEL
EDSFENMGAQLVKEVATKTNDVAGDGTTTATVLAQAMINEGMKNLAAGANPIILRRGMK
KATDCAVEAISSMSSAINGKDQIAKVAAISAGDDSVGEMVADAMDKVSKDGVITIEESKTM
QTELDLVEGMQFDRGYVSAYMATDMDKMEANLDNPYILITDKKISNIQEILPVLEQIVQSGS
RLLIIAEDIEGEALTTLVINKLRGTFTVVGVKAPGYGDRRKAMLQDIAILTGGTVISDELGLD
LKEATLDQLGRAKSVKIQKENTIIVDGEGNKAEIEARISQIKAQIAETTSEFDKEKLQERLAK
LAGGVAVIRVGAATETEMKEKKLRMEDALAATRAAVEEGIIAGGGSAYIHASKEVAKLAA
KLEGDERTGAQIILKALEAPLSCIAQNAGLEGAVIVNKVREKKTGVGFNALTEKYVDMVED
GILDPSKVTRSALQNATSVASTFLTTEAAVASIKEPAPAMPAGGPGGMGMM (SEQ ID NO:
28)

FIGURE 27

Cphy_3290 chaperonin Cpn10; K04078 chaperonin GroES

MKLVPLGDKVVLKQLVAEETTKSGIVLPGQAKEKPQQAEVVAVGPGGMVDGKEVTMQVK VGDKVIYSKYAGTEVKLDEEEFIVVKQNDIVAIVAD (SEQ ID NO: 29)

FIGURE 28

Cphy_3510

agacagcataagtcacatccagacaaatgtcctataggatgttagtaggggtttggagaattgcccgtaaggcaggttatttggctagatataatca
atccagttacaggatagtaggattgcaacccagtcgttttgaccagtttgtacaagaattttaatttgtcgaaatattgtggcaaatcaaatgaagttctt
tgatgaaatgtttagaaacatgacttagaatggggtacaaaaagtgaatttgtaagcaaaaagacttgacctttcctacgatagttgttataatcatctt
gttattggaacgattatatttacttatgcacattttagagtttttcgaattgttaatacatcattaacaatttaattatactcgttatgtgacgtaagtcaatata
atacaaaaccatatattttaa

(SEQ ID NO: 32)

FIGURE 29

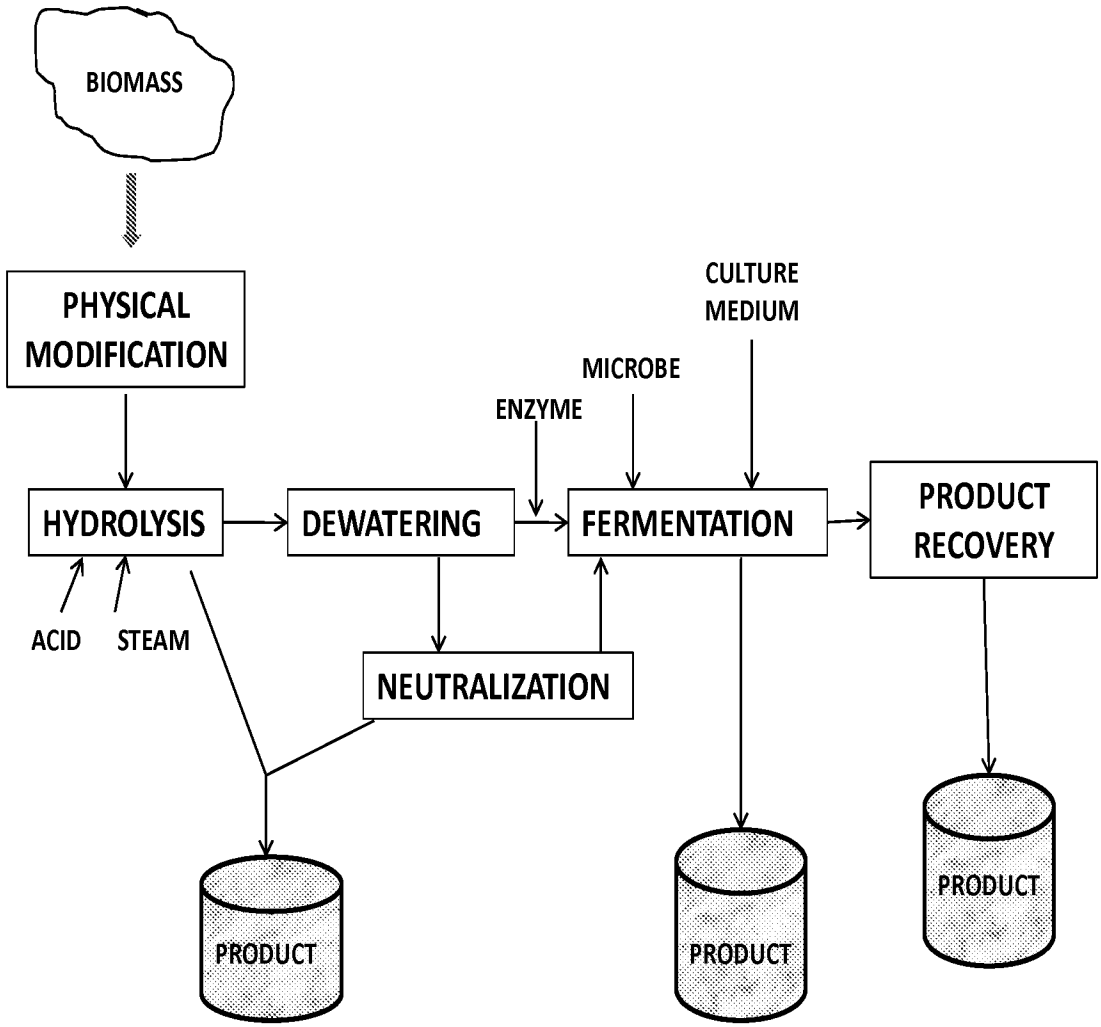

BIOMASS
PHYSICAL MODIFICATION
CULTURE MEDIUM
MICROBE
ENZYME
HYDROLYSIS
DEWATERING
FERMENTATION
PRODUCT RECOVERY
ACID
STEAM
NEUTRALIZATION
PRODUCT
PRODUCT
PRODUCT

FIGURE 30

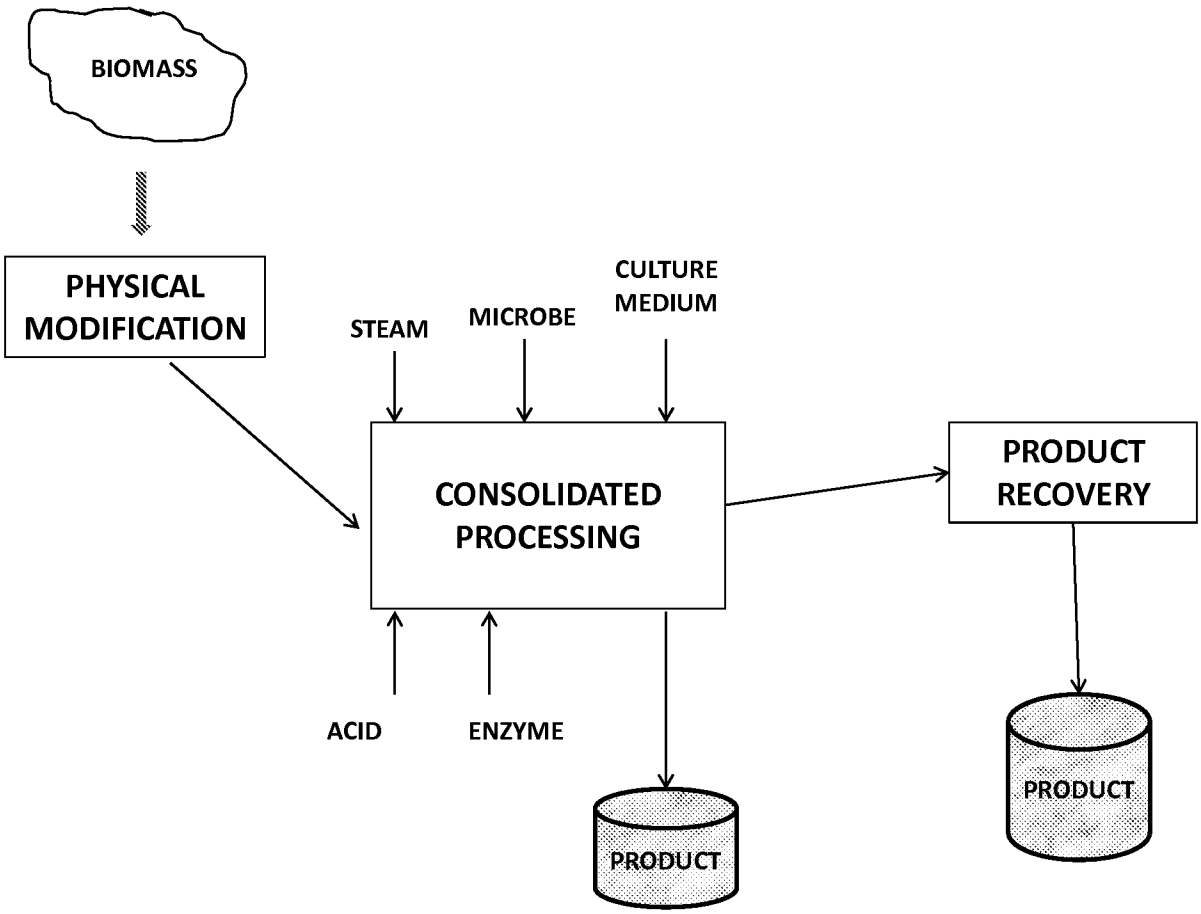
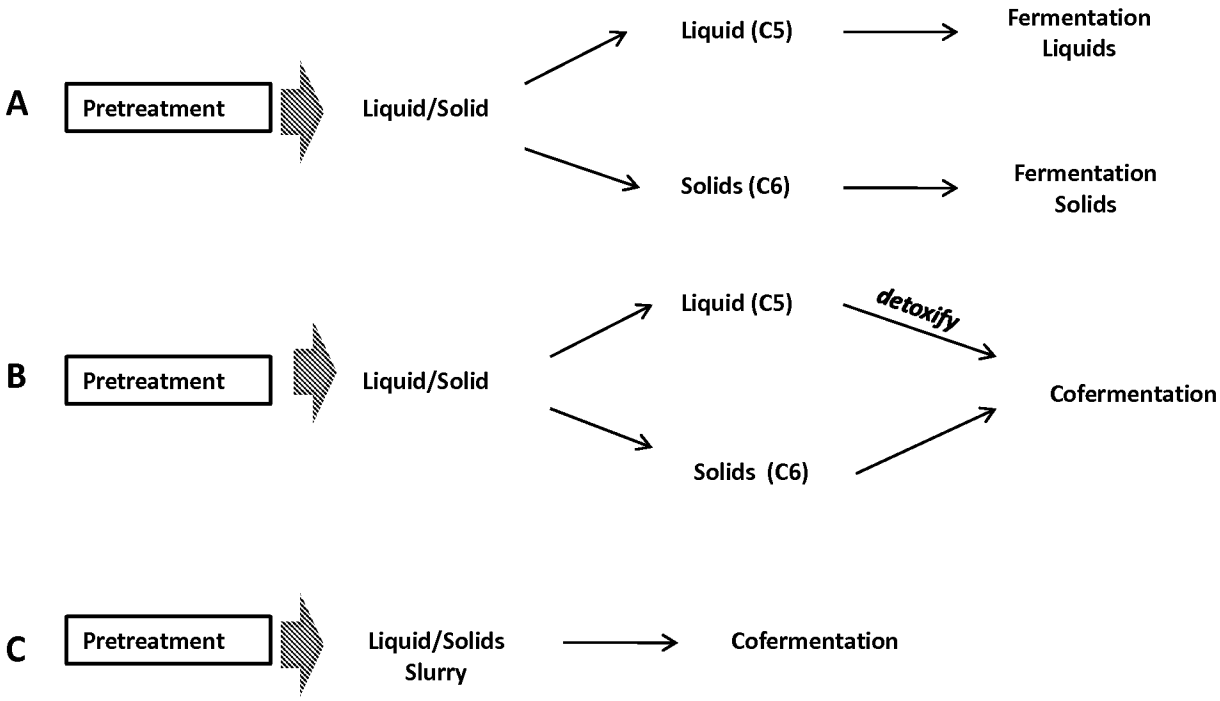

BIOMASS
PHYSICAL MODIFICATION
STEAM
MICROBE
CULTURE MEDIUM
CONSOLIDATED PROCESSING
PRODUCT RECOVERY
ACID
ENZYME
PRODUCT
PRODUCT

FIGURE 31


A
Pretreatment
Liquid/Solid
Liquid (C5)
Fermentation Liquids
Solids (C6)
Fermentation Solids
B
Pretreatment
Liquid/Solid
Liquid (C5)
detoxify
Cofermentation
Solids (C6)
C
Pretreatment
Liquid/Solids Slurry
Cofermentation