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**(54) Title:** ENZYMATIC DEGRADATION OF BIOMASS SUBSTRATES COMPRISING MANNAN

(57) **Abstract:** The present invention relates to processes comprising enzymatic degradation of mannan-containing materials for producing a hydrolyzate. The invention also relates to processes of producing a fermentation product from mannan-containing materials.

WO 2009/074685 A1

# ENZYMATIC DEGRADATION OF BIOMASS SUBSTRATES COMPRISING MANNAN

## TECHNICAL FIELD

The present invention relates to processes comprising enzymatic degradation of mannan-containing materials for producing a hydrolyzate. The invention also relates to processes of producing a fermentation product from mannan-containing materials.

## BACKGROUND OF THE INVENTION

Mannan is a non-starch polysaccharide which is a polymer of the monosaccharide mannose. Mannan is found in plant, fungal and bacterial cell walls. Mannan is present in significant amounts in certain plant residues, such as e.g. palm kernel cake and palm kernel meal.

If substantially complete enzymatic hydrolysis of mannan and cellulose in palm kernel cake/meal could be accomplished the palm kernel cake/meal will be a suitable substrate for the production of bio-ethanol, since the yeast *Saccharomyces cerevisiae,* is able to ferment mannose and glucose to ethanol. The residue obtained after enzymatic hydrolysis and fermentation would in addition make up an animal feed with higher protein content, than the un-treated palm kernel cake/meal.

Processes allowing partial hydrolysis of palm kernel meal are disclosed in EP1 345499, in US6797292, and in Düsterhoft et al (J. Sci. Food Agric. 63, 211-220, 1993) wherein 40-50% of the mannan is solubilised. The aim of the present invention is to provide improved processes, including fermentation processes, comprising enzymatic hydrolysis of both the mannan and the cellulose present in palm kernel cake/meal and similar mannan rich substrates.

## SUMMARY OF THE INVENTION

The aim of the present invention is to provide improved processes, including fermentation processes, comprising enzymatic hydrolysis of both the mannan and the cellulose present in palm kernel cake and similar mannan rich substrates.

In a first aspect the invention relates to a process for converting a mannan-containing material into a fermentation product, the process comprising; a) forming an aqueous slurry comprising the mannan-containing material, b) contacting the slurry with an enzyme composition comprising the enzyme activities cellulase, mannanase, and mannosidase and producing a soluble hydrolyzate, c) contacting the soluble hydrolyzate with a fermenting organism to produce a fermentation product.

In further aspects the invention relates to a composition comprising a cellulase, a mannanase, and a mannosidase, as well as to use of such a composition in a process for hydrolysis of a mannan-containing material comprising contacting said material with said composition.

## DETAILED DESCRIPTION OF THE INVENTION

Mannan-containing materials

The terms "mannan-containing material" used herein refers to a material comprising a significant amount of mannan. Any mannan-containing material is contemplated according to the present invention. In a preferred embodiment the mannan-containing material contains at least 1 wt-%, preferably at least 5 wt.-%, more preferably at least 10 wt-%, even more preferably at least 15 wt-% mannan, yet more preferably at least 20 wt-%, or most preferably at least 25 wt-%. It is to be understood that the mannan-containing material may also comprise other constituents such as cellulosic material, including cellulose and/or hemicellulose, and may also comprise other constituents such as proteinaceous material, starch, sugars, such as fermentable sugars and/or un-fermentable sugars.

Mannan and galacto-mannan is found in plant, fungal and bacterial cell walls. Mannan-containing material is generally found, for example, in the stems, leaves, fruits, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. It is understood herein that mannan-containing material may be in the form of plant cell wall material containing lignin, cellulose, and hemicellulose in a mixed matrix.

The mannan-containing material may be selected from the list consisting of herbaceous and/or woody crops, agricultural food and feed crops, animal feed products, tubers, roots, stems, legumes, cassava peels, cocoa pods, rice husks and/or hulls, rice bran, cobs, straw, hulls, husks, sugar beet pulp, locust bean pulp, vegetable pomaces, agricultural crop waste, straw, stalks, leaves, corn bran, husks, cobs, rind, shells, pods, wood waste, bark, shavings, sawdust, wood pulp, pulping liquor, waste paper, cardboard, wood waste, industrial or municipal waste water solids, manure, by-product from brewing and/or fermentation processes, wet distillers grain, dried distillers grain, spent grain, vinasse and bagasse.

In a preferred embodiment the mannan-containing material is derived from a plant within an *Aracaceae* sp. such as from a genus selected from the group consisting of *Cocos, Elaeis, Coffea,* and *Cyamopsis.* In a more preferred embodiment the mannan-containing material is derived from an *Aracaceae* sp. such as from a species selected from the group consisting of *Cocos nucifera* (coconut), *Elaeis guineensis, Elaeis malanococca* (oil palm), and *Coffea* sp. (coffee) and *Cyamopsis tetragonoloba* (guar bean)

The oil palm *(Elaeis* sp.) tree is grown for its fruits which each comprises an oily, fleshy outer layer (the pericarp), with a single seed (the kernel). Oil can be extracted from both the pericarp and the kernel. Extraction of palm kernel oil from palm kernels can either be done by mechanical extraction or by solvent extraction. The residue obtained after mechanical extraction is called palm kernel cake and the residue obtained after solvent extraction, is called palm kernel meal.

Palm kernel cake contains approximately 50 wt% of non-starch polysaccharides, approximately 20 wt% protein, and approximately 12 wt% lignin. The major polysaccharides in palm kernel cake are mannan (78% of total non-starch polysaccharides), cellulose (12%), arabinoxylan (3%), and 4-O-methyl-glucuronoxylan (3%).

Copra meal is the residue remaining after the coconut oil is pressed from copra, the albumen of Cocos nucifera (expelled copra meal) or the residue remaining after the coconut oil extraction using solvents (extracted copra meal). Copra meal contains 25-32% mannan.

Both oil palm residues and coconut residues are highly suitable as substrates for the present invention.

**Pretreatment**

The mannan-containing material may optionally be pretreated before hydrolysis and/or fermentation. The goal of pretreatment is to separate and/or release non-mannan constituents such as cellulose, hemicellulose and/or lignin and this way improve the rate of hydrolysis of the cellulose and hemicellulose compounds. Pretreatment methods such as wet-oxidation and alkaline pretreatment targets lignin, while dilute acid and auto-hydrolysis targets hemicellulose.

The mannan-containing material may be chemically, mechanically and/or biologically pretreated before hydrolysis and/or fermentation. Mechanical treatment (often referred to as physical treatment) may be used alone or in combination with subsequent or simultaneous hydrolysis, especially enzymatic hydrolysis.

Preferably, the chemical, mechanical and/or biological pretreatment is carried out prior to the hydrolysis and/or fermentation. Alternatively, the chemical, mechanical and/or biological pretreatment may be carried out simultaneously with hydrolysis, such as simultaneously with addition of one or more of the enzyme activities mentioned below, to release, e.g., fermentable sugars, such as mannose, glucose and/or maltose.

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

The chemical pretreatment may be an acid treatment, more preferably, a continuous dilute and/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 1-5, preferably pH 1-3.

Alkaline chemical pretreatment with base, e.g., NaOH, $Na_2CO_3$ and/or ammonia or the like, is also contemplated according to the invention. Pretreatment methods using ammonia are described in, e.g., WO 2006/110891, WO 2006/111899, WO 2006/111900, WO 2006/110901

(which are hereby incorporated by reference)

Wet oxidation techniques involve 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 dependent on the material to be pretreated.

Other examples of suitable pretreatment methods are described by Schell et al. (2003) Appl. Biochem and Biotechn. Vol. 105-108, p. 69-85, Mosier et al. Bioresource Technology 96 (2005) 673-686, Ahring et al. in WO2006032282 and WO200160752, Foody et al. in WO2006034590, and Ballesteros et al. in US publication no. 2002/0164730, which references are hereby all incorporated by reference.

The term "mechanical pretreatment" refers to any mechanical (or physical) treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin from the mannan-containing material. For example, mechanical pretreatment includes various types of milling, irradiation, steaming/steam explosion, wet oxidation, and other hydrothermal treatments.

Mechanical pretreatment includes comminution (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). In an embodiment of the invention high pressure means pressure in the range from 300 to 600 psi, preferably 400 to 500 psi, such as around 450 psi. In an embodiment of the invention high temperature means temperatures in the range from about 100 to 300 $^0$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.

As used in the present invention the term "biological pretreatment" refers to any biological pretreatment which promotes the degradation of the mannan-containing material. 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 hydrolysates 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).

In a preferred embodiment wherein the mannan-containing material is palm kernel meal or palm kernel cake the mannan-containing material is not subjected to a preatreatment.

**Hydrolysis**

The mannan-containing material is hydrolyzed to break down mannan, cellulose and hemicellulose into sugars and/or oligosaccharides. The hydrolysis may be performed prior to and/or simultaneously with a fermentation step.

According to the invention the mannan-containing material to be hydrolyzed is contacted by a cellulase, a mannanase and a mannosidase. The mannan-containing material is converted into a soluble hydrolyzate, in a process comprising a) forming an aqueous slurry comprising the biomass substrate, b) contacting the slurry with an enzyme composition comprising the enzyme activities cellulase, mannanase, and mannosidase and producing a soluble hydrolyzate.

In an embodiment one or more enzymes of galactomannanase, beta-xylosidase, endo-1,4-beta xylanase, beta-glucosidase, alpha-galactosidase, alpha-L-arabinofuranosidase, acetyl xylan esterase, ferulic acid esterase and alpha-glucuronidase are present. Also alpha-amylase, glucoamylase and/or the like may be present during hydrolysis and/or fermentation as the mannan-containing material may include some starch. Galactomannanase is preferably applied if the substrate comprises galactomannan.

The enzyme(s) used for hydrolysis is(are) capable of directly or indirectly converting mannan and other carbohydrate polymers into fermentable sugars which can be fermented into a desired fermentation product, such as ethanol. Suitable enzymes are described in the E nzymes"-section below.

The dry solids content during hydrolysis may be in the range from 5-50 wt-%, preferably 10-40 wt-%, preferably 20-30 wt-%. Hydrolysis may in a preferred embodiment be carried out as a fed batch process where the mannan-containing material (substrate) is fed gradually to an enzyme containing hydrolysis solution. The mannan-containing material may be supplied to the enzyme containing hydrolysis solution either in one or more distinct batches, as one or more distinct continuous flows or as a combination of one or more distinct batches and one or more distinct continuous flows.

Suitable hydrolysis time, temperature and pH conditions can readily be determined by one skilled in the art. The temperature should be decided with regard to the optimum temperatures of the applied enzymes. Generally a temperature between 25°C and 70$^0$C, preferably between 40$^0$C and 60°C, more preferably between 45°C and 55°C, especially around 50$^0$C will be suitable. The process is preferably carried out at a pH in the range from 3-8, preferably pH 4-6, especially around pH 5, e.g., in the range of pH 4.5 to 5.5.. Preferably,

hydrolysis is carried out for between 12 and 144 hours, preferable 16 to 120 hours, more preferably between 24 and 96 hours, such as between 32 and 48 hours.

Preferably the hydrolysis in step b) is allowed to proceed for a time and under conditions allowing that at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, or even at least 90% of the mannan content of the mannan-containing material is degraded to mannose and/or for a time and under conditions allowing that at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or even at least 95% of the cellulose content of the mannan-containing material is degraded to glucose.

During the hydrolysis of substrates such as oil palm or palm kernel cake, palm kernel meal and/ or copra cake residual oil is released and can easily be isolated from the soluble hydrolyzate. Thus in a preferred embodiment the process comprises isolating an oil fraction from the soluble hydrolyzate, e.g., where the mannan-containing material is derived from a plant comprising plant oil, such as a residue produced in a vegetable oil extraction process.

**Fermentation**

According to the invention the hydrolyzed mannan-containing material is fermented by at least one fermenting organism capable of fermenting fermentable sugars, such as mannose, glucose, and galactose directly or indirectly into a desired fermentation product. Accordingly, in an embodiment a mannan-containing material is converted into a soluble hydrolyzate, in a process comprising a) forming an aqueous slurry comprising the biomass substrate, b) contacting the slurry with an enzyme composition comprising the enzyme activities cellulase, mannanase, and mannosidase and producing a soluble hydrolyzate, and c) contacting the soluble hydrolyzate with a fermenting organism to produce a fermentation product.

Preferred for ethanol fermentation is yeast of the species *Saccharomyces cerevisiae,* preferably strains which are resistant towards high levels of ethanol, i.e., up to, e.g., about 10, 12 or 15 vol. % ethanol or more, such as 20 vol. % ethanol. The fermentation is preferably ongoing for between 8 to 96 hours, preferably 12 to 72, more preferable from 24 to 48 hours. In an embodiment the fermentation is carried out at a temperature between 20 to 40$^0$C, preferably 26 to 34°C, in particular around 32°C. In an embodiment the pH is from pH 3 to 6, preferably around pH 4 to 5.

According to the invention hydrolysis in step (b) and fermentation in step (c) may be carried out simultaneously or sequentially. In an embodiment there is no separate holding stage for the hydrolysis, meaning that the hydrolysing enzyme(s) and the fermenting organism are added together. When the fermentation (e.g., ethanol fermentation using *Saccharomyces* yeast) is performed simultaneous with hydrolysis the temperature is preferably between 26°C and 35°C, more preferably between 30$^0$C and 34°C, such as around 32°C. A temperature program comprising at least two holding stages at different temperatures may be applied according to the invention.

The process of the invention as well as the individual steps may be performed as a batch, fed-batch or as a continuous process/process step. Preferably the fermentation step is performed as a continuous fermentation.

Recovery

Subsequent to fermentation the fermentation product may be separated from the fermentation broth. The broth may be distilled to extract the fermentation product or the fermentation product may be extracted from the fermentation broth 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

The process of the invention may be used for producing any fermentation product. Especially contemplated 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., $H_2$ and $CO_2$); antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B12, beta-carotene); and hormones.

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 fuel alcohol/ethanol. However, in the case of ethanol it may also be used as potable ethanol.

Fermenting Organism

The term "fermenting organism" refers to any organism, including bacterial and fungal organisms, suitable for producing a desired fermentation product. Especially suitable fermenting organisms according to the invention are able to ferment, i.e., convert, C6 sugars, such as mannose and glucose, directly or indirectly into the desired fermentation product. Also suitable are fermenting organisms capable of converting C5 sugars such as xylose into a desired fermentation product. Examples of fermenting organisms include fungal organisms, especially yeast. Preferred yeast includes strains of *Saccharomyces* spp., in particular a strain of *Saccharomyces cerevisiae* or *Saccharomyces uvarum;* a strain of *Pichia,* preferably *Pichia stipitis,* such as *Pichia stipitis* CBS 5773; a strain of *Candida,* in particular a strain of *Candida utilis, Candida diddensii,* or *Candida boidinii.* Other contemplated yeast includes strains of *Zymomonas; Hansenula,* in particular *H. anomala; Klyveromyces,* in particular *K. fragilis;* and *Schizosaccharomyces,* in particular *S. pombe.*

Commercially available yeast includes, e.g., ETHANOL RED™ yeast (available from Fermentis/Lesaffre, USA), FALI (available from Fleischmann's Yeast, USA), SUPERSTART and THERMOSACC™ fresh yeast (available from Ethanol Technology, WI, USA), BIOFERM

AFT and XR (available from NABC - North American Bioproducts Corporation, GA, USA), GERT STRAND (available from Gert Strand AB, Sweden), and FERMIOL (available from DSM Specialties). ANQI YEAST (available from Anqi yeast (CHIFENG) CO., LTD, China).

Compositions

The present invention also relates to compositions suitable for use in the processes of the invention, said compositions comprising a cellulase, a mannanase, and a mannosidase. The composition may comprise additional enzymatic activities, such as acetylxylan esterase, alpha-glucuronidase, arabinofuranosidase, aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, ferulic acid esterase, galactomannanase alpha-galactosidase, beta-galactosidase, beta-xylosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, haloperoxidase, invertase, laccase, lipase, mannosidase, oxidase, pectinolytic enzyme, peptidoglutaminase, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase or xylosidase.

**Enzymes**

Even though not specifically mentioned in context of a process of the invention, it is to be understood that the enzymes (as well as other compounds) are used in an "effective amount".

Cellulases

The term "cellulases" as used herein are understood as comprising the cellobiohydrolases (EC 3.2.1 .91 ), e.g., cellobiohydrolase I and cellobiohydrolase II, as well as the endo-glucanases (EC 3.2.1 .4) and beta-glucosidases (EC 3.2.1 .21 ).

In order to be efficient, the digestion of cellulose and hemicellulose requires several types of enzymes acting cooperatively. At least three categories of enzymes are necessary to convert cellulose into fermentable sugars: endo-glucanases (EC 3.2.1 .4) that cut the cellulose chains at random; cellobiohydrolases (EC 3.2.1 .91 ) which cleave cellobiosyl units from the cellulose chain ends and beta-glucosidases (EC 3.2.1 .21) that convert cellobiose and soluble cellodextrins into glucose. Among these three categories of enzymes involved in the biodegradation of cellulose, cellobiohydrolases are the key enzymes for the degradation of native crystalline cellulose. The term "cellobiohydrolase I" is defined herein as a cellulose 1,4-beta-cellobiosidase (also referred to as exo-glucanase, exo-cellobiohydrolase or 1,4-beta-cellobiohydrolase) activity, as defined in the enzyme class EC 3.2.1 .91 , which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose and cellotetraose, by the release of cellobiose from the non-reducing ends of the chains. The definition of the term "cellobiohydrolase II activity" is identical, except that cellobiohydrolase II attacks from the

reducing ends of the chains.

Endoglucanases (EC No. 3.2.1 .4) catalyses endo hydrolysis of 1,4- beta -D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxy methyl cellulose and hydroxy ethyl 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 parts. The authorized name is endo-1,4- beta -D-glucan 4-glucano hydrolase, but the abbreviated term endoglucanase is used in the present specification.

The cellulases may comprise a carbohydrate-binding module (CBM) which enhances the binding of the enzyme to a cellulose-containing fiber and increases the efficacy of the catalytic active part of the enzyme. A CBM is defined as contiguous amino acid sequence within a carbohydrate-active enzyme with a discreet fold having carbohydrate-binding activity. For further information of CBMs see the CAZy internet server (Supra) or Tomme et al., (1995) in Enzymatic Degradation of Insoluble Polysaccharides (Saddler, J.N. & Penner, M., eds.), Cellulose-binding domains: classification and properties. pp. 142-163, American Chemical Society, Washington.

The cellulase activity may, in a preferred embodiment, be derived from a fungal source, such as 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.*

In a preferred embodiment the cellulases may a preparation as defined in co-pending application US application US 60/941 ,251 , which is hereby incorporated by reference. In a preferred embodiment the cellulase preparation comprising a polypeptide having cellulolytic enhancing activity (GH61A), preferably the one disclosed as SEQ ID NO:2 in WO 2005/074656. The cellulase preparation may further comprise a beta-glucosidase, such as the fusion protein disclosed in US 60/832,51 1. In an embodiment the cellulase preparation also comprises a CBH II, preferably *Thielavia terrestris* cellobiohydrolase II CEL6A. In an embodiment the cellulase preparation also comprises a cellulase enzymes preparation, preferably the one derived from *Trichoderma reesei.* In a preferred embodiment the cellulase preparation is Cellulase preparation A used in Example 1 and disclosed in co-pending US application US 60/941 ,251 .

In an embodiment the cellulase preparation is the commercially available product CELLUCLAST® 1.5L or CELLUZYME™ (Novozymes A/S, Denmark).

The cellulase may be dosed in the range from 0.1-100 FPU per gram dry solids (DS), preferably 0.5-50 FPU per gram DS, especially 1-20 FPU per gram DS.

Mannanases

In the context of the present invention a mannanase is a beta-mannanase and defined as an enzyme belonging to EC 3.2.1 .78.

Mannanases have been identified in several *Bacillus* organisms. For example, Talbot et al., Appl. Environ. Microbiol., Vol. 56, No. 11, pp. 3505-3510 (1990) describes a beta-mannanase derived from *Bacillus stearothermophilus* having an optimum pH of 5.5-7.5. Mendoza et al., World J. Microbiol. Biotech., Vol. 10, No. 5, pp. 551-555 (1994) describes a beta-mannanase derived from *Bacillus subtilis* having an optimum activity at pH 5.0 and 55°C. JP-03047076 discloses a beta-mannanase derived from *Bacillus sp.,* having an optimum pH of 8-10. JP-63056289 describes the production of an alkaline, thermostable beta-mannanase. JP-08051975 discloses alkaline beta-mannanases from alkalophilic *Bacillus sp.* AM-001. A purified mannanase from *Bacillus amyloliquefaciens* is disclosed in WO 97/11164. WO 94/25576 discloses an enzyme from *Aspergillus aculeatus,* CBS 101.43, exhibiting mannanase activity and WO 93/24622 discloses a mannanase isolated from *Trichoderma reesei.*

The mannanase may be derived from a strain of the genus *Bacillus,* such as the amino acid sequence having the sequence deposited as GENESEQP accession number AAY54122 and shown herein as SEQ ID NO:1 or an amino acid sequence which is homologous to this amino acid sequence.

A suitable commercial mannanase preparation is Mannaway® produced by Novozymes A/S.

Mannosidases

In the context of the present invention a mannosidase, such as a beta-mannosidase, is defined as an enzyme belonging to EC 3.2.1.25.

A suitable mannosidase preparation can be obtained from *Aspergillus niger,* such as e.g. a mannosidase preparation comprising the mannosidase having the amino acid sequence deposited as Swiss-Prot accession number A2QWU and shown herein as SEQ ID NO:2., or an amino acid sequence which is homologous to this amino acid sequence..

Hemicellulases

Any hemicellulase suitable for use in hydrolyzing hemicellulose may be used. Preferred hemicellulases include xylanases, arabinofuranosidases, acetyl xylan esterase, feruloyl esterase, glucuronidases, endo-galactanase, endo or exo arabinases, exo-galactanases, 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 ULTRAFLO L (available from Novozymes A/S, Denmark).

Arabinofuranosidase (EC 3.2.1.55) catalyses the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha -L-arabinosides.

Galactanase (EC 3.2.1 .89), arabinogalactan endo-1 ,4-beta-galactosidase, catalyses the endohydrolysis of 1,4-D-galactosidic linkages in arabinogalactans.

Pectinase (EC 3.2.1 .15) catalyses the hydrolysis of 1,4-alpha-D-galactosiduronic linkages in pectate and other galacturonans.

Xyloglucanase catalyses the hydrolysis of xyloglucan.

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 dry solids (DS), more preferably from about 0.05 to 0.5 wt.-% of DS.

Alpha-Amylase

According to the invention an alpha-amylase may be used. In a preferred embodiment the alpha-amylase is an acid alpha-amylase, e.g., fungal acid alpha-amylase or bacterial acid alpha-amylase. The term "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

According to the invention the bacterial alpha-amylase is preferably derived from the genus *Bacillus.*

In a preferred embodiment the *Bacillus* alpha-amylase is 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 99/19467, the *Bacillus amyloliquefaciens* alpha-amylase SEQ ID NO:5 in WO 99/19467 and the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO:3 in WO 99/19467 (all sequences hereby incorporated by reference). In an embodiment of the invention 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 NOS: 1, 2 or 3, respectively, in WO 99/19467.

The *Bacillus* alpha-amylase may also be a variant and/or hybrid, especially one described in any of WO 96/23873, WO 96/23874, WO 97/41213, WO 99/19467, WO 00/60059, and WO 02/10355 (all documents hereby incorporated by reference). Specifically contemplated alpha-amylase variants are disclosed in US patent nos. 6,093,562, 6,297,038 or US patent no. 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 99/19467 or deletion of amino acids R179 and G180 using SEQ ID NO:3 in WO 99/19467 for numbering (which reference is hereby incorporated by reference). 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 11 8 1* + G182 * + N193F) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO:3 disclosed in WO 99/19467.

Fungal Alpha-Amylase

Fungal alpha-amylases include alpha-amylases derived from a strain of the genus *Aspergillus,* such as, *Aspergillus oryzae, Aspergillus niger* and *Aspergillis kawachii* alpha-amylases.

A preferred acidic fungal alpha-amylase is a Fungamyl-like alpha-amylase which is derived from a strain of *Aspergillus oryzae.* According to the present invention, the term "Fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high identity, i.e. at least 70%, at least 75%, at least 80%, at least 85% at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity to the mature part of the amino acid sequence shown in SEQ ID NO:10 in WO 96/23874.

Another preferred acidic alpha-amylase is derived from a strain *Aspergillus niger.* In a preferred embodiment the acid fungal alpha-amylase is the one from *A. niger* disclosed as "AMYA_ASPNG" in the Swiss-prot/TeEMBL database under the primary accession no. P56271 and described in WO 89/01969 (Example 3).

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 2004/0551 78 incorporated by reference) or *Meripilus giganteus.*

In a preferred embodiment the alpha-amylase is derived from *Aspergillus kawachii* and disclosed by Kaneko et al. J. Ferment. Bioeng. 8 1:292-298(1996) "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.

Glucoamylases

A glucoamylase used according to the invention 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* G 1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1 102), or variants thereof, such as those disclosed in WO 92/00381 , WO 00/04136 and WO 01/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 84/02921 , *A. oryzae* glucoamylase (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or 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.

Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii)* glucoamylase (see US patent no. 4,727,026 and (Nagasaka,Y. et al. (1998) "Purification and properties of the raw-starch-degrading glucoamylases from *Corticium rolfsii,* Appl Microbiol Biotechnol 50:323-330), *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (US patent no. Re. 32,153), *Talaromyces duponti, Talaromyces thermophilus* (US patent no. 4,587,215).

Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium,* in particular *C. thermoamylolyticum* (EP 135,138), and *C. thermohydrosulfuricum* (WO 86/01831) and *Trametes cingulata* disclosed in WO 2006/069289 (which is hereby incorporated by reference).

Also hybrid glucoamylase are contemplated according to the invention. Examples the hybrid glucoamylases disclosed in WO 2005/045018. Specific examples include the hybrid glucoamylase disclosed in Table 1 and 4 of Example 1 (which hybrids are hereby incorporated by reference.).

Contemplated are also glucoamylases which exhibit a high identity to any of above mention glucoamylases, i.e., at least 70%, at least 75%, at least 80%, at least 85% at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity to the mature enzymes sequences.

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.).

Glucoamylases may in an embodiment 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.

Animal Feed

The solids remaining after enzymatic hydrolysis and fermentation have an improved nutritional value as an animal feed compared to the un-treated manna-containing material, e.g., un-treated palm kernel cake/meal. During hydrolysis and fermentation the protein content is increased through the removal of polysaccharides just as the digestibility of the remaining polysaccharides is increased. Furthermore, the protein composition is improved by the yeast residues.

Accordingly, the solids remaining after the process of the first aspect are very suitable

as an animal feed or for inclusion in an animal feed composition.

The term animal includes all animals, including human beings. Examples of animals are non-ruminants, and ruminants. Ruminant animals include, for example, animals such as sheep, goats, horses, and cattle, e.g. beef cattle, cows, and young calves. In a particular embodiment, the animal is a non-ruminant animal. Non-ruminant animals include mono-gastric animals, e.g. pigs or swine (including, but not limited to, piglets, growing pigs, and sows); poultry such as turkeys, ducks and chicken (including but not limited to broiler chicks, layers); young calves; and fish (including but not limited to salmon, trout, tilapia, catfish and carps; and crustaceans (including but not limited to shrimps and prawns).

The term feed or feed composition means any compound, preparation, mixture, or composition suitable for, or intended for intake by an animal.

## MATERIALS & METHODS

### Chemicals

Arabinose, galactose, glucose, xylose, mannose, and succinic acid were purchased from Merck (Darmstadt, Germany). Sulphuric acid was from Bie & Berntsen A/S (Rødovre, Denmark).

### Substrate

Palm kernel cake was from Indonesia and having a dry matter content of 96 wt%.

### Enzymes

Cellulase composition A is a cellulolytic composition comprising a polypeptide having cellulolytic enhancing activity (GH61A) disclosed as SEQ ID NO:2 in WO 2005/074656; an *Aspergillus oryzae* beta-glucosidase (in the fusion protein disclosed in US 60/832,51 1), and an cellulolytic enzymes preparation derived from *Trichoderma reesei* comprising cellobiohydrolase I and cellobiohydrolase II, endo-glucanase and beta-glucosidase, as well as endo-1 ,4- beta-xylanase and beta-xylosidase sideactivities.

Mannanase composition B comprises a *Bacillus* mannanase having the sequence deposited as GENESEQP accession number AAY54122 and shown herein as SEQ ID NO:1 (NP001840).

Mannosidase composition C comprising a mannosidase derived from *Aspergillus niger* and having the sequence deposited as SWISSPROT accession number A2QWU9 and shown herein as SEQ ID NO:2.

### Determination of identity

The relatedness between two amino acid sequences or between two nucleotide

sequences is described by the parameter "identity".

The degree of identity between two amino acid sequences may be 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.

The degree of identity between two nucleotide sequences may be 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.

Amino acid sequences or nucleotide sequences which exhibit a high identity to a particular amino acid or nucleotide sequence, i.e., at least 60%, at least 70%, at least 75%, at least 80%, at least 85% at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity, are consider homologous to that particular amino acid or nucleotide sequence.

**High Performance Anion Exchange Chromatography (HPAEC)**

Hydrolysates (10 µl) were applied onto a Dionex BioLC system fitted with a Dionex CarboPac™ PA1 analytical column (4 x 250 mm)(Dionex Corporation, Sunnyvale, CA, USA) combined with a CarboPac™ PA1 precolumn (4 x 50 mm). The monosaccharides were separated isocratically with 0.001 M KOH for 40 min, flow: 1 mL-min$^{-1}$. Monosaccharides were detected by a pulsed electrochemical detector in the pulsed amperiometric detection mode. The potential of the electrode was programmed for +0.1 V (t = 0-0.4 s) to -2.0 V (t = 0.41-0.42 s) to 0.6 V (t = 0.43 s) and finally -0.1 V (t = 0.44-0.50 s), while integrating the resulting signal from t = 0.2-0.4 s. A mixture of arabinose, galactose, glucose, xylose, and mannose (concentration of each component: 0.0025-0.075 g-L$^{-1}$) was used as standard.

**EXAMPLES**

**Example 1**

**Determination of Monosaccharide Composition and Content**

Palm kernel cake was degraded using sulphuric acid hydrolysis following the standard procedure from the US National Renewable Energy Laboratory (NREL)(Laboratory analytical procedure 002 (1996)). The samples were then centrifuged (20800 g, 10 min), and the monosaccharide composition and content (g-L$^{-1}$) was determined by HPAEC, see above. The values are the mean of three determinations and are reported in g/kg palm kernel cake DM.

The polysaccharide material in palm kernel cake was composed of primarily mannose and glucose, with the net acid hydrolysis yield of mannose and glucose being 358 g/kg DM and -86.6 g/kg DM, respectively (Table 1). Summation of the monosaccharides resulting after acid hydrolysis indicated that the carbohydrates made up >51 wt% of the palm kernel cake DM (Table 1).

**Table 1.** Monosaccharide composition of palm kernel cakel determined by acid hydrolysis and HPAEC. Results are expressed as g-kg$^{-1}$ palm kernel cake DM ± S.D

| Arabinose | Galactose | Glucose | Xylose | Mannose |
|---|---|---|---|---|
| 14.6 ± 1.97 | 24.9 ± 3.03 | 87.8 ± 2.71 | 33.0 ± 6.10 | 358 ± 32.8 |

**Example 2**

**Enzymatic hydrolysis of palm kernel cake**

Palm kernel cake was incubated with a mixture of cellulase preparation A, mannanase preparation B and mannosidase preparation C added to a total E/S level of 2.5 wt% (25 mg EP/g DM) per assay at pH 5 and 50°C. The ratio E/S relate to the weight of enzyme protein (E) added in percent per weight of the palm kernel cake DM (S). The substrate level was always 5 wt% dry matter (DM), and the total reaction volume was kept constant in all experiments. To evaluate possible synergistic among the enzyme preparations, 1:1 mixtures, and 1:1:1 mixture of the enzyme preparations were added to a total E/S level of 5 wt% E/S and 7.5 wt% E/S, respectively. These hydrolyzates were compared with those obtained with addition of 2.5 wt% E/S of the individual enzyme preparations under the same reaction conditions. All samples were withdrawn after exactly 24 h of reaction and heated immediately at 100$^0$C for 10 min to halt the enzyme reaction. The samples were then centrifuged (20800 g, 10 min), and the levels of arabinose, galactose, glucose, xylose, and mannose were determined by HPAEC analysis. The values are the mean of three determinations and are reported in g/kg palm kernel cake DM.

The 1:1 mixtures of cellulase preparation A: mannanase preparation B induced a significantly elevated release of glucose as compared with both the corresponding individual enzyme treatments and the hypothetical, calculated sum of the glucose release catalyzed by the individual preparations. After 24 hours of treatment, -95 wt% of the available glucose, was released which was -71 wt% more than the theoretical no-interaction situation, and thus there was a synergistic effect between the enzyme activities in the two enzyme preparations (Table 2).

The 1:1:1 mixture of cellulase preparation A: mannanase preparation B: mannosidase preparation C also induced a significantly elevated release of glucose

compared with the corresponding individual enzyme treatments and the hypothetical, calculated sum of glucose. After 24 hours the mixture released >100 wt% of the glucose released by acid hydrolysis with $H_2SO_4$.

Table 2. Glucose released from palm kernel cake after treatments with 2.5 wt% (25 g EP/kg DM) of cellulase preparation A, mannanase preparation B and mannosidase preparation C either alone or in 1:1 mixtures, or a 1:1:1 mixture for 24 hours at pH 5, 50°C. Results are expressed as g·kg-1 palm kernel cake DM ± S.D.

| **Enzyme name** | **Hypothetical sum** | **Obtained experimentally** |
|---|---|---|
| No enzyme | - | 0.00 |
| Cellulase prep. A | - | 20.6±1.7 |
| Mannanase prep. B | - | 0.00±0.0 |
| Mannosidase prep C | - | 11.2±0.6 |
| Mannanase prep. B: Mannosidase prep. C | 11.2 | 22.7±1.5 |
| Cellulase prep. A: Mannosidase prep. C | 31.8 | 33.5±7.8 |
| Cellulase prep. A: Mannanase prep. B | 20.6 | 82.5±2.0 |
| Cellulase prep. A :Mannanase prep. B: Mannosidase prep. C | 31.8 | 88.1±1.6 |

The 1:1 mixtures of mannosidase preparation C:mannanase preparation B, mannosidase preparation C:Cellulase preparation A, and Cellulase preparation A:Mannanase preparation B all induced an significantly elevated release of mannose as compared with both the corresponding individual enzyme treatments and the hypothetical, calculated sum of the mannose release catalyzed by the individual preparation. After 24 hours of treatment, the Mannanase preparation B:mannosidase preparation C mixture released -83 wt% of the available mannose, which was ~11 wt% more than the theoretical no-interaction situation, and thus there was a synergistic effect between the mannan-degrading activities in the two enzyme preparations (Table 3).

The 1:1:1 mixture of cellulase preparation A: mannanase preparation B:mannosidase preparation C induced a significantly elevated release of mannose compared with the corresponding individual enzyme treatments and the hypothetical, calculated sum of mannose. But the mannose released by this mixture was not significantly higher then the amount of mannose released by the 1:1 mixture of mannanase preparation B and mannosidase preparation C (Table 3).

**Table 3.** Mannose released from palm kernel cake after treatments with 2.5 wt% (25 g EP/kg DM) of cellulase preparation A, mannanase preparation B and mannosidase preparation C either alone or in 1:1 mixtures, or a 1:1:1 mixture for 24 hours at pH 5, 50°C Results are expressed as $g \cdot kg^{-1}$ palm kernel cake DM ± S.D.

| Enzyme name | Hypothetical sum | Obtained experimentally |
|---|---|---|
| No enzyme | - | 0.00±0.0 |
| cellulase prep. A | - | 18.5±0.6 |
| Mannanase prep. B | - | 161±29 |
| Mannosidase prep. C | - | 136±1.5 |
| Mannanase prep. B: Mannosidase prep. C | 297 | 334±1.5 |
| Cellulase prep. A: Mannosidase prep. C | 154.5 | 210±15 |
| Cellulase prep. A: Mannanase prep. B | 179.5 | 201±11 |
| Cellulase prep. A: Mannanase prep. B: Mannosidase prep. C | 315.5 | 335±6.5 |

## Example 3

### Enzymatic saccharification and fermentation of palm kernel cake

A 35 wt% dry matterD(M) palm kernel cake (PKC) suspension was incubated 24 hours at pH 5 and 50°C with various mixtures of mannanase preparation B and mannosidase preparation C. In some experiments also cellulase preparation A was added. After this hydrolysis bakers yeast was added to part of the samples and the mixture was fermented for 156 hours. The other part of the samples was hydrolyzed further for 156 hours without yeast added.

The fermented samples were withdrawn after 156 hours of fermentation and centrifuged at 20800 g, for 10 min. The level of ethanol was determined by HPLC. The values are the mean of three determinations and are reported in g/kg palm kernel cake DM.

All hydrolyzed samples were withdrawn after exactly 24 + 156 hours of reaction and heated to $100^0$C for 10 min to halt the enzyme reaction. The samples were then centrifuged (20800 g, 10 min), and the levels of glucose and mannose were determined by HPAEC analysis. The values are the mean of three determinations and are reported in g/kg palm kernel cake DM. The results are shown in table 4.

**Table 4.** Comparison of sugars released by hydrolysis of palm kernel cake alone and ethanol formed by simultaneous hydrolysis/saccharification and fermentation

| Enzyme dosage; mg EP/kg PKC DM | | | Mannose formed after 180 hydrolysis hours g/kg PKC DM | Glucose formed after 180 hours hydrolysis g/kg PKC DM | Ethanol formed after 24 hydrolysis + 156 hours fermentation g/kg PKC DM |
|---|---|---|---|---|---|
| mannanase preparation B | mannosidase preparation C | cellulase preparation A | | | |

| | | | | | |
|---|---|---|---|---|---|
| 171 | 85 | 0 | 240 | 5 | 120 |
| 171 | 85 | 324 | 240 | 20 | 130 |
| 171 | 255 | 0 | 295 | 5 | 145 |
| 171 | 255 | 324 | 300 | 20 | 160 |
| 342 | 510 | 648 | 320 | 40 | 180 |

Table 4 shows that, like glucose, mannose is fully fermentable by bakers yeast and that the ethanol formation in all cases is close to the theoretically possible yield (51% of released monosaccharides).

Mannanase is the main responsible for solubilisation of the otherwise insoluble palm kernel cake mannan where mannosidase prefers the solubilised mannose oligomers as substrate. But the results indicate synergy between mannanase and mannosidase as an increase in mannosidase results in a larger increase of solubilised mannan than an increase in mannanase.

**CLAIMS**

1. A process for converting a mannan-containing material into a fermentation product, the process comprising

a) forming an aqueous slurry comprising the mannan-containing material,

b) contacting the slurry with an enzyme composition comprising the enzyme activities cellulase, mannanase, and mannosidase and producing a soluble hydrolyzate.

c) contacting the soluble hydrolyzate with a fermenting organism to produce a fermentation product.

2. The process of claim 1, wherein the enzyme composition further comprises one or more enzyme activities selected from the list consisting of galactomannanase, beta-xylosidase, endo-1 ,4-beta xylanase, beta-glucosidase, alpha-galactosidase, beta-galactosidase, alpha-L-arabinofuranosidase, acetyl xylan esterase, ferulic acid esterase and alpha-glucuronidase.

3. The process of any of claims 1 or 2, wherein the enzyme composition further comprises one or more enzyme activities selected from the list consisting of alpha-amylase and amyloglycosidase.

4. The process of any of claims 1 to 3, wherein the cellulase is derived from *Trichoderma reesei.*

5. The process of any of claims 1 to 4, wherein the mannosidase is derived from *Aspergillus niger.*

6. The process of any of claims 1 to 5, wherein the mannosidase is derived from *Aspergillus niger* and has the sequence shown in SEQ ID NO:2.

7. The process of any of claims 1 to 6, wherein the mannanase is derived from *Bacillus* sp., e.g., a mannanase having the sequence shown in SEQ ID NO:1

8. The process of any of claims 1 to 7, wherein the mannan-containing material comprises plant material derived from an *Aracaceae* sp. such as *Cocos mucifera, Elaeis guineensis, Elaeis malanococca,* an *Coffea* sp., an *Cyamopsis* sp. such as Cyamopsis *tetragonoloba* (guar bean)

9. The process of any of claims 1 to 8, wherein the mannan-containing material comprises coffee waste, guar meal, palm kernel cake, palm kernel meal and/ or copra cake.

10. The process of any of claims 1 to 9 comprising isolating an oil fraction from the soluble hydrolyzate.

11. The process of any of claims 1 to 10, comprising recovery of the fermentation product from the fermentation medium resulting from step (c), e.g. by distillation.

12. The process of any of claims 1 to 11, wherein the pretreatment is acid pretreatment is carried out using an organic acid, preferably sulphuric acid, acetic acid, citric acid, tartaric acid, succinic acid, and/or mixtures thereof.

13. The process of any of claims 1 to 12, wherein the fermenting organism in step c) is a yeast.

14. The process of any of claims 1 to 13, wherein the fermentation product is an alcohol, preferably ethanol.

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/067465

**A. CLASSIFICATION OF SUBJECT MATTER**
INV. C12P7/08 C12P19/02 A23K1/00

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
C12P A23K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**EPO-Internal**, **WPI** Data, BIOSIS, FSTA, **CHEM ABS** Data, **Sequence Search**

**C. DOCUMENTS** CONSIDERED TO **BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|---|---|---|
| X | BALDRIAN P ET AL: "Degradation of lignocellulose by Pleurotus ostreatus in the presence of copper, manganese, lead and zinc" RESEARCH IN MICROBIOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 156, no. 5-6, 1 June 2005 (2005-06-01), pages 670-676, XP004932778 ISSN: 0923-2508 page 671, column 1, paragraph 2 - page 672, column 1, last paragraph page 673, column 2, last paragraph - page 674, column 2, paragraph 3; table 1 ----- -/- | 1,2 |

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

"L' document which may throw doubts on pπonty claιm(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

*O* document referring to an oral disclosure, use, exhibition or other means

'P' document published pπor to the international filing date but later than the priority date claimed

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

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

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

'&' document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 5 May 2009 | 12/05/2009 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P B 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel (+31-70) 340-2040,<br>Fax (+31-70) 340-3016 | De Kok, Ad |

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/067465

**C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| X | ONC L G A ET AL: "Enzyme producti on and profi l e by Aspergi l l us niger - Duri ng sol i d substrate fermentation usi ng palm kernel cake as substrate. " APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, vol . 118, no. 1-3, July 2004 (2004-07) , pages 73-79, XP008091838 ISSN: 0273-2289 abstract page 74, paragraph 2 - page 78, paragraph 2 | 1 , 3,5,8, 9 |
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| A | GUEBITZ G M ET AL: "Mannan-degradi ng enzymes from Scleroti um rol fsi i : Characteri sati on and synergi sm of two endo beta-mannanases and a beta-mannosidase" BIORESOURCE TECHNOLOGY , vol . 58, no. 2 , 1996 , pages 127-135 , XP002481256 ISSN: 0960-8524 abstract page 127 - page 132 | 1 ,8,9 |
| A | CHARRIER MARYVONNE ET AL: "Mannan-degrading enzymes puri fied from the crop of the brown garden snai l Hel i x aspersa mul ler (gastropoda pulmonata)" JOURNAL OF EXPERIMENTAL ZOOLOGY, vol . 290, no. 2 , 1 July 2001 (2001-07-01) , pages 125-135, XP002481257 ISSN: 0022-104X page 125 - page 131 | 1 |
| A | DATABASE WPI Week 199637 Thomson Scientific , London , GB ; AN 1996-365490 XP002481258 & JP 08 173055 A (HOSHIDA S) 9 July 1996 ( 1996-07-09) abstract | 1 , 5 ,6,8, 9 |

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# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/067465

**C(Continuation). DOCUMENTS** CONSIDERED TO **BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|---|---|---|
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