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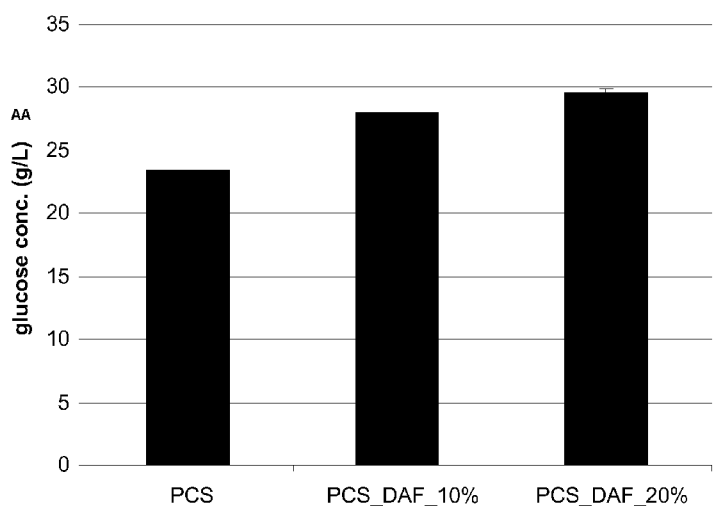
(54) Title: IMPROVEMENT OF ENZYMATIC HYDROLYSIS OF PRETREATED LIGNOCELLULOSE-CONTAINING MA-
TERIAL WITH DISSOLVED AIR FLOTATION SLUDGE

FIGURE 1

(57) Abstract: A method for producing
a fermentation product from a lignocel-
lulose-containing material comprises
pretreating the lignocellulose-containing
material; introducing treated dissolved
air flotation sludge to the pretreated lig-
nocellulose-containing material; expos-
ing the pretreated lignocellulose-con-
taining material to a hydrolyzing en-
zyme; and fermenting with a fermenting
organism to produce a fermentation
product.



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IMPROVEMENT OF ENZYMATIC HYDROLYSIS OF PRETREATED LIGNOCELLULOSE-CONTAINING MATERIAL WITH DISSOLVED AIR FLOTATION SLUDGE

FIELD

[0001] Methods for producing fermentation products from lignocellulose-containing material, and more particularly, methods for increasing the efficiency of producing fermentation products from lignocellulose-containing material by treating the material with dissolved air flotation sludge are disclosed.

BACKGROUND

[0002] Lignocellulose-containing material, or biomass, may be used to produce fermentable sugars, which in turn may be used to produce fermentation products such as renewable fuels and chemicals. Lignocellulose-containing material is a complex structure of cellulose fibers wrapped in a lignin and hemicellulose sheath. Production of fermentation products from lignocellulose-containing material includes pretreatment, hydrolysis, and fermentation of the lignocellulose-containing material.

[0003] Conversion of lignocellulose-containing material into renewable fuels and chemicals often involves physical, biological, chemical and/or enzymatic treatment of the biomass. In particular, enzymes hydrolyze cellulose to D-glucose, which is a simple fermentable sugar. Due to the high lignin content in many lignocellulose-containing materials, high doses of enzyme are needed to degrade the cellulose with high yields. This may be because lignin and lignin derivatives inhibit the hydrolyzing enzymes. Such inhibition may occur in at least two ways: the lignin or lignin derivatives preferentially bind with the enzyme thereby preventing the enzyme from binding with or hydrolyzing cellulose, and/or the lignin or lignin derivatives cover portions of the cellulose thereby reducing enzyme access to cellulose. Consequently, when processing high lignin content biomass, fewer enzymes may be available to degrade cellulose because the lignin or its derivatives may scavenge the enzyme or block its activity. Even for the enzymes that are available to degrade cellulose, often the available enzyme cannot contact the cellulose because lignin is covering the cellulose. Thus, the effectiveness of the process for digesting cellulose is reduced. In addition, the costs of enzymes are high. Thus, when the amount of

enzymes needed to degrade cellulose is high, the processing costs are high and economically unfeasible.

[0004] Reduction in the amount of enzyme needed to obtain a satisfactory sugar yield can have a significant impact on process economics. Therefore, improving the efficiency of enzyme use is a major need in the bioconversion process. Several factors are thought to influence enzymatic hydrolysis of cellulose. These factors include lignin content, hemicellulose content, acetyl content, surface area of cellulose and cellulose crystallinity. It is generally understood that the lignin present in complex substrates has a negative effect on enzyme hydrolysis.

[0005] The exact role of lignin and lignin derivatives in limiting hydrolysis has been difficult to define. However, it is known that removing the effect of lignin and its derivatives increases hydrolysis of cellulose and increases fermentable sugar yield. This action may open more cellulose surface area for enzymatic attack and may reduce the amount of enzyme that is non-specifically adsorbed on the lignocellulosic substrate. Compounds used to remove the effect of lignin and its derivatives may make cellulose more accessible to enzymatic degradation, thus decreasing the amount of enzyme necessary and increasing the ethanol yield in the biomass to ethanol process.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] FIG. 1 is a chart showing the effect of dissolved air flotation sludge on the glucose yield from hydrolysis of washed PCS.

[0007] FIG. 2 is a chart showing the effect of dissolved air flotation sludge on the percent glucose conversion from hydrolysis of washed PCS.

SUMMARY

[0008] Methods for producing fermentation products from lignocellulose-containing material by pretreating and/or hydrolyzing the material in the presence of treated dissolved air flotation sludge are disclosed.

[0009] Also disclosed are methods for producing a fermentation product from a lignocellulose-containing material including pretreating the lignocellulose-containing material; introducing treated dissolved air flotation sludge to the pretreated lignocellulose-containing material; exposing the pretreated lignocellulose-containing

material to a hydrolyzing enzyme; and fermenting with a fermenting organism to produce a fermentation product. In one aspect, the treated dissolved air flotation sludge may be introduced to the lignocellulose-containing material prior to exposing the lignocellulose-containing material to an effective amount of a hydrolyzing enzyme.

[0010] Additionally, methods for enhancing enzymatic hydrolysis of a lignocellulose-containing material are disclosed. Such methods comprise introducing an effective lignin blocking amount of dissolved air flotation sludge to the lignocellulose-containing material and exposing the lignocellulose-containing material to a hydrolyzing enzyme.

DETAILED DESCRIPTION

[0011] An improved and more efficient method for enzymatically hydrolyzing lignin-containing biomass by using treated dissolved air flotation sludge as a lignin blocker is disclosed.

[0012] Lignin is a phenolic polymer that can be derived by the dehydrogenative polymerization of coniferyl alcohol and/or sinapyl alcohol and is found in the cell walls of many plants. As used herein, the term "lignin" refers to the intact structure of the lignin polymer as well as any derivative fragments or compounds of the intact polymer that result from disruption of the lignin structure, including soluble lignin derivatives, condensed lignin and insoluble precipitated lignin. It is believed that lignin derivatives vary in their interaction with treated dissolved air flotation sludge. For example, it is believed that insoluble precipitated lignin and condensed lignin have the ability to adsorb treated dissolved air flotation sludge from aqueous solutions.

[0013] As used herein, the term "biomass slurry" refers to the aqueous biomass material that undergoes enzymatic hydrolysis. Biomass slurry is produced by mixing biomass, e.g., corn stover, bagasse, etc., with water, buffer, and other pretreatment materials. The biomass may be pretreated prior to hydrolysis.

[0014] As used herein, the term "lignin blocking" means the reduction or elimination of the deleterious effects of lignin on the process of converting biomass to a fermentation product. In addition, as used herein, the term "effective lignin blocking amount" means any amount useful in facilitating lignin blocking.

[0015] In one embodiment, the method utilizes treated dissolved air flotation sludge. Without being bound by any particular theory, it is believed that the treated dissolved air flotation sludge may preferentially bind with lignin more readily than cellulose. A biomass slurry may be treated with treated dissolved air flotation sludge, for example, by introducing treated dissolved air flotation sludge directly into the pretreated biomass slurry. It is surmised that the treated dissolved air flotation sludge preferentially binds with lignin in the pretreated slurry thereby covering lignin that has precipitated onto the surface of the cellulose, thus impeding the precipitated lignin from binding hydrolyzing enzymes. Cellulose-hydrolyzing enzymes may then hydrolyze cellulose more efficiently. Without treatment of the lignin-containing biomass slurry with treated dissolved air flotation sludge, lignin may bind a portion of the cellulose-hydrolyzing enzymes rendering them unable to hydrolyze cellulose, or may cover portions of the cellulose, rendering it inaccessible to hydrolyzing enzymes.

[0016] Without being bound by any particular theory, it is believed that lignin operates in multiple ways to inhibit enzymes from hydrolyzing cellulose in biomass. Lignin limits the degree to which cellulose and hemicellulose can be converted to monomeric sugars by cellulolytic and hemicellulolytic enzymes. The focus of many research activities has been directed to understanding the nature of lignin in cell walls and developing pretreatment processes that are effective in removing it. By understanding the mode in which lignin inhibits enzymatic activity, it is possible to reduce the detrimental effects traditionally caused by the lignin component of biomass. As will be described in further detail below, lignocellulose-containing material or biomass may be pretreated prior to being hydrolyzed. For example, pretreatment may take the form of steam pretreatment, alkaline pretreatment, acid pretreatment, or some combination of these. Steam pretreatment physically breaks up the structure of the biomass, i.e., at least partially breaks the bonds connecting the lignin, cellulose, and hemicellulose. Alkaline pretreatment generally includes treatment of the biomass with an alkaline material such as ammonium. Alkaline pretreatment chemically alters the biomass. With respect to the lignin component of the biomass, it is believed that alkaline pretreatment at least partially degrades the lignin thereby forming lignin derivatives and small phenolic fragments that may adversely affect enzyme performance, and yeast growth and fermentative capacity.

Acid pretreatment also chemically alters the lignin component of the biomass thereby forming lignin derivatives including condensed lignin that precipitates on the cellulose fiber surface. The condensed lignin inhibits enzymes from reaching the cellulose by covering the cellulose fiber surface. Other lignin derivatives formed during acid pretreatment include small phenol containing fragments and compounds that may inhibit enzyme function.

[0017] It is further believed that treatment of biomass slurry with treated dissolved air flotation sludge is effective, at least in part, through binding lignin, thus reducing and/or inhibiting non-productive adsorption of cellulose hydrolyzing enzymes to lignin. In addition, it is thought that the dissolved air flotation sludge acts, advantageously, as a surfactant for the enzyme. It is believed that a surfactant improves substrate accessibility, improves enzyme stability, and reduces non-productive lignin binding. It is believed that these advantages may be due to the surfactant keeping the enzyme in solution thus potentially keeping the enzyme away from lignin, stabilizing the enzyme, and extending the productive life of the enzyme. The treatment of biomass slurry with treated dissolved air flotation sludge thus improves processing of lignin containing substrates by inhibiting lignin from binding to the enzymes and improving enzyme hydrolysis. Treated dissolved air flotation sludge can reduce enzyme load and/or improve enzyme performance because the enzyme is not as adversely affected by lignin and thus more of the enzyme remains available to more effectively hydrolyze the biomass slurry. In addition, the productive life of the enzyme is extended through the surfactant effect of the dissolved air flotation sludge.

[0018] The present method reduces enzyme loading in hydrolysis of lignin containing biomass slurry. The amount of enzyme that is needed to provide hydrolysis is significantly reduced through adding treated dissolved air flotation sludge to the biomass slurry. Reduction in enzyme loading reduces the overall costs of the biomass conversion processes.

[0019] According to one embodiment, the method enhances enzymatic hydrolysis of cellulose using treated dissolved air flotation sludge. This method includes the steps of treating a lignin containing biomass slurry with treated dissolved air flotation sludge to provide a treated biomass slurry having a blocked lignin component and exposing the treated biomass slurry to an effective amount of a hydrolyzing enzyme.

The dissolved air flotation sludge may be added directly to the biomass slurry during or after pretreatment, or before or during hydrolysis. It is preferred that the dissolved air flotation sludge be added to the biomass slurry prior to the addition of the cellulose hydrolyzing enzyme and fermenting organism.

Dissolved Air Flotation Sludge

[0020] Dissolved air flotation is a water treatment process widely used in industries such as food processing and oil refineries. Dissolved air flotation clarifies wastewaters by removing suspended matter, such as oil or solids. Large quantities of light solids and hydrophobic material, such as fat, oil, and grease, are removed from dissolved air flotation units as sludge. This sludge may be used in the process being described in the instant application.

[0021] The dissolved air flotation process works by dissolving air in wastewater under pressure and then releasing the air at atmospheric pressure in a flotation tank or basin. The released air forms tiny bubbles which adhere to the suspended matter causing the suspended matter to float to the surface of the water where it may then be removed by a skimming device.

[0022] Dissolved air flotation is very widely used in treating industrial wastewater effluents from oil refineries, petrochemical and chemical plants, natural gas processing plants and similar industrial facilities. It is also used in treatment of wastewater from agricultural processes.

[0023] Agricultural wastewater treatment relates to the treatment of wastewaters produced in the course of agricultural activities. Agricultural processes may generate wastewaters that include animal wastes, silage liquor, pesticide run off and surpluses, milking parlor wastes including milk, slaughtering waste, vegetable washing water, and fire water. Wastewaters of agricultural processes typically contains the following constituents: a strong organic content, a high solids concentration, high nitrate and phosphorus content, antibiotics, synthetic hormones, often high concentrations of parasites and their eggs, and spores of various bacteria. It may also contain large volumes of wash-down water and cleaning and disinfection chemicals.

[0024] Dissolved air flotation sludge for use in the present invention may comprise waste from wastewater treatment related to an agricultural process. More

specifically, it may comprise waste from wastewater treatment related to the slaughtering process. An example of dissolved air flotation sludge that may be used is dissolved air flotation sludge from wastewater treatment in a pig slaughtering process. Using dissolved air flotation sludge to improve enzymatic hydrolysis is economically beneficial. It increases the production of fermentation products while at the same time recycling and using natural waste from agricultural processes that would otherwise have to be treated using costly treatment processes and/or discarded. In addition, it is believed that adding dissolved air flotation sludge to improve hydrolysis of the biomass slurry may reduce or alleviate the need to add nitrogen to the biomass slurry for the fermentation process. Typically, nitrogen is added to the biomass slurry after hydrolysis to improve the fermentation process by improving the conditions for the fermenting organism. However, the dissolved air flotation sludge contains enough nitrogen to reduce or alleviate the need to add more nitrogen to the biomass slurry after hydrolysis to improve the fermentation process.

[0025] It is contemplated that the dissolved air flotation sludge may be treated or processed prior to being introduced to the biomass slurry in order to kill live organisms in the sludge. If the live organisms are not killed, it is possible that they may consume simple sugars resulting from the hydrolysis process thereby reducing the amount of sugar available for fermentation. Treatment or processing may include enzymatic methods, thermal methods, mechanical methods, chemical methods, or a combination of methods. The dissolved air flotation sludge may be autoclaved prior to being introduced to the biomass slurry. For example, the dissolved air flotation sludge may be autoclaved at 121°C for 20 minutes.

[0026] It is envisioned that first treating biomass slurry with treated dissolved air flotation sludge and then adding the cellulose hydrolyzing enzyme provides the highest efficiency in cellulose conversion. The treated dissolved air flotation sludge treatment of biomass slurry may also occur simultaneously with the addition of a cellulose-hydrolyzing enzyme to the biomass slurry.

[0027] Without being bound by any particular theory, it is believed that nonspecific binding of treated dissolved air flotation sludge to lignin decreases unproductive binding of enzymes to lignin surfaces or inhibition of enzyme activity due to interactions with lignin. Thus, use of treated dissolved air flotation sludge in a

process for lignocellulose conversion advantageously facilitates a lowering of the enzyme loading used to achieve the same target conversion percentage.

Lignocellulose-Containing Material

[0028] "Lignocellulose" or "lignocellulose-containing material" means material primarily consisting of cellulose, hemicellulose, and lignin. Such material is often referred to as "biomass."

[0029] Biomass is a complex structure of cellulose fibers wrapped in a lignin and hemicellulose sheath. The structure of biomass is such that it is not susceptible to enzymatic hydrolysis. In order to enhance enzymatic hydrolysis, the biomass has to be pretreated, *e.g.*, by acid hydrolysis under adequate conditions of pressure and temperature, in order to break the lignin seal, saccharify and solubilize the hemicellulose, and disrupt the crystalline structure of the cellulose. The cellulose can then be hydrolyzed enzymatically, *e.g.*, by cellulolytic enzyme treatment, to convert the carbohydrate polymers into fermentable sugars which may be fermented into a desired fermentation product, such as ethanol. Hemicellulolytic enzyme treatments may also be employed to hydrolyze any remaining hemicellulose in the pretreated biomass.

[0030] The biomass may be any material containing lignocellulose. In a preferred embodiment, the biomass contains at least about 30 wt. %, preferably at least about 50 wt. %, more preferably at least about 70 wt. %, even more preferably at least about 90 wt. %, lignocellulose. It is to be understood that the biomass may also comprise other constituents such as proteinaceous material, starch, and sugars such as fermentable or un-fermentable sugars or mixtures thereof.

[0031] Biomass is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. Biomass includes, but is not limited to, herbaceous material, agricultural residues, forestry residues, municipal solid wastes, waste paper, and pulp and paper mill residues. It is to be understood that biomass may be in the form of plant cell wall material containing lignin, cellulose, and hemicellulose in a mixed matrix.

[0032] Other examples of suitable biomass include corn fiber, rice straw, pine wood, wood chips, bagasse, paper and pulp processing waste, corn stover, corn cobs, hard wood such as poplar and birch, soft wood, cereal straw such as wheat straw, rice

straw, switch grass, Miscanthus, rice hulls, municipal solid waste (MSW), industrial organic waste, office paper, or mixtures thereof.

[0033] In a preferred embodiment, the biomass is selected from one or more of corn stover, corn cobs, corn fiber, wheat straw, rice straw, switch grass, and bagasse.

Pretreatment

[0034] The biomass may be pretreated in any suitable way. In accordance with the present invention, pretreatment may include the introduction of dissolved air flotation sludge to the biomass.

[0035] Pretreatment is carried out before hydrolysis or fermentation. The goal of pretreatment is to separate or release cellulose, hemicellulose, and lignin and thus improving the rate or efficiency of hydrolysis. Pretreatment methods including wet-oxidation and alkaline pretreatment target lignin release, while dilute acid treatment and auto-hydrolysis target hemicellulose release. Steam explosion is a pretreatment method that targets cellulose release.

[0036] The pretreatment step may include a step wherein dissolved air flotation sludge is added to the biomass. As indicated previously, biomass is typically in the form of biomass slurry when dissolved air flotation sludge is added. If dissolved air flotation sludge is added to the biomass slurry during pretreatment, the remainder of the pretreatment process remains conventional. However, dissolved air flotation sludge may alternatively be added after pretreatment and before hydrolysis or during the hydrolysis step such that the pretreatment step is a conventional pretreatment step using techniques well known in the art.

[0037] Dissolved air flotation sludge may be added in an amount of between about 1 to 40% w/w dissolved air flotation sludge/lignocellulose-containing material. Preferably, it may be added in an amount of between about 5 to 20% w/w dissolved air flotation sludge/lignocellulose-containing material. In a preferred embodiment, biomass pretreatment takes place in aqueous slurry. The biomass may be present during pretreatment in an amount between about 10-80 wt. %, preferably between about 20-70 wt. %, especially between about 30-60 wt. %, such as around about 50 wt. %.

Chemical, Mechanical and/or Biological Pretreatment

[0038] The biomass may be pretreated chemically, mechanically, biologically, or any combination thereof, before or during hydrolysis.

[0039] Preferably the chemical, mechanical or biological pretreatment is carried out prior to hydrolysis. Alternatively, the chemical, mechanical or biological pretreatment may be carried out simultaneously with hydrolysis, such as simultaneously with addition of one or more cellulolytic enzymes, or other enzyme activities, to release, *e.g.*, fermentable sugars, such as glucose or maltose.

[0040] In one embodiment, the pretreated biomass may be washed or detoxified in another way. However, washing or detoxification is not required. In a preferred embodiment, the pretreated biomass is washed or detoxified.

Chemical Pretreatment

[0041] The phrase "chemical pretreatment" refers to any chemical pretreatment which promotes the separation or release of cellulose, hemicellulose, or lignin. Examples of suitable chemical pretreatment methods include treatment with, for example, dilute acid, lime, alkaline, organic solvent, ammonia, sulfur dioxide, or carbon dioxide. Further, wet oxidation and pH-controlled hydrothermolysis are also considered chemical pretreatment.

[0042] In a preferred embodiment, the chemical pretreatment is acid treatment, more preferably, a continuous dilute or mild acid treatment such as treatment with sulfuric acid, or another organic acid such as acetic acid, citric acid, tartaric acid, succinic acid, hydrogen chloride or mixtures thereof. Other acids may also be used. Mild acid treatment means that the treatment pH lies in the range from about pH 1-5, preferably about pH 1-3. In a specific embodiment the acid concentration is in the range from 0.1 to 2.0 wt. % acid and is preferably sulfuric acid. The acid may be contacted with the biomass and the mixture may be held at a temperature in the range of about 160-220°C, such as about 165-195°C, for periods ranging from minutes to seconds, *e.g.*, 1-60 minutes, such as 2-30 minutes or 3-12 minutes. Addition of strong acids such as sulfuric acid may be applied to remove hemicellulose. The addition of strong acids enhances the digestibility of cellulose.

[0043] Other chemical pretreatment techniques are also contemplated according to the invention. Cellulose solvent treatment has been shown to convert about 90% of cellulose to glucose. It has also been shown that enzymatic hydrolysis could be

greatly enhanced when the lignocellulose structure is disrupted. Alkaline H_2O_2 , ozone, organosolv (using Lewis acids, FeCl_3 , $(\text{Al})_2\text{SO}_4$ in aqueous alcohols), glycerol, dioxane, phenol, or ethylene glycol are among solvents known to disrupt cellulose structure and promote hydrolysis (Mosier et al., 2005, Bioresource Technology 96: 673-686).

[0044] Alkaline chemical pretreatment with base, e.g., NaOH , Na_2CO_3 and ammonia or the like, is also contemplated according to the invention. Pretreatment methods using ammonia are described in, e.g., WO 2006/110891, WO 2006/11899, WO 2006/11900, WO 2006/110901, which are hereby incorporated by reference.

[0045] Wet oxidation techniques involve the use of oxidizing agents such as sulphite based oxidizing agents or the like. Examples of solvent pretreatments include treatment with DMSO (dimethyl sulfoxide) or the like. Chemical pretreatment is generally carried out for 1 to 60 minutes, such as from 5 to 30 minutes, but may be carried out for shorter or longer periods of time depending on the material to be pretreated.

[0046] Other examples of suitable pretreatment methods are described by Schell et al., 2003, Appl. Biochem and Biotechn. Vol. 105-108, p. 69-85, and Mosier et al., 2005, Bioresource Technology 96: 673-686, and U.S. Application Publication No. 2002/0164730, each of which are hereby incorporated by reference.

Mechanical Pretreatment

[0047] The phrase "mechanical pretreatment" refers to any mechanical or physical pretreatment which promotes the separation or release of cellulose, hemicellulose, or lignin from biomass. For example, mechanical pretreatment includes various types of milling, irradiation, steaming/steam explosion, and hydrothermolysis.

[0048] Mechanical pretreatment includes comminution, i.e., mechanical reduction of the size. Comminution includes dry milling, wet milling and vibratory ball milling. Mechanical pretreatment may involve high pressure and/or high temperature (steam explosion). "High pressure" means pressure in the range from about 300 to 600 psi, preferably 400 to 500 psi, such as around 450 psi. High temperature means temperatures in the range from about 100 to 300°C, preferably from about 140 to 235°C. In a preferred embodiment, mechanical pretreatment is a batch-process, steam gun hydrolyzer system which uses high pressure and high temperature as

defined above. A Sunds Hydrolyzer (available from Sunds Defibrator AB (Sweden) may be used for this.

Combined Chemical and Mechanical Pretreatment

[0049] In a preferred embodiment, the biomass is pretreated both chemically and mechanically. For instance, the pretreatment step may involve dilute or mild acid treatment and high temperature and/or pressure treatment. The chemical and mechanical pretreatments may be carried out sequentially or simultaneously, as desired.

[0050] Accordingly, in a preferred embodiment, the biomass is subjected to both chemical and mechanical pretreatment to promote the separation or release of cellulose, hemicellulose or lignin.

[0051] In a preferred embodiment pretreatment is carried out as a dilute or mild acid pretreatment step. In another preferred embodiment pretreatment is carried out as an ammonia fiber explosion step (or AFEX pretreatment step).

Biological Pretreatment

[0052] The phrase "biological pretreatment" refers to any biological pretreatment which promotes the separation or release of cellulose, hemicellulose, or lignin from the biomass. Biological pretreatment techniques can involve applying lignin-solubilizing microorganisms. See, for example, Hsu, T.-A., 1996, Pretreatment of biomass, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C.E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh, P., and Singh, A., 1993, Physicochemical and biological treatments for enzymatic/microbial conversion of lignocellulosic biomass, *Adv. Appl. Microbiol.* 39: 295-333; McMillan, J.D., 1994, Pretreating lignocellulosic biomass: a review, in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M.E., Baker, J.O., and Overend, R.P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, chapter 15; Gong, C.S., Cao, N.J., Du, J., and Tsao, G.T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson, L., and Hahn-Hagerdal, B., 1996, Fermentation of lignocellulosic hydrolyzates for ethanol

production, *Enz. Microb. Tech.* 18: 312-331; and Vallander, L., and Eriksson, K.-E. L., 1990, Production of ethanol from lignocellulosic materials: State of the art, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95.

Hydrolysis

[0053] Before the pretreated biomass, preferably in the form of biomass slurry, is fermented it may be hydrolyzed to break down cellulose and hemicellulose into fermentable sugars. In a preferred embodiment, the pretreated material is hydrolyzed, preferably enzymatically, before fermentation.

[0054] The dry solids content during hydrolysis may be in the range from about 5-50 wt. %, preferably about 10-40 wt. %, preferably about 20-30 wt. %. Hydrolysis may in a preferred embodiment be carried out as a fed batch process where the pretreated biomass (*i.e.*, the substrate) is fed gradually to, *e.g.*, an enzyme containing hydrolysis solution.

[0055] In a preferred embodiment hydrolysis is carried out enzymatically. According to the invention, the pretreated biomass slurry may be hydrolyzed by one or more cellulolytic enzymes, such as cellulases or hemicellulases, or combinations thereof.

[0056] In a preferred embodiment, hydrolysis is carried out using a cellulolytic enzyme preparation comprising one or more polypeptides having cellulolytic enhancing activity. In a preferred embodiment, the polypeptide(s) having cellulolytic enhancing activity is of family GH61A origin. Examples of suitable and preferred cellulolytic enzyme preparations and polypeptides having cellulolytic enhancing activity are described in the "Cellulolytic Enzymes" section and "Cellulolytic Enhancing Polypeptides" section below.

[0057] As the biomass may contain constituents other than lignin, cellulose and hemicellulose, hydrolysis and/or fermentation may be carried out in the presence of additional enzyme activities such as protease activity, amylase activity, carbohydrate-generating enzyme activity, and esterase activity such as lipase activity.

[0058] Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions which can readily be determined by one skilled in the art. In a preferred embodiment, hydrolysis is carried out at suitable, preferably optimal, conditions for the enzyme(s) in question.

[0059] Suitable process time, temperature and pH conditions can readily be determined by one skilled in the art. Preferably, hydrolysis is carried out at a temperature between 25 and 70°C, preferably between 40 and 60°C, especially around 50°C. Hydrolysis is preferably carried out at a pH in the range from pH 3-8, preferably pH 4-6, especially around pH 5. In addition, hydrolysis is typically carried out for between 12 and 192 hours, preferably 16 to 72 hours, more preferably between 24 and 48 hours.

Fermentation

[0060] Fermentable sugars from pretreated and/or hydrolyzed biomass may be fermented by one or more fermenting organisms capable of fermenting sugars, such as glucose, xylose, mannose, and galactose directly or indirectly into a desired fermentation product. The fermentation conditions depend on the desired fermentation product and fermenting organism and can easily be determined by one of ordinary skill in the art.

[0061] Especially in the case of ethanol fermentation, the fermentation may be ongoing for between 1-48 hours, preferably 1-24 hours. In an embodiment, the fermentation is carried out at a temperature between about 20 to 40°C, preferably about 26 to 34°C, in particular around 32°C. In one embodiment, the pH is greater than 5. In another embodiment, the pH is from about pH 3-7, preferably 4-6. However, some, e.g., bacterial fermenting organisms have higher fermentation temperature optima. Therefore, in an embodiment, the fermentation is carried out at temperature between about 40-60°C, such as 50-60°C. The skilled person in the art can easily determine suitable fermentation conditions.

[0062] Fermentation can be carried out in a batch, fed-batch, or continuous reactor. Fed-batch fermentation may be fixed volume or variable volume fed-batch. In one embodiment, fed-batch fermentation is employed. The volume and rate of fed-batch fermentation depends on, for example, the fermenting organism, the identity and concentration of fermentable sugars, and the desired fermentation product. Such fermentation rates and volumes can readily be determined by one of ordinary skill in the art.

SSF, HHF and SHF

[0063] Hydrolysis and fermentation may be carried out as a simultaneous hydrolysis and fermentation step (SSF). In general, this means that combined/simultaneous hydrolysis and fermentation are carried out at conditions (e.g., temperature and/or pH) suitable, preferably optimal, for the fermenting organism(s) in question.

[0064] The hydrolysis step and fermentation step may be carried out as hybrid hydrolysis and fermentation (HHF). HHF typically begins with a separate partial hydrolysis step and ends with a simultaneous hydrolysis and fermentation step. The separate partial hydrolysis step is an enzymatic cellulose saccharification step typically carried out at conditions (e.g., at higher temperatures) suitable, preferably optimal, for the hydrolyzing enzyme(s) in question. The subsequent simultaneous hydrolysis and fermentation step is typically carried out at conditions suitable for the fermenting organism(s) (often at lower temperatures than the separate hydrolysis step).

[0065] The hydrolysis and fermentation steps may also be carried out as separate hydrolysis and fermentation, where the hydrolysis is taken to completion before initiation of fermentation. This is often referred to as "SHF".

Recovery

[0066] Subsequent to fermentation, the fermentation product may optionally be separated from the fermentation medium in any suitable way. For instance, the medium may be distilled to extract the fermentation product, or the fermentation product may be extracted from the fermentation medium by micro or membrane filtration techniques. Alternatively, the fermentation product may be recovered by stripping. Recovery methods are well known in the art.

Fermentation Products

[0067] The present invention may be used for producing any fermentation product. Preferred fermentation products include alcohols (e.g., ethanol, methanol, butanol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, gluconic acid); ketones (e.g., acetone); amino acids (e.g., glutamic acid); gases (e.g., H₂ and CO₂); antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B12, beta-carotene); and hormones.

[0068] Other products include consumable alcohol industry products, e.g., beer and wine; dairy industry products, e.g., fermented dairy products; leather industry products and tobacco industry products. In a preferred embodiment, the fermentation product is an alcohol, especially ethanol. The fermentation product, such as ethanol, obtained according to the invention, may preferably be used as fuel alcohol/ethanol. However, in the case of ethanol, it may also be used as potable ethanol.

Enzymes

[0069] Even if not specifically mentioned in the context of a method or process of the invention, it is to be understood that the enzyme(s) as well as other compounds are used in an effective amount. One or more enzymes may be used.

[0070] The phrase "cellulolytic activity" as used herein is understood as comprising enzymes having cellobiohydrolase activity (EC 3.2.1.91), e.g., cellobiohydrolase I and cellobiohydrolase II, as well as endo-glucanase activity (EC 3.2.1.4) and beta-glucosidase activity (EC 3.2.1.21).

[0071] The cellulolytic activity may, in a preferred embodiment, be in the form of a preparation of enzymes of fungal origin, such as from a strain of the genus *Trichoderma*, preferably a strain of *Trichoderma reesei*; a strain of the genus *Humicola*, such as a strain of *Humicola insolens*; or a strain of *Chrysosporium*, preferably a strain of *Chrysosporium lucknowense*.

[0072] The cellulolytic enzyme preparation may contain one or more of the following activities: enzyme, hemienzyme, cellulolytic enzyme enhancing activity, beta-glucosidase activity, endoglucanase, cellobiohydrolase, or xylose isomerase.

[0073] The enzyme may be a composition as defined in PCT/US2008/065417, which is hereby incorporated by reference. For example, the cellulolytic enzyme preparation comprises a polypeptide having cellulolytic enhancing activity, preferably a family GH61A polypeptide, preferably the one disclosed in WO 2005/074656 (Novozymes). The cellulolytic enzyme preparation may further comprise a beta-glucosidase, such as a beta-glucosidase derived from a strain of the genus *Trichoderma*, *Aspergillus* or *Penicillium*, including the fusion protein having beta-glucosidase activity disclosed in WO 2008/057637. The cellulolytic enzyme preparation may also comprise a CBH II enzyme, preferably *Thielavia terrestris* cellobiohydrolase II CEL6A. The cellulolytic enzyme preparation may also comprise

cellulolytic enzymes, preferably one derived from *Trichoderma reesei* or *Humicola insolens*.

[0074] The cellulolytic enzyme preparation may also comprising a polypeptide having cellulolytic enhancing activity (GH61A) disclosed in WO 2005/074656; a beta-glucosidase (fusion protein disclosed in WO 2008/057637) and cellulolytic enzymes derived from *Trichoderma reesei*.

[0075] The cellulolytic enzyme may be the commercially available product CELLUCLAST® 1.5L or CELLUZYME™ available from Novozymes A/S, Denmark or ACCELERASE™ 1000 (from Genencor Inc., USA).

[0076] A cellulolytic enzyme may be added for hydrolyzing pretreated biomass slurry. The cellulolytic enzyme may be dosed in the range from 0.1-100 FPU per gram total solids (TS), preferably 0.5-50 FPU per gram TS, especially 1-20 FPU per gram TS. In another embodiment, at least 0.1 mg cellulolytic enzyme per gram total solids (TS), preferably at least 3 mg cellulolytic enzyme per gram TS, such as between 5 and 10 mg cellulolytic enzyme(s) per gram TS is(are) used for hydrolysis.

Endoglucanase (EG)

[0077] One or more endoglucanases may be present during hydrolysis. The term "endoglucanase" means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. No. 3.2.1.4), which catalyses endo-hydrolysis 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. Endoglucanase activity may be determined using carboxymethyl cellulose (CMC) hydrolysis according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268.

[0078] Endoglucanases may be derived from a strain of the genus *Trichoderma*, preferably a strain of *Trichoderma reesei*; a strain of the genus *Humicola*, such as a strain of *Humicola insolens*; or a strain of *Chrysosporium*, preferably a strain of *Chrysosporium lucknowense*.

Cellobiohydrolase (CBH)

[0079] One or more cellobiohydrolases may be present during hydrolysis. The term "cellobiohydrolase" means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91), 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.

[0080] Examples of cellobiohydrolases are mentioned above including CBH I and CBH II from *Trichoderma reesei*; *Humicola insolens* and CBH II from *Thielavia terrestris* cellobiohydrolase (CELL6A).

[0081] Cellobiohydrolase activity may be determined according to the procedures described by Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279 and by van Tilbeurgh *et al.*, 1982, *FEBS Letters* 149: 152-156; van Tilbeurgh and Claeysens, 1985, *FEBS Letters* 187: 283-288. The Lever *et al.* method is suitable for assessing hydrolysis of cellulose in corn stover and the method of van Tilbeurgh *et al.* is suitable for determining the cellobiohydrolase activity on a fluorescent disaccharide derivative.

Beta-glucosidase

[0082] One or more beta-glucosidases may be present during hydrolysis. The term "beta-glucosidase" means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. For purposes of the present invention, beta-glucosidase activity is determined according to the basic procedure described by Venturi *et al.*, 2002, *J. Basic Microbiol.* 42: 55-66, except different conditions were employed as described herein. One unit of beta-glucosidase activity is defined as 1.0 μ mole of p-nitrophenol produced per minute at 50°C, pH 5 from 4 mM p-nitrophenyl-beta-D-glucopyranoside as substrate in 100 mM sodium citrate, 0.01% TWEEN® 20.

[0083] The beta-glucosidase may be of fungal origin, such as a strain of the genus *Trichoderma*, *Aspergillus* or *Penicillium*. The beta-glucosidase may be derived from *Trichoderma reesei*, such as the beta-glucosidase encoded by the *bgl1* gene (see Fig. 1 of EP 562003). The beta-glucosidase may be derived from *Aspergillus oryzae* (recombinantly produced in *Aspergillus oryzae* according to WO 2002/095014), *Aspergillus fumigatus* (recombinantly produced in *Aspergillus oryzae* according to

Example 22 of WO 2002/095014) or *Aspergillus niger* (1981, J. Appl. Vol 3, pp 157-163).

Hemicellulase

[0084] Hemicellulose can be broken down by hemienzymes and/or acid hydrolysis to release its five and six carbon sugar components. The lignocellulose derived material may be treated with one or more hemicellulases. Any hemicellulase suitable for use in hydrolyzing hemicellulose, preferably into xylose, may be used.

[0085] Preferred hemicellulases include xylanases, arabinofuranosidases, acetyl xylan esterase, feruloyl esterase, glucuronidases, endo-galactanase, mannases, endo or exo arabinases, exo-galactanses, and mixtures of two or more thereof. Preferably, the hemicellulase for use in the present invention is an exo-acting hemicellulase, and more preferably, the hemicellulase is an exo-acting hemicellulase which has the ability to hydrolyze hemicellulose under acidic conditions of below pH 7, preferably pH 3-7. An example of hemicellulase suitable for use in the present invention includes VISCOZYME™ (available from Novozymes A/S, Denmark).

[0086] The hemicellulase may be a xylanase. The xylanase may preferably be of microbial origin, such as of fungal origin (e.g., *Trichoderma*, *Meripilus*, *Humicola*, *Aspergillus*, *Fusarium*) or from a bacterium (e.g., *Bacillus*). The xylanase may be derived from a filamentous fungus, preferably derived from a strain of *Aspergillus*, such as *Aspergillus aculeatus*; or a strain of *Humicola*, preferably *Humicola lanuginosa*. The xylanase may preferably be an endo-1,4-beta-xylanase, more preferably an endo-1,4-beta-xylanase of GH10 or GH11. Examples of commercial xylanases include SHEARZYME™ and BIOFEED WHEAT™ from Novozymes A/S, Denmark.

[0087] The hemicellulase may be added in an amount effective to hydrolyze hemicellulose, such as, in amounts from about 0.001 to 0.5 wt. % of total solids (TS), more preferably from about 0.05 to 0.5 wt. % of TS.

[0088] Xylanases may be added in amounts of 0.001-1.0 g/kg DM (dry matter) substrate, preferably in the amounts of 0.005-0.5 g/kg DM substrate, and most preferably from 0.05-0.10 g/kg DM substrate.

Xylose Isomerase

[0089] Xylose isomerases (D-xylose ketoisomerase) (E.C. 5.3.1.5.) are enzymes that catalyze the reversible isomerization reaction of D-xylose to D-xylulose. Glucose isomerases convert the reversible isomerization of D-glucose to D-fructose. However, glucose isomerase is sometimes referred to as xylose isomerase.

[0090] A xylose isomerase may be used in the method or process and may be any enzyme having xylose isomerase activity and may be derived from any sources, preferably bacterial or fungal origin, such as filamentous fungi or yeast. Examples of bacterial xylose isomerases include the ones belonging to the genera *Streptomyces*, *Actinoplanes*, *Bacillus* and *Flavobacterium*, and *Thermotoga*, including *T. neapolitana* (Vieille et al., 1995, Appl. Environ. Microbiol. 61 (5), 1867-1875) and *T. maritime*. Examples of fungal xylose isomerases are derived species of *Basidiomycetes*.

[0091] A preferred xylose isomerase is derived from a strain of yeast genus *Candida*, preferably a strain of *Candida boidinii*, especially the *Candida boidinii* xylose isomerase disclosed by, e.g., Vongsuvanlert et al., 1988, Agric. Biol. Chem., 52(7): 1817-1824. The xylose isomerase may preferably be derived from a strain of *Candida boidinii* (*Kloeckera 2201*), deposited as DSM 70034 and ATCC 48180, disclosed in Ogata et al., Agric. Biol. Chem, Vol. 33, p. 1519-1520 or Vongsuvanlert et al., 1988, Agric. Biol. Chem, 52(2), p. 1519-1520.

[0092] In one embodiment, the xylose isomerase is derived from a strain of *Streptomyces*, e.g., derived from a strain of *Streptomyces murinus* (U.S. Patent No. 4,687,742); *S. flavovirens*, *S. albus*, *S. achromogenus*, *S. echinatus*, *S. wedmorensis* all disclosed in U.S. Patent No. 3,616,221. Other xylose isomerases are disclosed in U.S. Patent No. 3,622,463, U.S. Patent No. 4,351,903, U.S. Patent No. 4,137,126, U.S. Patent No. 3,625,828, HU patent no. 12,415, DE patent 2,417,642, JP patent no. 69,28,473, and WO 2004/044129, each incorporated by reference herein. The xylose isomerase may be either in immobilized or liquid form. Liquid form is preferred. Examples of commercially available xylose isomerases include SWEETZYME™ T from Novozymes A/S, Denmark. The xylose isomerase is added in an amount to provide an activity level in the range from 0.01-100 IGIU per gram total solids.

Alpha-Amylase

[0093] One or more alpha-amylases may be used. Preferred alpha-amylases are of microbial, such as bacterial or fungal origin. The most suitable alpha-amylase is determined based on process conditions but can easily be done by one skilled in the art.

[0094] The preferred alpha-amylase may be an acid alpha-amylase, e.g., fungal acid alpha-amylase or bacterial acid alpha-amylase. The phrase "acid alpha-amylase" means an alpha-amylase (E.C. 3.2.1.1) which added in an effective amount has activity optimum at a pH in the range of 3 to 7, preferably from 3.5 to 6, or more preferably from 4-5.

Bacterial Alpha-Amylase

[0095] As indicated above, the alpha-amylase may be of *Bacillus* origin. The *Bacillus* alpha-amylase may preferably be derived from a strain of *B. licheniformis*, *B. amyloliquefaciens*, *B. subtilis* or *B. stearothermophilus*, but may also be derived from other *Bacillus* sp. Specific examples of contemplated alpha-amylases include the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 4 in WO 1999/19467, the *Bacillus amyloliquefaciens* alpha-amylase SEQ ID NO: 5 in WO 1999/19467 and the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 3 in WO 1999/19467 (all sequences hereby incorporated by reference). In an embodiment, the alpha-amylase may be an enzyme having a degree of identity of at least 60%, preferably at least 70%, more preferred at least 80%, even more preferred at least 90%, such as at least 95%, at least 96%, at least 97%, at least 98% or at least 99% to any of the sequences shown in SEQ ID NO: 1, 2 or 3, respectively, in WO 1999/19467 (hereby incorporated by reference).

[0096] The *Bacillus* alpha-amylase may also be a variant and/or hybrid, especially one described in any of WO 1996/23873, WO 1996/23874, WO 1997/41213, WO 1999/19467, WO 2000/60059, and WO 2002/10355 (all documents hereby incorporated by reference). Specifically contemplated alpha-amylase variants are disclosed in U.S. Patent No. 6,093,562, 6,297,038 or 6,187,576 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-amylase (BSG alpha-amylase) variants having a deletion of one or two amino acid in positions R179 to G182, preferably a double deletion disclosed in WO 1996/023873 – see e.g., page 20, lines 1-10 (hereby incorporated by reference), preferably corresponding to delta(181-

182) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO: 3 disclosed in WO 1999/19467 or deletion of amino acids R179 and G180 using SEQ ID NO: 3 in WO 1999/19467 for numbering. Even more preferred are *Bacillus* alpha-amylases, especially *Bacillus stearothermophilus* alpha-amylase, which have a double deletion corresponding to delta(181-182) and further comprise a N193F substitution (also denoted I181* + G182* + N193F) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO:3 disclosed in WO 1999/19467.

Bacterial Hybrid Alpha-Amylase

[0097] One or more bacterial hybrid alpha-amylases may be used. A hybrid alpha-amylase specifically contemplated comprises 445 C-terminal amino acid residues of the *Bacillus licheniformis* alpha-amylase (shown in SEQ ID NO: 4 of WO 1999/19467) and the 37 N-terminal amino acid residues of the alpha-amylase derived from *Bacillus amyloliquefaciens* (shown in SEQ ID NO: 5 of WO 1999/19467), with one or more, especially all, of the following substitution:

48A+T49I+G107A+H156Y+A181T+N190F+I201F+A209V+Q264S (using the *Bacillus licheniformis* numbering in SEQ ID NO: 4 of WO 1999/19467). Also preferred are variants having one or more of the following mutations (or corresponding mutations in other *Bacillus* alpha-amylase backbones): H154Y, A181T, N190F, A209V and Q264S and/or deletion of two residues between positions 176 and 179, preferably deletion of E178 and G179 (using the SEQ ID NO: 5 numbering of WO 1999/19467).

Fungal Alpha-Amylase

[0098] One or more fungal alpha-amylases may be used. Fungal alpha-amylases include alpha-amylases derived from a strain of the genus *Aspergillus*, such as, *Aspergillus oryzae*, *Aspergillus niger* and *Aspergillus kawachii* alpha-amylases.

[0099] A preferred acidic fungal alpha-amylase is a Fungamyl-like alpha-amylase, which is derived from a strain of *Aspergillus oryzae*. The phrase "Fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high identity, *i.e.*, more than 70%, more than 75%, more than 80%, more than 85% more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100%

identity to the mature part of the amino acid sequence shown in SEQ ID NO: 10 in WO 1996/23874.

[00100] Another preferred acidic alpha-amylase is derived from a strain *Aspergillus niger*. The acid fungal alpha-amylase may be the one from *A. niger* disclosed as "AMYA_ASPNG" in the Swiss-prot/TrEMBL database under the primary accession no. P56271 and described in WO 1989/01969 (Example 3). A commercially available acid fungal alpha-amylase derived from *Aspergillus niger* is SP288 (available from Novozymes A/S, Denmark).

[00101] The fungal alpha-amylase may also be a wild-type enzyme comprising a starch-binding domain (SBD) and an alpha-amylase catalytic domain (*i.e.*, none-hybrid), or a variant thereof. In an embodiment, the wild-type alpha-amylase may be derived from a strain of *Aspergillus kawachii*.

[00102] Other contemplated wild-type alpha-amylases include those derived from a strain of the genera *Rhizomucor* and *Meripilus*, preferably a strain of *Rhizomucor pusillus* (WO 2004/055178 incorporated by reference) or *Meripilus giganteus*.

[00103] The alpha-amylase may be derived from *Aspergillus kawachii* as disclosed by Kaneko et al., 1996, J. Ferment. Bioeng. 81:292-298, "Molecular-cloning and determination of the nucleotide-sequence of a gene encoding an acid-stable alpha-amylase from *Aspergillus kawachii*"; and further as EMBL:#AB008370.

Fungal Hybrid Alpha-Amylase

[00104] One or more fungal hybrid alpha-amylases may be used. The fungal acid alpha-amylase may be a hybrid alpha-amylase. Examples of fungal hybrid alpha-amylases include the ones disclosed in WO 2005/003311 or U.S. Application Publication No. 2005/0054071 (Novozymes) or US patent application no. 60/638,614 (Novozymes), which are hereby incorporated by reference. A hybrid alpha-amylase may comprise an alpha-amylase catalytic domain (CD) and a carbohydrate-binding domain/module (CBM), such as a starch binding domain, and optionally a linker.

[00105] Specific examples of contemplated hybrid alpha-amylases include those disclosed in Table 1 to 5 of the examples in US patent application no. 60/638,614, including Fungamyl variant with catalytic domain JA118 and *Athelia rolfsii* SBD (SEQ ID NO:100 in US 60/638,614), *Rhizomucor pusillus* alpha-amylase with *Athelia rolfsii* AMG linker and SBD (SEQ ID NO: 101 in US application no. 60/638,614), *Rhizomucor*

pusillus alpha-amylase with *Aspergillus niger* glucoamylase linker and SBD (which is disclosed in Table 5 as a combination of amino acid sequences SEQ ID NO:20, SEQ ID NO:72 and SEQ ID NO:96 in US application no. 11/316,535) or as V039 in Table 5 in WO 2006/069290, and *Meripilus giganteus* alpha-amylase with *Athelia rolfsii* glucoamylase linker and SBD (SEQ ID NO:102 in US application no. 60/638,614). Other specifically contemplated hybrid alpha-amylases are any of the ones listed in Tables 3, 4, 5, and 6 in Example 4 in US application no. 11/316,535 and WO 2006/069290, each hereby incorporated by reference.

[00106] Other specific examples of contemplated hybrid alpha-amylases include those disclosed in U.S. Application Publication no. 2005/0054071, including those disclosed in Table 3 on page 15, such as *Aspergillus niger* alpha-amylase with *Aspergillus kawachii* linker and starch binding domain.

[00107] Contemplated are also alpha-amylases which exhibit a high identity to any of above mention alpha-amylases, *i.e.*, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature enzyme sequences.

[00108] An acid alpha-amylases may according to the invention be added in an amount of 0.1 to 10 AFAU/g DS, preferably 0.10 to 5 AFAU/g DS, especially 0.3 to 2 AFAU/g DS.

Commercial Alpha-Amylase Products

[00109] Preferred commercial compositions comprising alpha-amylase include MYCOLASE from DSM, BAN™, TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ X and SAN™ SUPER, SAN™ EXTRA L (Novozymes A/S) and CLARASE™ L-40,000, DEX-LO™, SPEZYME™ FRED, SPEZYME™ AA, and SPEZYME™ DELTA AA (Genencor Int.), and the acid fungal alpha-amylase sold under the trade name SP288 (available from Novozymes A/S, Denmark).

Carbohydrate-Source Generating Enzyme

[00110] The phrase "carbohydrate-source generating enzyme" includes glucoamylase (being glucose generators), beta-amylase and maltogenic amylase (being maltose generators). A carbohydrate-source generating enzyme is capable of producing a

carbohydrate that can be used as an energy-source by the fermenting organism(s) in question, for instance, when used in a process for producing a fermentation product such as ethanol. The generated carbohydrate may be converted directly or indirectly to the desired fermentation product, preferably ethanol. A mixture of carbohydrate-source generating enzymes may be present. Especially contemplated mixtures are mixtures of at least a glucoamylase and an alpha-amylase, especially an acid amylase, even more preferred an acid fungal alpha-amylase.

Glucoamylase

[00111] One or more glucoamylases may be used. A glucoamylase may be derived from any suitable source, e.g., derived from a microorganism or a plant. Preferred glucoamylases are of fungal or bacterial origin selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al., 1984, EMBO J. 3 (5), p. 1097-1102), and variants thereof, such as those disclosed in WO 1992/00381, WO 2000/04136 and WO 2001/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 1984/02921, *A. oryzae* glucoamylase (Agric. Biol. Chem., 1991, 55 (4), p. 941-949), and variants or fragments thereof. Other *Aspergillus* glucoamylase variants include variants with enhanced thermal stability: G137A and G139A (Chen et al., 1996, Prot. Eng. 9, 499-505); D257E and D293E/Q (Chen et al., 1995, Prot. Eng. 8, 575-582); N182 (Chen et al., 1994, Biochem. J. 301, 275-281); disulphide bonds, A246C (Fierobe et al., 1996, Biochemistry, 35, 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al., 1997, Protein Eng. 10, 1199-1204).

[00112] Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii*), glucoamylase (see U.S. Patent No. 4,727,026 and Nagasaka et al., 1998, "Purification and properties of the raw-starch-degrading glucoamylases from *Corticium rolfsii*, Appl Microbiol Biotechnol 50:323-330), and *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO 1999/28448), *Talaromyces leycettanus* (U.S. Patent No. Re. 32,153), *Talaromyces duponti*, and *Talaromyces thermophilus* (U.S. Patent No. 4,587,215).

[00113] Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP 135,138) and *C.*

thermohydrosulfuricum (WO 1986/01831), and *Trametes cingulata* disclosed in WO 2006/069289 (which is hereby incorporated by reference).

[00114] Hybrid glucoamylases are also contemplated. Examples of the hybrid glucoamylases are disclosed in WO 2005/045018. Specific examples include the hybrid glucoamylase disclosed in Table 1 and 4 of Example 1 of WO 2005/045018, which is hereby incorporated by reference, to the extent it teaches hybrid glucoamylases.

[00115] Contemplated are also glucoamylases that exhibit a high identity to any of the above mentioned glucoamylases, *i.e.*, more than 70%, more than 75%, more than 80%, more than 85% more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature enzymes sequences.

[00116] Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL, SPIRIZYME™ B4U and AMG™ E (from Novozymes A/S); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from Genencor Int.).

[00117] Glucoamylases may be added in an amount of 0.02-20 AGU/g DS, preferably 0.1-10 AGU/g DS, especially between 1-5 AGU/g DS, such as 0.5 AGU/g DS.

Beta-amylase

[00118] One or more beta-amylases may be used. The term "beta-amylase" (E.C 3.2.1.2) is the name traditionally given to exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers. Maltose units are successively removed from the non-reducing chain ends in a step-wise manner until the molecule is degraded or, in the case of amylopectin, until a branch point is reached. The maltose released has the beta anomeric configuration, hence the name beta-amylase.

[00119] Beta-amylases have been isolated from various plants and microorganisms (W.M. Fogarty and C.T. Kelly, Progress in Industrial Microbiology, vol. 15, pp. 112-115, 1979). These beta-amylases are characterized by having optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from 4.5 to 7. A

commercially available beta-amylase from barley is NOVOZYM™ WBA from Novozymes A/S, Denmark and SPEZYME™ BBA 1500 from Genencor Int., USA.

Maltogenic amylase

[00120] One or more maltogenic amylases may be used. The amylase may also be a maltogenic alpha-amylase. A maltogenic alpha-amylase (glucan 1,4-alpha-maltohydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose and amylopectin to maltose in the alpha-configuration. A maltogenic amylase from *Bacillus stearothermophilus* strain NCIB 11837 is commercially available from Novozymes A/S. Maltogenic alpha-amylases are described in U.S. Patent Nos. 4,598,048, 4,604,355 and 6,162,628, which are hereby incorporated by reference. The maltogenic amylase may be added in an amount of 0.05- 5 mg total protein/gram DS or 0.05- 5 MANU/g DS.

Proteases

[00121] A protease may be added during hydrolysis, fermentation or simultaneous hydrolysis and fermentation. The protease may be added to deflocculate the fermenting organism, especially yeast, during fermentation. The protease may be any protease. In a preferred embodiment, the protease is an acid protease of microbial origin, preferably of fungal or bacterial origin. An acid fungal protease is preferred, but also other proteases can be used.

[00122] Suitable proteases include microbial proteases, such as fungal and bacterial proteases. Preferred proteases are acidic proteases, *i.e.*, proteases characterized by the ability to hydrolyze proteins under acidic conditions below pH 7.

[00123] Contemplated acid fungal proteases include fungal proteases derived from *Aspergillus*, *Mucor*, *Rhizopus*, *Candida*, *Coriolus*, *Endothia*, *Enthomophtra*, *Irpex*, *Penicillium*, *Sclerotium* and *Torulopsis*. Especially contemplated are proteases derived from *Aspergillus niger* (see, *e.g.*, Koaze et al., 1964, Agr. Biol. Chem. Japan, 28, 216), *Aspergillus saitoi* (see, *e.g.*, Yoshida, 1954, J. Agr. Chem. Soc. Japan, 28, 66), *Aspergillus awamori* (Hayashida et al., 1977, Agric. Biol. Chem., 42(5), 927-933, *Aspergillus aculeatus* (WO 1995/02044), or *Aspergillus oryzae*, such as the pepA protease; and acidic proteases from *Mucor pusillus* or *Mucor miehei*.

[00124] Also contemplated are neutral or alkaline proteases, such as a protease derived from a strain of *Bacillus*. For example, protease contemplated for the invention is derived from *Bacillus amyloliquefaciens* and has the sequence obtainable at Swissprot as Accession No. P06832. Also contemplated are the proteases having at least 90% identity to amino acid sequence obtainable at Swissprot as Accession No. P06832 such as at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% identity.

[00125] Further contemplated are the proteases having at least 90% identity to amino acid sequence disclosed as SEQ ID NO:1 in WO 2003/048353 such as at 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% identity.

[00126] Also contemplated are papain-like proteases such as proteases within E.C. 3.4.22.* (cysteine protease), such as EC 3.4.22.2 (papain), EC 3.4.22.6 (chymopapain), EC 3.4.22.7 (asclepain), EC 3.4.22.14 (actinidain), EC 3.4.22.15 (cathepsin L), EC 3.4.22.25 (glycyl endopeptidase) and EC 3.4.22.30 (caricain).

[00127] In an embodiment, the protease may be a protease preparation derived from a strain of *Aspergillus*, such as *Aspergillus oryzae*. In another embodiment, the protease may be derived from a strain of *Rhizomucor*, preferably *Rhizomucor meihei*. In another contemplated embodiment, the protease may be a protease preparation, preferably a mixture of a proteolytic preparation derived from a strain of *Aspergillus*, such as *Aspergillus oryzae*, and a protease derived from a strain of *Rhizomucor*, preferably *Rhizomucor meihei*.

[00128] Aspartic acid proteases are described in, for example, Hand-book of Proteolytic Enzymes, Edited by A.J. Barrett, N.D. Rawlings and J.F. Woessner, Academic Press, San Diego, 1998, Chapter 270). Suitable examples of aspartic acid protease include, e.g., those disclosed in R.M. Berka et al., Gene, 96, 313 (1990)); (R.M. Berka et al., Gene, 125, 195-198 (1993)); and Gomi et al., Biosci. Biotech. Biochem. 57, 1095-1100 (1993), which are hereby incorporated by reference.

[00129] Commercially available products include ALCALASE®, ESPERASE™, FLAVOURZYME™, PROMIX™, NEUTRASE®, RENNILASE®, NOVOZYME™ FM 2.0L, and NOVOZYME™ 50006 (available from Novozymes A/S, Denmark) and GC106™ and SPEZYME™ FAN from Genencor Int., Inc., USA.

[00130] The protease may be present in an amount of 0.0001-1 mg enzyme protein per g DS, preferably 0.001 to 0.1 mg enzyme protein per g DS. Alternatively, the protease may be present in an amount of 0.0001 to 1 LAPU/g DS, preferably 0.001 to 0.1 LAPU/g DS and/or 0.0001 to 1 mAU-RH/g DS, preferably 0.001 to 0.1 mAU-RH/g DS.

[00131] The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention as well as combinations of one or more of the embodiments. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

[00132] Various references are cited herein, the disclosures of which are incorporated by reference in their entireties. The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

MATERIALS & METHODS

Methods:

Identity

The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity".

For purposes of the present invention, the degree of identity between two amino acid sequences is determined by the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters are Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

For purposes of the present invention, the degree of identity between two nucleotide sequences is determined by the Wilbur-Lipman method (Wilbur and Lipman, 1983, *Proceedings of the National Academy of Science USA* 80: 726-730) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty

of 10 and gap length penalty of 10. Pairwise alignment parameters are Ktuple=3, gap penalty=3, and windows=20.

Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute under the standard conditions 37°C, pH 4.3, substrate: maltose 23.2 mM, buffer: acetate 0.1 M, reaction time 5 minutes.

An autoanalyzer system may be used. Mutarotase is added to the glucose dehydrogenase reagent so that any alpha-D-glucose present is turned into beta-D-glucose. Glucose dehydrogenase reacts specifically with beta-D-glucose in the reaction mentioned above, forming NADH which is determined using a photometer at 340 nm as a measure of the original glucose concentration.

<u>AMG incubation:</u>	
Substrate:	maltose 23.2 mM
Buffer:	acetate 0.1 M
pH:	4.30 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Enzyme working range:	0.5-4.0 AGU/mL

<u>Color reaction:</u>	
GlucDH:	430 U/L
Mutarotase:	9 U/L
NAD:	0.21 mM
Buffer:	phosphate 0.12 M; 0.15 M NaCl
pH:	7.60 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Wavelength:	340 nm

A folder (EB-SM-0131.02/01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Alpha-amylase activity (KNU)

The alpha-amylase activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (*i.e.*, at 37°C +/- 0.05; 0.0003 M Ca²⁺; and pH 5.6) dextrinizes 5260 mg starch dry substance Merck Amylum solubile.

A folder EB-SM-0009.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Acid alpha-amylase activity (AFAU)

When used according to the present invention the activity of an acid alpha-amylase may be measured in FAU-F (Fungal Alpha-Amylase Unit) or AFAU (Acid Fungal Alpha-amylase Units).

Determination of FAU-F

FAU-F Fungal Alpha-Amylase Units (Fungamyl) is measured relative to an enzyme standard of a declared strength.

Reaction conditions	
Temperature	37°C
pH	7.15
Wavelength	405 nm
Reaction time	5 min
Measuring time	2 min

A folder (EB-SM-0216.02) describing this standard method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

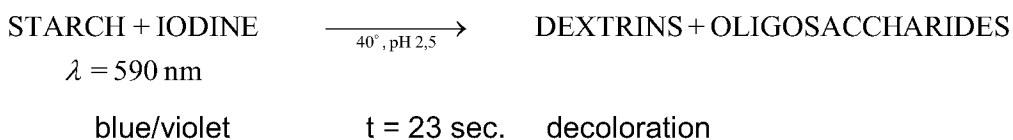
Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard. 1 AFAU is

defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

Acid alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucanohydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the starch molecule to form dextrans and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

ALPHA - AMYLASE



Standard conditions/reaction conditions:

Substrate:	Soluble starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I ₂):	0.03 g/L
CaCl ₂ :	1.85 mM
pH:	2.50 ± 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

A folder EB-SM-0259.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Measurement of Cellulase Activity Using Filter Paper Assay (FPU assay)**1. Source of Method**

1.1 The method is disclosed in a document entitled "Measurement of Cellulase Activities" by Adney and Baker, 1996, Laboratory Analytical Procedure, LAP-006, National Renewable Energy Laboratory (NREL). It is based on the IUPAC method for measuring cellulase activity (Ghose, 1987, Measurement of Cellulase Activities, *Pure & Appl. Chem.* 59: 257-268).

2. Procedure

2.1 The method is carried out as described by Adney and Baker, 1996, *supra*, except for the use of a 96 well plates to read the absorbance values after color development, as described below.

2.2 Enzyme Assay Tubes:

2.2.1 A rolled filter paper strip (#1 Whatman; 1 X 6 cm; 50 mg) is added to the bottom of a test tube (13 X 100 mm).

2.2.2 To the tube is added 1.0 mL of 0.05 M Na-citrate buffer (pH 4.80).

2.2.3 The tubes containing filter paper and buffer are incubated 5 min. at 50°C ($\pm$ 0.1°C) in a circulating water bath.

2.2.4 Following incubation, 0.5 mL of enzyme dilution in citrate buffer is added to the tube. Enzyme dilutions are designed to produce values slightly above and below the target value of 2.0 mg glucose.

2.2.5 The tube contents are mixed by gently vortexing for 3 seconds.

2.2.6 After vortexing, the tubes are incubated for 60 mins. at 50°C ($\pm$ 0.1°C) in a circulating water bath.

2.2.7 Immediately following the 60 min. incubation, the tubes are removed from the water bath, and 3.0 mL of DNS reagent is added to each tube to stop the reaction. The tubes are vortexed 3 seconds to mix.

2.3 Blank and Controls

2.3.1 A reagent blank is prepared by adding 1.5 mL of citrate buffer to a test tube.

2.3.2 A substrate control is prepared by placing a rolled filter paper strip into the bottom of a test tube, and adding 1.5 mL of citrate buffer.

2.3.3 Enzyme controls are prepared for each enzyme dilution by mixing 1.0 mL of citrate buffer with 0.5 mL of the appropriate enzyme dilution.

2.3.4 The reagent blank, substrate control, and enzyme controls are assayed in the same manner as the enzyme assay tubes, and done along with them.

2.4 *Glucose Standards*

2.4.1 A 100 mL stock solution of glucose (10.0 mg/mL) is prepared, and 5 mL aliquots are frozen. Prior to use, aliquots are thawed and vortexed to mix.

2.4.2 Dilutions of the stock solution are made in citrate buffer as follows:

G1 = 1.0 mL stock + 0.5 mL buffer = 6.7 mg/mL = 3.3 mg/0.5 mL

G2 = 0.75 mL stock + 0.75 mL buffer = 5.0 mg/mL = 2.5 mg/0.5 mL

G3 = 0.5 mL stock + 1.0 mL buffer = 3.3 mg/mL = 1.7 mg/0.5 mL

G4 = 0.2 mL stock + 0.8 mL buffer = 2.0 mg/mL = 1.0 mg/0.5 mL

2.4.3 Glucose standard tubes are prepared by adding 0.5 mL of each dilution to 1.0 mL of citrate buffer.

2.4.4 The glucose standard tubes are assayed in the same manner as the enzyme assay tubes, and done along with them.

2.5 *Color Development*

2.5.1 Following the 60 min. incubation and addition of DNS, the tubes are all boiled together for 5 mins. in a water bath.

2.5.2 After boiling, they are immediately cooled in an ice/water bath.

2.5.3 When cool, the tubes are briefly vortexed, and the pulp is allowed to settle. Then each tube is diluted by adding 50 microL from the tube to 200 microL of ddH₂O in a 96-well plate. Each well is mixed, and the absorbance is read at 540 nm.

2.6 *Calculations (examples are given in the NREL document)*

2.6.1 A glucose standard curve is prepared by graphing glucose concentration (mg/0.5 mL) for the four standards (G1-G4) vs. A₅₄₀. This is fitted using a linear regression (Prism Software), and the equation for the line is used to determine the glucose produced for each of the enzyme assay tubes.

2.6.2 A plot of glucose produced (mg/0.5 mL) vs. total enzyme dilution is prepared, with the Y-axis (enzyme dilution) being on a log scale.

2.6.3 A line is drawn between the enzyme dilution that produced just above 2.0 mg glucose and the dilution that produced just below that. From this line, it is determined the enzyme dilution that would have produced exactly 2.0 mg of glucose.

2.6.4 The Filter Paper Units/mL (FPU/mL) are calculated as follows:

$$\text{FPU/mL} = 0.37 / \text{enzyme dilution producing 2.0 mg glucose}$$

Protease Assay method - AU(RH)

The proteolytic activity may be determined with denatured hemoglobin as substrate. In the Anson-Hemoglobin method for the determination of proteolytic activity denatured hemoglobin is digested, and the undigested hemoglobin is precipitated with trichloroacetic acid (TCA). The amount of TCA soluble product is determined with phenol reagent, which gives a blue color with tyrosine and tryptophan.

One Anson Unit (AU-RH) is defined as the amount of enzyme which under standard conditions (*i.e.*, 25°C, pH 5.5 and 10 min. reaction time) digests hemoglobin at an initial rate such that there is liberated per minute an amount of TCA soluble product which gives the same color with phenol reagent as one milliequivalent of tyrosine.

The AU(RH) method is described in EAL-SM-0350 and is available from Novozymes A/S Denmark on request.

Proteolytic Activity (AU)

The proteolytic activity may be determined with denatured hemoglobin as substrate. In the Anson-Hemoglobin method for the determination of proteolytic activity denatured hemoglobin is digested, and the undigested hemoglobin is precipitated with trichloroacetic acid (TCA). The amount of TCA soluble product is determined with phenol reagent, which gives a blue color with tyrosine and tryptophan.

One Anson Unit (AU) is defined as the amount of enzyme which under standard conditions (*i.e.*, 25°C, pH 7.5 and 10 min. reaction time) digests hemoglobin at an initial rate such that there is liberated per minute an amount of TCA soluble product which gives the same color with phenol reagent as one milliequivalent of tyrosine.

A folder AF 4/5 describing the analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Protease assay method (LAPU)

1 Leucine Amino Peptidase Unit (LAPU) is the amount of enzyme which decomposes 1 microM substrate per minute at the following conditions: 26 mM of L-leucine-p-nitroanilide as substrate, 0.1 M Tris buffer (pH 8.0), 37°C, 10 minute reaction time.

LAPU is described in EB-SM-0298.02/01 available from Novozymes A/S Denmark on request.

Determination of Maltogenic Amylase activity (MANU)

One MANU (Maltogenic Amylase Novo Unit) may be defined as the amount of enzyme required to release one micro mole of maltose per minute at a concentration of 10 mg of maltotriose (Sigma M 8378) substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37°C for 30 minutes.

Materials:

Yeast Preparation:

RED STAR™ available from Red Star/Lesaffre, USA

Cellulolytic Preparation A:

Cellulolytic composition comprising a polypeptide having cellulolytic enhancing activity (GH61A) disclosed in WO 2005/074656; a beta-glucosidase (fusion protein disclosed in WO 2008/057637), and cellulolytic enzymes preparation derived from *Trichoderma reesei*. Cellulase preparation A is disclosed in co-pending application PCT/US2008/065417.

Biomass Substrate:

Unwashed pre-treated corn stover (PCS): Acid-catalyzed, steam-exploded obtained from The National Renewable Energy Laboratory, Golden, CO.

Examples

[00133] The effect of adding dissolved air flotation sludge to biomass slurry was tested. Dissolved air flotation slurry was added to washed pretreated corn stover (PCS) slurry prior to hydrolysis in varying amounts. The sugar content was measured at 72 hours after the start of hydrolysis.

[00134] Dissolved air flotation sludge samples were obtained from a pig slaughtering facility in Clinton, NC. Cellulase preparation A was used for hydrolysis. The dissolved air flotation sludge was autoclaved at 121° C for 20 minutes. The treated dissolved air flotation sludge was added to washed pretreated corn stover (PCS) slurry and mixed in amounts of 0.10 g dissolved air flotation sludge/g biomass slurry and 0.20 g dissolved air flotation sludge/g biomass slurry, as shown in Table 1. The mixtures were hydrolyzed by Cellulase preparation A in an amount of 6.0 mg

protein/g total solids at 50° C for 72 hours. In addition, a control of PCS only and dissolved air flotation sludge only was hydrolyzed under the same conditions.

[00135] The content of released sugar was determined by PHBA method and confirmed by HPLC (High Pressure Liquid Chromatography). As shown in Figures 1 and 2, respectively, the addition of treated dissolved air flotation sludge to the enzymatic hydrolysis process increased the final sugar yield and the glucose conversion percentage. When 10% w/w dissolved air flotation sludge/lignocellulose-containing material was added to PCS slurry before hydrolysis, the sugar yield increased from about 23.4 g/L to about 27.5 g/L, and the carbohydrate conversion rate improved from about 69% to about 83%. When 20% w/w dissolved air flotation sludge/lignocellulose-containing material was added to PCS slurry before hydrolysis, the sugar yield increased from about 23.4 g/L to about 29.7 g/L, and the carbohydrate conversion rate improved from about 69% to about 87%. For the dissolved air flotation sludge only control, during the 72 hours of hydrolysis, less than 0.5 g/L of glucose was produced, thus indicating that improved hydrolysis of PCS can be attributed to the favorable interaction between dissolved air flotation sludge and PCS, i.e., the ability of dissolved air flotation sludge to reduce the harmful effects of lignin during hydrolysis.

Sample ID	components	PCS (g, dry wt)	Additives (g, dry wt)	NS50073 (mg EP/g TS)
Control	PCS only	1	0	6
DA F_10%	PCS+DAF	1	0.10	6
DAF_20%	PCS+DAF	1	0.20	6

Table 1. Experimental setup for enzymatic hydrolysis

What is claimed is:

1. A method for producing a fermentation product from a lignocellulose-containing material, comprising:
 - (a) pretreating the lignocellulose-containing material;
 - (b) introducing dissolved air flotation sludge to the pretreated lignocellulose-containing material;
 - (c) exposing the pretreated lignocellulose-containing material to a hydrolyzing enzyme; and
 - (d) fermenting with a fermenting organism to produce a fermentation product.
2. The method of claim 1, wherein the dissolved air flotation sludge is introduced to the lignocellulose-containing material prior to exposing the lignocellulose-containing material to a hydrolyzing enzyme.
3. The method of claim 1, wherein the dissolved air flotation sludge is introduced to the lignocellulose-containing material at the same time as exposing the lignocellulose-containing material to a hydrolyzing enzyme.
4. The method of any of claims 1-3, wherein the dissolved air flotation sludge is introduced to the lignocellulose-containing material in an amount of between about 1 to about 40% w/w dissolved air flotation sludge/lignocellulose-containing material.
5. The method of any of claims 1-4, wherein the dissolved air flotation sludge is introduced to the lignocellulose-containing material in an amount of between about 5 to about 20% w/w dissolved air flotation sludge/lignocellulose-containing material.
6. The method of any of claims 1-3, wherein the dissolved air flotation sludge comprises waste from an agricultural wastewater treatment process.
7. The method of claim 6, wherein the dissolved air flotation sludge comprises waste from a wastewater treatment process associated with slaughtering of animals.

8. The method of any of claims 1-7, wherein the hydrolyzing enzyme to which the lignocellulose-containing material is exposed comprises Cellulase preparation A, or one or more components thereof.
9. The method of any of claims 1-8, wherein the dissolved air flotation sludge is treated prior to being introduced to the pretreated lignocellulose-containing material.
10. The method of claim 9, wherein the dissolved air flotation sludge is treated using methods including enzymatic methods, thermal methods, mechanical methods, chemical methods, or combinations of these methods.
11. The method of claim 9, wherein the dissolved air flotation sludge treatment is autoclaving.
12. The method of any of claims 1-11, wherein the lignocellulose-containing material is pretreated using acid pretreatment.
13. The method of any of claims 1-12, wherein the lignocellulose-containing material is selected from the group consisting of corn stover, corn cobs, corn fiber, switch grass, wheat straw, rice straw, bagasse, and combinations thereof.
14. A method for enhancing enzymatic hydrolysis of a lignocellulose-containing material, comprising:
 - (a) introducing an effective lignin blocking amount of dissolved air flotation sludge to the lignocellulose-containing material, and
 - (b) exposing the lignocellulose-containing material to a hydrolyzing enzyme.
15. The method of claim 14, wherein the dissolved air flotation sludge is introduced to the lignocellulose-containing material prior to exposing the lignocellulose-containing material to a hydrolyzing enzyme.

16. The method of claim 14, wherein the dissolved air flotation sludge is introduced to the lignocellulose-containing material at the same time as exposing the lignocellulose-containing material to a hydrolyzing enzyme.
17. The method of any of claims 14-16, wherein the dissolved air flotation sludge is treated prior to being introduced to the lignocellulose-containing material.
18. The method of any of claims 14-17, wherein the lignocellulose-containing material is selected from the group consisting of corn stover, corn cobs, corn fiber, switch grass, wheat straw, rice straw, bagasse, and combinations thereof.
19. A fermentation product made according to a method comprising:
- (a) pretreating the lignocellulose-containing material;
 - (b) introducing dissolved air flotation sludge to the pretreated lignocellulose-containing material;
 - (c) exposing the pretreated lignocellulose-containing material to an effective amount of a hydrolyzing enzyme; and
 - (d) fermenting with a fermenting organism to produce a fermentation product.
20. A mixture comprising:
- (a) a lignocellulose-containing material;
 - (b) dissolved air flotation sludge; and
 - (c) a hydrolyzing enzyme.

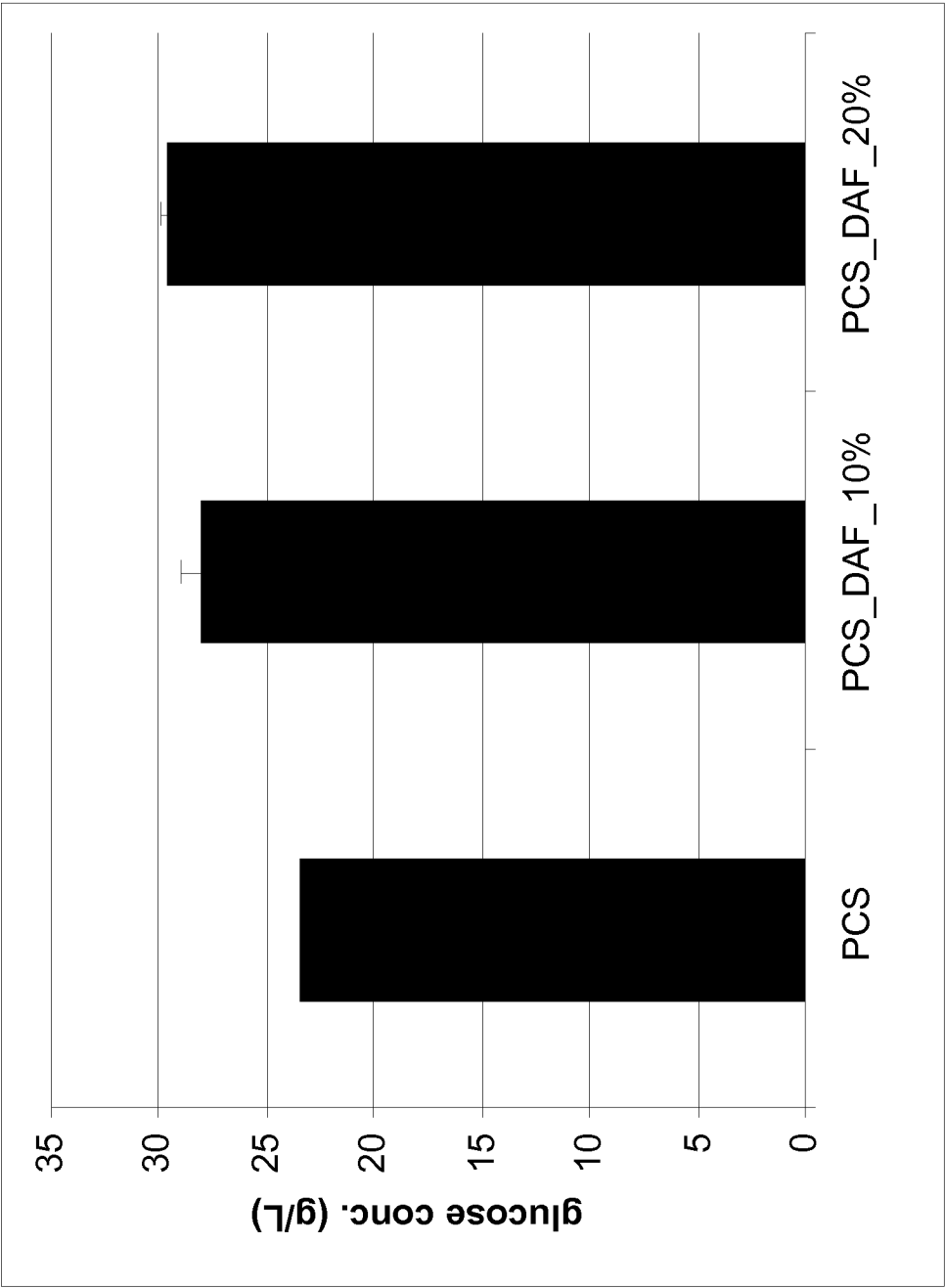


FIGURE 1

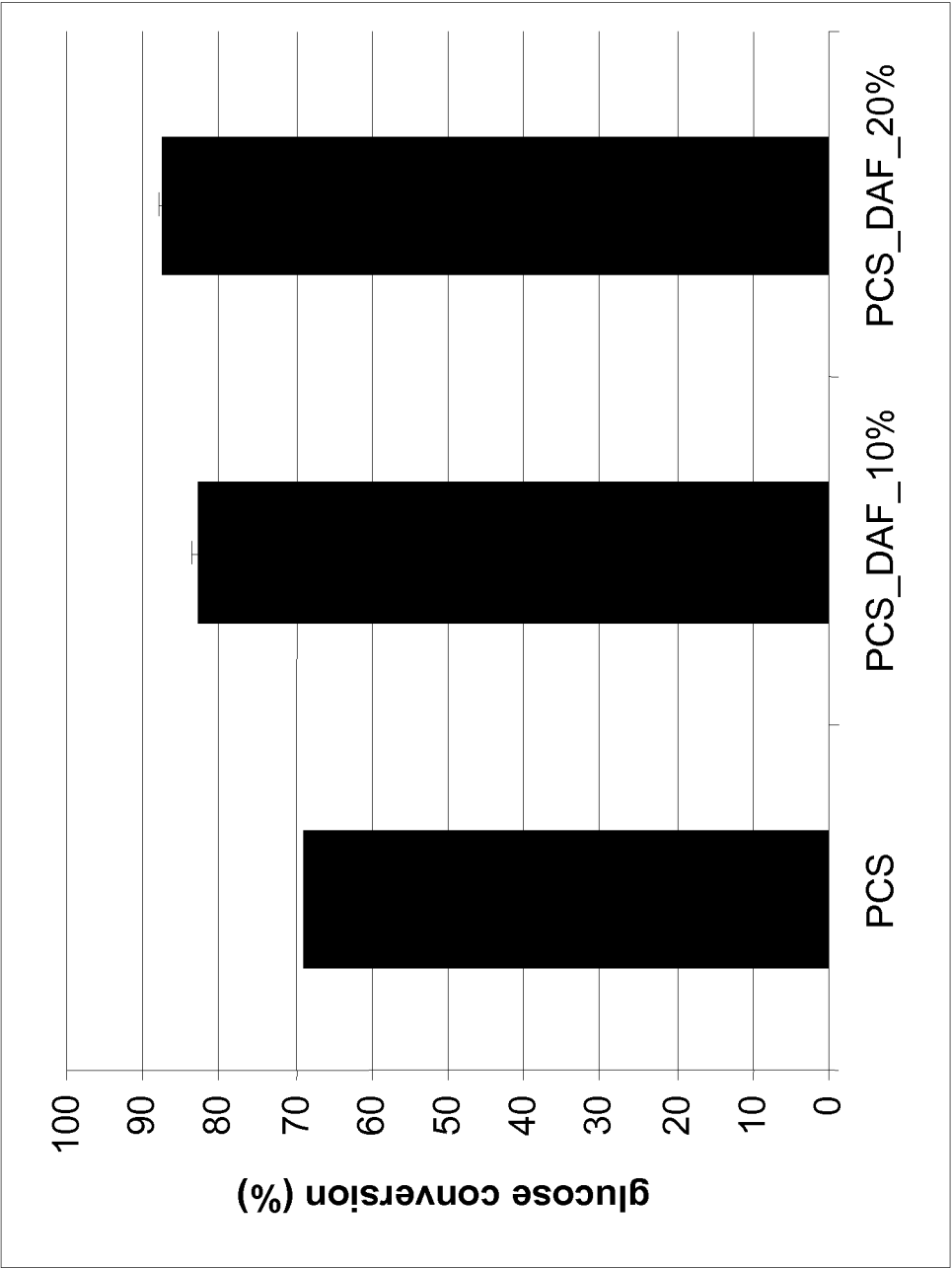


FIGURE 2