ENZYMATIC PROCESSING AND ANTIOXIDANT ACTIVITY OF AGRICULTURAL WASTE AUTOHYDROLYSIS LIQUORS

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This paper presents the production of xylooligosaccharides from autohydrolysis liquors of wheat straw and sunflower stalk, as wells as the antioxidant activity of these autohydrolysis liquors. The autohydrolysis liquors (raw or refined by ultrafiltration) of both wastes were subjected to the action of two different endoxylanase to produce xylooligosaccharides with low degree of polymerization that are potentially useful as food additives. *Trichoderma reseei* xylanase led to the highest proportion of oligomers with degree of polymerization values in the range 1–3, while *Aspergillus niger* xylanase gave mainly oligomers with polymerization degree in the range 2-3. *T reseei* xylanase gave a higher increase in equivalent xylose concentration and produced more monosaccharide than *A.niger* xylanase. Membranes with a nominal MWCO 1 kDa did not reject antioxidant-related and other low molar mass compounds, and most of these compounds were recovered with monosaccharides in the permeate of the membrane.

Keywords: Xylooligosaccharides; Autohydrolysis; Xylanase; Antioxidant

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INTRODUCTION

Xylan is a major component of plant hemicellulose that could potentially be an appropriate starting material for production of a variety of chemicals and compounds (Saha 2003). Depolymerization of xylan gives xylose and xylooligosaccharides (XOs). XOs show potential for practical applications in pharmaceuticals, feed formulations, agricultural purposes, and food applications. From nutritional point of view, XOs behave as non-digestible oligosaccharides. When consumed as a part of a diet, XOs have prebiotic properties due to their preferential utilization by *Bifidobacteria*. Additionally, they have various physiological importance such as reducing cholesterol, maintaining the gastrointestinal health, improving the biological availability of calcium, reducing risk of colon cancer, and having a cytotoxic effect on human leukemia cells (Ando et al. 2004).

Treatment of xylan-containing lignocellulosic biomass at high temperatures (140-220 ^{o}C), a process known as autohydrolysis, results in water autoionisation, which causes both xylan depolymerization (to give xylooligomers and xylose) and deacetylation of xylans (to give acetic acid, which increases the hydronium concentration in the reaction medium). The autohydrolysis process converts the xylan into soluble products such as XOs, high weight compounds related with the structure, monosaccharides, monosac-

charide decomposition products, and noncarbohydrate components, and leaves both cellulose and lignin in the solid phase (Vegas et al. 2008).

The molecular weight distribution of XOs produced through autohydrolysis shows the presence of a significant proportion of compounds with high degree of polymerization (DP) (MW 1000-3000 g/mol) and very low DP compounds (<MW 300 g/mol). However, the preferred DP range of XOs to be used as active ingredients of functional foods is 2-6, owing to the utilization of these compounds by *Bifidobacteria*. The high-DP compounds can be transferred into low-molecular weight compounds by the action of enzymes (Moure et al. 2006; Vegas et al. 2008). However, a high amount of low DP compounds (monosaccharides and degradation products) may cause inhibition effects to enzyme activity; therefore they have to be removed before enzymatic hydrolysis. Vegas et al. (2008) used membrane purification techniques to remove the undesired compound in autohydrolysis liquors, and they successfully concentrated the high DP compound and reduced the DP of them by the action of endo-xylanase.

Xylanase hydrolyzes β-1,4 glycosidic linkages in xylan, xylan fragments, and high molecular weight XOs to products with lower DP. In nature, xylanolytic enzyme systems consist of endoxylanase (1,4-ß-D-xylan xylohydrolases, EC 3.2.1.8), β-D-xylosidases (1,4-ß-xyloside xylanohydrolases, EC 3.2.1.37), and debranching enzymes (esterases) (Bakir, 2002). Since the presence of monosaccharides is undesirable in prebiotic oligosaccharides, the enzyme complex should have high endo-xylanase activity and should not result in xylose generation.

Autohydrolysis processes not only break down the xylan to smaller oligomers, but also cleave the β-1-4 alkyl-aryl linkages in lignin and form soluble phenolic compounds (Garrote et al. 2004; Nabarlatz et al. 2007). These phenolics, considered as the byproducts of the autohydrolysis process, might have potential application as food additives. Previous studies showed that phenolics in the autohydrolysis liquors had high antioxidant activity (Garrote et al. 2004; Moure et al. 2005).

This research deals with the enzymatic processing of sunflower stalk and wheat straw autohydrolysis liquors using xylanase. Due to their lignocellulosic nature (composed of hemicellulose, cellulose, and lignin), both agricultural wastes represent an abundant and cheap renewable resource for production of xylooligosaccharide (XO) and other compounds. The main objectives were to produce lower DP XO from autohydrolysis liquors of these wastes with two different commercial xylanase and assess the antioxidant activity of the byproducts of autohydrolysis.

EXPERIMENTAL

Materials

An Aminex HPX 87H column (dimension: 300x7.8 mm; average particle size: 25 μm) was purchased from Bio-Rad Laboratories, CA, USA. Xylooligosaccharide standards, xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexose (X6) were obtained from Megazyme, Ireland. An endo-xylanase from *Aspergillus niger* and *Trichoderma reseei* was obtained from Orbazyme, Turkey. Aluminum-backed silica gel Thin Layer Chromatography Plates were from Merck

KGaA, Germany. All the other chemicals were analytical grade obtained either from Sigma Chemical Company, MO, USA, or Merck KGaA, Germany.

Samples of sunflower stalk and wheat straw were collected from local farmer in Turkey, dried and fractioned to particle size of 5-10 mm.

Autohydrolysis

Sunflower stalk and wheat straw were mixed with water (liquid/solid ratio of 8:1 kg/kg) in a batch stainless steel reactor with a volume of 100 ml. The reactor was equipped with a teflon (PTFE) internal cup, manometer, and automatic temperature controller, connected to an electric heating apparatus. The treatment of the sample was carried out at 160 °C for 1 h. Solid fractions obtained were recovered by filtration, washed with water, air-dried, and subjected to moisture determination to measure the amount of dissolved substrate. The reducing sugar contents were quantified in the filtrate from the xylan suspension. Samples of liquors from autohydrolysis treatment were filtered through 0.20 μm membranes and used for direct HPLC determination of monosaccharides. A second sample of liquors was subjected to acid hydrolysis with 4g/100g sulfuric acid, and the increase in the concentrations of monosaccharides provided a measure of the oligomer concentrations (Garrote et al., 1999).

Analysis of Raw Materials and Autohydrolysis Liquors

Moisture, ash, and lignin (Klason and acid soluble) content of sunflower stalk and wheat straw were determined according to the method provided by ASTM (1993). Protein content of the agricultural waste was measured by the Kjeldahl method (Protein=6.25xN). The polysaccharides in the stalk were hydrolyzed according to Browning (1967), and the monosaccharide composition was determined. Ground agricultural waste (300mg) was mixed with 72g/100g sulfuric acid (3 ml), and the mixture was held at 30 °C for 1 h with stirring. The concentration of acid in the mixture was adjusted to 4g/100g by adding water, and the mixture was refluxed for 2 h. The sugars in the aliquot of the hydrolysate were assayed by HPLC as described below. The monosaccharides present in the hydrolysate were converted to percent monosaccharides: D-glucose to glucan, D-xylose to xylan, and D-arabinose to araban. The amount of acetyl groups present in the reaction products was determined by measuring acetic acid concentration by HPLC as described below.

Uronic acid content of agricultural wastes and autohydrolysis liquors were determined with the m-hydroxydiphenyl method (Melton and Smith 2002). Reducing sugars were quantified with the DNS method (Miller 1959) by using xylose as a standard. Furfural in the hydrolysate was determined with a UV spectrophotometer (adding 1 volume ethanol into the sample) at 277 nm (William 1997).

The monosaccharides and acetic acid were determined with an HPLC system equipped with a refractive index detector (Perkin Elmer Series 200), and column oven (Perkin Elmer Series 200) on Aminex HPX 87H (Biorad) column after hydrolysis with sulfuric acid (Pellerin et al. 1991). Neutral sugars were eluted at 45 ^{o}C with 5 mmol/l H_2SO_4 as the mobile phase (flow rate of 0.5 ml/min) from an Aminex HPX 87H.

Refining of Autohydrolysis Liquors

Autohydrolysis liquors were refined by two-step ultrafiltration (UF) for removal of undesired compounds. The 45 ml of hydrolysate was applied to a UF system (Amicon stirred cell-Model 8050, MA, USA) having 1 kDa-molecular weight cut-off (MWCO) membrane, and it was concentrated to achieve a volume reduction ratio of 3. The concentrate was diafiltrated two times, and discontinuous diafiltration was carried out as follows: distilled water was added to the concentrated solutions to restore the initial feed volume. This solution was concentrated again up to reach a volume reduction ratio of 3.

Enzymatic Xylan Hydrolysis

Commercial enzyme concentrates were assayed for the desired enzyme activities according to endo-xylanase activity (Bailey et al. 1992). One unit is defined as the quantity of enzymes which liberates 1.0 μmol of xylose per minute under described conditions.

The processing scheme for XO production is given in Figure 1. The pHs and temperatures for the enzymatic hydrolysis were as follows: *T.reseei* xylanase, 4.6 and 50 ^{o}C; *A. niger* xylanase, 5.5 and 40 ^{o}C. Enzymatic hydrolysis of raw and refined autohydrolysis liquors were conducted by mixing 1 ml of 8 U ml^{-1} of *T.reseei* xylanase or *A. niger* xylanase with 10 ml of hydrolysate and allowing the incubation to proceed. A 1-ml sample was taken at regular intervals and heated to 100 ^{o}C for 5 min to inactivate the enzyme. The hydrolysis reaction was monitored by measuring the reducing sugars formed with the dinitrosalicylic acid (DNS) method. The reaction products were further analyzed by thin layer chromatography on silica plates to determine the degree of polymerization (DP) of the XOs. The solvent system was ethyl acetate: acetic acid:water (the volumetric ratio 2:2:1), with detection with ethanol-sulfuric acid (the volumetric ratio 18:2) (Chen et al. 1997).

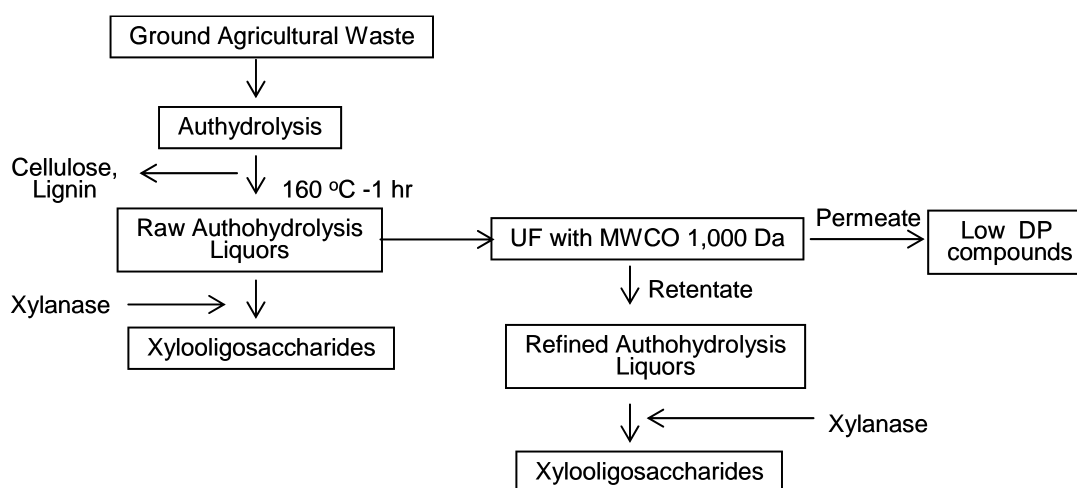

Figure 1. The processing scheme of XO production from sunflower stalk and wheat straw

FRAP Assay

The solutions of 300mM acetate buffer (pH 3.6), 10 mmol/l TPTZ in 40 mmol/l HCl, and 20 mmol/l $FeCl_3.6H_2O$ were prepared, and fresh working solutions were used by mixing stock solutions in a respective ratio of 10:1:1 to prepare the FRAP reagent. The FRAP reagent (2850µL) was mixed with 150µL extract or standard, and the mixtures were kept at room temperature in darkness for 30 min, and then absorbance of ferrous tripyridyltriazine complex was measured at 593nm with a spectrophotometer. A standard calibration curve was plotted using Trolox at a concentration range between 10 and 50µM (Thaipong et al. 2006).

DPPH Assay

The DPPH assay procedure described by Thaipong et al. (2006) was used to determine antioxidant activities of extracts. The DPPH solution (120 mmol/l) was prepared to obtain an absorbance of 1.10±0.02 units at 515nm. Samples (150µL) were allowed to react with the DPPH working solution (2850µL) in the dark. Then the absorbance was taken at 515nm wavelength. The linear standard curve between 10 and 50µM Trolox was used to express the results in Trolox equivalents (Thaipong et al. 2006).

RESULTS AND DISCUSSION

Sunflower stalk and wheat straw, major agricultural wastes in Turkey, are generally incorporated into soil or burned in the field. Utilization of the wastes for industrial purposes is important for economic and ecological reasons. Previous studies showed that alkali-extracted xylans from sunflower stalk and wheat straw were appropriate substrate for production of XOs (Akpinar et al. 2007, 2009a,b). Table 1 shows the chemical composition of sunflower stalk and wheat straw. The major component was determined as glucan (36.3-36.8 g/100g), followed by xylan (22.0-23.1 g/100g) and klason lignin (19.8-26.1 g/100g).

Table 1. Composition of the Raw Material, Expressed as Percent of Dry Weight

Components	Content (g/100 g wheat straw)	Content (g/100 g sunflower stalk)
Glucan	36.8	36.3
Xylan	23.1	22.0
Arabinan	1.84	0.66
Acetyl groups	1.24	2.83
Uronic acid	3.20	6.20
Klason lignin	19.8	26.1
Acid soluble lignin	1.56	1.37
Proteins	2.70	1.11
Ash	5.46	3.13
Others (by diff.)	4.24	0.30

The xylan type polysaccharide occurs in several structural varieties in plants, and even in different plant tissues within the same plant. Xylan from higher plants has β-(1→4) linked xylopyranoside units as the backbone, usually substituted with different sugar such as arabinose, glucose, and *O*-acetyl groups (Ebringrova and Heinze 2000). Wheat straw showed higher arabinose content, whereas sunflower stalk showed higher uronic acid and acetic acid contents.

Other components of wheat straw and sunflower stalk (ash, acid soluble lignin, and protein) have also been determined. Like most of the non-wood fibers, the ash content of these plant materials (3-5 g/100g) was markedly higher than that of wood species (Agrupis and Maekawa 1999). Protein and acid soluble lignin contents of wheat straw were found to be higher than those of sunflower stalk. The rest of the components (extractives such as hot water, cold water or ethanol extractives) were of minor importance for this study, and are reported as "others".

It was observed that formation of toxic decomposition products at high autohydrolysis temperatures resulted in the complete inhibition of the enzymatic reaction (Yuan et al. 2004). Therefore, autohydrolysis was accomplished at 160 ^{o}C for 1 h with the severity factor of 3.5 (calculated as followed: R_0 = t* exp(T-100)/14.75). Table 2 presents the composition of wheat straw and sunflower stalk autohydrolysis liquors. Based on the autohydrolysis of 100 kg wheat straw and sunflower stalk, 22 and 21 kg of dissolved substrate were obtained, respectively. The concentrations of XOs (calculated as the sum of XOs and XO substituent) in the autohydrolysis liquors of wheat straw and sunflower stalk were 2.7 and 2.1 g/l, respectively. In addition to XOs, glucooligosaccharides coming from starch fractions of the raw materials were present, and their level in the autohydrolysis liquors of wheat straw and sunflower stalk were 0.5 and 0.2 g/l, respectively.

Table 2. Composition of the Raw Sunflower Stalk and Wheat Straw Autohydrolysis Liquors

Components	Content (g/l wheat straw liquor)	Content (g/l sunflower stalk liquor)
Glucose	0.035±0.012	0.027±0.007
Xylose	0.086±0.037	0.078±0.015
Arabinose	0.211±0.048	0.006±0.001
Glucooligosaccharides	0.458±0.024	0.202±0.055
Xylooligosaccharides	2.733±0.320	2.068±0.491
Arabinooligosaccharides	0.154±0.017	0.026±0.016
Acetyl groups linked to oligosaccharides	0.061±0.030	0.179±0.049
Uronic acid	0.460±0.069	1.164±0.100
Furfural	0.252±0.003	0.202±0.007

The autohydrolysis process yields a significant amount of very low DP compounds (monosaccharides and monosaccharides dehydration products). Accumulation of monomers may inhibit the endoxylanase activity, which is called end-product inhibition (Reilly 1981). Due to the presence of undesired compounds in raw autohydrolysis liquors

(monosaccharides and furfural), a refining process (diafiltration and concentration through 1 kDa membrane) was carried out. Table 3 lists compositional data for refined wheat straw and sunflower stalk autohydrolysis liquors. Undesired compounds (monosaccharides from 0.331-0.111 g/l to 0.038-0.016 g/l and furfural from 0.252-0.202 g/l to 0.112-0.134 g/l in refined both autohydrolysis liquor) were reduced, while the target compounds (XOs from 2.733-2.068 g/l to 3.214-4.587 g/l) were increased with this treatment. The refining process eliminated more than 90 g/100g of monosaccharides and almost 50 g/100g of furfural from autohydrolysis liquors of wheat straw and sunflower stalk.

Table 3. Composition of the Refined Sunflower Stalk and Wheat Straw Autohydrolysis Liquors

Components	Content (g/l wheat straw liquor)	Content (g/l sunflower stalk liquor)
Glucose	0.002±0.002	0.002±0.002
Xylose	0.012±0.001	0.013±0.001
Arabinose	0.024±0.001	0.001±0.001
Glucooligosaccharides	0.319±0.072	0.339±0.012
Xylooligosaccharides	3.214±0.668	4.587±0.350
Arabinooligosaccharides	0.135±0.020	0.035±0.000
Acetyl groups linked to oligosaccharides	0.099±0.000	0.376±0.039
Uronic acid	0.960±0.029	1.808±0.043
Furfural	0.112±0.005	0.134±0.003

The preferred DP range of XOs, used as active ingredients of functional foods is 2-6, owing to the utilization of these compounds by *Bifidobacteria*. In order to obtain XO in the DP range 2-6 as the product from autohydrolysis, an extra treatment with a hydrolytic reagent such as an enzyme or an acid is needed. To achieve high-molecular weight xylan depolymerization products, two commercial endo-xylanase preparations were employed in experiments: *Trichoderma reseei* xylanase and *Aspergillus niger* xylanase.

The autohydrolysis solutions from wheat straw and sunflower stalk were treated with the two commercial xylanases at a loading of 8 U/ml. The time course of the concentration of equivalent xylose (measured by the DNS assay) in the reaction media shows the extent of the enzymatic hydrolysis, and the increase in the amount of reducing sugar along the hydrolysis process provides the comparative evaluation of results obtained with different enzymes and/or substrates. The data in Figure 2 A and B show that *T. reseei* xylanase yielded higher increase in equivalent xylose concentration during the hydrolysis process of wheat straw and sunflower stalk autohydrolysis liquors. *T. reseei* xylanase produced mainly low molecular-weight oligomers (Fig. 3). The major reaction products corresponded to oligomers of DP 2–3 with monosaccharides, and the relative proportions of the monosaccharides increased with time. *A. niger* xylanase yielded the products with DP range 2 to 5, and the relative proportions of DP2 increased with the time (Fig. 3).

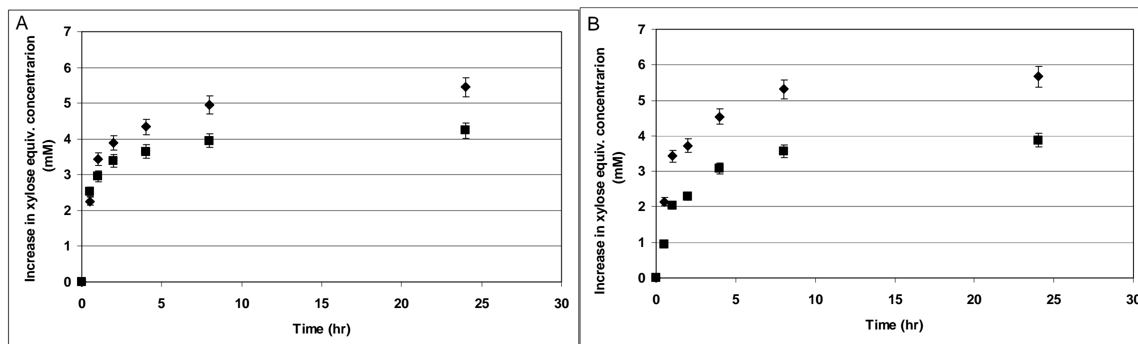

Figure 2. A: XO production with xylanase from raw sunflower stalk autohydrolysis liquor. B: XO production with xylanase from raw wheat straw autohydrolysis liquor (each data point was average of four replicate determinations and the error bars show the data ranges) (*Trichoderma reseei*:◆, *Aspergillus niger*:■).

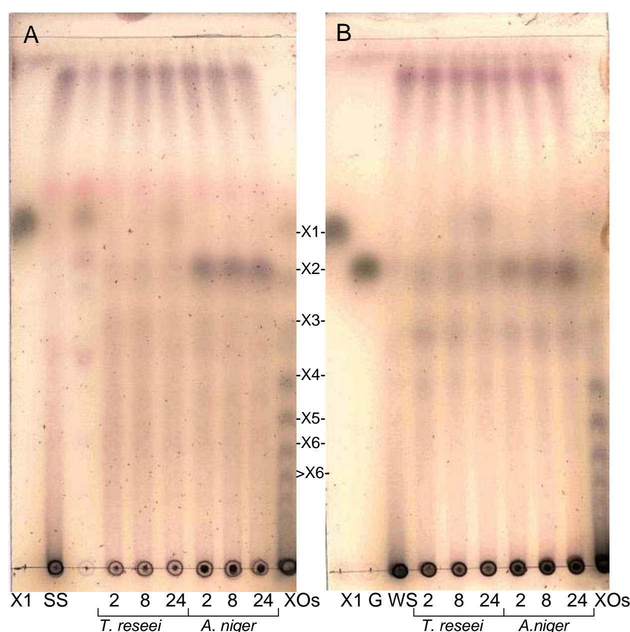

Figure 3. Thin layer chromatogram of XOs from raw sunflower stalk and wheat straw autohydrolysis liquors produced by *A. niger* and *T. reseei* xylanases at different times. A: Raw sunflower stalk autohydrolysis liquor; B: raw wheat straw autohydrolysis liquor; SS: raw sunflower stalk autohydrolysis liquor; WS: raw wheat straw autohydrolysis liquor; G: glucose; X1: xylose; X2: xylobiose; X3: xylotriose, X4: xylotetraose; X5: xylopentaose; X6: xylohexose; >X6: longer chain oligosaccharide; XOs: xylooligosaccharides standard.

The refined autohydrolysis solutions were treated with the same xylanase at a loading of 8 U/ml. The hydrolysis profile determined for *T. reseei* and *A. niger* xylanase showed similarities with respect to the processing of raw autohydrolysis liquors (Fig. 4 A and B). *T. reseei* xylanase produced mainly low molecular-weight oligomers with DP in

the range 1 to 3. Again, the proportion of oligomers with DP in the range 2–3 was higher for *A. niger* xylanase (Fig. 5).

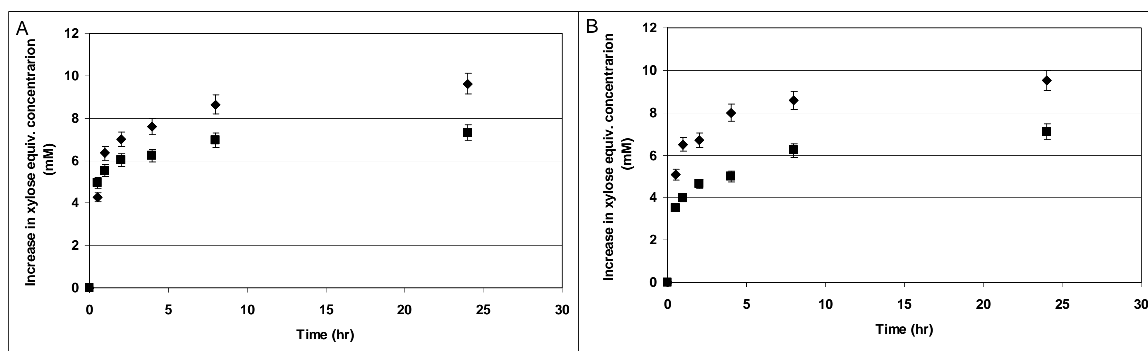

Figure 4. A: XO production with xylanase from refined sunflower stalk autohydrolysis liquor. B: XO production with xylanase from refined wheat straw autohydrolysis liquor (each data point was average of four replicate determinations and the error bars show the data ranges) (*Trichoderma reseei*:◆, *Aspergillus niger*:■).

Endo-xylanases have been classified into glycoside hydrolase families designated as 5, 8, 10, 11, and 43, based on their structure and amino acid sequences (Kolenova et al. 2006). Most of the endo-xylanases belong to the glycosyl hydrolase families 10 and 11 (GHF 10 and GHF 11) (Tenkanen, 2000), which have different physicochemical properties (including structure, molecular weight, pI, and thermal stability) (Biely et al. 1997), and have different specificities for different xylans. This difference is mainly determined by the degree of substitution of the xylan. In this research two commercial endo-xylanase preparations were used: *T. reseei* xylanase from the GH 10 family and *A. niger* xylanase from the GH 11 family. In general, GH-10 xylanases require a lower number of contiguous unsubstituted xylose residues in the main chain and produce oligosaccharides having a low DP, while GH-11 xylanases tend to be more specific for xylan and form larger oligosaccharides (Biely et al. 1997; Courtin and Delcour 2001). The data in Figure 1 and 3 show that *T. reseei* xylanase from the GH 10 family led to higher increases in equivalent xylose concentration and produced more monosaccharide than *A. niger* xylanase from GH 11 family. The findings from this research are in agreement with the literature information cited above concerning the general properties of GH-10 and GH-11 xylanase.

The autohydrolysis liquors can be used for the production of antioxidant compounds. The presence of these compounds in the autohydrolysis liquors is undesirable, because they decrease the purity of XOs or inhibit the microbial metabolism of them. Generally, ethyl acetate treatments have been employed to separate non-saccharide fractions with antioxidant properties from autohydrolysis liquors containing xylooligosaccharide. However, this method is not practical and economical for the processing of large scale autohydrolysis liquors.

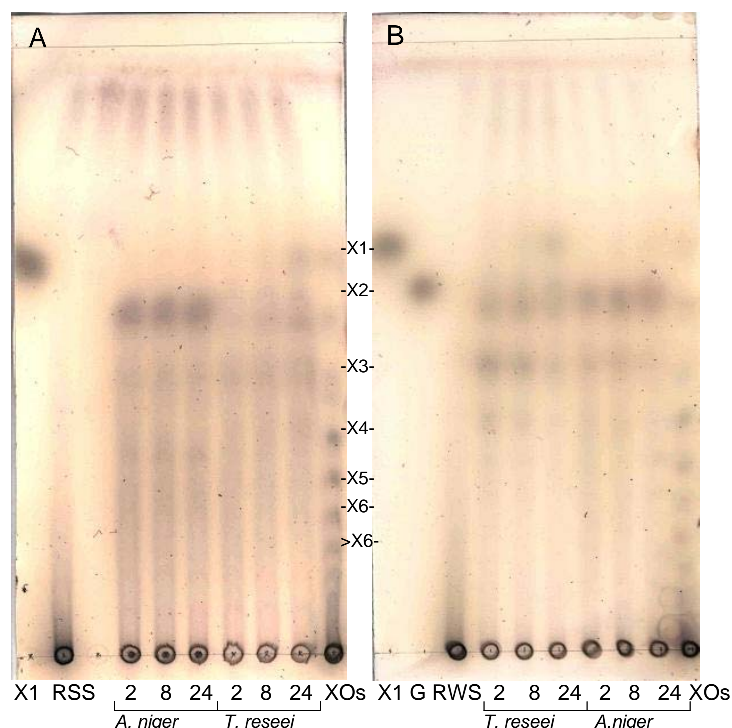

Figure 5. Thin layer chromatogram of XOs from refined sunflower stalk and wheat straw autohydrolysis liquors produced by *A. niger* and *T. reseei* xylanases at different times. A: Refined sunflower stalk autohydrolysis liquor; B: refined wheat straw autohydrolysis liquor; RSS: refined sunflower stalk autohydrolysis liquor; WWS: refined wheat straw autohydrolysis liquor; G: glucose; X1: xylose; X2: xylobiose; X3: xylotriose, X4: xylotetraose; X5: xylopentaose; X6: xylohexose; >X6: longer chain oligosaccharide; XOs: xylooligosaccharides standard.

Membrane separations can be considered as an alternative approach. Ultrafiltration and nanofiltration, which are well known membrane separation processes, are the most promising methods for refining and concentrating oligosaccharides and separating molecules with different molecular weight. The size-dependent selection mechanism of the membrane process results in a varying concentration of molecules with different molecular weight (Li et al. 2004).

The antioxidant activities of feed and permeates were evaluated using the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging capacity test (Brand-Williams et al. 1995) and FRAP based on the reduction of Fe^{3+}-TPTZ to a blue colored Fe^{2+}-TPTZ (Benzie and Strain 1996). Table 4 shows results for the antioxidant activity of raw autohydrolysis liquors and permeates obtained from the ultrafiltration of wheat straw and sunflower stalk liquors. The antioxidant activities of raw sunflower stalk and wheat straw autohydrolysis liquors were in the range of those produced from autohydrolysis treatment of different lignocellulosic materials (Conde et al. 2009a,b). It was found that raw sunflower stalk autohydrolysis liquor had higher antioxidant activity than wheat straw. The membranes with a nominal MWCO of 1 kDa did not reject antioxidant-related and other low molar mass compounds, which represent phenolics in the raw autohydrolysis liquors of wheat straw and sunflower stalk, indicating that most of these compounds were recovered in the permeate of the membrane.

Table 4. Recovery of Antioxidant Activity in the Permeate of 1 kDa MWCO Membrane

	Antioxidant activity test	Initial Feed (μmol Trolox)	MWCO 1kDa permeate (μmol Trolox)
SS	DPPH	3.12	1.94
WS		2.80	1.87
SS	FRAP	3.30	2.66
WS		2.88	2.45

CONCLUSIONS

1. Sunflower stalk and wheat straw were used as source materials to produce xylooligosaccharide by autohydrolysis followed by enzymatic hydrolysis.
2. Ultrafiltration using a commercial 1 kDa membrane is a suitable process for the purification of xylooligosaccharide solutions obtained by autohydrolysis of lignocellulosic biomass, forming low molecular weight sugars, phenolics, and other lignin-related compounds.
3. These results show that autohydrolysis-treated sunflower stalk and wheat straw can be used for production of value-added products such as prebiotics and antioxidants.

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