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(54) Title: HYDROLYSIS OF ARABINOXYLAN

(57) Abrégé/Abstract:
The present invention relates to a process for enzymatic hydrolysis of arabinoxylan, and an enzyme composition suitable for use in such a process.

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(54) **Title:** HYDROLYSIS OF ARABINOXYLAN

(57) **Abstract:** The present invention relates to a process for enzymatic hydrolysis of arabinoxylan, and an enzyme composition suitable for use in such a process.

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HYDROLYSIS OF ARABINOXYLAN

SEQUENCE LISTING

The present invention comprises a sequence listing.

FIELD OF THE INVENTION

The present invention relates to a process for enzymatic hydrolysis of arabinoxylan, and an enzyme composition suitable for use in such a process.

BACKGROUND OF THE INVENTION

Arabinoxylan, a polysaccharide composed of xylose and arabinose, is part of the water soluble and insoluble fibre present in cereals, in particular in the cell walls. Hydrolysis of arabinoxylan is an important prerequisite for improved utilization of cereal hemicellulose, e.g. in the ethanol fermentation industry and other cereal-based industries.

Arabinoxylan consist of alpha-L-arabinofuranose residues attached as branch-points to a beta-(1-4)-linked xylose polymeric backbone. The xylose residues may be mono-substituted in the C2- or C3-position or di-substituted at both the C2- and C3-position. In addition, ferulic acid and p-coumaric acid may be covalently linked to arabinoxylan via esterification at the C5 position of some of the arabinosyl units. These substitutions on the xylan backbone retard the actions of xylanases and the complete hydrolysis of arabinoxylan thus requires both side-group cleaving and depolymerising activities. The major products of hydrolysis of arabinoxylan are the C5 sugars xylose and arabinose.

A process for hydrolysis of arabinoxylan using synergistic interactions amongst enzymes present commercial enzyme compositions from *Humicola insolence* and *Trichoderma reesei* has previously been described by the present inventors in Sørensen, H.R. et al. (Biotechnology and Bioengineering, Vol. 81, No. 6, March 20, 726-731, 2003). Enzyme catalyzed hydrolysis of > 50% of the soluble part of the wheat endosperm arabinoxylan could be achieved, but only low monosaccharide yields were obtained with similar enzymatic treatments on insoluble wheat arabinoxylan. However, since the arabinoxylan degrading enzyme activities are present as side-activities in commercial preparations having other enzyme activities as their main activity, high dosage levels of 5 - 10 wt% of the enzyme preparation per weight of the substrate has to be added for obtaining efficient hydrolysis. Such high enzyme addition levels are not feasible for use in full scale production applications and improved processes for hydrolysis of arabinoxylan are thus needed.

SUMMARY OF THE INVENTION

The inventors have now found improved processes for hydrolysis of arabinoxylan and an enzyme composition suitable for use in such a process. In the process of the invention an arabinoxylan containing substrate is contacted with an enzyme having activity towards di-substituted xyloses, e.g. such as an alpha-L-arabinofuranosidase of Glycoside Hydrolase Family 43 (GH43), and an enzyme having activity towards C2- or C3-position mono-substituted xyloses, e.g. such as an alpha-L-arabinofuranosidase of Glycoside Hydrolase Family 51, 54 or 62 (GH51, GH54 or GH62).

Accordingly the invention provides in a first aspect a process comprising adding to an arabinoxylan containing substrate an enzyme having activity towards di-substituted xyloses, and, an enzyme having activity towards C2- or C3-position mono-substituted xyloses.

The invention provides in a second aspect a composition for hydrolysis of arabinoxylan said composition comprising an enzyme having activity towards di-substituted xyloses, and, an enzyme having activity towards C2- or C3-position mono-substituted xyloses.

The invention provides in further aspects uses of the composition of the second aspect.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1A-C show arabinoxylan polymers:

Fig. 1A shows intact arabinoxylan. Arabinofuranosyl residues linked alpha(1→3) (mono-substituted) and alpha(1→2) and alpha(1→3) (di-substituted) to internal xyloses.

Fig. 1B shows di-substituted arabinoxylan. Arabinofuranosyl residues linked alpha(1→2) and alpha(1→3) (di-substituted) to internal xyloses.

Fig. 1C shows singly substituted arabinoxylan. Arabinofuranosyl residues linked alpha(1→2) and alpha(1→3)(mono-substituted) to internal xyloses

Fig. 2 A-C show arabinoxylo-oligosaccharides:

Fig. 2A shows arabinosyl groups linked to internal C-3. Arabinofuranosyl residues linked alpha(1→3) (mono-substituted) and alpha(1→2) and alpha(1→3) (di-substituted) to internal xyloses.

Fig. 2B shows arabinosyl groups linked to terminal C-3. Arabinofuranosyl residues linked alpha(1→3) (mono-substituted) to terminal xyloses and alpha(1→2) and alpha(1→3) (di-substituted) to internal xyloses

Fig. 2C shows arabinosyl groups linked to internal C-2. Arabinofuranosyl residues linked alpha(1→2) and alpha(1→3)(mono-substituted) to internal xyloses

DETAILED DESCRIPTION OF THE INVENTION

In the description and claims which follows the following are definitions of some of the technical terms which are employed.

The numbering of Glycoside Hydrolase Families applied in this disclosure follows the concept of Coutinho, P.M. & Henrissat, B. (1999) *CAZy - Carbohydrate-Active Enzymes server* or alternatively Coutinho, P.M. & Henrissat, B. 1999; The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In *"Genetics, Biochemistry and Ecology of Cellulose Degradation"*., K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita and T. Kimura eds., Uni Publishers Co., Tokyo, pp. 15-23, and Bourne, Y. & Henrissat, B. 2001; Glycoside hydrolases and glycosyltransferases: families and functional modules, *Current Opinion in Structural Biology* 11:593-600.

The term "granular starch" in context of the present invention is understood as raw uncooked starch, i.e. starch that has not been subjected to a gelatinization.

The term "biomass" means in context of the present invention all hemicellulose containing materials. Biomass is a very heterogeneous and chemically complex resource comprising byproducts from agricultural and industrial processing of all forms of plant material. The biomass may be any plant-derived organic matter including herbaceous and woody energy crops, agricultural food and feed crops, agricultural crop waste and residues such as straw, stalks, leaves, corn bran, husks, cobs, rind, shells, and pods, wood waste such as bark, shavings, sawdust, wood pulp and pulping liquor. The biomass may include biomass from waste, such as waste paper, cardboard, construction and demolition wood waste. The biomass may also include sludge or solids recovered from industrial or municipal waste water treatment as well as from animal manure.

The "arabinoxylan containing substrate" to be treated in the process of the present invention may be obtained from any vegetable source, in particular be obtained from tubers, roots, stems, legumes, cereals or whole grain. Preferred are hemicellulose containing agricultural waste products (i.e. residues and/or by-products) such as cassava peels, cocoa pods, rice husks and/or hulls, rice bran from rice polishing, cobs, straw, hulls and/or husks from cereal grain, pressed sugar cane stalk, sugar beet pulp, locust bean pulp or other vegetable or fruit pomaces. The substrate may be any biomass.

Preferred is a substrate obtained from cereal grain, e.g. such as milled grain or by-products from processing of cereal grain, e.g. an arabinoxylan containing by-product from wet- or dry-milling of cereal, The cereal grain may be any cereal grain though preferred is a cereal grain selected from the group consisting of corn (maize), wheat, barley, oat, rice, sorghum and millet. Most preferred for the present invention is an arabinoxylan containing substrate derived from wheat.

The arabinoxylan containing substrate may be the grist or mash of a brewing and/or fermentation process, or it may be a by-product from a brewing and/or fermentation process, e.g. wet or dried distillers grain, spent grain, vinasse, bagasse etc.

Arabinoxylan containing substrates usually comprise both water soluble and water in-soluble arabinoxylan. Contemplated for the aspects of the present invention is substrates comprising both water soluble arabinoxylan and/or water in-soluble arabinoxylan.

Processes

The process of the first aspect wherein an arabinoxylan containing substrate with enzyme activities comprising an enzyme having activity towards di-substituted xyloses, and an enzyme having activity towards C2- or C3-position mono-substituted xyloses is particular suitable for the production of linear xylose polymers (xylan homopolymer) with little or no arabinose side groups. In a preferred embodiment the enzyme having activity towards di-substituted xyloses is an alpha-L-arabinofuranosidase of GH43, and an enzyme having activity towards C2- or C3-position mono-substituted xyloses is an alpha-L-arabinofuranosidase of GH51, GH54 or/or GH62, more preferably a GH51.

When the two arabinofuranosidases are added to an arabinoxylan solution the resulting products will be high molecular weight linear xylose polymers and arabinose molecules. This will allow for an easy separation of the linear xylose polymer by known techniques (ultrafiltration or solvent precipitation of the xylan in an ethanol solution) from arabinose.

The linear xylose polymers may be further partially digested with enzyme activities, such as a beta-xylosidase, and/or an endo-1,4-beta-xylanase, to yield xylo-oligosaccharides, which also have dietary applications. Preferably the beta-xylosidase is a beta-xylosidase of GH3, and/or preferably the endo-1,4-beta-xylanase is an endo-1,4-beta-xylanase of GH10 or GH11.

When an endo-1,4-beta-xylanase is added to the purified linear xylose polymers (purified as described above) the resulting products will be xylo-oligosaccharides essentially free of arabinose side groups. The size of the oligosaccharides can be controlled by the dose of the endo-1,4-beta-xylanase as well as by the length of the reaction time.

When both an endo-1,4-beta-xylanase and a beta-xylosidase are added to the purified linear xylose polymers the resulting product will be xylose.

Thus the invention provides a process for obtaining a linear xylose polymer product essentially free of arabinose substituents, a process for obtaining a xylo-oligosaccharide product essentially free of arabinose side groups and a process for separating xylose and arabinose in a simpler way than previous technology (ion exchange chromatography).

Furthermore the invention provides a linear xylose polymer product of high molecular weight and essentially free of arabinose side groups and a xylo-oligosaccharide product essentially free of arabinose side groups.

Preferably the linear xylose polymer product or xylo-oligosaccharide product comprises at least 50%, at least 60%, at least 70%, at least 90%, at least 80%, at least

90%, at least 95%, such as at least 98% polymer by weight of the product which polymer has a degree of polymerization of at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120 at least 150, at least 200, at least 300, at least 500, at least 1000, at least 2000, at least 5000, or at least 10000.

Preferably the linear xylose polymer product or xylo-oligosaccharide product comprising at least 50%, at least 60%, at least 70%, at least 90%, at least 80%, at least 90%, at least 95%, such as at least 98% polymer by weight of the product which polymer has a degree of polymerization of at of less than 5000, less than 2500, less than 1500, less than 1000, less than 500, less than 100, less than 75, less than 50, less than 25, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, and preferably less than 4.

Preferably the linear xylose polymer product or xylo-oligosaccharide product comprises at least 50%, at least 60%, at least 70%, at least 90%, at least 80%, at least 90%, at least 95%, such as at least 98% polymer by weight of the product which polymer has a degree of polymerization selected from the group consisting of the intervals from 3 to 10, from 11 to 25, from 26 to 50, from 51 to 100, from 101 to 200, from 201 to 500, from 501 to 1000, from 1001 to 5000, and from 5001 to 10000.

The linear xylose polymers produced may be used as a food additive, e.g. as a bulking agent, a low calorie fat replacer or dietary fiber, such as a non soluble dietary fibre. Applications will e.g. be in cakes, extruded snacks, other cereal products, and confectionary. Technical applications will include additive to paper and pulp products, plastic materials (films), where plasticizers might be added, and as a sizing agent.

The xylo-oligosaccharide product will have applications as dietary fibres, such as soluble dietary fibres. These dietary fibres may be used for increasing the amount of bifidus-bacteria in the lower gut. Applications will e.g. be in yoghurt, ice cream, and soft drinks.

An embodiment of the first aspect, wherein further enzyme activities are present, such as a beta-xylosidase of GH3, and/or an endo-1,4-beta-xylanase of GH10 is particularly useful when more complete hydrolysis of arabinoxylan is wanted. Apart from releasing C5 sugars the hydrolysis of arabinoxylan also makes associated glucose polymers such as starch and cellulase more accessible for the action of the appropriate enzymes. This is particularly useful when degradation of complex substrates are required, e.g. in brewing or in hydrolysis of starch or biomass for fuel ethanol production, or in animal feed composition.

Xylose and/or arabinose released during enzymatic hydrolysis of arabinoxylan in the process of the first aspect and/or second aspect may be used as a source of xylose and/or arabinose as such, or as raw material for chemical/enzymatic synthesis or fermentation processes, e.g. for production of xylitol, xylaric acid, xylonic acids, arabonic acid, arabinoic acid, 2,3-butanediol, lactic acid, lactonic acid, furans and/or ethanol.

For degradation of even more complex substrates, or where a more complete degradation is required, the presence of even further enzyme activities may be desired. In a preferred embodiment the enzyme activity/activities further comprise an acetyl xylan esterase (EC 3.1.1.72) and/or a feruloyl esterase (EC 3.1.1.73) and/or an alpha-glucuronidiase (EC 3.2.1.139).

In an embodiment of the process of the first aspect the enzyme activity/activities further comprise an enzyme selected from the list consisting of an acetyl xylan esterase, a feruloyl esterase, an alpha-amylase, a glucoamylase, a phytase and a protease.

In brewing and other fermentation processes based on cereal grist arabinoxylans can be extracted from cell walls with hot water and may form solutions of high viscosity. If in brewing processes malts are used which are not adequately modified during malting, malt extracts can contain high levels of arabinoxylans and other polysaccharides causing an increase in viscosity of the extracts. The difficulties associated with the filtration of such extracts can significantly slow down the brewing process. In an embodiment of the present invention the arabinoxylan containing substrate to be contacted with the composition of the invention is a mash of a beer brewing process, whereby e.g. the viscosity of the mash is reduced and/or further polysaccharides released.

In an embodiment of the present invention the process is any ethanol process, based on enzymatic hydrolysis of gelatinized or granular starch, e.g. on granular starch as described in WO2004080923 or WO2004081193. By contacting the mash with the composition of the invention the viscosity of the mash may be reduced. Also further polysaccharides may be released, not only as C5 sugars but also as glucose when the break-down of arabinoxylan leaves the starch more accessible to amyloytic enzymes usually present during such processes. An additional enzyme which advantageously may be applied in a starch-based ethanol process is an enzyme selected from the list consisting of beta-glucanase, alpha-amylase, glucoamylase, CGTase, phytase and protease.

The process of present invention may be any ethanol process, comprising enzymatic hydrolysis of biomass and/or effluent from pre-treatment of biomass. An additional enzyme which advantageously may be applied in a biomass-based ethanol process is an enzyme selected from the list consisting of beta-glucanase, cellulase, cellobiohydrolase, and beta-glucosidase.

In a fermentation process the arabinoxylan hydrolysate may advantageously be contacted with a yeast or another fermenting organism capable of utilizing C5 sugars. Alternatively, the arabinoxylan hydrolysate may be contacted with a xylose isomerase (EC 5.3.1.5) for isomerization of xylose into xylulose which is fermentable to ethanol using a *Saccharomyces* yeast.

The composition of the invention may also be used in processing of a cereal raw material intended for use as a feed/food product or the composition may be applied as a feed/food additive. Such enzyme-based feed/food additives can be incorporated into a cereal-based feed/food product which includes one or more of wheat, barley, triticale, rye, rice and corn. The feed/food additive has the advantage of improving the feed/food conversion ratio and/or increasing the digestibility of the cereal-based feed/food product in which it is included. The composition of the invention used as feed/food additive may preferably be used together with a phytase.

The present invention furthermore relates to composition for treating an arabinoxylan containing substrate, said composition comprising an enzyme having activity towards di-substituted xyloses, e.g. such as an alpha-L-arabinofuranosidase of GH43, and an enzyme having activity towards C2- or C3-position substituted xyloses, e.g. such as an alpha-L-arabinofuranosidase of GH51, GH54 or GH62.

The present invention further relates to compositions comprising an alpha-L-arabinofuranosidase of GH43, an alpha-L-arabinofuranosidase of GH51, GH54 or GH62, a beta-xylosidase, and/or an endo-1,4-beta-xylanase, as well as to composition comprising the aforementioned activities and an enzyme selected from the group consisting of alpha-amylase, CGTase, glucoamylase, phytase, protease, beta-glucanase, cellulase, cellobio-hydrolase, and/or beta-glycosidase.

The composition may comprise alpha-L-arabinofuranosidase of GH43 in an amount of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 70%, or even at least 80% w/w of total arabinofuranosidase enzyme protein present in the composition. More preferably the composition may comprise alpha-L-arabinofuranosidase of GH43 in an amount of at least 5%, such as at least 10% at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 70% w/w of total enzyme protein present in the composition.

The composition may be used for treatment of an arabinoxylan containing substrate, e.g. in a fermentation process, e.g. for reduction of viscosity of a slurry and/or solution comprising an arabinoxylan containing substrate. The composition may be used for producing a feed/food product, e.g. for producing or modifying a nutritional/dietary fibre and/or for producing a xylose, arabinose and/or linear xylose or for producing derivatives of xylose, arabinose by fermentation, enzymatic processing or chemical synthesis.

The invention furthermore provides a process wherein an arabinoxylan containing substrate and/or a biomass is contacted with an enzyme arabinofuranosidase capable of releasing arabinose from di-substitued xyloses. Preferably the enzyme capable of releasing arabinose from di-substitued xyloses is an arabinofuranosidase. Preferably the alpha-L-arabinofuranosidase is an alpha-L-arabinofuranosidase of GH43. The alpha-L-arabinofuranosidase of GH43 is preferably derived of bacterial, of fungal or of plant origin. Preferably the arabinoxylan containing substrate and/or the biomass is selected from the list consisting of herbaceous and/or woody energy 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.

Enzymes

Alpha-L-arabinofuranosidase having activity towards di-substituted xyloses

The enzyme having activity towards di-substituted xyloses, e.g. such as an alpha-L-arabinofuranosidase of GH43, may be of microbial origin, e.g. derivable from a strain of a filamentous fungus (e.g., *Humicola, Aspergillus, Trichoderma, Fusarium, Penicillum*) or from a bacteria (e.g. *Bacillus, Bifidobacterium*). A suitable such enzyme may be selected by the assay for alpha-arabinofuranosidase activity on di-substituted arabinoxylan in the Methods section.

Preferably the alpha-L-arabinofuranosidase of GH43 is derived from *Humicola insolens*. Most preferably the alpha-L-arabinofuranosidase of GH43 is the polypeptide shown as SEQ ID NO:1, more preferably the polypeptide shown as amino acids 19-558 of SEQ ID NO:1, or even more preferably a polypeptide which has at least 75%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity with the amino acid sequence shown as amino acids 19-558 of SEQ ID NO:1 (hereinafter "homologous polypeptides").

The alpha-L-arabinofuranosidase of GH43 may also derived from *Bifidobacterium adolescenti*. More preferably the alpha-L-arabinofuranosidase of GH43 is the enzyme described by Van Laere, 1997, in Appl.Microbiol.Biotechnol, 47, 231-235 and/or by Van den Broek, 2005, in Applied Microbiology and Biotechnology.

An enzyme having activity towards di-substituted xyloses, e.g. such as an alpha-L-arabinofuranosidase of GH43, may be added in amounts of 0.001-1.0 g/kg DM 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.

Alpha-L-arabinofuranosidase having activity towards mono-substituted xyloses

The enzyme having activity towards C2- and/or C3-position mono-substituted xyloses, e.g. such as an alpha-L-arabinofuranosidase of GH51, GH54 or GH62, may be of microbial origin, such as derivable from a strain of a filamentous fungus (e.g., *Meripilus, Humicola, Aspergillus, Trichoderma, Fusarium, Penicillum*) or from a bacteria (e.g. *Bacillus*). Preferably the enzyme is an alpha-L-arabinofuranosidase of GH51, and even more preferably the alpha-L-arabinofuranosidase GH51 is derived from *Meripilus giganteus*. The polypeptide may preferably have at least 75%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity with the amino acid sequence shown as amino acids 17-643 of SEQ ID NO:2 (hereinafter "homologous polypeptides"). More preferably the alpha-L-arabinofuranosidase is the polypeptide shown as SEQ ID NO:2, even more preferably the polypeptide shown as amino acids 17-643 of SEQ ID NO:2.

Alpha-L-arabinofuranosidase of GH51, GH54 or GH62 may be added in amounts of 0.001-1.0 g/kg DM 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

Beta-xylosidase

The beta-xylosidase of is preferably a beta-xylosidase of GH3. The beta-xylosidase may be of microbial origin, such as derivable from a strain of a filamentous fungus (e.g., *Trichoderma, Meripilus, Humicola, Aspergillus, Fusarium* or from a bacteria (e.g. *Bacillus*). Preferably the beta-xylosidase is a beta-xylosidase of GH3 derived from *Trichoderma reesei* and more preferably the beta-xylosidase of GH3 is the polypeptide shown as SEQ ID NO:3 or a polypeptide which has at least 75%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity with the amino acid sequence shown as amino acids of SEQ ID NO:3 (hereinafter "homologous polypeptides"). Beta-xylosidase of GH3 may be added in amounts of 0.001-1.0 g/kg DM 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

Endo-1,4-beta-xylanase

The endo-1,4-beta-xylanase is preferably an endo-1,4-beta-xylanase of GH10 or GH11. The endo-1,4-beta-xylanase may be of microbial origin, such as derivable from a strain of a filamentous fungus (e.g., *Trichoderma, Meripilus, Humicola, Aspergillus, Fusarium*) or from a bacteria (e.g. *Bacillus*). The endo-1,4-beta-xylanase is preferably an endo-1,4-beta-xylanase of GH10 derived from *Humicola insolens* and more preferably the endo-1,4-beta-xylanase of GH10 is the polypeptide shown as SEQ ID NO:4, more preferably as amino acids 17-389 of SEQ ID NO:4, or even more preferably a polypeptide which has at least 75%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity with the amino acid sequence shown as amino acids 17-389 of SEQ ID NO:4 (hereinafter "homologous polypeptides").

Endo-1,4-beta-xylanase of GH10 may be added in amounts of 0.001-1.0 g/kg DM 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.

MATERIALS AND METHODS

Enzymes used

A GH43 alpha-L-arabinofuranosidase from *H. insolens* (SEQ ID NO:1), a GH51 alpha -L-arabinofuranosidase from *M. giganteus* (SEQ ID NO:2), a GH3 beta-xylosidase from *Trichoderma reesei* (SEQ ID NO:3) and a GH10 endo-1,4-beta-xylanase from *H. insolens* (SEQ ID NO:4). The aforementioned enzymes were cloned using basic molecular techniques (Ausubel *et al.*, 2003, Curr. Prot. Mol.Biol., John Wiley & Sons, Cambridge, USA, Christgau et al. 1995, Curr. Genet. 27, 135-141).

Ultraflo ™ L and Celluclast ™ 1.5 L are commercial enzyme compositions, and available from Novozymes A/S. Ultraflo L is derived from *Humicola insolence* and comprises cellulases and hemicellulases. Celluclast 1.5 L is derived from *Trichoderma reesei* and comprises cellobiohydrolases and endoglucanases.

Bio-Feed Wheat L is a commercial xylanase for feed application and available from Novozymes A/S. Bio-Feed Wheat L is derived from *Termomyces lanuginosus.*

Chemicals and substrates

Arabinose and xylose were purchased from Merck (Darmstadt, Germany). Water soluble and water insoluble wheat arabinoxylans were obtained from Megazyme (Bray, County Wicklow, Ireland). The ethanol fermentation effluent, "vinasse", was provided by Tate & Lyle, Amylum UK (Greenwich, UK).

Soluble wheat arabinoxylan substrate

Medium viscosity water-soluble wheat arabinoxylan was obtained from Megazyme (Bray, County Wicklow, Ireland). Monosaccharide contents after acid hydrolysis (0.4 N HCl, 2 h, 100°C) and HPAEC were: Arabinose 275.8 mg/g, xylose 479.2 mg/g (= A:X 0.58), with only traces of galactose and glucose. According to the product sheet the starch, beta-glucan, protein, moisture, and ash contents by weight were <0.1%, <0.1%, 0.9%, 1.9%, and 2.2%, respectively.

Wheat vinasse substrate

Wheat vinasse, a by-product from industrial ethanol fermentation, was provided by Tate & Lyle, Amylum UK, (Greenwich, UK). The dry matter content of the vinasse was 9.02 wt%. Monosaccharide contents after acid hydrolysis (0.4 N HCl, 2 h, 100 °C) and HPAEC were: Arabinose 82,9 g/kg DM vinasse, xylose 119 g/kg DM vinasse mg/g, galactose 21,6 g/kg DM vinasse, and 78,2 g/kg DM vinasse. Organic acids, protein, ash, and ferulic acid constituted ~30%, ~16%, ~11%, and 0.2% by weight, respectively of the dry matter.

Preparation of specific arabinoxylan polymers and oligosaccharides

Doubly substituted arabinoxylan was prepared by incubating soluble wheat arabinoxylan (1g) in 0.1 M acetate buffer (100 mL), pH 6.0 with 0.167 g α-L-arabinofuranosidase from *Meripilus giganteus* (GH51)$\cdot kg^{-1}$ water soluble wheat arabinoxylan for 48 hours at 30°C. Singly substituted arabinoxylan was prepared by incubating water soluble wheat arabinoxylan (1g) in 0.1 M acetate buffer (42 mL), pH 6.0 with 0.147 g α-L-arabinofuranosidase from *Humicola insolens* (GH43) $\cdot kg^{-1}$ water soluble wheat arabinoxylan for 48 hours at 30°C. To halt the enzymatic reactions the mixtures were heated to 100°C for 10 min. Arabinoxylan polymers were precipitated by addition of ethanol (126 ml). The precipitates were filtered (Miracloth) and dryed in vacuum

Oligosaccharides containing arabinosyl groups linked to terminal (1→3) were prepared by incubating the water insoluble wheat arabinoxylan (1g) in 0.1 M acetate buffer (100 mL), pH 6.0 with 6.67 g Shearzyme (xylanase GH10) $\cdot kg^{-1}$ water insoluble wheat arabinoxylan for 2 hours at 30°C. Oligosaccharides containing arabinosyl groups linked to internal (1→3) were prepared by incubating water insoluble wheat arabinoxylan (1g) in 0.1 M acetate buffer (100 mL), pH 6.0 with 0.03 g Pentopan Mono (xylanase GH11) $\cdot kg^{-1}$ water insoluble wheat arabinoxylan for 2 hours at 30°C. Oligosaccharides containing arabinosyl groups linked to internal (1→2) were prepared by incubating water insoluble wheat arabinoxylan (1g) in 0.1 M acetate buffer (100 mL), pH 6.0 with 0.03 g Pentopan Mono (xylanase GH11) $\cdot kg^{-1}$ water insoluble wheat arabinoxylan and alpha-L-arabinofuranosidase from *H. insolens* (GH43) $\cdot kg^{-1}$ water soluble wheat arabinoxylan for 2 hours at 30°C. To halt the enzymatic reactions the mixtures were heated to 100°C for 10 min. The arabinoxylo-oligosaccharides were concentrated on a rotary evaporator and evaluated by ^{1}H-NMR.

Substrate analysis

Contents of arabinose and xylose in arabinoxylan containing substrates were determined by acid hydrolysis with hydrochloric acid (0.4 N HCl, 2 hours, 100°C) followed by HPAEC (Sørensen et al., 2003). All yields, including enzymatic hydrolysis yields, are reported as mg per g substrate dry matter or as relative yields in percent.

Assay for activity towards alpha-L-arabinofuranosidase activity

Alpha-L-arabinofuranosidase activity may be assessed as described by Poutanen et al. (Appl. Microbiol. Biotechnol. 1988, 28, 425-432) using 5 mM p-nitrophenyl alpha-L-arabinofuranoside as substrates. The reactions may be carried out in 50 mM citrate buffer at pH 6.0, 40°C with a total reaction time of 30 min. The reaction is stopped by adding 0.5 ml of 1 M sodium carbonate and the liberated p-nitrophenol is measured at 405 nm. Activity is expressed in U/ml.

Assay for alpha-arabinofuranosidase activity on di-substituted arabinoxylan

Medium viscosity water-soluble wheat arabinoxylan (Megazyme, Bray, Ireland) was treated with an alpha-arabinofuranosidase of GH51 from *Meripilus giganteus* (SEQ ID NO:2) to remove single alpha-arabinofuranosyl substituents attached to the C(O)-3 arabinose of the arabinoxylan in order to produce an di-substituted arabinoxylan substrate with arabinofuranosyl substituents attached to both C(O)-2,3 of the xylose residues. The substrate was dialysed and freeze dried.

A 0.1% solution of the di-substituted arabinoxylan was prepared and the alpha-arabinofuranosidase activity was measured by mixing 0.1 ml enzyme, 0.9 ml buffer (0.12 M Succinic acid, pH 6.0) and 1.0 ml substrate solution in an eppendorf tube. The eppendorf tube was incubated at 60°C for 1 hour with shaking. The amount of liberated arabinose was measured by HPAEC (high-performance anion-exchange chromatography).

HPAEC

Hydrolysates (10 μl) were applied onto a Dionex BioLC™ system fitted with a Dionex CarboPac™ PA1 guard 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 10 mM KOH for 15 min, flow: 1 $mL \cdot min^{-1}$. 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 and xylose (concentration of each component: 0.0025-0.1 $g \cdot L^{-1}$) was used as standard.

^{1}H-NMR analysis

All degradation products were lyophilized twice from 99.9% D_2O and re-dissolved in 99.9% D_2O. Some hydrolysates were dialyzed (Spectra/Por membrane molecular weight cut-off 1000) to remove free arabinose prior to the spectral analysis. The ^{1}H-NMR spectra were recorded at 30°C in a Varian Mercury-VX instrument operated at 400 MHz and equipped with a 4-nucleus auto-switchable probe. Data were collected over 128-512 scans and the HDO signal was used as a reference signal (4.67 ppm).

EXAMPLES

Example 1

Enzymatic hydrolysis of water-insoluble arabinoxylan

Water-insoluble wheat arabinoxylan substrate (0.05 g) dissolved in 50 ml double de-ionized water per assay (0.1% DM) was incubated with a composition of the invention, or with 10 wt% of a 50:50 mixture of Ultraflo and Celluclast 1.5 L. E/S relate to the weight of enzyme preparation (E) added in percent per weight of substrate (S).

The composition of the invention comprised 0.075 g GH51 alpha-arabinofuranosidase from *M. giganteus*/kg DM arabinoxylan, 0.075 g GH43 alpha-arabinofuranosidase from *H. insolens*/kg DM arabinoxylan, 0.075 g beta-xylosidase from *T. reesei*/kg DM arabinoxylan, and 0.075 g xylanase from *H. insolens*/kg DM arabinoxylan.

The treatments were performed for 24 hours at pH 5 and 50°C. Samples were withdrawn after 24 hours and immediately heated at 100°C for 10 min. The samples were filtered (0.2 microM filter) and the levels of arabinose, and xylose were determined by HPAEC. Enzymatic hydrolysis experiments were performed in triplicate and the mean values reported are in percentage of the amounts released by acid hydrolysis. Results are presented in Table 1.

Table 1. Arabinose and xylose released from water insoluble wheat arabinoxylan by enzymatic hydrolysis. Numbers are in weight percent of the amount of each monosaccharide released by acid hydrolysis of the water-soluble wheat arabinoxylan samples.

Enzyme	Arabinose	Xylose
Celluclast 1.5 L: Ultraflo L	43	56.7
Composition of the invention	57	64

Enzymatic hydrolysis of water-soluble arabinoxylan

Water-soluble wheat arabinoxylan substrate (0.05 g) dissolved in 50 ml double de-ionized water per assay (0.1% DM) was incubated with a composition of the invention, or with 10 wt% of a 50:50 mixture of Ultraflo and Celluclast 1.5 L. E/S relate to the weight of enzyme preparation (E) added in percent per weight of substrate (S).

The composition of the invention comprised 0.080 g GH51 alpha-arabinofuranosidase from *M. giganteus*/kg DM arabinoxylan, 0.080 g GH43 alpha-arabinofuranosidase from *H. insolens*/kg DM arabinoxylan, 0.16 g beta-xylosidase from *T. reesei*/kg DM arabinoxylan, and 0.080 g xylanase from *H. insolens*/kg DM arabinoxylan.

The treatments were performed for 24 hours at pH 5 and 50°C. Samples were withdrawn after 24 hours and immediately heated at 100°C for 10 min. The samples were filtered (0.2 microM filter) and the levels of arabinose, and xylose were determined by HPAEC. Enzymatic hydrolysis experiments were performed in triplicate and the mean values reported are in percentage of the amounts released by acid hydrolysis. Results are presented in Table 2.

Table 2. Arabinose and xylose released from water soluble wheat arabinoxylan by enzymatic hydrolysis. Numbers are in weight percent of the amount of each monosaccharide released by acid hydrolysis of the water-soluble wheat arabinoxylan samples.

Enzyme	**Arabinose**	**Xylose**
Celluclast 1.5 L: Ultraflo L	25	51
Composition of the invention	116	107

Example 2

The hydrolysis of vinasse was performed according to the method described for water soluble arabinoxylan in Example 1 except that the dosage of beta-xylosidase was 0,050 g/kg DM vinasse and the substrate level was 5 wt% DM. Samples were withdrawn after 24 h and heated immediately at 100°C for 10 min to halt the enzyme reaction, centrifuged (14000 rpm, 10 min), filtered (0.2 microM filter) and subjected to HPAEC analysis to determine the levels of arabinose and xylose, see below. Enzymatic hydrolysis experiments were performed in duplicate and the mean values reported are in percentage of the amounts released by acid hydrolysis. Results are presented in Table 2.

Table 3. Arabinose and xylose released from vinasse by enzymatic hydrolysis. Numbers are in weight percent of the amount of each monosaccharide released by acid hydrolysis of the water-soluble wheat arabinoxylan samples.

Enzyme	Arabinose	Xylose
Celluclast 1.5 L:Ultraflo L	77	75
Composition of the invention	103	81

Example 3

Wheat arabinoxylan comprises arabinofuranoside as a monosubstituent linked to the 3-position of internal xylose and arabinofuranoside linked to the 3- and 2-position on di-substituted xylose, respectively. Substrates were produced comprising only one of the 3 types of arabinofuranoside linkages. The activity of the arabinofuranosidases towards these substrates was investigated.

Table 4. Activity on selected arabinoxylan polymers, incubation at pH 6, 40°C for 2 hours.

		Enzyme			
Substrate	**Linkage**	***H. insolens* (GH43)**	***Bifidobacterium adolescentis* (GH43)**	***H. insolens* (GH51)**	***M. giganteus* (GH51)**
Intact arabinoxylan	Mono-substituted (1→3)	-	-	x	xx
	Di-substituted (1→2)	-	-	-	-
	Di-substituted (1→3)	xx	x	-	-
Di-substituted arabinoxylan	Di-substituted (1→2)	-	-	-	-
	Di-substituted (1→3)	xx	xx	-	-
Mono-substituted arabinoxylan	Mono-substituted (1→2)	-	-	xx	xx
	Mono-substituted (1→3)	-	-	xx	xx

xx refers to more than 75% hydrolysis, x(x) to 50-75% hydrolysis, x to 25-50% hydrolysis and (x) to 5-25% hydrolysis. – refers to no detectable hydrolysis

Example 4

Soluble wheat arabinoxylan was incubated with 0.1 g enzyme protein per kg DM of alpha -L-arabinofuranosidase from *H. insolens* (GH43), *B. adolescentis* (GH43), *H. insolens* (GH51), and *M. giganteus* (GH51). The released arabinose as mg per g water soluble wheat arabinoxylan, their hypothetical sum, and their arabinose release after treatment with 0.2 g enzyme protein per kg DM of a 50:50 mixture of alpha-L-arabinofuranosidases from from *H. insolens* (GH43), *Bi. sp.* (GH43), *H. insolens* (GH51), and *M. giganteus* (GH51) was measured. Results are expressed as the average of triplicate determinations, coefficient of variation on mean < 6.4.

Tabel 5. Released arabinose from soluble wheat arabinoxylan treated with alpha - L-arabinofuranosidase at two different temperature and pH conditions.

	pH 6, 40°C	pH 5, 50°C
H. insolens (GH43)	128.0 a	147.0 a
M. giganteus (GH51)	48.15 c	121.0 b
B. adolescentis (GH43)	63.43 b	4.833 d
H. insolens (GH51)	20.75 d	18.47 c

Values within a column not sharing a common letter index differ with statistical significance (P<0.05).

Tabel 6. Released arabinose from soluble wheat arabinoxylan treated with 50%:50% mixtures of alpha -L-arabinofuranosidases at pH 5, 50°C.

	pH 5, 50°C	pH 5, 50°C
H. insolens (GH43) and *H. insolens* (GH51)	-	168.3 b
H. insolens (GH43) and *M. Giganteus* (GH51)	-	289.0 a
B. adolescentis (GH43) and *H. insolens* (GH51)	-	17.43 d
B. adolescentis (GH43) and *M. giganteus* (GH51)	-	131.0 c

Values within a column not sharing a common letter index differ with statistical significance (P<0.05).

Example 5

Spent grain was obtained from a pilsner brewing process using hammer milled barley malt. The spent grain was freeze dried to a dry matter content of 96.1 % w/w and milled. The spent grain material was suspended 5 g dry matter/100 ml succinic acid - sodium succinate buffer pH 5.0 and subjected to hydrolysis by two treatments: 1) a conventional treatment using a 50:50 mixture of Celluclast 1.5 L + Ultraflo L with 6.5 g enzyme protein per kg spent grain dry matter and 2) a treatment of the invention applying a 25:25:25:25 blend on protein weight basis of the GH43 alpha-L-arabinofuranosidase from *H. insolens* (SEQ ID NO:1), the GH51 alpha -L-arabinofuranosidase from *M. giganteus* (SEQ ID NO:2), the GH3 beta-xylosidase from *Trichoderma reesei* (SEQ ID NO:3) and the GH10 endo-1,4-beta-xylanase from *H. insolens* (SEQ ID NO:4). An enzyme dosage equivalent to 0.6 g enzyme protein per kg spent grain dry matter was used.

The hydrolysis was performed in Ependorfer tubes incubated in a Thermomixer Compact at 1000 rpm for 16 hours at 50°C. The samples were cooked for 10 minutes, centrifuged for 10 minutes at 14000 x g and the soluble phase was analysed carbohydrates on HPLC. HPLC was performed on a Dionex BioLC using a GS50 Gradient Pump, AS50

Autosampler, and an ED40 Elektrochemical detector. The concentration of released arabinose and xylose was measured.

Table 7. Spent grain: Results of HPLC-analyses of released arabinose and xylose in g/litre.

	Arabinose	Xylose
Control, no enzyme	0.00	0.00
1) Conventional treatment	1.19	1.98
2) Treatment of the invention	1.48	2.12

Example 6

An arabinoxylan containing solution was obtained by cooking wheat straw at 190°C followed by separating the liquid from the straw by filtration. In all experiments 1.5 g of the liquid was further diluted to 2.0 g by addition of acid/base for pH adjustment, by addition of enzyme solution and by addition of deionzed water. The liquid was incubated with 2.5 g enzyme protein per liter reaction volume with either the enzyme mix of the invention, with the conventional cellulose blend consisting of a 50:50 mixture of Ultraflo and Celluclast 1.5 L (mix ratio based on protein content).

The composition of the invention comprised a 10:10:5:25 blend on protein weight basis of the alpha-arabinofuranosidase from *H. insolens* (SEQ ID NO:1), the alpha-arabinofuranosidase from *M. giganteus* (SEQ ID NO:2), the beta-xylosidase from *T. reesei* (SEQ ID NO:3) and the xylanase from *H. insolens* (SEQ ID NO:4). The treatments were performed for 24 hours at three pH levels (4, 5, 6) and at two temperatures (40, 50°C). Samples were withdrawn after 24 hours and immediately heated at 100°C for 10 min. The samples were filtered (0.2 microM filter) and the levels of arabinose, and xylose were determined by HPAEC. All Enzymatic hydrolysis experiments were performed in duplicate and the mean values reported are in percentage of the amounts released by acid hydrolysis. Results are presented in Table 8.

Table 8. Arabinose and xylose released from water soluble wheat arabinoxylan by enzymatic hydrolysis. Numbers are in weight percent of the amount of each monosaccharide released by acid hydrolysis of the water soluble wheat arabinoxylan.

Temperature	pH	Arabinose		Xylose	
[°C]		Reference	Invention	Reference	Invention
40	4	63	95	66	72
40	5	70	91	71	78
40	6	76	94	79	88
50	4	60	93	71	72
50	5	66	92	81	88
50	6	87	99	87	87

DEMANDES OU BREVETS VOLUMINEUX

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CECI EST LE TOME __1___ DE __2___

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JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS

1. A process for enzymatic hydrolysis of arabinoxylan comprising
contacting an arabinoxylan containing substrate, with a composition comprising:
    a. a fungal alpha-L-arabinofuranosidase of glycoside hydrolase family 43 having activity towards di-substituted xyloses,
    b.an alpha-L-arabinofuranosidase of glycoside hydrolase family 51 having activity towards C2- or C3-position mono-substituted xyloses, and
hydrolyzing the arabinoxylan,
wherein the alpha-L-arabinofuranosidase of glycoside hydrolase family 43 has at least 90% identity with the amino acid sequence shown as amino acids 19-558 of SEQ ID NO: 1.

2. The process of claim 1, wherein the composition further comprise, a beta-xylosidase, an endo-1,4-beta-xylanase or both.

3. The process of claim 2, wherein the beta-xylosidase is a beta-xylosidase of glycoside hydrolase family 3.

4. The process of any of one claims 2 to 3, wherein the endo-1,4-beta-xylanase is an endo-1,4-beta-xylanase of glycoside hydrolase family 10 or glycoside hydrolase family 11.

5. The process of any one of claims 1 to 4, wherein the composition further comprises an acetyl xylan esterase, feruloyl esterase, alpha-amylase, CGTase, glucoamylase, phytase, protease, beta-glucanase, cellulase, cellobio-hydrolase, beta-glycosidase, or a combination thereof.

6. The process of any one of claims 1 to 5, which process comprises producing a hydrolysate comprising linear xylose, xylo-oligosaccharide, xylose, arabinose or a combination thereof.

7. The process of any one of claims 1 to 6, which process comprises contacting the arabinoxylan containing substrate with a yeast and producing a fermentation product.

8. The process of any one of claims 1 to 7, wherein the substrate is derived from a cereal.

9. The process of claim 8, wherein the cereal is wheat, rye, barley, corn, rice, sorghum or

millet.

10. The process of any one of claims 1 to 9, wherein the substrate is a by-product from a wet-milling process, from a fermentation process, or from sugar production.

11. The process of any one of claims 1 to 10, which process further comprises contacting the arabinoxylan containing substrate with a yeast utilizing C5 sugars.

12. The process of any one of claims 1 to 11, wherein the alpha-L-arabinofuranosidase of glycoside hydrolase family 43 is present in an amount of at least 5% w/w of total enzyme protein present in the composition.

13. A composition for hydrolysis of arabinoxylan, said composition comprising:
a. a fungal alpha-L-arabinofuranosidase of glycoside hydrolase family 43 having activity towards di-substituted xyloses, and
b. an alpha-L-arabinofuranosidase of glycoside hydrolase family 51 having activity towards C2- or C3-position mono-substituted xyloses
wherein the alpha-L-arabinofuranosidase of glycoside hydrolase family 43 has at least 90% identity with the amino acid sequence shown as amino acids 19-558 of SEQ ID NO: 1.

14. The composition of claim 13, wherein the composition further comprises a beta-xylosidase, an endo-1,4-beta-xylanase or both.

15. The composition of claim 14, wherein the beta-xylosidase is a beta-xylosidase of glycoside hydrolase family 3.

16. The composition of any one of claims 14 to 15, wherein the endo-1,4-beta-xylanase is an endo-1,4-beta-xylanase of glycoside hydrolase family 10 or glycoside hydrolase family 11.

17. The composition of any one of claims 13 to 16, wherein the composition further comprise an acetyl xylan esterase, feruloyl esterase, alpha-amylase, CGTase, glucoamylase, phytase, protease, beta-glucanase, cellulase, cellobiohydrolase, beta-glycosidase or a combination thereof.

18. The composition of any one of claims 13 to 17, wherein the alpha-L-arabinofuranosidase

of glycoside hydrolase family 43 is present in an amount of at least 5% w/w of total enzyme protein present in the composition.

19. Use of the composition of any one of claims 13 to 18 for treatment of an arabinoxylan containing substrate.

20. Use according to claim 19 in a fermentation process.

21. Use according to any one of claims 19 to 20 for viscosity reduction of a slurry or solution comprising an arabinoxylan containing substrate.

22. Use according to any one of claims 19 to 21 for producing an animal feed product or a food product.

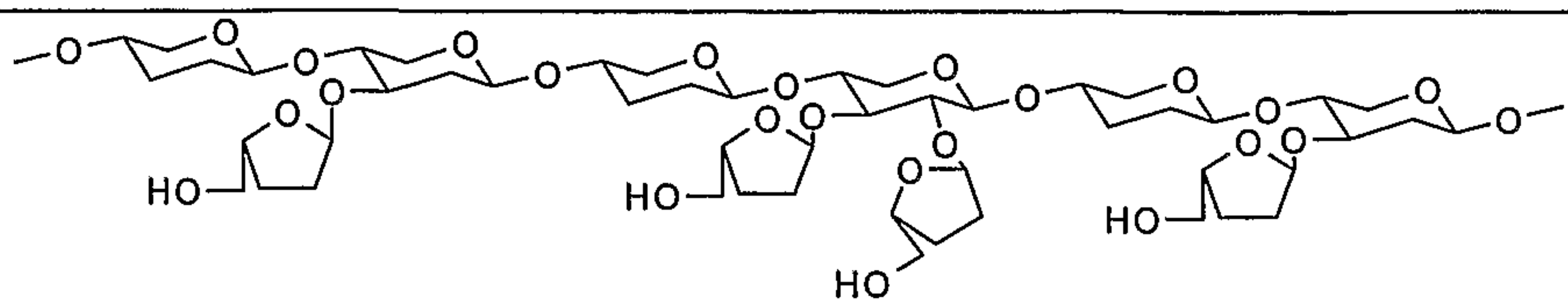
23. Use according to any one of claims 19 to 22 for producing xylose, arabinose, xylo-oligosaccharides, linear xylose or combination thereof.

24. Use according to any one of claims 19 to 23 for producing a dietary fiber.

25. Use according to any one of claims 19 to 24 for producing alcoholic beverages, fuel or potable ethanol.

Fig. 1

A



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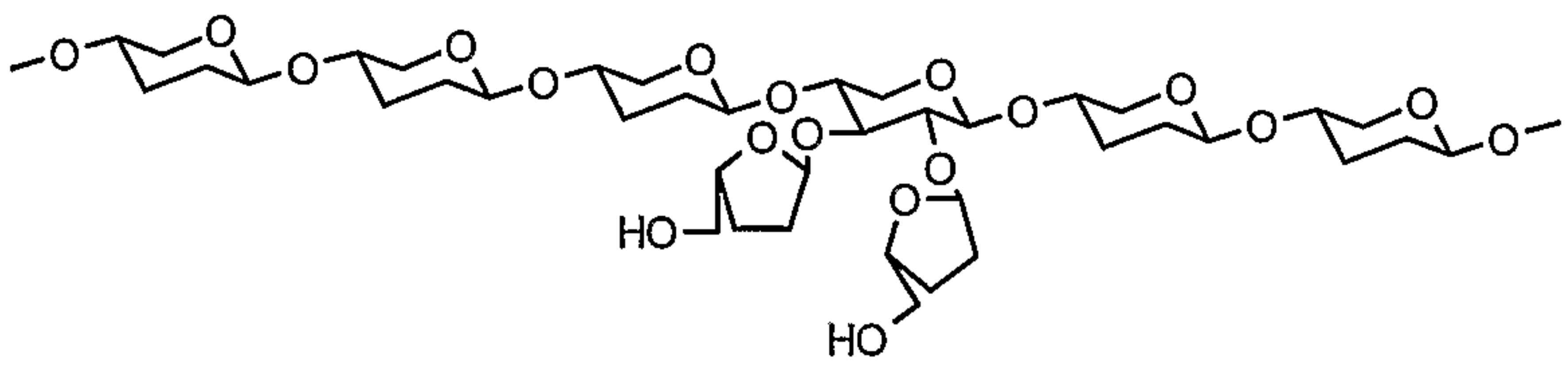

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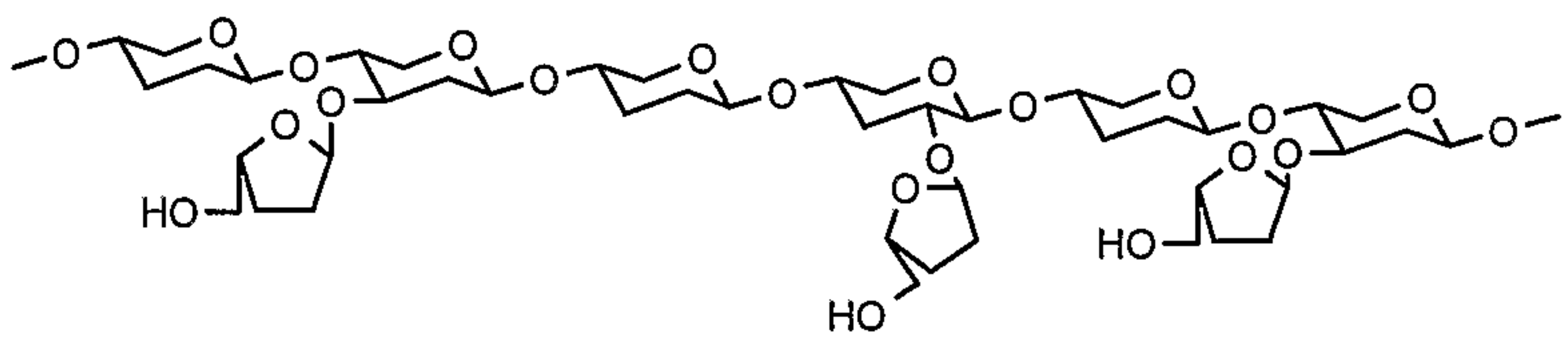
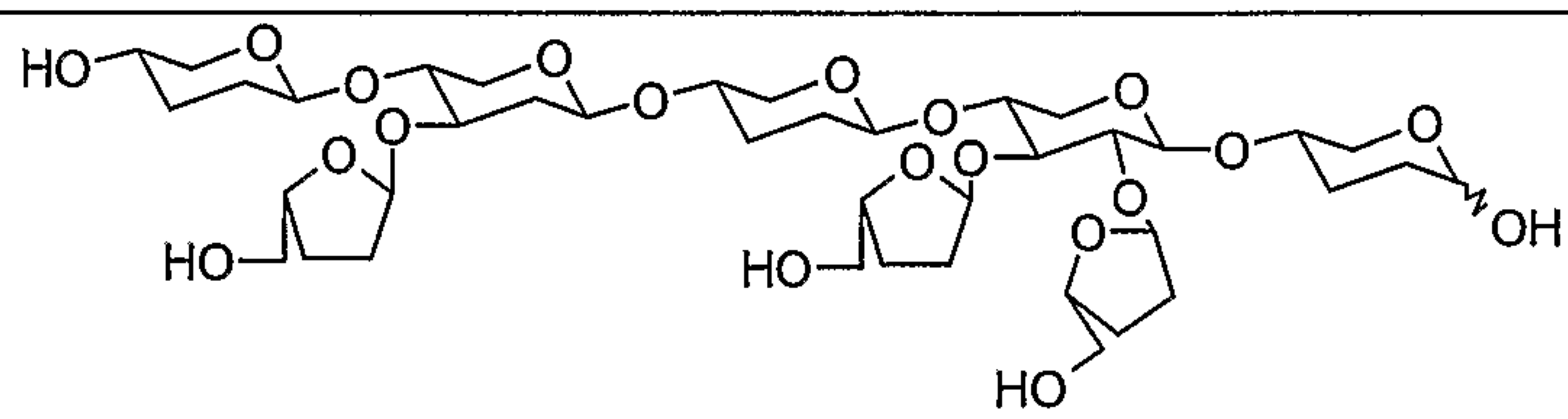


Fig. 2

A



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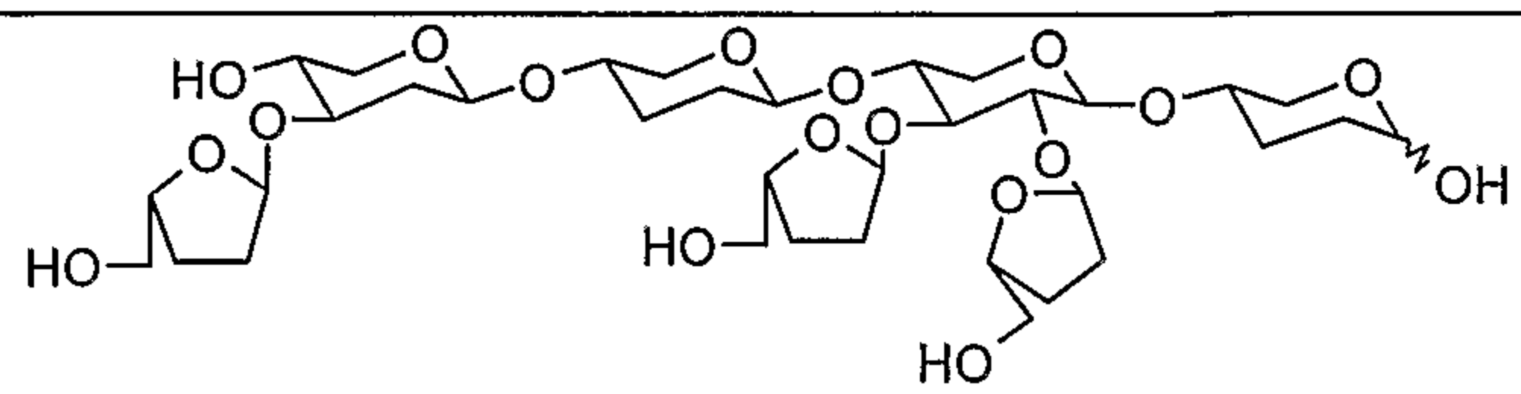

C