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FIG. 1A

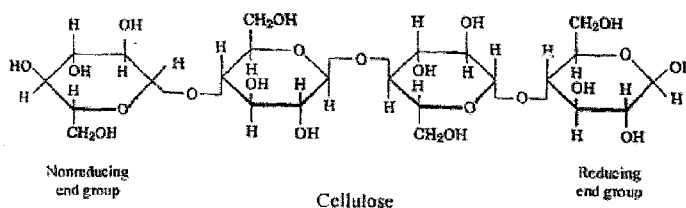
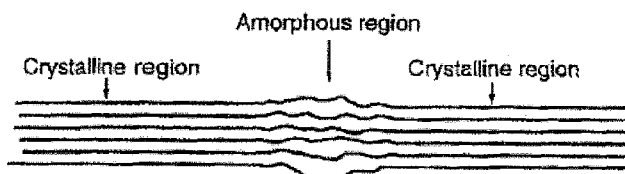


FIG. 1B



Panel B from Beguin and Aubert, 1994.

(57) Abstract: The present invention provides a novel process for ethanol fermentation.



PROCESSES FOR PLANT POLYSACCHARIDE CONVERSION

RELATED APPLICATIONS

- [0001] This application claims benefit of priority from U.S. Provisional Application 61/156,158, filed on February 27, 2009, incorporated herein by reference in its entirety..

FIELD OF THE INVENTION

- [0002] The present invention is in the field of biofuel production with involves the enzymatic degradation of plant cell wall polysaccharides, which is then used to for ethanol or oil production.

BACKGROUND

- [0003] Cellulases and related enzymes have been utilized in food, beer, wine, animal feeds, textile production and laundering, pulp and paper industry, and agricultural industries. Various such uses are described in the paper "Cellulases and related enzymes in biotechnology" by M. K. Bhat (Biotechnical Advances 18 (2000) 355-383), the subject matter of which is hereby incorporated by reference in its entirety.
- [0004] The cell walls of plants are composed of a heterogenous mixture of complex polysaccharides that interact through covalent and noncovalent means. Complex polysaccharides of higher plant cell walls include, for example, cellulose (β -1,4 glucan) which generally makes up 35-50% of carbon found in cell wall components. Cellulose polymers self associate through hydrogen bonding, van der Waals interactions and hydrophobic interactions to form semi-crystalline cellulose microfibrils. These microfibrils also include noncrystalline regions, generally known as amorphous cellulose. The cellulose microfibrils are embedded in a matrix formed of hemicelluloses (including, *e.g.*, xylans, arabinans, and mannans), pectins (*e.g.*, galacturonans and galactans), and various other β -1,3 and β -1,4

glucans. These matrix polymers are often substituted with, for example, arabinose, galactose and/or xylose residues to yield highly complex arabinoxylans, arabinogalactans, galactomannans, and xyloglucans. The hemicellulose matrix is, in turn, surrounded by polyphenolic lignin.

[0005] The complexity of the matrix makes it difficult to degrade by microorganisms as lignin and hemicellulose components must be degraded before enzymes can act on the core cellulose microfibrils. Ordinarily, a consortium of different microorganisms is required to degrade cell wall polymers to release the constituent monosaccharides. For saccharification of plant cell walls, the lignin must be permeabilized and hemicellulose removed to allow cellulose-degrading enzymes to act on their substrate. For industrial saccharification of cell walls, large amounts of primarily fungal cellulases are added to processed feedstock that has been treated with dilute sulfuric acid at high temperature and pressure to permeabilize the lignin and partially saccharify the hemicellulose constituents.

[0006] The oldest methods studied to convert lignocellulosic materials to saccharides are based on acid hydrolysis (see, *e.g.*, review by Grethlein, Chemical Breakdown Of Cellulosic Materials, J. Appl. Chem. Biotechnol. 28:296-308 (1978)). This process can involve the use of concentrated or dilute acids. For example, U.S. Pat. Nos. 5,221,537 and 5,536,325, incorporated by reference herein in their entireties, describe a two-step process for the acid hydrolysis of lignocellulosic material to glucose. These processes have numerous disadvantages including, for example, recovery of the acid, the specialized materials of construction required, the need to minimize water in the system, and the high production of degradation products which can inhibit the fermentation to ethanol.

[0007] To overcome the problems of the acid hydrolysis process, cellulose conversion processes are being developed using enzymatic hydrolysis. See, for example, U.S. Pat. No. 5,916,780, incorporated by reference herein in its entirety, which discloses enzymatic hydrolysis with a pre-treatment step to break down the

integrity of the fiber structure and make the cellulose more accessible to attack by cellulase enzymes in the treatment phase.

[0008] U.S. Pat. No. 6,333,181, incorporated by reference herein in its entirety, discloses production of ethanol from lignocellulosic material by treatment of a mixture of lignocellulose, cellulose, and an ethanologenic microorganism with ultrasound.

[0009] Various attempts have been made to reduce the costs of industrial fermentation, particularly in utilization of less expensive enzyme systems and substrates. However, despite the development of numerous approaches, there remains a need in the art for processes that economically and efficiently degrade plant materials, such as lignocellulosic materials, into fermentable sugars that may then be feed to organisms for production of biofuels, such as ethanol and/or oils.

[0010] The description herein of disadvantages and problems associated with known compositions, and methods is in no way intended to limit the scope of the embodiments described in this document to their exclusion. Indeed, certain embodiments may include one or more known compositions, compounds, or methods without suffering from the so-noted disadvantages or problems.

[0011] All headings and sub-headings are used herein for convenience only and should not be construed as limiting the invention in any way.

SUMMARY OF THE INVENTION

[0012] The present invention provides for methods related to a consolidated bio-processing approach for the manufacture of ethanol and other valuable products formed from sugars. The methods of the present invention significantly reduce the need for costly and potentially toxic thermo-chemical pretreatment and substantially reduces the need for cellulase loading from external sources. The methods of the present invention may be deployed with substantially lower upfront capital investment and lower operating expenses per unit of production.

- [0013] According to some preferred embodiments, the invention provides for methods of producing ethanol from a plant material comprising: providing a liquid pulp suspension comprising lignocellulose materials; adding to the pulp a culture of a saccharifying microorganism; incubating the mixture of pulp and saccharifying microorganism for a period of time sufficient to induce lignocellulase enzyme production; collecting the solids from the liquid pulp suspension; transferring the solids to a hypotonic solution containing a fermenting organism; fermenting the solution, thereby producing a biofuel. Biofuels include ethanol, butanol, isobutanol, acetic acid, acetone and oil. In a preferred aspect of this embodiment, the biofuel is ethanol.
- [0014] According to some preferred embodiments, the liquid pulp suspension is at least 1% saline.
- [0015] According to some preferred embodiments, the hypotonic solution is less than 0.9% saline.
- [0016] According to some preferred embodiments, the saccharifying microorganism is *Saccharophagus degradans*.
- [0017] According to some preferred embodiments, the *Saccharophagus degradans* is grown until it reaches an OD₆₀₀ of at least 10 prior to the adding step.
- [0018] According to some preferred embodiments, the collecting step comprises a dewatering step.
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- [0019] According to some preferred embodiments, the fermenting organism is yeast.
- [0020] A further aspect of the invention is directed to a method for the degradation of substances comprising cellulose.
- [0021] Further aspects of the invention are directed to utilization of cellulose by degrading substances and/or waste in, for example, food industry, animal feeds, textile production and laundering, pulp and paper industry,

and agricultural industries.

- [0022] Some embodiments of this invention are directed to a method for producing ethanol from lignocellulosic material, comprising combining lignocellulosic material with a saccharifying microorganism expressing one or more compounds listed in FIGS. 4-11, preferably cellulase cel5A listed in FIG. 4 (*e.g.*, 2 or more; 3 or more; 4 or more; 5 or more; 6 or more; 7 or more; 8 or more; 9 or more; 10 or more; 12 or more; 15 or more; 20 or more; 30 or more; *etc.* including up to all of the compounds listed in FIGS. 4-11), to obtain saccharides and converting the saccharides to produce ethanol. The initial incubation phase may be conducted in an aquatic or marine environment, such as under water.
- [0023] Conversion of sugars to ethanol and recovery may be accomplished by, but are not limited to, any of the well-established methods known to those of skill in the art. For example, through the use of an ethanologenic microorganism, such as yeast, *Zymomonas*, *Erwinia*, *Klebsiella*, *Xanthomonas*, and *Escherichia*, preferably *Escherichia coli* K011 and *Klebsiella oxytoca* P2.
- [0024] Some embodiments of this invention are directed to ethanol produced by combining lignocellulosic material with a saccharifying microorganism expressing of one or more compounds listed in FIGS. 4-11, and subsequently lysing the saccharifying microorganism to release the saccharifying enzymes to converting the polysaccharides to fermentable sugars to produce ethanol. Conversion of sugars to ethanol and recovery may be accomplished by, but are not limited to, any of the well-established methods known to those of skill in the art. For example, through the use of an ethanologenic microorganism, such as yeast, *Zymomonas*, *Erwinia*, *Klebsiella*, *Xanthomonas*, and *Escherichia*, preferably *Escherichia coli* K011 and *Klebsiella oxytoca* P2.
- [0025] Further embodiments of this invention are directed to a method for producing ethanol from lignocellulosic material, comprising contacting lignocellulosic material with a microorganism expressing an effective saccharifying amount of

one or more compounds listed in FIGS. 4-11, preferably cellulase cel5A listed in FIG. 4, to obtain saccharides and converting the saccharides to produce ethanol. The contact may be conducted in an aquatic or marine environment, such as under water. The microorganism may be *S. degradans* or a recombinant microorganism containing a chimeric gene comprising at least one polynucleotide encoding a polypeptide comprising an amino acid sequence of at least one of the compounds listed in FIGS. 4-11; wherein the gene is operably linked to regulatory sequences that allow expression of the amino acid sequence by the microorganism. The recombinant microorganism, may be a bacteria or yeast, such as *Escherichia coli*. In some aspects of the present invention, the recombinant microorganism is an ethanologenic microorganism, such as microorganisms from the species *Zymomonas*, *Erwinia*, *Klebsiella*, *Xanthomonas*, or *Escherichia*, preferably *Escherichia coli* K011 or *Klebsiella oxytoca* P2.

- [0026] Further aspects of the present invention are directed to ethanol produced by contacting lignocellulosic material with a microorganism expressing an effective saccharifying amount of one or more compounds listed in FIGS. 4-11 to obtain saccharides and converting the saccharides to produce ethanol.
- [0027] A further aspect of the invention is directed to a method for producing ethanol from lignocellulosic material, comprising contacting lignocellulosic material with an ethanologenic microorganism expressing an effective saccharifying amount of one or more compounds listed in FIGS. 4-11 to produce ethanol. The ethanologenic microorganism expresses an effective amount of one or more compounds listed in FIGS. 4-11 to saccharify the lignocellulosic material and an effective amount of one or more enzymes or enzyme systems which, in turn, catalyze (individually or in concert) the conversion of the saccharides (*e.g.*, sugars such as xylose and/or glucose) to ethanol. The one or more enzymes or enzyme systems of the ethanologenic organism may be expressed naturally or by, but not limited to, any of the methods known to those of skill in the art. For example, release of the one or more enzymes or enzyme systems may be obtained through

the use of ultrasound. In some aspects of the present invention, the ethanologenic microorganism is transformed in order to be able to express one or more of the compounds listed in FIGS. 4-11. In some aspects of the present invention, the ethanologenic microorganism is from the species yeast, *Zymomonas*, *Erwinia*, *Klebsiella*, *Xanthomonas*, or *Escherichia*, preferably *Escherichia coli* K011 or *Klebsiella oxytoca* P2.

[0028] Other aspects, features, and advantages of the invention will become apparent from the following detailed description, which when taken in conjunction with the accompanying figures, which are part of this disclosure, and which illustrate by way of example the principles of this invention.

BRIEF DESCRIPTION OF FIGURES

- [0029] FIG. 1A shows the chemical formula of cellulose.
- [0030] FIG. 1B illustrates the physical structure of cellulose.
- [0031] FIG. 2A illustrates the degradation of cellulose fibrils.
- [0032] FIG. 2B shows the chemical representation of cellulose degradation to cellobiose and glucose.
- [0033] FIG. 3 shows SDS-PAGE and Zymogram analysis of 2-40 culture supernatants.
- [0034] FIG. 4 lists the predicted cellulases of *S. degradans* 2-40.
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- [0035] FIG. 5 lists the predicted xylanases, xylosidases and related accessories of *S. degradans* 2-40.
- [0036] FIG. 6 lists the predicted pectinases and related accessories of *S. degradans* 2-40.
- [0037] FIG. 7 lists the arabinanases and arabinogalactanases of *S. degradans* 2-40.
- [0038] FIG. 8 lists the mannanases of *S. degradans* 2-40.

- [0039] FIG. 9 lists the laminarinases of *S. degradans* 2-40.
- [0040] FIG. 10 lists selected carbohydrate-binding module proteins of *S. degradans* 2-40.
- [0041] FIG. 11 lists the recombinant proteins of *S. degradans* 2-40 and a comparison of predicted vs. observed molecular weights thereof.
- [0042] FIG 12 provides an illustration of a consolidated bio-processing approach for the manufacture of ethanol and other valuable products formed from the sugars released from the degradation of non-fermentable plant polysaccharides or starches according preferred embodiments of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

- [0043] The methods of the present invention may be used to process plant material to many useful organic products, chemicals, and fuels. In addition to ethanol, some commodity and specialty chemicals that can be produced from plant material include xylose, acetone, acetate, glycine, lysine, organic acids (*e.g.*, lactic acid), 1,3-propanediol, butanediol, glycerol, ethylene glycol, furfural, polyhydroxyalkanoates, *cis*, *cis*-muconic acid, and animal feed. Potential coproduction benefits extend beyond the synthesis of multiple organic products from fermentable carbohydrate.
- [0044] Conventional methods used to process the plant material in accordance with the methods of the present invention are well understood to those skilled in the art. The methods of the present invention may be implemented using any conventional biomass processing apparatus configured to operate in accordance with the invention.
- [0045] According to preferred embodiments, the invention provides a method of producing sugars such as monosaccharides and/or disaccharides (*e.g.*, glucose, fructose, and/or sucrose) from biodegradable materials containing plant cell wall polysaccharides and/or non-edible plant parts (*e.g.*, plant biomasses or recently

dead material from photosynthetic plants). According to some embodiments, the method of producing sugar from biodegradable materials comprising the following steps: providing a liquid pulp suspension comprising biodegradable materials; adding to the pulp suspension a culture containing one or more marine γ -proteobacterium having complete saccharifying enzyme systems for degrading plant cell wall polysaccharides; incubating the mixture of pulp and bacteria for a period of time sufficient to induce saccharifying enzyme production; collecting the solids from the liquid pulp suspension; transferring the solids to a hypotonic solution.

[0046] According to preferred embodiments, the invention also provides a method of producing ethanol from biodegradable materials containing plant cell wall polysaccharides and/or non-edible plant parts (*e.g.*, plant biomasses or recently dead material from photosynthetic plants). According to some embodiments, the method of producing ethanol from biodegradable materials comprising the following steps: providing a liquid pulp suspension comprising biodegradable materials; adding to the pulp suspension a culture containing one or more marine γ -proteobacterium having complete enzyme systems; incubating the mixture of pulp and bacteria for a period of time sufficient to induce saccharifying enzyme production; collecting the solids from the liquid pulp suspension; and transferring the solids to a hypotonic solution. In this method, the one or more marine γ -proteobacterium having complete saccharifying enzyme systems is used to produce enzymes that degrade one or more plant materials, and the simpler sugars that result from the degradation process can then be converted to ethanol. These embodiments of the invention are also used to make sugar by omitting steps to convert sugars to ethanol.

[0047] The ethanol is produced by any way known in the art. In one embodiment, the ethanol is produced from the degradation product of the bacterium by a fermentation organism (*e.g.*, yeast).

- [0048] The methods of the invention provide for the degradation of biodegradable materials into sugars and/or ethanol. Accordingly to some embodiments, the biodegradable materials are plant materials or a plant biomass. According to preferred embodiments, the biodegradable materials are lignocellulose biomass materials.
- [0049] Accordingly to some embodiments, the source of the polysaccharides or carbohydrates for the production of sugars and/or chemicals including ethanol can be any plant biomass containing cell wall polysaccharides. There are four main categories of plant biomass, which are: (1) wood residues (including sawmill and paper mill discards), (2) municipal paper waste, (3) agricultural residues (including corn stover and sugarcane bagasse), and (4) dedicated energy crops (which are mostly composed of fast growing tall, woody grasses).
- [0050] Any suitable plant cell wall biomass may be used in a fermentation process of the present invention. The plant cell wall biomass is generally selected based on the desired fermentation product(s) and the process employed, as is well known in the art. Examples of substrates suitable for use in the methods of the present invention, include plant cell wall polysaccharide containing materials, such as wood or plant residues, or low molecular sugars obtained from processed plant cell wall polysaccharides that can be metabolized by the fermenting microorganism, and which may be supplied by direct addition to the fermentation media.
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- [0051] In preferred embodiments, the plant or biodegradable material can be any material containing lignocellulose. The majority of carbohydrates in plants are in the form of lignocellulose, which is composed of mainly cellulose, hemicellulose, pectin, and lignin. Lignocellulose is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. Hydrolysis of these polymers releases a mixture of neutral sugars including glucose, xylose, mannose, galactose, and arabinose. The lignocellulosic material can also be, but

is not limited to, herbaceous material, agricultural residues, forestry residues, municipal solid wastes, waste paper, and pulp and paper mill residues.

[0052] According to preferred embodiments, preferred sources of plant biomass or lignocellulose material is switchgrass, alfalfa, corn stover, corn fiber, rice straw, sugar cane, sugar beet, sweet sorghum, starch (*e.g.*, corn/maize), paper material, wood pulp, pulp processing waste, woody or herbaceous plants, fruit pulp, vegetable pulp, pumice, grains, distillers grains, herbaceous material, agricultural residues, forestry residues, municipal solid waste, waste paper, pulp and paper mill residues, and/or mixtures and combinations thereof. Exemplary plant materials also include, but are not limited to, those derived from wood, such as wood pulp, as well as non-woody fibers from cotton, from straws and grasses, such as rice and esparto, from canes and reeds, such as bagasse, from bamboos, from stalks with bast fibers, such as jute, flax, kenaf, cannabis, linen and ramie, and from leaf fibers, such as abaca and sisal. It is also possible to use mixtures of one or more plant materials.

[0053] Preferably, the biodegradable materials or plant biomass of the present invention contain at least 30% of material that contain lignocellulose. Preferably, the biodegradable materials or plant biomass of the present invention contain at least 40% of material that contain lignocellulose. Preferably, the biodegradable materials or plant biomass of the present invention contain at least 50% of material that contain lignocellulose. According to some preferred embodiments, the biodegradable materials or plant biomass of the present invention contain at least 60% of material that contain lignocellulose. According to some preferred embodiments, the biodegradable materials or plant biomass of the present invention contain at least 70% of material that contain lignocellulose. According to some preferred embodiments, the biodegradable materials or plant biomass of the present invention contain at least 75% of material that contain lignocellulose. According to some preferred embodiments, the biodegradable materials or plant biomass of the present invention contain at least 80% of material that contain

lignocellulose. According to some preferred embodiments, the biodegradable materials or plant biomass of the present invention contain at least 85% of material that contain lignocellulose. According to some preferred embodiments, the biodegradable materials or plant biomass of the present invention contain at least 90% of material that contain lignocellulose. According to some preferred embodiments, the biodegradable materials or plant biomass of the present invention contain at least 95% of material that contain lignocellulose.

[0054] Preferred ranges include, but are not limited to, the following: from about 50% to 100% lignocellulose material; from about 60% to 100% lignocellulose material; from about 70% to 100% lignocellulose material; from about 75% to 100% lignocellulose material; from about 50% to 100% lignocellulose material; from about 80% to 100% lignocellulose material; from about 85% to 100% lignocellulose material; from about 90% to 100% lignocellulose material; from about 95% to 100% lignocellulose material; from about 50% to 95% lignocellulose material; from about 60% to 95% lignocellulose material; from about 70% to 95% lignocellulose material; from about 75% to 95% lignocellulose material; from about 80% to 95% lignocellulose material; from about 70% to 95% lignocellulose material; from about 75% to 95% lignocellulose material; from about 80% to 95% lignocellulose material; from about 85% to 95% lignocellulose material; from about 50% to 90% lignocellulose material; from about 60% to 90% lignocellulose material; from about 70% to 90% lignocellulose material; from about 75% to 90% lignocellulose material; from about 80% to 90% lignocellulose material; from about 50% to 80% lignocellulose material; from about 50% to 80% lignocellulose material; from about 60% to 80% lignocellulose material; and from about 70% to 80% lignocellulose material.

Pulp

[0055] According to some embodiments, the biodegradable materials may be used as-is or may be converted to a pulp. Preferably, the biodegradable materials are

converted to a pulp, which is then brought into contact with the saccharifying microorganisms. The advantage of creating a pulp is that it creates a larger surface area that is exposed to the digestive processes.

[0056] Pulping is the process of converting the biodegradable materials, plant biomass, or lignocellulosic materials to separated pulp fibers or fibrous mass. The pulp of the biodegradable materials or plant biomass may be prepared using any method known in the art. For example, the material being processed may be ground using mechanical techniques to create a pulp, such as grinding the material into fibers by disk refiners or grindstones. Chemical processes may also be used. The dominant chemical process, the Kraft process, uses a solution of sodium hydroxide and sodium sulfide.

[0057] Fractionation of the material into its component polymer families (cellulose, hemicellulose, pectin, lignin) is not required to perform the methods of the present invention. However, according to some embodiments, fractionation treatments (e.g., steam explosion) may be preferred in order to speed the degradation process.

[0058] Preferably, the degradation of the plant material occurs without chemical pretreatments of the plant material. Thus, according to some embodiments, the pulp is produced in the absence of a pretreatment process.

[0059] According to some embodiments, the pulp material is subjected to a pretreatment process to facilitate mechanical or chemical degradation of the pulp materials prior to the addition of the saccharifying microorganisms (e.g., *S. degradans*).

Thus, the plant biomass or lignocellulosic materials may be used as is or may be subjected to a pretreatment using conventional methods known in the art. For example, physical pretreatment techniques can include various types of milling, irradiation, steaming/steam explosion, and hydrothermolysis. Chemical pretreatment techniques can include dilute acid, alkaline, organic solvent, ammonia, sulfur dioxide, carbon dioxide, and pH-controlled hydrothermolysis. Biological pretreatment techniques can involve applying lignin-solubilizing

microorganisms.

[0060] According to preferred embodiments, the pulp materials are maintained in a pulp suspension that is suitable for hosting the saccharifying marine microorganisms, without lysing or otherwise damaging or killing the saccharifying microorganisms.

According to some embodiments, the pulp or plant biomass is maintained (for example, in a holding vessel or tank) as a pulp suspension or slurry in aqueous liquid that is at least 1% salt. Preferably, the pulp or plant biomass is maintained as a pulp suspension or slurry in aqueous liquid that is at least 1% salt to at least 10% salt (e.g., at least 1.5%, 2%, 2.5%, 3%, 5%, 6%, 7%, 8%, or 10% salt).

According to some embodiments, the pulp or plant biomass is maintained as a pulp suspension or slurry in aqueous liquid that is at least 1% salt to at least 5% salt (e.g., at least 1.5%, 2%, 2.5%, 3%, 5% salt). According to some embodiments, the pulp or plant biomass is maintained as a pulp suspension or slurry in aqueous liquid that is at least 1% salt to at least 3% salt (e.g., at least 1.5%, 2%, 2.5%, 3% salt). In alternative embodiments, the salt is sea salt, sodium chloride or mixtures of sodium chloride and other common salts, for example, potassium chloride.

[0061] Sterilization: According to preferred embodiments, the pulp is subjected to one or more sterilization techniques prior to bringing the pulp into contact with the saccharifying microorganisms into contact with the pulp. The sterilization techniques may include any heat or chemical sterilization process known in the art.

Saccharifying Microorganisms

[0062] According to some embodiments, the saccharifying microorganisms of the present invention are a marine γ -proteobacterium having complete saccharifying enzyme systems for degrading plant cell wall polysaccharides. According to preferred embodiments, the marine γ -proteobacterium having complete saccharifying

enzyme systems is *Saccharophagus degradans*. The preferred strain of *Saccharophagus degradans* is the *S. degradans* strain 2-40 having the American Type Culture Collection accession number 43961. *S. degradans* is further described in: WO 2008/136997; WO 2008/033330; U.S. Patent Publication No. 2005/0136426; U.S. Patent Publication No. 2007/0292929; U.S. Patent No. 7,384,772; and U.S. Patent No. 7,365,180; the disclosures of which are incorporated herein by reference in their entireties.

[0063] The term “complete saccharifying enzyme systems” refers to the ability of the marine γ -proteobacterium generally refers the ability of the saccharifying microorganism to convert polysaccharides into monosaccharides or other fermentable sugars. Thus, a saccharifying microorganism can be said to have a complete saccharifying enzyme system where the microorganism is capable of saccharifying at least 70% (*e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99%) of the carbohydrate material contained in the pulp, plant biomass, or biodegradable material. For example, the microorganism is capable of saccharifying at least 70 - 95% of the lignocellulose material.

[0064] A saccharifying microorganism can be said to have a complete saccharifying enzyme system where the degradation of a plant substrate (*e.g.*, corn leaves) by a saccharifying microorganism that results in residual lignocellulose material or convertible starch of less than 30%, less than 20%, less than 15%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 2% and less than 1%.

[0065] It is understood that marine γ -proteobacterium may be modified to achieve a complete saccharifying enzyme system.

[0066] The marine γ -proteobacterium for use in the present invention is preferably able to grow or tolerate an environment having at least 1% sea salt. According to some embodiments, the marine γ -proteobacterium for use in the present invention is

able to grow or tolerate an environment having at least 2% sea salt. According to some embodiments, the marine γ -proteobacterium for use in the present invention is able to grow or tolerate an environment having at least 3% sea salt. According to some embodiments, the marine γ -proteobacterium for use in the present invention is able to grow or tolerate an environment having at least 5% sea salt. According to some embodiments, the marine γ -proteobacterium for use in the present invention is able to grow or tolerate an environment having at least 8% sea salt. According to some embodiments, the marine γ -proteobacterium for use in the present invention is able to grow or tolerate an environment having at least 10% sea salt.

[0067] *Saccharophagus degradans* (formerly *Microbulbifer degradans*) is a bacterium that is representative of an emerging group of marine bacteria that degrade complex polysaccharides. *S. degradans* is a marine γ -proteobacterium that was isolated from decaying *Spartina alterniflora*, a salt marsh cord grass in the Chesapeake Bay watershed. Consistent with its isolation from decaying plant matter, *S. degradans* is able to degrade many complex polysaccharides, including cellulose, pectin, xylan, and chitin, which are common components of the cell walls of higher plants. *S. degradans* is also able to depolymerize algal cell wall components, such as agar, agarose, and laminarin, as well as protein, starch, pullulan, and alginic acid. In addition to degrading this plethora of polymers, *S. degradans* can utilize each of the polysaccharides as the sole carbon source. Therefore, *S. degradans* is not only an excellent model of microbial degradation of insoluble complex polysaccharides (ICPs) but can also be used as a paradigm for complete metabolism of these ICPs. ICPs are polymerized saccharides that are used for form and structure in animals and plants. ICPs are insoluble in water and therefore are difficult to break down.

[0068] *S. degradans* is a highly pleomorphic, Gram-negative bacterium that is aerobic, generally rod-shaped, and motile by means of a single polar flagellum. Previous work has determined that *S. degradans* can degrade at least 10 different

carbohydrate polymers (CP), including agar, chitin, alginic acid, carboxymethylcellulose (CMC), β -glucan, laminarin, pectin, pullulan, starch and xylan. In addition, it has been shown to synthesize a true tyrosinase. 16S rDNA analysis shows that *S. degradans* is a member of the gamma-subclass of the phylum Proteobacteria, related to *Microbulbifer hydrolyticus* and to *Teridinobacter sp.*, cellulolytic nitrogen-fixing bacteria that are symbiotes of shipworms.

[0069] *Saccharophagus degradans* is a marine bacterium capable of degrading all of the polymers found in the higher plant cell wall using secreted and surface-associated enzymes. This bacterium has the unusual ability to saccharify whole plant material without chemical pretreatments. For example, this bacterium is able to utilize as sole carbon sources glucose, Avicel, oat spelt xylan, newsprint, whole and pulverized corn leaves, and pulverized *Panicum vigatum* leaves, indicating the production of synergistically-acting hemicellulases, pectinases, cellulases, and possibly ligninases. Analysis of the genome sequence predicts this bacterium produces at least 12 endoglucanases, 1 cellobiohydrolase, 2 cellodextrinases, 3 cellobiases, 7 xylanases, 10 "arabinases", 5 mannases, and 14 pectinases.

[0070] *Saccharophagus degradans* is a marine bacterium with complete cellulase and xylanase systems, as well as a number of other systems containing plant-wall active carbohydrases. That is, *S. degradans* has a complete complement of enzymes, suitably positioned, to degrade plant cell walls. Thus, *S. degradans* can play a significant role in the marine carbon cycle, functioning as a "super-degrader" that mediates the breakdown of carbohydrates from various algal, plantal, and invertebrate sources.

Enzyme Systems

[0071] The predominant polysaccharide in the primary cell wall of biomass is cellulose. The second most abundant is hemi-cellulose. The third is pectin. The secondary cell wall, produced after the cell has stopped growing, also contains other

polysaccharides and is strengthened by polymeric lignin covalently cross-linked to hemicellulose. Cellulose is a homopolymer of anhydrocellobiose and thus a linear beta-(1-4)-D-glucan, while hemicelluloses include a variety of compounds, such as xylans, xyloglucans, arabinoxylans, and mannans in complex branched structures with a spectrum of substituents. Although generally polymorphous, cellulose is found in plant tissue primarily as an insoluble crystalline matrix of parallel glucan chains. Hemicelluloses usually hydrogen bond to cellulose, as well as to other hemicelluloses, which help stabilize the cell wall matrix.

[0072] Cellulose is a polymer of the simple sugar glucose covalently bonded by beta-1,4-linkages. Many microorganisms produce enzymes that hydrolyze beta-linked glucans. These enzymes include endoglucanases, cellobiohydrolases, glucohydrolases and beta-glucosidases.

[0073] Hemicellulose exists as short branched chains of sugar monomers. Sugars that make up hemicellulose include various hexoses (glucose, mannose and galactose), pentoses (D-xylose and L-arabinose), and other minor sugars. Hemicellulose forms a series of crosslinks with cellulose and pectin to form a rigid cell wall. Unlike cellulose, hemicellulose is mostly amorphous, relatively weak and susceptible to hydrolyzation.

[0074] Similar to cellulose degradation, hemicellulose hydrolysis requires coordinated action of many enzymes, which can be placed into three general categories, the endo-acting enzymes that attack internal bonds within the polysaccharide chain, the exo-acting enzymes that act processively from either the reducing or nonreducing end of the polysaccharide chain, and the accessory enzymes (acetylerases and esterases that hydrolyze lignin glycoside bonds).

[0075] *S. degradans* is effective in degrading to simple sugars plant material rich in cellulose and hemicellulose. Enzyme systems for degrading these two types of carbohydrates are described in greater detail below.

[0076] *S. degradans* expresses many enzymes for the degradation of cellulose to simple sugars. For example, in the presence of corn leaves, the cellulolytic enzymes shown in Table 1, below were increased.

[0077] **TABLE 1. Predicted cellulases and accessory enzymes of *S. degradans* strain 2-40 and evidence supporting their identification.**

Name	Predicted function	Module(s)	MM(kDa)
Cel5A	Endo-1,4- β -glucanase (EC 3.2.1.4)	GH5/CBM6/CBM6/CBM6/GH5	127.2
Cel5B	Endo-1,4- β -glucanase	LPB/PSL(47)/CBM6/GH5	60.8
Cel5C	Endo-1,4- β -glucanase	LPB/PSL(47)/GH5	49.1
Cel5D	Endo-1,4- β -glucanase	CBM2/PSL(58)/CBM10/PSL(36)/GH5	65.9
Cel5E	Endo-1,4- β -glucanase	CBM6/CBM6/GH5	72.6
Cel5F	Endo-1,4- β -glucanase	GH5	42.0
Cel5G	Endo-1,4- β -glucanase	GH5/PSL(21)/CBM6/PSL(32)/Y95	67.9
Cel5H	Endo-1,4- β -glucanase	GH5/PSL(32)/CBM6/EPR(16)	66.9
Cel5I	Endo-1,4- β -glucanase	CBM2/PSL(33)/CBM10/PSL(58)/GH5	77.2
Cel5J	Endo-1,4- β -glucanase	GH5/CBM6/CBM6	65.2
Cel6A	Cellobiohydrolase (EC 3.2.1.91)	CBM2/PSL(43)/CBM2/PSL(85)/GH6	81.9
Cel9A	Endo-1,4- β -glucanase	GH9	62.7
Cel9B	Endo-1,4- β -glucanase	GH9/PSL(54)/CBM10/PSL(50)/CBM2	89.5
Ced3A	Cellodextrinase (EC 3.2.1.74)	LPB/GH3/PLP	116.0
Ced3B	Cellodextrinase	LPB/GH3	92.9
Bgl1A	Cellobiase (EC 3.2.1.21)	GH1	52.8
Bgl1B	Cellobiase	GH1	49.8
Bgl3C	Cellobiase	LPB/GH3/UNK(511)	95.4
Cep94A	Cellobiose phosphorylase (EC 2.4.1.20)	GH94	91.7
Cep94B	Cellodextrin phosphorylase (EC 2.4.1.49)	GH94	88.7

[0078] Moreover, the following enzymes were induced as shown below in Table 2 in *S. degradans* when exposed to Avicel, microcrystalline cellulose in a chemically pure form, for 10 hours.

[0079] **TABLE 2 Fold increase in enzymes after 10 hours growth of *S. degradans* strain 2-40 on Avicel.**

Basal Expression	Fold Increase after 10 h Growth on Avicel		
	Low (<5)	Medium (5-25)	High (>25)
Low (<1% GK)		<i>cel5C</i> <i>cel5D</i> <i>ced3A</i>	<i>cel5E</i> <i>cel5F</i> <i>cel5H</i> <i>cel6A</i> <i>cel9B</i>
Medium (2-10%)	<i>cel5A</i> <i>cel5G</i>	<i>cel5B</i> <i>cel5J</i> <i>cel9A</i> <i>ced3B</i>	<i>cel5I</i> <i>bgl3C</i> <i>cep94A</i>
High (>10%)	<i>bgl1A</i> <i>bgl1B</i>	<i>cep94B</i>	
MAX EXPRESSION: CON 2H 4-10H 24H			

[0080] The data shows that *cel5A*, *cel5G*, *cel9A*, *cel5B*, *ced3B*, *bgl1A* and *cep94B* are constitutively expressed in *S. degradans* 2-40. After 2 hours of growth on Avicel, *cel9A* expression increases. This is followed by an increase in *cel5F* expression at 4 hours, and increases in *cel5H* and *cel5I* expression at 10 hours. *Cel5I* continues to be overexpressed even at 24 hours of culture on Avicel.

[0081] *S. degradans* expresses many enzymes for the degradation of hemicellulose to simple sugars. *S. degradans* produces many hemicellulases that are used to break down hemicellulose to simpler sugars. As shown in Figure 6, expression of *xyn10A*, *xyn10B*, *xyn10D*, *xyn11A* and *xyn11B* is induced in *S. degradans* 2-40 grown on xylan, containing hemicellulose. Moreover, as shown in Figure 7, expression of *xyn10A*, *xyn10B*, *xyn10D*, *xyn11A* and *xyn11B* was shown after 10 hours of culture of *S. degradans* 2-40 on xylan. However, at 2 hours, the greatest increases in expression were for *xyn11A* and *xyn11B*, while the greatest increases in expression at 4 hours of culture of *S. degradans* 2-40 on xylan was *xyn10A*.

[0082] Thus, Xyn10a, Xyn10b, Xyn10d, Xyn11a And Xyn11b are all important for

hemicellulose break down to simpler sugars. However, particular emphasis should be placed on the importance of Xyn10a, Xyn10b, Xyn11a And Xyn11b.

- [0083] The bacterium used according to the methods of the invention may be *Saccharophagus degradans* or it may be a modified bacterium that expresses enzymes upregulated in *Saccharophagus degradans* in response to the presence of the given plant material. In specific embodiments of the invention the aqueous mixture of bacteria and one or more plant materials comprises at least 1% salt and/or at most 10% salt. These embodiments of the invention are also used to make sugar by omitting steps to convert sugars to ethanol.
- [0084] According to some embodiments, the marine γ -proteobacterium is modified to express enzymes that are efficient to degrade the plant polysaccharides in the given plant material. Thus, a marine γ -proteobacterium may be modified to optimally express the enzymes that most efficiently degrade a given plant material. The modified bacterium may be able to degrade the given plant material at a faster rate than a non-modified bacterium. For example, enzymes that are increased in expression by *S. degradans* in the presence of corn leaves are likely necessary for the digestion of corn leaves to sugar. Thus, the enzymes that were increased over 20 fold, *i.e.* *cel5F*, *cel5H* are effective in degradation of corn leaves.
- [0085] It has also been shown that *Cel5I* and *Cel5H* are particularly important for the degradation of cellulose. *Cel5I* is induced over 500 fold and *cel5H* over 100 fold when *S. degradans* 2-40 is exposed to cellulose (Figure 4). Moreover, *cel5H* is expressed over 500 fold when *S. degradans* 2-40 is exposed to cellodextrins, such as cellobiose, cellotraose and cellodextrin (Figure 5). Thus, either of these proteins could be used to efficiently break down cellulose to simple sugars.
- [0086] The agarase, chitinase and alginase systems have been generally characterized. Zymogram activity gels indicate that all three systems are comprised of multiple depolymerases and multiple lines of evidence suggest that at least some of these

depolymerases are attached to the cell surface. Activity assays reveal that the majority of 2-40 enzyme activity resides with the cell fraction during logarithmic growth on carbohydrate polymer (CP), while in later growth phases the bulk of the activity is found in the supernatant and cell-bound activity decreases dramatically. Growth on CP is also accompanied by dramatic alterations in cell morphology. Glucose-grown cultures of 2-40 are relatively uniform in cell size and shape, with generally smooth and featureless cell surfaces. However, when grown on agarose, alginate, or chitin, 2-40 cells exhibit novel surface structures and features.

- [0087] These exo- and extra-cellular structures (ES) include small protuberances, larger bleb-like structures that appear to be released from the cell, fine fimbriae or pili, and a network of fibril-like appendages which may be tubules of some kind. Immunoelectron microscopy has shown that agarases, alginases and/or chitinases are localized in at least some types of 2-40 ES. The surface topology and pattern of immunolocalization of 2-40 enzymes to surface protuberances are very similar to what is seen with cellulolytic members of the genus *Clostridium*.
- [0088] Further, degradation enzymes with higher expression in *S. degradans* when exposed to a particular plant material, may be constitutively and/or over-expressed in an engineered bacterium, thus making a bacterium that is effective in the degradation of the particular plant material. In certain embodiments of the invention, mixtures of proteins that are shown to be induced in *S. degradans* in the presence of corn leaves in Table 1, are introduced into a bacteria so that they are constitutively expressed. In other embodiments, these proteins are also introduced so they are expressed at a high rate. These engineered bacteria are then used to degrade plant material, in this example, corn leaves. In one embodiment the bacterium to be engineered is *S. degradans*. In another embodiment, the bacterium to be engineered is *E. coli*.
- [0089] The cell walls of higher plants are comprised of a variety of carbohydrate polymer (CP) components. These CP interact through covalent and non-covalent means, providing the structural integrity plants require to form rigid cell walls and resist

turgor pressure. The major CP found in plants is cellulose, which forms the structural backbone of the cell wall. See Fig. 1A. During cellulose biosynthesis, chains of poly-1,3- 1,4-D-glucose self associate through hydrogen bonding and hydrophobic interactions to form cellulose microfibrils which further self-associate to form larger fibrils. Cellulose microfibrils are somewhat irregular and contain regions of varying crystallinity. The degree of crystallinity of cellulose fibrils depends on how tightly ordered the hydrogen bonding is between its component cellulose chains. Areas with less-ordered bonding, and therefore more accessible glucose chains, are referred to as amorphous regions. The relative crystallinity and fibril diameter are characteristic of the biological source of the cellulose. The irregularity of cellulose fibrils results in a great variety of altered bond angles and steric effects which hinder enzymatic access and subsequent degradation.

[0090] The general model for cellulose depolymerization to glucose involves a minimum of three distinct enzymatic activities. Endoglucanases cleave cellulose chains internally to generate shorter chains and increase the number of accessible ends, which are acted upon by exoglucanases. These exoglucanases are specific for either reducing ends or non-reducing ends and frequently liberate cellobiose, the dimer of cellulose (cellobiohydrolases). The accumulating cellobiose is cleaved to glucose by cellobiases ((3-1,4-glucosidases). In many systems an additional type of enzyme is present: cellodextrinases are 6-1,4- glucosidases which cleave glucose monomers from cellulose oligomers, but not from cellobiose. Because of the variable crystallinity and structural complexity of cellulose, and the enzymatic activities required for its degradation, organisms with "complete" cellulase systems synthesize a variety of endo and/or exo-acting 6-1,4-glucanases.

[0091] For example, *Cellulomonas fimi* and *Thermomonospora fusca* have each been shown to synthesize six cellulases while *Clostridium thermocellum* has as many as 15 or more. Presumably, the variations in the shape of the substrate-binding pockets and/or active sites of these numerous cellulases facilitate complete cellulose degradation. Organisms with complete cellulase systems are believed to be capable of efficiently using plant biomass as a carbon and energy source while mediating cellulose degradation. The ecological and evolutionary role of

incomplete cellulose systems is less clear, although it is believed that many of these function as members of consortia (such as ruminal communities) which may collectively achieve total or near-total cellulose hydrolysis. Thus, a saccharifying microorganism can be said to have a “total” or “near-total” cellulose hydrolysis enzyme system where the microorganism is capable of saccharifying at least 70% (*e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99%) of the cellulose material contained in the pulp, plant biomass, or biodegradable material.

- [0092] In the plant cell wall, microfibrils of cellulose are embedded in a matrix of hemicelluloses (including xylans, arabinans and mannans), pectins (galacturonans and galactans), and various beta-1,3 and beta-1,4 glucans. These matrix polymers are often substituted with arabinose, galactose and/or xylose residues, yielding arabinoxylans, galactomannans and xyloglucans—to name a few. The complexity and sheer number of different glycosyl bonds presented by these non-cellulosic CP requires specific enzyme systems which often rival cellulase systems in enzyme count and complexity. Because of its heterogeneity, plant cell wall degradation often requires consortia of microorganisms.

Growth of the Bacterium

- [0093] According to preferred embodiment, the saccharifying microorganisms are added to the pulp or other suspension of biodegradable materials during the logarithmic growth phase of the saccharifying organism(s). The saccharifying microorganisms are generally grown to a high density and transferred during logarithmic growth phase to a vessel containing the pulp materials. A saccharifying organism goes through different stages of growth including a lag phase, logarithmic phase, a stationary phase and a death phase. The length of the lag phase may vary depending on nutrition, growth conditions, temperature, and inoculation density. Also the lag phase may depend on whether or not the saccharifying organisms were acclimatized or directly added to a growth medium. Generally the lag phase

is 6 to 9 hours.

[0094] According to some embodiment, the saccharifying microorganism (*e.g.*, *S. degradans*) is grown until it reaches an OD₆₀₀ from about 5 to about 30. In other specific embodiments, the saccharifying microorganism is grown until it reaches an OD₆₀₀ from about 10 to about 25 (*e.g.*, 12, 15, 17, 18, 20, 21, 22, or 24). In other specific embodiments, the the saccharifying microorganism is grown until it reaches an OD₆₀₀ greater than 10. In other specific embodiments, the saccharifying microorganism is grown until it reaches an OD₆₀₀ greater than 15. In other specific embodiments, the saccharifying microorganism is grown until it reaches an OD₆₀₀ greater than 20. In other specific embodiments, the saccharifying microorganism is grown until it reaches an OD₆₀₀ greater than 25.

[0095] Growth of the saccharifying microorganisms may be achieved by any method known in the art. For example, continuous and fed-batch cultivation may be used. Any growth medium may be used that is known to art for the cultivation of marine bacterium. According to one embodiment, a growth medium containing glucose, yeast extract, ammonia, and Instant Ocean™ at pH 7.0-7.5 is used. According to one embodiment, a growth medium containing glucose, yeast extract, ammonia, and Tryptone™ at pH 7.0-7.5 is used. According to some preferred embodiments, a standard batch culture should produce an optical density at 600 nm (OD) of at least 10 in less than 24 h. According to some preferred embodiments, a standard batch culture should produce an optical density at 600 nm (OD) of between 10 to 25 (*e.g.*, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24) in less than 12 to 24 h (preferably less than 12, less than 18, or less than 24 hours).

Induction of Enzyme Production

[0096] According to some embodiments, the pulp materials are combined with the saccharifying microorganisms in a holding vessel, a combined holding/ saccharification (H/S) vessel, or combined holding/ saccharification/ fermentation

(H/S/F) vessel. The pulp or plant biomass become infused or impregnated with the saccharifying microorganisms at this stage. The saccharifying microorganisms may be added to the vessel using any method known in the art. This includes adding the saccharifying microorganisms directly from the growth medium. Alternatively, the saccharifying microorganisms may be isolated by centrifugation, resuspended, and added to the vessel.

[0097] According to some embodiments, the saccharifying microorganisms are added at a ratio of 1 gram per 1 kilogram of pulp or plant biomass. According to some embodiments, the saccharifying microorganisms are added at a ratio of 0.5 gram per 1 kilogram of pulp or plant biomass. According to some embodiments, the saccharifying microorganisms are added at a ratio of 2 gram per 1 kilogram of pulp or plant biomass. According to some embodiments, the ratio of the mass of saccharifying microorganisms to plant solids may be: 1:100,000; 1:50,000; 1:25,000; 1:10,000; 1:5,000; 1:1,000; 1:500; or 1:100.

[0098] According to some embodiments, this infusion or impregnation stage comprises incubating the saccharifying microorganisms in the presence of a given plant material for a period sufficient to induce lignocellulase enzyme or saccharifying enzyme production. Induction of carbohydrate digesting enzymes in *S. degradans* in the presence of the corresponding carbohydrate is described in U.S. Patent Publication No. 2009/0117619, filed on April 30, 2008 and incorporated by reference herein in its entirety. According to some embodiments, the methods of the present invention comprise incubating *Saccharophagus degradans* in the presence of a given plant material for a period sufficient to induce saccharifying enzyme production. The pattern of lignocellulase enzyme or saccharifying enzyme induction is dependent on the composition of the plant cell wall polysaccharides, starches, or lignocellulose material contained pulp or plant biomass. Induction of specific enzymes can be assessed by qRT-PCR or any other method known in the art to obtain such information. Nomenclature for specific enzymes is explained in further detail in U.S. Application No. 11/121,154, filed

on May 4, 2005 and published as U.S. Publication No. 2006/0105914, which is incorporated by reference herein in its entirety.

- [0099] According to some embodiments, the period of time sufficient to induce saccharifying enzyme production is from about 1 hour to about 24 hours; preferably, from about 6 hour to about 24 hours; more preferably, from about 12 hour to about 24 hours. According to some embodiments, the period of time sufficient to induce saccharifying enzyme production is at least 6 hours. According to some embodiments, the period of time sufficient to induce saccharifying enzyme production is at least 8 hours. According to some embodiments, the period of time sufficient to induce saccharifying enzyme production is at least 10 hours. According to some embodiments, the period of time sufficient to induce saccharifying enzyme production is at least 12 hours. According to some embodiments, the period of time sufficient to induce saccharifying enzyme production is at least 18 hours. According to some embodiments, the period of time sufficient to induce saccharifying enzyme production is at least 24 hours.
- [0100] The expression of the saccharifying enzyme, and also its enzymatic activity, may be measured to ensure proper incubation time. Enzymatic activity may be measured using any method known in the art. Expression of enzymes may be measured using any known method in the art. The enzymes that undergo increased expression may be dependent on the given plant material present in the biodegradable materials.
- [0101] The expression of the saccharifying enzymes is preferably induced from about 2 to about 20 fold over the basal expression levels (*e.g.*, the level of expression in standard growth media absent a plant substrate). According to some embodiments, the saccharifying enzymes are induced from about 5 to about 50 fold over the basal expression levels. According to some embodiments, the saccharifying enzymes are induced from about 5 to about 20 fold over the basal

expression levels. According to some embodiments, the saccharifying enzymes are induced from about 10 to about 20 fold over the basal expression levels. According to some embodiments, the saccharifying enzymes are induced at least 50 fold over the basal expression levels. According to some embodiments, the saccharifying enzymes are induced at least 20 fold over the basal expression levels. According to some embodiments, the saccharifying enzymes are induced at least 10 fold over the basal expression levels. According to some embodiments, the saccharifying enzymes are induced at least 5 fold over the basal expression levels. According to some embodiments, the saccharifying enzymes are induced at least 2 fold over the basal expression levels.

- [0102] According to preferred embodiments, the induction of one or more (*e.g.*, 2, 3, 4, *etc.*) of the following cellulolytic enzymes is achieved according to the guidelines above: cel5A, cel5G, cel9A, cel5B, ced3B, bgl1A, cep94B, and/or their equivalents.
- [0103] According to preferred embodiments, the induction of one or more (*e.g.*, 2, 3, 4, *etc.*) of the following hemicellulolytic enzymes is achieved according to the guidelines above: Xyn10a, Xyn10b, Xyn10d, Xyn11a, Xyn11b, and/or their equivalents. According to preferred embodiments, the induction of one or more (*e.g.*, 2, 3, 4, *etc.*) of the following hemicellulolytic enzymes is achieved according to the guidelines above: Xyn10a, Xyn10b, Xyn11a, Xyn11b, and/or their equivalents.
- [0104] *S. degradans* synthesizes complete multi-enzyme systems that degrade the major structural polymers of plant cell walls.

Saccharification and Fermentation

- [0105] According to preferred embodiments, the pulp or plant biomass impregnated with the saccharifying microorganisms according to the guidelines above is then prepared for saccharification and fermentation. Preferably, the saline broth holding the pulp or slurry is drained, thereby leaving the pulp solids for

saccharification and fermentation. According to some embodiments, the pulp solids may be transferred to a saccharification vessel or a combined saccharification / fermentation (S/F) vessel. According to some embodiments, the pulp solids may be transferred to a saccharification vessel or combined saccharification / fermentation (S/F) vessel.

[0106] According to preferred embodiments, the pulp solids are brought into contact with a hypotonic solution. The term “hypotonic” as used in the present invention refers a tonicity that is relative to the saccharifying microorganism. That is, when the saccharifying microorganism is brought into contact with a hypotonic solution, the net movement of water is into the cell resulting in the lysis of the cell and release of the saccharifying enzymes.

[0107] According to preferred embodiments, the hypotonic solution has a salt concentration of less than 1.0%, preferably less than 0.9% salt, and more preferably less than 0.5% salt. Suitable ranges include the following: from about 0.0% salt to less than about 0.9% salt; from about 0.1% salt to less than about 0.9% salt; from about 0.3% salt to less than about 0.9% salt; from about 0.5% salt to less than about 0.9% salt; from about 0.0% salt to less than about 0.8% salt; from about 0.1% salt to less than about 0.8% salt; from about 0.3% salt to less than about 0.8% salt; from about 0.5% salt to less than about 0.8% salt; from about 0.0% salt to less than about 0.7% salt; from about 0.1% salt to less than about 0.7% salt; from about 0.3% salt to less than about 0.7% salt; from about 0.5% salt to less than about 0.7% salt; from about 0.0% salt to less than about 0.6% salt; from about 0.1% salt to less than about 0.6% salt; from about 0.3% salt to less than about 0.6% salt; from about 0.0% salt to less than about 0.5% salt; and from about 0.1% salt to less than about 0.5% salt. The hypotonic solution may be water or other aqueous liquid (*e.g.*, thin-stillage).

[0108] According to preferred embodiments, the hypotonic solution is the fermentation broth or fermentation medium, which preferably contains the fermentation

organism.

- [0109] The hypotonic solution may be combined with the pulp solids using any method known in the art, such as an in-line feed means. According to some embodiments, the addition of the aqueous liquid by an in-line feed means may be accomplished at any point during the transfer of the pulp solids to the S/F vessel.
- [0110] Fermentation. The fermentation process may be carried out using any method known in the art. Fermentation may, therefore, be understood as comprising shake flask cultivation, small- or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the fermentation of fermentable sugars into ethanol.
- [0111] In the fermentation step, sugars, released from the plant cell wall polysaccharides are fermented to one or more organic substances, *e.g.*, ethanol, by a fermentation organism, such as yeast, or fermenting organisms. According to preferred embodiments, the fermentation is carried out simultaneously with the enzymatic hydrolysis in the same vessels, again under controlled pH, temperature and mixing conditions. When saccharification and fermentation are performed simultaneously in the same vessel, the process is generally termed simultaneous saccharification and fermentation. The most widely used process in the art is the simultaneous saccharification and fermentation (SSF) process where there is no holding stage for the saccharification, meaning that the adding of the fermenting microorganism and lysis of the saccharifying microorganism occur in the same vessel.
- [0112] During the fermentation stage, the combining of the impregnated pulp and fermenting organisms may be accomplished in a number of ways that one skilled in the art would readily be able to determine. A fermenting organism goes through different stages of growth including a lag phase, logarithmic phase, a stationary phase and a death phase. The length of the lag phase may vary depending on nutrition, growth conditions, temperature, and inoculation density.

Also the lag phase may depend on whether or not the fermenting organism, such as yeast were acclimatized or directly added to a fermenter. Generally the lag phase is 6 to 9 hours. If a fermenting organism such as yeast can be kept in an active growth state, production of end products such as alcohol and particularly ethanol could be increased and fermentation time potentially decreased.

- [0113] Therefore, in some embodiments the initial fermentation is conducted for a period of time that corresponds to the lag phase of the fermenting organism. In other embodiments, the initial fermentation step is conducted for a period of time between 2 to 40 hours, also between 2 to 30 hours, also between 2 to 25 hours, also between 5 and 20 and between 2 and 15 hours. In some embodiments, the initial fermentation time is greater than 2, 3, 4, 5, 6, 7, 8, 9, 10 or 15 hours but less than 36 hours.
- [0114] In some embodiments, the initial fermentation is conducted at a temperature of at least about 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, and 75 °C and also at a temperature of less than 70 °C, less than 65 °C. and less than 60 °C. In other embodiments, the temperature will be between about 5-65 °C, about 10-65 °C, about 20-65 °C, about 20-60 °C, about 20-55 °C, about 25-50 °C, about 25-45 °C, about 30-45 °C, about 30- 40 °C and about 35-45 °C.
- [0115] In some embodiments, the initial fermentation is conducted at a pH of between pH 3.0 and 7.0, between pH 3.0 and 6.5, between pH 3.0 and 6.0, between pH 3.0 and 5.0, between pH 3.5 and 5.5, between pH 3.5 to 5.0, or between pH 3.5 and 4.5. The exact temperature and pH used in accordance with any of the fermentation steps of the instant process depends upon the specific fermentable substrate and further may depend upon the particular plant variety, enzymes that are being used and the fermenting organism.
- [0116] In some embodiments the total fermentation time of the fermentation process will be for about 24 to 168 hours, 24 to 144 hours, 24 to 108 hours; 24 to 96 hours, 36

to 96 hours, 36 to 72 hours and 48 to 72 hours. In a preferred aspect, the fermentation proceeds for 24-96 hours, such as typically 35-60 hours. In another preferred aspect, the temperature is generally between 26-40 °C, in particular about 32 °C, and the pH is generally from pH 3 to 6, preferably from about pH 4 to about 5. The fermenting organism are preferably applied in amounts of 10^5 to 10^{12} , preferably from 10^7 to 10^{10} , especially 5×10^7 viable cells count per ml of fermentation broth. During the ethanol producing phase the cell count (*e.g.*, yeast cell count) should preferably be in the range from 10^7 to 10^{10} , especially around 2×10^8 .

[0117] Recovery. Following the fermentation, the organic substance of interest is recovered from the mash by any method known in the art. Such methods include, but are not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, distillation, or extraction. For example, in an ethanol fermentation, the alcohol is separated from the fermented plant cell wall polysaccharides and purified by conventional methods of distillation. Ethanol with a purity of up to about 96 vol. % ethanol can be obtained, which can be used as, *e.g.*, fuel ethanol; drinking ethanol, *i.e.*, potable neutral spirits; or industrial ethanol. According to preferred methods of ethanol production, following the fermentation the mash is distilled to extract the ethanol.

[0118] The yield of glucose (percent of the total solubilized solids) from a fermentable substrate may be at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% and 98%. However, in a preferred embodiment, the glucose is continually produced and substantially all of the glucose is used in the process to produce an end-product, such as ethanol. In further embodiments, the final mash will include less than 1.0%, less than 0.8%, less than 0.5%, less than 0.2%, less than 0.15%, less than 0.1%, and less than 0.05% monosaccharides (w/v).

- [0119] While the preferred end-product is an alcohol and particularly ethanol, other end-products may be obtained and these include without limitation, glycerol, ASA intermediates, 1,3 -propanediol, butanol, isobutanol, acetic acid, oil, enzymes, antimicrobials, organic acids, amino acids and antibiotics.
- [0120] In some embodiments, the yield of ethanol will be greater than 8%, 10%, 12%, 14%, 16%, 18% and 20% by volume. In other embodiments, at least 50%, 60%, 70%, 80% of the final ethanol yield is produced in the first 20, 22, 24, 26, 28 or 30 hours. In certain embodiments, the yield of ethanol will be greater than 16% and at least 50% of the final ethanol will be produced in the first 20 hours. The ethanol obtained according to the fermentation process may be used as a fuel ethanol, potable ethanol or industrial ethanol.
- [0121] The mash at the end of the fermentation may include from 0 to 30% residual starch. In some embodiments, the mash may include at least 1%, 2%, 4%, 6%, 8%, 10%, 12% but less than 30%, less than 20% and less than 15% residual starch.
- [0122] In some embodiments, the fermentation process will have a higher carbon conversion efficiency when compared with other fermentation processes under essentially the same fermentation conditions of for example, fermentable substrate, pH, temperature, time of fermentation and the like. The carbon conversion efficiency may be defined as an increase in the conversion of carbon in the fermentable substrate directly into an end- product, such as alcohol without losing carbon as a by-product. In some embodiments, the increase in carbon conversion efficiency when the fermentation process is used compared to another fermentation process using the same raw material under essentially the same conditions will be at least 2%, at least 5%, at least 7%, at least 10%, at least 15% and at least 20%. In some embodiments, the increased carbon conversion efficiency is reflected in the higher residual starch levels at the end of a fermentation, which yields approximately the same amount of ethanol as the

process to which it is being compared.

[0123] The term “fermenting microorganism” refers to any microorganism suitable for use in a desired fermentation process. Suitable fermenting microorganisms according to the invention are able to ferment, *i.e.*, convert, sugars, such as glucose, xylose, arabinose, mannose, galactose, or oligosaccharides, directly or indirectly into the desired fermentation product(s). Examples of fermenting microorganisms include fungal organisms, such as yeast. Preferred yeast includes strains of *Saccharomyces spp.*, and in particular, *Saccharomyces cerevisiae*. Commercially available yeast include, *e.g.*, Red Star®/Lesaffre Ethanol Red, FALI, SUPERSTART, GERT, and FERMIOL. Other microorganisms may also be used depending the fermentation product(s) desired. These other microorganisms include Gram positive bacteria, *e.g.*, *Lactobacillus* such as *Lactobacillus lactis*, *Propionibacterium* such as *Propionibacterium freudenreichii*; *Clostridium sp.* such as *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium diolis*, *Clostridium acetobutylicum*, and *Clostridium thermocellum*; Gram negative bacteria, *e.g.*, *Zymomonas* such as *Zymomonas mobilis*; and filamentous fungi, *e.g.*, *Rhizopus oryzae*. Bacteria that can efficiently ferment glucose to ethanol include, for example, *Zymomonas mobilis* and *Clostridium thermocellum*.

[0124] According to preferred embodiments, the yeast is a *Saccharomyces sp.*, *Saccharomyces cerevisiae*, *Saccharomyces distaticus*, *Saccharomyces uvarum*, *Kluyveromyces*, *Kluyveromyces marxianus*, *Kluyveromyces fragilis*, *Candida*, *Candida pseudotropicalis*, *Candida brassicae*, *Clavispora*, *Clavispora lusitaniae*, *Clavispora opuntiae*, *Pachysolen*, *Pachysolen tannophilus*, *Bretanomyces*, or *Bretanomyces clauseni*.

[0125] It is well known in the art that the organisms described above can also be used to produce other organic substances, as described herein. Other examples might be clostridial strains for butanol or isobutanol production, algae for oil production,

various bacteria for acetic acid production. According to some embodiments, the algae are used for the production of oils, which may then be used as a source to produce non-petroleum-based diesel fuel (e.g., biodiesel). Preferably, the alga is selected from *spirogyra*, *cladophora*, *oedogonium*, or a combination thereof. The production of biodiesel may be performed using any known method in the art. According to preferred embodiments, the saccharifying enzymes are inactivated using a heat and/or chemical process prior to the addition of algae.

- [0126] A fermentation stimulator may also be used to improve the fermentation process, and in particular, the performance of the fermenting microorganism, such as, rate enhancement and ethanol yield. A “fermentation stimulator” refers to stimulators for growth of the fermenting microorganisms, in particular, yeast. Preferred fermentation stimulators for growth include vitamins and minerals. Examples of vitamins include multivitamins, biotin, pantothenate, nicotinic acid, meso-inositol, thiamine, pyridoxine, para-aminobenzoic acid, folic acid, riboflavin, and Vitamins A, B, C, D, and E. Examples of minerals include minerals and mineral salts that can supply nutrients comprising P, K, Mg, S, Ca, Fe, Zn, Mn, and Cu.
- [0127] The term “endoglucanase” is defined herein as an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase, which catalyses endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Endoglucanases digest the cellulose polymer at random locations, opening it to attack by cellobiohydrolases.
- [0128] The exo-1,4-beta-D-glucanases include both cellobiohydrolases and glucohydrolases.
- [0129] The term “cellobiohydrolase” is defined herein as a 1,4-beta-D-glucan cellobiohydrolase, which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellooligosaccharides, or any beta-1,4-linked glucose

containing polymer, releasing cellobiose from the reducing or non-reducing ends of the chain. Cellobiohydrolases sequentially release molecules of cellobiose from the ends of the cellulose polymer.

[0130] The term “glucohydrolase” is defined herein as a 1,4-beta-D-glucan glucohydrolase, which catalyzes the hydrolysis of 1,4-linkages (O-glycosyl bonds) in 1,4-beta-D-glucans so as to remove successive glucose units. Glucohydrolases liberate molecules of glucose from the ends of the cellulose polymer.

[0131] The term “beta-glucosidase” is defined herein as a beta-D-glucoside glucohydrolase, which catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. Cellobiose is a water-soluble beta-1,4-linked dimer of glucose. Beta-glucosidases hydrolyze cellobiose to glucose.

[0132] Analysis of zymograms and proteomic analyses of cultures may be used to reveal the identity of enzymes that are induced during growth on a particular substrate (e.g., glucose, Avicel, oat spelt xylan, newsprint, whole and pulverized corn leaves, pulverized *Panicum virgatum* leaves, or any other known substrate). Induction of specific enzymes can be assessed by qRT-PCR. Nomenclature for specific enzymes is explained in further detail in U.S. Application No. 11/121,154, filed on May 4, 2005 and published as U.S. Publication No. 2006/0105914, which is incorporated herein in its entirety.

[0133] The term “fermentation medium” will be understood to refer to a medium before the fermenting microorganism(s) is(are) added, such as, a medium resulting from a saccharification process, as well as a medium used in a simultaneous saccharification and fermentation process (SSF).

[0134] A “fermentable sugar” refers to mono- or disaccharides, which may be converted in a fermentation process by a microorganism in contact with the fermentable sugar to produce an end product. In some embodiments, the fermentable sugar is

metabolized by the microorganism and in other embodiments the expression and/or secretion of enzymes by the microorganism achieves the desired conversion of the fermentable sugar.

- [0135] As used herein, “monosaccharide” refers to a monomeric unit of a polymer such as starch wherein the degree of polymerization is 1 (*e.g.*, glucose, mannose, fructose and galactose).
- [0136] As used herein the term “starch” refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin with the formula $(C_6H_{10}O_5)_x$, wherein x can be any number.
- [0137] The term “cellulose” refers to any cellulose-containing material. In particular, the term refers to the polymer of glucose (cellobiose) with the formula $(C_6H_{10}O_5)_x$, wherein x can be any number.
- [0138] The term “slurry” refers to an aqueous mixture containing insoluble solids (may be used interchangeably with “pulp”).
- [0139] The term “mash” refers to a mixture of a fermentable substrate in liquid used in the production of a fermented product and is used to refer to any stage of the fermentation from the initial mixing of the fermentable substrate or inoculated pulp and fermenting organisms through the completion of the fermentation run. Sometimes the terms “mash”, “fermentation broth”, and “fermentation medium” are used interchangeably. In some embodiments the term fermentation broth means a fermentation medium, which includes the fermenting organisms.
- [0140] The terms “saccharifying enzyme” and “starch hydrolyzing enzymes” refer to any enzyme that is capable of converting starch to mono- or oligosaccharides.
- [0141] The term “vessel” includes but is not limited to tanks, vats, bottles, flasks, bags, bioreactors and the like. In one embodiment, the term refers to any receptacle suitable for conducting the saccharification and/or fermentation processes

encompassed by the invention.

[0142] “A”, “an” and “the” include plural references unless the context clearly dictates otherwise.

[0143] Numeric ranges are inclusive of the numbers defining the range.

[0144] The headings provided herein are not limitations of the various aspects or embodiments of the invention, which can be had by reference to the specification as a whole.

[0145] The following examples are illustrative, but not limiting, of the methods and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in therapy and that are obvious to those skilled in the art are within the spirit and scope of the embodiments.

[0146] Example 1: Zymograms

[0147] All activity gels were prepared as standard SDS-PAGE gels with the appropriate carbohydrate polymer (CP) substrate incorporated directly into the separating gel. Zymograms are cast with 8% polyacrylamide concentration and the substrate dissolved in dH₂O and/or gel buffer solution to give a final concentration of 0.1% (HE-cellulose), 0.15% (barley β -glucan), or 0.2% (xylan). Gels are run under discontinuous conditions according to the procedure of Laemmli with the exception of an 8 minute treatment at 95 °C in sample buffer containing a final concentration of 2% SDS and 100 mM dithiothreitol (DTT). After electrophoresis, gels are incubated at room temperature for 1 hour in 80 ml of a renaturing buffer of 20 mM PIPES buffer pH 6.8 which contains 2.5% Triton X-100, 2 mM DTT and 2.5 mM CaCl₂. The calcium was included to assist the refolding of potential calcium-binding domains such as the tsp3s of Lam16A.

[0148] After the 1 hour equilibration, gels were placed in a fresh 80 ml portion of

renaturing buffer and held overnight at 4 °C with gentle rocking. The next morning gels were equilibrated in 80 ml of 20 mM PIPES pH 6.8 for 1 hour at room temperature, transferred to a clean container, covered with the minimal amount of PIPES buffer and incubated at 37 °C for 4 hours. Following incubation gels were stained for 30 minutes with a solution of either 0.25% Congo red in dH₂O (HE-cellulose, β -glucan and xylan) or 0.01% Toluidine blue in 7% acetic acid. Gels were destained with 1M NaCl for Congo red and dH₂O for Toluidine blue until clear bands were visible against a stained background.

[0149] Example 2: Nelson-Somogyi Reducing-Sugar Assays

[0150] Saccharifying enzyme activity is assayed using a modification of the Nelson-Somogyi reducing sugar method adapted for 96-well microtiter plates, using 50 μ l reaction volumes (Green, Clausen et al. 1989). Test substrates include avicel, CMC, phosphoric-acid swollen cellulose (PASC), Barley glucan, laminarin, and xylan dissolved at 1% in 20 mM PIPES pH 6.8 (Barley glucan and laminarin, 0.5%). Barley glucan, laminarin and xylan assays are incubated 2 hours at 37° C.; avicel, CMC and PASC assays were incubated 36 hours at 37° C. Samples are assayed in triplicate, corrected for blank values, and levels estimated from a standard curve. Enzymatic activity is calculated, with one unit (U) defined as 1 μ M of reducing sugar released/minute and reported as specific activity in U/mg protein.

[0151] Example 3: Genotyping Methods

[0152] The growth rate of *S. degradans* was cultured on different substrates (Figure 1). The basic media was composed of (2.3% Instant Ocean, 0.05% Yeast Extract, 0.05% NH₄Cl, 15mM Tris, pH 6.8). The final concentration of each carbon source were 0.2% for Glucose, Xylose, Cellobiose, Arabinose, Xylan, Avicel and 1.0% for Newsprint, Switchgrass, and corn leaves. *S. degradans* grew on all plant material it was grown on.

[0153] A Zymogram was performed to find which glucanases were induced during growth on various cell wall polymers (Figure 2). Cells were grown to an OD₆₀₀ of 0.3-0.5 in media containing glucose as the sole carbon source, harvested and transferred to the same volume of media containing the indicated inducer. Samples were removed at the indicated times and proteins in samples normalized to OD₆₀₀ were fractionated by standard SDS-PAGE in which either 0.1% barley β -glucan or HE cellulose was included in the resolving gel. Gels were incubated in refolding buffer (20 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] buffer [pH 6.8], 2.5% Triton X-100, 2 mM dithiothreitol, 2.5 mM CaCl₂) for 1 hour at room temperature and then held overnight in fresh refolding buffer at 4°C. The gels were transferred to PIPES buffer, incubated at 37 °C, and stained in 0.25% Congo red. Calculated masses are shown on the left in kDa. Different glucanases were expressed in the presence of different plant materials or carbohydrate sources used.

[0154] The expression of cellulolytic enzymes during growth on glucose and cell wall polymers was determined (Figure 3). *S. degradans* was cultured on glucose to OD₆₀₀ 0.3-0.4, harvested and transferred to the same volume medium containing the indicated substrate. After 10 hours the RNA was isolated using the RNA PROTECT™ Bacteria Reagent (Qiagen) and RNEASY™ Mini kit (Qiagen). The cDNA was synthesized using the QIANTITECT™ Reverse Transcription Kit. The 120-200bp fragments of each indicated gene or two control genes for Guanylate kinase and Dihydrofolate reductase were amplified using the SYBR Green™ master mix kit (Roche) and a LIGHT CYCLER™ 480 (Roche). The bars shown with numbers above them are presented at 1/10 scale. Different cellulolytic enzymes were induced by different plant materials or carbohydrate sources.

[0155] Example 4. Measurement of increase in expression of Xyn10A, Xyn10B, Xyn11A and Xyn11B in response to growth of *S. degradans* on Xylan.

[0156] Primers were designed for six target genes: *xyn10A-D* and *xyn11A-B* along with two house keeping genes: dihydrofolate reductase and guanylate kinase. *S.*

degradans was cultured in glucose media until OD₆₀₀ reached 0.370-0.400. The 0 hour time point was taken and the cultures were transferred to xylan media for 10 hour time course experiments. A second culture was transferred back to glucose as a control. Samples were taken at 0, 2, 4 and 10 hours from both the xylan and glucose cultures.

- [0157] RNA from each sample was purified using RNeasy ProtectTM bacteria reagent (Qiagen) and RNeasy MiniKit. The isolated mRNA was transformed using QuantiTectTM reverse transcriptase and expression patterns were analyzed using LightCycler ProTM pRT-PCR.
- [0158] As shown in Figure 6, *xyn10A*, *xyn10B*, *xyn10D*, *xyn11A* and *xyn11B* all had greater mRNA expression at 2, 4, and 10 hours after exposure to xylan. The increases were the greatest for *xyn10A*, *xyn10B*, *xyn11A* and *xyn11B*. As shown in Figure 7, the highest fold induction of mRNA expression at 2 hours of culture of *S. degradans* on xylan, was for *xyn11A* and *xyn11B*. *Xyn10A* had the highest induction at 4 hours. At 10 hours, *xyn10A*, *xyn10B*, *xyn11A* and *xyn11B* all had higher fold induction.
- [0159] As *S. degradans* increases expression of these proteins when it is exposed to xylan, and with the sequence homology these proteins have to known hemicellulase genes, Xyn10A, Xyn10B, Xyn10D, Xyn11A and Xyn11B are functional hemicellulases that can be used to break down hemicellulose.

[0160] Example 5: Ethanol Production

- [0161] Pulping: The plant material is ground using mechanical techniques to create a pulp in order to increase the surface area of the material and expose the complex polysaccharide polymers of the cell walls to digestion. Fractionation of the material into its component polymer families (cellulose, hemicellulose, pectin, lignin) is not necessary, but some pretreatments may speed up the process (*e.g.*, steam explosion). The pulp undergoes heat sterilization prior to the addition of the

Saccharophagus degradans bacterium.

- [0162] Growth of the bacterium: The *Saccharophagus degradans* bacterium is grown to a high density using a growth medium containing glucose, yeast extract, ammonia, and Instant Ocean™ at pH 7.0-7.5. In standard batch culture, an optical density at 600 nm (OD) of 10 in less than 24 h is achieved. Tryptone™ may also be added.
- [0163] Induction of enzyme production: The cellulases, hemicellulases, etc that this *S. degradans* is capable of producing are not expressed under these conditions. Rather, *S. degradans* recognizes the polymers in the pulp to induce expression of the enzymes necessary to digest those polymers.
- [0164] For induction, the bacterium is harvested from the growth medium and undergoes continuous centrifugation to separate the growth medium from the bacterial cells. The separated growth medium can be recycled by passage through sand and charcoal filters and then sterilized by heat, UV or chlorine dioxide to grow more bacteria first recycling loop for the water). The harvested bacterial cells (cell paste) are suspended in an induction medium containing Instant Ocean™, ammonia, and the pulp material.
- [0165] Capture of the enzymes: The intact bacterial cells and secreted enzymes produced during are collected by adsorption to the pulp material. The bacterial cells and the secreted enzymes adsorb to the pulp material. Because there is little lysis of the bacterial cells during the induction phase and digestion of the material is minimal during the adsorption step, the material together with adsorbed enzymes and bacterial cells remains as a suspended solid in the mixture.
- [0166] The enzymes and adsorbed cells are collected by collecting the solids from the medium using continuous centrifugation or filtration coupled with a mechanical dewatering of the material. The medium released during solids collection can be recycled using a sand and charcoal filter in conjunction with in line sterilization.
- [0167] Simultaneous saccharification/fermentation: The material collected with the

adsorbed bacteria and enzymes is transferred into a fermentation tank containing water, ethanol, corn steep liquor, MgSO_4 and *Saccharomyces cerevisiae* (yeast) as the fermentative organism. A continuous fermentation process is used to generate ethanol concentrations excess of 10%. The transfer of the material and adsorbed bacteria into the water-ethanol mixture causes the lysis of the bacteria adsorbed to the material. This releases the enzymes contained by the bacteria directly into the material where they can depolymerize and degrade the material to its constituent sugars. The enzymes so produced and the lysis conditions have been demonstrated to be compatible with yeast and do not infer with yeast alcoholic fermentation. The added ethanol also serves as an antimicrobial agent to limit the growth of contaminating microorganisms. The enzymes of the bacterium and yeast are tolerant of added ethanol up to 15% v/v.

- [0168] While the invention has been described with reference to particularly preferred embodiments and examples, those skilled in the art recognize that various modifications may be made to the invention without departing from the spirit and scope thereof.
- [0169] All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety.

What is claimed is:

1. A method of producing a biofuel from a plant material comprising
 - (a) providing a liquid pulp suspension comprising lignocellulose materials;
 - (b) adding to the pulp a culture of *Saccharophagus degradans*;
 - (c) incubating the mixture of pulp and *Saccharophagus degradans* for a period of time sufficient to induce lignocellulase enzyme production;
 - (d) collecting the solids from the liquid pulp suspension;
 - (e) transferring the solids to a hypotonic solution containing a fermenting organism; and
 - (f) fermenting the solution, thereby producing the biofuel.
2. The method of claim 1, wherein the biofuel is selected from the group consisting of ethanol, butanol, isobutanol, acetic acid, oil and acetone.
3. The method of claim 2, wherein the biofuel is ethanol.
4. The method of claim 1, wherein the liquid pulp suspension is at least 1% salt.
5. The method of claim 1, wherein the hypotonic solution is less than 0.9% salt.
6. The method of claim 1, wherein the *Saccharophagus degradans* is grown until it reaches an OD600 of at least 10 prior to the adding step.
7. The method of claim 1, wherein the collecting step comprises a dewatering step.
8. The method of claim 1, wherein the fermenting organism is yeast.

9. A method of producing sugar from a plant material comprising:
 - (a) providing a liquid pulp suspension comprising carbohydrates;
 - (b) adding to the pulp a culture of *Saccharophagus degradans*;
 - (c) incubating the mixture of pulp and *Saccharophagus degradans* for a period of time sufficient to induce saccharolytic enzyme production;
 - (d) collecting the solids from the liquid pulp suspension; and
 - (e) transferring the solids to a hypotonic solution, thereby producing sugar from the plant material.
10. The method of claim 9, wherein the liquid pulp suspension is at least 1% salt.
11. The method of claim 9, wherein the hypotonic solution is less than 0.9% salt.
12. The method of claim 9, wherein the *Saccharophagus degradans* is grown until it reaches an OD600 of at least 10 prior to the adding step.
13. The method of claim 9, wherein the collecting step comprises a dewatering step.
14. A method for producing a biofuel from lignocellulosic material comprising
 - (a) providing a liquid pulp suspension comprising lignocellulose materials;
 - (b) adding to the pulp a culture of a saccharifying microorganism expressing one or more compounds listed in Figures 4-11;
 - (c) incubating the mixture of pulp and saccharifying microorganism for a period of time sufficient to induce lignocellulase enzyme production;
 - (d) collecting the solids from the liquid pulp suspension;

- (e) transferring the solids to a hypotonic solution containing a fermenting organism; and
- (f) fermenting the solution, thereby producing the biofuel.

15. The method of claim 14, wherein the biofuel is selected from the group consisting of ethanol, butanol, isobutanol, acetic acid, oil and acetone.
16. The method of claim 15, wherein the biofuel is ethanol.
17. The method of claim 15, wherein cel5A is the compound expressed.
18. The method of claim 15, wherein 5 or more of the compounds listed in Figures 4-11 are expressed.
19. The method of claim 15, wherein 5 or more of the compounds listed in Figures 4-11 are expressed.
20. The method of claim 14, wherein the liquid pulp suspension is at least 1% salt.
21. The method of claim 14, wherein the hypotonic solution is less than 0.9% salt.
22. The method of claim 14, wherein the saccharifying microorganism is grown until it reaches an OD600 of at least 10 prior to the adding step.
23. The method of claim 14, wherein the collecting step comprises a dewatering step.
24. The method of claim 14, wherein the fermenting organism is yeast.

FIG. 1A

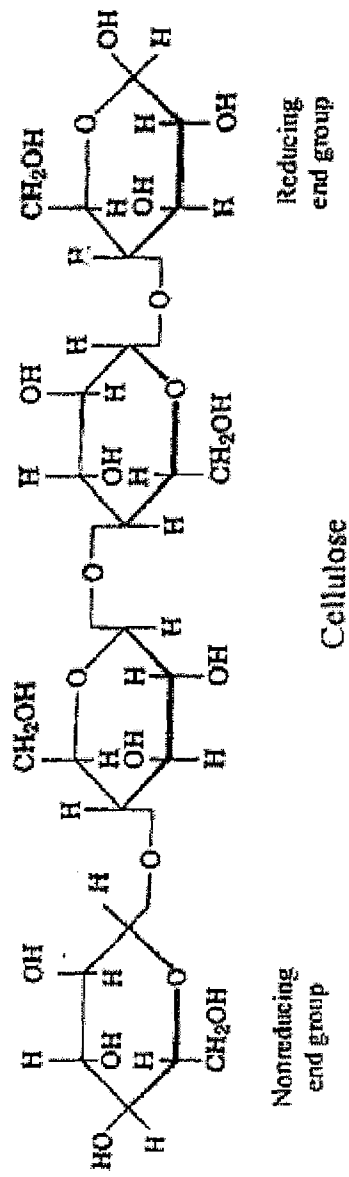
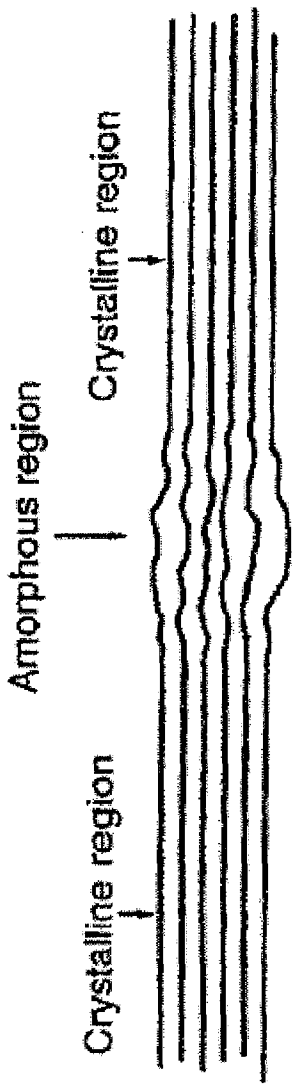
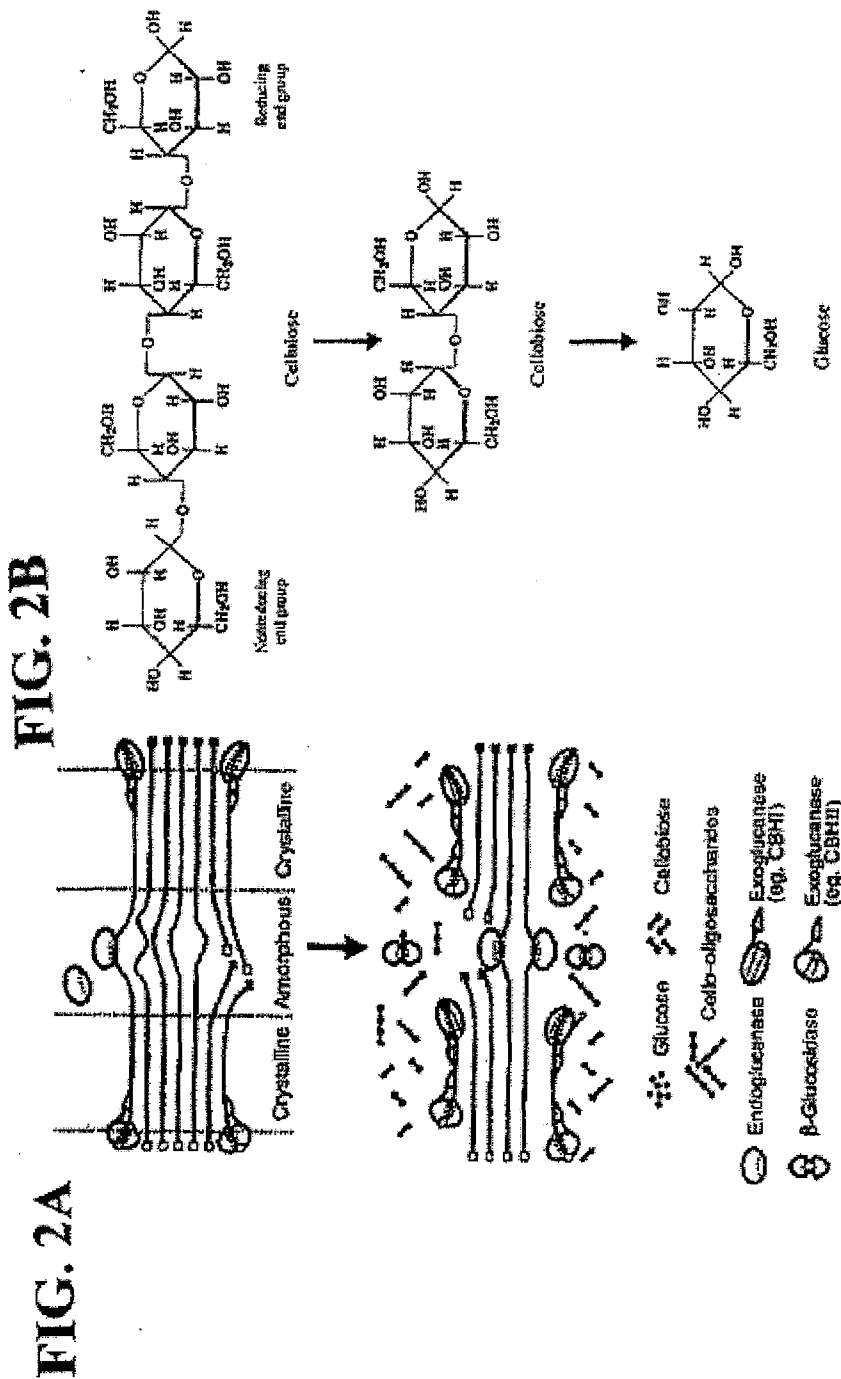


FIG. 1B

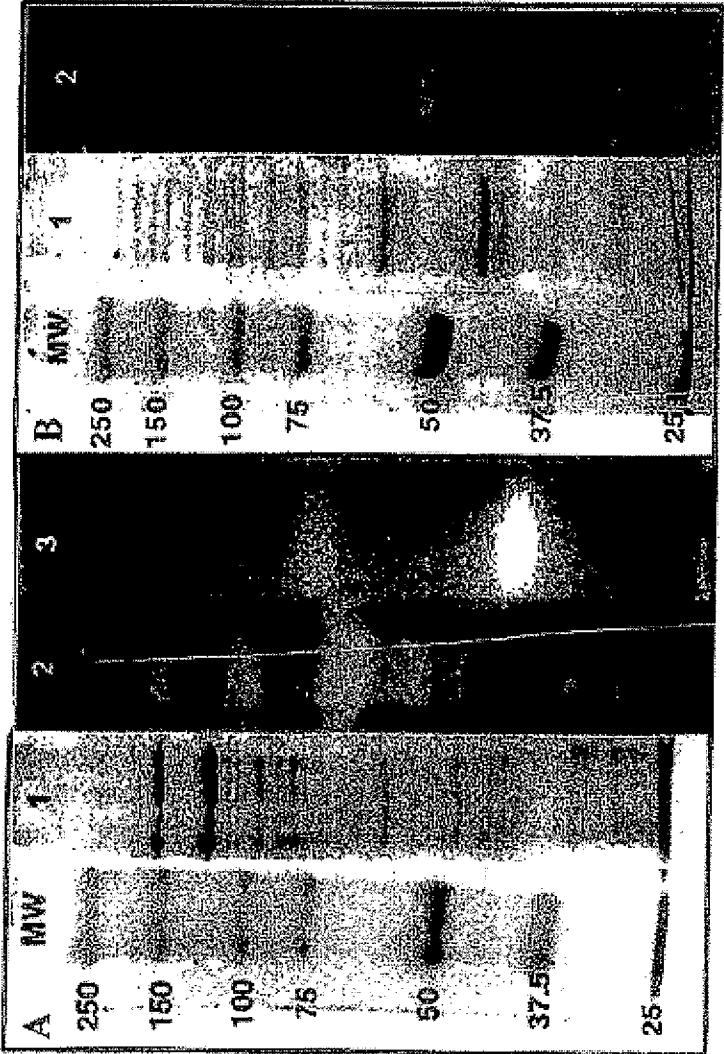


Panel B from Beguin and Aubert, 1994.



From Lynd, Weimer et al., 2002.

FIG. 3



SDS-PAGE and Zymogram analysis of 2-40 culture supernatants.

FIG. 4

Predicted cellulases of *M. degradans* 2-40. Cellulase depolymerases and accessories as predicted by CAZyme analysis and sequence-based functional predictions as described in the text.

Name	MS	amino acids	MW	Predicted function	Modules	Refseq accession
cel5A		1,167	127.2	endo 1,4-glucanase	GH5/CBM6/CBM6/CBM6/GH5	ZP_00067013.1
cel5B		566	60.8	endo 1,4-glucanase	LPB/PSL(47)/CBM6/GH5	ZP_00064853.1
cel5C		451	49.1	endo 1,4-glucanase	LPB/PSL(47)/GH5	ZP_00067454.1
cel5D		621	65.9	endo 1,4-glucanase	CBM2/PSL(58)/CBM10/PSL(36)/GH5	ZP_00064986.1
cel5E		673	72.6	endo 1,4-glucanase	CBM6/CBM6/GH5	ZP_00066079.1
cel5F		365	42.0	endo 1,4-glucanase	GH5	ZP_00066536.1
cel5G		638	67.9	endo 1,4-glucanase	GH5/PSL(21)/CBM6/PSL(32)/Y95	ZP_00066178.1
cel5H	av	630	66.9	endo 1,4-glucanase	GH5/PSL(32)/CBM6/EPR(16)	ZP_00068260.1
cel5I	av	725	77.2	endo 1,4-glucanase	CBM2/PSL(33)/CBM10/PSL(58)/GH5	ZP_00067367.1
cel5J		610	65.2	endo 1,4-glucanase	GH5/CBM6/CBM6	ZP_00064857.1
cel6A		791	81.9	non-reducing end cellobiohydrolase	CBM2/PSL(43)/CBM2/PSL(85)/GH6	ZP_00064659.1
cel9A		578	62.7	endo-1,4-glucanase	GH9	ZP_00065765.1
cel9B	av	867	89.5	endo-1,4-glucanase	GH9/PSL(54)/CBM10/PSL(50)/CBM2	ZP_00065776.1
ced3A	av, cm, xn	1,072	116.0	cellodextrinase	LPB/GH3/PLP	ZP_00064860.1
ced3B	xn	862	92.9	cellodextrinase	LPB/GH3	ZP_00068210.1
bgl1A		461	52.8	cellobiase	[†] GH1	ZP_00067238.1
bgl1B		444	49.8	cellobiase	[†] GH1	ZP_00066838.1
bgl3C	av, cm, xn	866	95.4	β -glucosidase	LPB/GH3/UNK(511)	ZP_00066570.1
cep94A		811	91.7	cellobiose phosphorylase	[†] GH94	ZP_00067911.1
cep94B	cm	788	88.7	cellodextrin phosphorylase	[†] GH94	ZP_00068287.1

FIG. 5

Predicted xylanases, xylosidases and related accessories.

Name	MS	amino acids	MW	Predicted function	Modules	Refseq accession
xyn10A		574	61.7	β -1,4-xylanase	GH10/PSR(29)/Y94/PSL(21)/CBM5	ZP_00066395.1
xyn10B		619	65.0	β -1,4-xylanase	CBM2/PSL(42)/CBM10/PSL(48)/GH10	ZP_00066073.1
xyn10C		374	42.3	β -1,4-xylanase	GH10	ZP_000664983.1
xyl/arb43G-		1,186	129.6	β -xylosidase/ α -L-arabino	GH43/CBM6/Y94/PSL(24)/CBM2/PSL(22)/CBM22/GH10	ZP_00067229.1
xyn10D				furanosidase/ β -1,4-xylanase		
xyn10E	xn	670	75.2	β -1,4-xylanase	LPB/EPR(47)/GH10	ZP_00067456.1
xyn11A		275	30.4	β -1,4-xylanase	GH11	ZP_00066908.1
xyn11B-		767	80.8	bifunctional β -1,4-	GH11/PSL(10)/PSL(19)/CE4/CBM10	ZP_00067071.1
axe4A				xylanase/acetylxylosterase		
axe2A		360	40.9	acetylxylosterase	CE2	ZP_00064735.1
xyl31A	xn	973	110.2	α -xylosidase for xyloglucans	LPB/GH31	ZP_00064863.1
xyl3A	av, cm, xn	893	97.6	β -xylosidase	LPB/GH3	ZP_00067626.1
xyl43L		317	36.1	α -xylosidase	GH43	ZP_00067829.1
xyl/arb43H		577	63.8	bifunctional β -xylosidase/ α -L-	LPB/GH43	ZP_00065724.1
				arabinofuranosidase		
xyl/arb43I		566	62.6	bifunctional β -xylosidase/ α -L-	LPB/GH43	ZP_00066469.1
				arabinofuranosidase		
xyl/arb43J		317	35.1	bifunctional β -xylosidase/ α -L-	LPB/GH43	ZP_00066992.1
				arabinofuranosidase		
xyl/arb43K		385	42.6	bifunctional β -xylosidase/ α -L-	LPB/GH43	ZP_00315109.1
				arabinofuranosidase		
agu67A		738	82.0	α -1,2-glucuronidase	GH67	ZP_00315039.1

Fig. 6

Predicted pectinases and accessories.

Name	amino acids	MW	Predicted function	Modules	Refseq accession
pel1A	1,316	136.4	pectate lyase	PL1/PSL(35)/PSL(29)/FN3/PSL(65)/PL1	ZP_00067832.1
pel1B	427	46.1	pectate lyase	PL1	ZP_00067840.1
pel1C	769	78.9	pectate lyase	PL1/PSL(33)/PSL(23)/FN3	ZP_00067834.1
pel1D	594	63.3	pectate lyase	LPB/EPR(24)/PL1	ZP_00067182.1
pel1E	425	45.4	pectate lyase	LPB/PL1	ZP_00064693.1
pel1F	772	81.5	polysaccharide lyase (pectate?)	CBM2/PSL(45)/PSL(31)/PL1	ZP_00064697.1
pel3A	511	52.3	pectate lyase	CBM2/PSL(30)/PSL(20)/PL3	ZP_00064694.1
pel3B	452	46.1	pectate lyase	PSL(24)/FN3/PSL(20)/PL3	ZP_00065735.1
pel3C	424	42.9	pectate lyase	PSL(19)/FN3/PSL(18)/PL3	ZP_00067017.1
pel3D	392	41.6	pectate lyase	CBM13/PL3	ZP_00067887.1
pel9A	733	75.6	pectate lyase	PSL(47)/FN3/FN3/PL9	ZP_00066061.1
pel10A	700	73.3	pectate lyase	CBM2/PSL(54)/PSL(28)/PL10	ZP_00068232.1
pel10B	574	60.8	pectate lyase	LPB/PSL(46)/PL10	ZP_00066059.1
pgl28A	463	50.9	polygalacturonanase	LPB/GH28	ZP_00067822.1
pes8A	1,081	115.1	pectin methylesterase	LPB/EPR(23)/CE8	ZP_00067183.1
pes8B	389	42.4	pectin methylesterase	LPB/CE8	ZP_00067831.1
rgl11A	914	97.6	rhamnogalacturonan lyase	PL11/PSL(33)/CBM2	ZP_00066475.1

FIG. 7

Predicted arabinanases and arabinogalactanases of 2-40. The bifunctional xylosidase/arabinanases in table 2 are also expected to function as accessories to these enzymes

Name	amino acids	MW	Predicted function	Modules	Refseq accession
arb43A	789	85.0	endo-1,5-a-L-arabinanase	CBM13/GH43	ZP_00066674.1
arb43B	362	40.7	exo-1,5-a-L-arabinofuranosidase	LPB/GH43	ZP_00066990.1
arb43C	314	35.9	exo-1,5-a-L-arabinofuranosidase	^t GH43	ZP_00066981.1
arb43D	472	51.1	endo-1,5-a-L-arabinanase	CBM13/GH43	ZP_00068164.1
arb43E	346	38.6	endo-1,5-a-L-arabinanase	LPB/GH43	ZP_00066989.1
arb43F	607	67.2	exo-1,5-a-L-arabinofuranosidase	GH43	ZP_00066994.1
arb51A	534	59.4	exo-1,5-a-L-arabinofuranosidase	GH51	ZP_00315975.1
arg53A	397	44.5	arabinogalactanase	GH53	ZP_00068112.1
arg53B	476	52.6	arabinogalactanase	GH53/CBM13	ZP_00065874.1
arg53C	650	71.1	arabinogalactanase	GH53	ZP_00068237.1

FIG. 8

Predicted mannanases of 2-40.					
Name	MS	amino acids	MW	Predicted function	Modules
man26A		506	54.9	mannanase	LPB/PSL(52)/CBM10/GH26
man5N		561	57.6	mannanase	GH5/PSL(32)/CBM10/PSR(44)/CBM2
man5O		507	52.6	mannanase	GH5/PSL(23)/CBM10/CBM10
man5P	cm	457	50.9	mannanase	GH5
man5Q	ag	850	92.3	mannanase	LQAC/GH5
agl27A		408	46.5	α -galactosidase	GH27
					Refseq accession
					ZP_00065857.1
					ZP_00065570.1
					ZP_00065784.1
					ZP_00065642.1
					ZP_00064894.1
					ZP_00066516.1

FIG. 9

Predicted laminarinases of 2-40.					Modules	Refseq accession.
Name	amino acids	MW	Predicted function			
lam16A	1,707	163.3	B-1,3-glucanase	GH16/CBM6/CBM6/TSP3/TSP3/TSP3/COG3488	ZP_00066839.1	
lam16B	1,441	158.6	B-1,3-glucanase	GH16/CBM6/CBM6/EPR(56)/CBM32/CBM32	ZP_00066081.1	
lam16C	1,184	129.1	B-1,3-glucanase	GH16/CBM4/CBM32/CBM32	ZP_00066789.1	
lam16D	722	77.7	B-1,3-glucanase	GH16/CBM32/PSR(48)/TMR	ZP_00067027.1	
lam16E	569	61.4	B-1,3-glucanase	CBM6/CBM6/GH16	ZP_00065780.1	
lam16F	742	80.2	B-1,3-glucanase	LPB/GH16	ZP_00066288.1	
lam16G	877	94.2	catalytic residues missing	LPB/GH16/CBM6/CBM6	ZP_00068290.1	
lam81A	1,238	133.1	B-1,3-glucanase	LPB/CADG/GH81/FN3/FN3	ZP_00068322.1	

FIG. 10

Selected carbohydrate-binding module (CBM) proteins. Function is unknown for proteins containing CBM domains but lacking another well-characterized domain.

Name	MS	amino acids	MW	Predicted function	Modules	Refseq accession
cbm2A		354	37.9	cbm2a binds crystalline cellulose	CBM2/EPR(48)/UNK(174)	ZP_00067766.1
cbm2B	av	1,042	112.1	cbm2a	CBM2/UNK(914)	ZP_00067765.1
cbm2C	xu	933	97.5	cbm2a	CBM2/PSL(58)/Y94/PSL(25)/UNK(577)	ZP_00066396.1
cbm2D-cbm10A		558	56.3	cbm2a	CBM2/PSL(43)/CBM10/PSL(101)/UNK(231)	ZP_00065777.1
cbm2E		781	85.0	cbm2a	CBM2/PSL(18)/PSL(18)/UNK(471)	ZP_00065695.1
cbm2F		787	84.8	cbm2a	CBM2/PSL(33)/PSL(17)/UNK(544)	ZP_00066068.1
cbm6A		681	72.5	cbm6 binds glycan chains	LPB/PSL(28)/CBM6/UNK(476)	ZP_00068207.1
cbm6B		630	67.2	cbm6	CBM6/CBM6/UNK(217)	ZP_00066418.1
cbm6C		550	59.8	cbm6	CBM6/UNK(216)	ZP_00065386.1
cbm6D		500	54.6	cbm6	CBM6/UNK(262)	ZP_00068152.1
cbm6E		473	53.0	cbm6	LPB/UNK(~300)/CBM6	ZP_00066788.1
cbm6F		465	50.4	cbm6	UNK(~300)/CBM6	ZP_00065421.1
cbm6G-cbm16B		1,024	111.4	cbm6; cbm16 unknown	CBM6/CBM16/UNK(704)	ZP_00066336.1
cbm6H-cbm32F		912	97.5	cbm6; cbm32 binds monos/oligos (1-3)	UNK(~500)/CBM6/CBM32	ZP_00066663.1
cbm16A-cbm32E		552	58.456	cbm16; cbm32	CBM16/PTR/CBM32/PTR/UNK(232)	ZP_00068306.1
cbm32A	xn	1,028	111.9	cbm32; COG3488 thiol-oxidoreductase	CBM32/CBM32/UNK(251)/COG3488	ZP_00065611.1
cbm32B		557	59.5	cbm32	UNK(~100)/CBM32/CBM32/CBM32	ZP_00066007.1
cbm32C		674	73.6	Zn-protease; Cbm32; FN3 unknown	ZDP/CBM32/FN3/CBM32	ZP_00067813.1
cbm32D		818	88.8	Cbm32; FCL eel fucollectin-like domain	CBM32/FCL/UNK(521)	ZP_00065873.1

FIG. 11

Recombinant proteins and comparison of predicted vs. observed molecular weights. Proteins cloned and expressed for functional characterization as described in text.

Name	amino acids	MW (kDa)	PAGE MW	Predicted function	Modules
rLam16A	1,738	186.9	~240	β -1,3-glucanase	GH16/CBM6/CBM6/TSP3/TSP3/TSP3/COG3488
rLam16D	761	82.2	~90	β -1,3-glucanase	GH16/CBM32/PSL(48)/TMR
rCel5A	1,117	121.9	~130	β -1,4-endoglucanase	GH5/CBM6/CBM6/CBM6/GH5
rGly3D	1,591	174.3	~180	β -glycosidase	CBM32/CBM32/CBM32/GH3/CBM32
rGly5K	892	97.8	~115	(1,3 or 1,4) endoglucanase	GH5/CBM6/CBM6/CBM13
rGly5M	885	96.5	~125	(1,3 or 1,4) endoglucanase	LPB/EPR(34)/GH5/FTN3/CBM6
rGly9C	663	72.9	~70	glycanase <25% ID to cellulases	GH9
rGly43M	1,198	130.3	~135	β -glycosidase	LPB/GH43/LGL/LGL
rCbm2A	340	36.6	~45	binds crystalline cellulose	CBM2/EPR(48)/UNKNOWN(174)
rCbm2B	1,031	110.9	~125	binds crystalline cellulose	CBM2/UNKNOWN(914)
rCbm2C	950	99.3	~140	binds crystalline cellulose	CBM2/PSL(58)/Y94/PSL(25)/UNKNOWN(577)
rCbm2D-cbm10A	578	58.7	15-25 [†]	binds crystalline cellulose	CBM2/PSL(43)/CBM10/PSL(101)/UNKNOWN(231)

FIG. 12

