JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enzymatic Production of Xylooligosaccharides from Cotton Stalks

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Xylooligosaccharide (XO) production was performed from xylan, which was obtained by alkali extraction from cotton stalk, a major agricultural waste in Turkey. Enzymatic hydrolysis was selected to prevent byproduct formation such as xylose and furfural. Xylan was hydrolyzed using a commercial xylanase preparation, and the effects of pH, temperature, hydrolysis period, and substrate and enzyme concentrations on the XO yield and degree of polymerization (DP) were investigated. Cotton stalk contains about 21% xylan, the composition of which was determined as 84% xylose, 7% glucose, and 9% uronic acid after complete acid hydrolysis. XOs in the DP range of 2−7 (X6 ≈ X5 > X2 > X3) were obtained with minor quantities of xylose in all of the hydrolysis conditions used. Although after 24 h of hydrolysis at 40 °C, the yield was about 53%, the XO production rate leveled off after 8−24 h of hydrolysis. XO yield was affected by all of the parameters investigated; however, none of them affected the DP of the end product significantly, except the hydrolysis period. Enzyme hydrolysis was maintained by the addition of fresh substrate after 72 h of hydrolysis, indicating the persistence of enzyme activity. The optimal hydrolysis conditions were determined as 40 °C, pH 5.4, and 2% xylan. The obtained product was fractionated via ultrafiltration by using 10, 3, and 1 kDa membranes. Complete removal of xylanase and unhydrolyzed xylan was achieved without losing any oligosaccharides having DP 5 or smaller by 10 kDa membrane. After a two-step membrane processing, a permeate containing mostly oligosaccharides was obtained.

KEYWORDS: Xylooligosaccharides; cotton stalk; xylan; enzymatic hydrolysis; agricultural waste

INTRODUCTION

Cotton stalk with an annual generation rate of 1.5 million tons is a widely distributed and abundant agricultural waste found in Turkey (*1*). It can be used as animal feed, but generally the stalks are incorporated into soil or burned in the field after harvesting. Because of its lignocellulosic nature, cotton stalk has the potential to be used as a renewable raw material for a variety of chemical productions such as ethanol, glucose, xylose, xylitol, and xylooligosaccharides (XOs). The hemicellulose content of cotton stalk was reported as between 15 and 20% (*2*), and thus it could potentially be an appropriate starting material for the production of xylooligosaccharides.

XOs are sugar oligomers produced during the hydrolysis of xylan, which is the major component of plant hemicellulose, a heteropolysaccharide with a homopolymeric backbone of xylopyranose moieties (*3*). XOs are high value-added ingredients for functional foods, and they have potential uses as prebiotics. They have various physiological important actions such as reducing cholesterol, maintaining gastrointestinal health, and improving the biological availability of calcium. Besides, because they are moderately sweet, are stable over a wide range of pH and temperatures, and can inhibit starch retrogradation, they improve the nutritional and sensory properties of food (*4*).

Production of XOs from xylan-rich lignocellulosic materials generally includes chemical methods, enzymatic methods, and a combination of these methods (*5*). The production of XO with chemical methods can be accomplished by steam, diluted solutions of mineral acids, or alkaline solutions. Extraction of xylan with steam or acid produces large amounts of monosaccharides and their dehydration product (*6*−*8*). Steam or hydrolytic degradation of xylan, known as autohydrolysis, involves the deacetylation of xylans to produce acetic acid, which hydrolyzes the hemicellulose (*9*−*11*). This method eliminates the use of corrosive chemicals for the extraction of xylan. However, it requires special equipment that can be operated at high temperatures. The production of XOs with direct enzymatic treatment of xylan-containing materials is the

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10.1021/jf063580d CCC: $37.00
Published on Web 06/08/2007

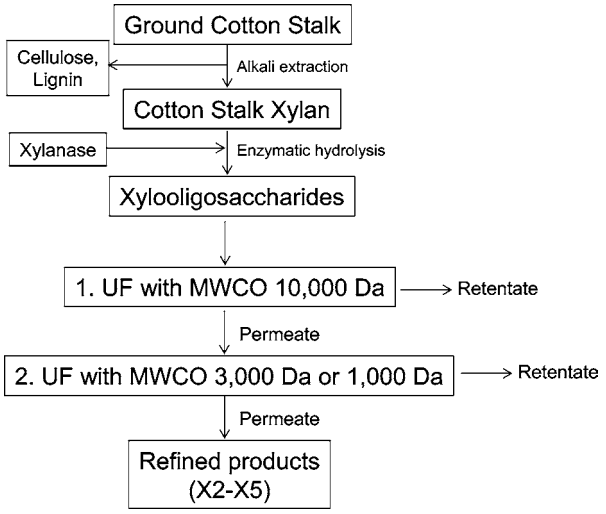

Figure 1. Processing scheme of XO production from cotton stalk.

only suitable method for susceptible materials such as citrus peels (*12*). To produce XOs with chemical and enzymatic methods, xylan is generally extracted with an alkaline such as KOH or NaOH from suitable lignocellulosic materials, and extracted xylan is converted to XOs by xylanase enzyme having low exo-xylanase and/or β-xylosidase activity. In contrast to autohydrolysis, this method is more desirable, because it does not produce undesirable byproducts or high amount of monosaccharides and does not require special equipment. Therefore, there are many papers that describe production of XOs by enzymatic hydrolysis xylan from oat spelt (*13*), beechwood (*14*), corn cob (*15*−*17*), wheat straw (*18*, *19*) and hardwood (*20*).

After the production of oligosaccharides, the most important step is a refining step, so the high molecular weight polysaccharides and low molecular weight sugars that do not have beneficial properties are separated from the main product. For this purpose, vacuum evaporation, solvent extraction, or chromatographic methods can be used (*12*). However, these methods are not practical and economical for the large-scale production of xylooligosaccharides. An alternative method might be membrane separations. Ultrafiltration and nanofiltration, well-known membrane separation processes, are the most promising methods for refining and concentrating oligosaccharides. The size-dependent selection of mechanism of the membrane process results in the various concentrations of molecules with different molecular weights. Membrane separations have been used for the preparation of several oligosaccharides such as fructooligosaccharides (*21*), chitooligosaccharides (*22*), pectic oligosaccharides (*23*), soybean oligosaccharides (*24*), and maltooligosaccharides (*25*). Recently, membrane technologies have been used successfully for processing XOs produced by enzymatic hydrolysis and autohydrolysis (*6*, *8*, *19*, *26*).

Because there is no information reported about utilization of cotton stalk for XO production, the driving force of this study was to test the suitability of cotton stalk for enzymatic preparation of XOs. In light of this, this paper deals with the characterization of cotton stalk and cotton stalk xylan (CSX), which was extracted from cotton stalk, and the optimization of the production conditions of XOs from CSX, with commercial xylanase preparation and membrane fractionation of them (**Figure 1**).

MATERIALS AND METHODS

Materials. A commercial xylanase, Veron 191, produced from *Aspergillus niger* (AB Enzymes, Darmstadt, Germany), was used for the hydrolysis of xylan. Ultrafiltration disc membranes were purchased from Millipore-Amicon, Bedford, MA. Aluminum-backed silica gel 60 thin layer chromatography plates were from Sigma Chemical Co., St. Louis, MO. An MCarb67C column (dimensions = 300 × 7.8 mm; average particle size = 25 μm) was purchased from MetaCarb, Varian Inc., Harbor City, CA. Xylooligosaccharide standards, xylobiose (X2), xylotriose (X3), xylotetraose (X4), and xylopentaose (X5), were obtained from Megazyme, Bray, Ireland. All other chemicals were of analytical grade obtained either from Sigma Chemical Co. or Merck KGaA, Darmstadt, Germany.

General Methods. Uronic acid and total neutral sugars were determined with *m*-hydroxydiphenyl (*27*) and phenol/sulfuric acid reagents (*28*). Reducing sugars were quantified according to the dinitrosalicylic acid (DNS) method (*29*) by using xylose as a standard. Protein concentration was determined following the Bradford method (*30*) using bovine serum albumin (BSA) as standard. Neutral monosaccharides were determined by HPLC on the MCarb67C column after hydrolysis of sulfuric acid (*31*). Moisture, ash, cellulose, hemicellulose, and lignin were determined according to the method provided by ASTM (*32*).

Extraction of Xylan. Xylan was extracted according to the method of Zilliox and Debeire (*18*), with slight modification. The ground cotton stalk was swollen at 60 °C for 16 h in water. Xylan from 2 g of cotton stalk was extracted with 17 mL of 24% KOH including 1% (w/v) $NaBH_4$ for 3 h at 35 °C. The extract was centrifuged at 10000*g* for 20 min followed by filtration on Whatman no. 1 filter paper. The xylan was precipitated in 2 volumes of cold ethanol and 0.2 volume of acetic acid and filtered on coarse filter paper. After filtration, the solid was dried in a vacuum oven at 60 °C to yield 0.4 g of xylan from 2 g of cotton stalk.

Xylan Hydrolysis. The protein content of the commercial enzyme solution was assessed by the Bradford method with BSA as a standard (*30*). Commercial enzyme concentrates were assayed for the desired enzyme activities according to endo-xylanase activity (*33*).

Hydrolysis of CSX was conducted by mixing 1 mL of 1.1 units/mL of commercial xylanase preparation with 10 mL of 2% CSX in 50 mM citrate buffer, pH 5.4, and allowing the incubation to proceed at 40 °C. A 1 mL sample was taken at regular intervals and heated to 100 °C for 5 min to inactivate the enzyme. The results of hydrolysis reaction were monitored by measuring the reducing sugars formed with the DNS method. One unit is defined as the quantity of enzymes that liberates 1.0 μmol of xylose per minute under described conditions. 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 was 2:2:1), with detection with ethanol/sulfuric acid (the volumetric ratio was 18:2) (*13*).

To optimize pH, temperature, substrate and enzyme concentrations, and reaction time on the production of oligosaccharides, the reaction was performed using the method described above with different pH values (4.5, 5.44, and 6.5); temperature (30, 40, 45, 50 °C); substrate concentration (in the range of 1−10%); enzyme concentration (0, 0.35, 0.70, 1.1, 2.2, 4.4 units/mL), and reaction time (in the range of 0−72 h).

Membrane Separations. For batch UF processing, four sets of 10 mL of 2% xylan substrates, which had already been incubated with 1 mL of 1.1 units/mL xylanase, were used. The reaction was terminated after 8 h of incubation. The 10 mL of hydrolysate was applied to the UF system having a 10 kDa molecular weight cutoff (MWCO) membrane to separate high molecular weight polysaccharides and enzymes. The oligosaccharide-containing permeate obtained from UF was next batch-processed by a second UF having membranes with 3 or 1 kDa molecular weight cutoffs. The maximum allowed membrane operating pressure was 4.3 bar, as recommended by the manufacturer. The reducing sugar and total sugar in retentate and permeate after both UF processes were analyzed with phenol/sulfuric acid method. The results were further analyzed by HPLC as described below to determine the DP of the XOs.

HPLC Separation of XOs. XOs were chromatographed on a (Varian Prostar) HPLC system equipped with a differential refractometer (model 350) and column oven (model 510). Before injection, samples were filtered through a 0.20 μm filter. Aliquots of filtered sample (10 μL)

Table 1. Chemical Composition of Cotton Stalk

component	content[a] (g/100 g of cotton stalk)
ash	3.81 ± 0.09
xylan	21.42 ± 1.96
cellulose	35.68 ± 0.76
lignin	27.83 ± 1.05
others (by diff)	11.26

[a] Mean ± standard deviation of at least three replicate determinations.

Table 2. Composition of Cotton Stalk Xylan

CSX	content[a] (%)	CSX	content[a] (%)
arabinose		galactose	
xylose	83.60 ± 0.66	glucose	7.12 ± 0.73
mannose		uronic acid	9.28 ± 1.29

[a] Mean ± standard deviation of at least three replicate determinations.

were injected onto the HPLC system. XOs were eluted using distilled−deionized water as the mobile phase from an ion-mediated stationary phase in the calcium form (MCarb67C). The column (300 × 7.8 mm), which was preceded by its complimentary deashing cartridge (MetaCarb), was used at 80 °C and a flow rate of 0.5 mL/min. A complete analysis of XOs was carried out in 30 min. Computing integrators determined the start, retention time, and end of the peak and integrated the area under each peak as a function of the height and width of the peak. The concentration of an oligosaccharide was quantified using average peak areas compared with a mixture of standard oligosaccharides (X1−X5) and expressed as milligrams per milliliter of oligosaccharide.

RESULTS AND DISCUSSION

To convert cotton stalk, generated in huge quantities, into more valuable products, initially its composition was determined. As shown in **Table 1**, concentrations of cellulose, xylan, and lignin were determined on a dry basis as 35.68, 21.42, and 27.83%, respectively, consistent with the literature (*34*). To get the maximum benefit possible from cotton stalk, separation and individual utilization of these three polymers were considered. After polymer separation, composition of the CSX was determined to indicate a suitable application area. As observed from **Table 2**, arabinose, mannose, and galactose were not detected in CSX. The major component of CSX on dry basis was xylose, 83.6%, together with 7.12 and 9.28% of glucose and uronic acid, respectively. This is similar to a common species of hardwood xylan (glucuronoxylan) (*35*). The simple structure of glucuronoxylan can be effectively converted by enzymatic hydrolysis to XOs. Therefore, enzymatic XO production from CSX and its fractionation via ultrafiltration were investigated in this study, considering the advantages of enzymatic production such as the lack of toxic byproduct formation, the ease of recovery, and the high-level purity of the final product and also the importance of nondigestible oligosaccharides for health and relatively higher market prices of the XOs ($22 U.S. $/kg) (*36*).

After alkali extraction of xylan from cotton stalk, enzymatic hydrolysis was performed (**Figure 1**). The effects of hydrolysis period, pH, temperature, and substrate and enzyme concentrations on XO production were studied. The investigation was performed on both XO yield and product range (DP). **Figure 2** illustrates the effect of pH on XO production from CSX. The hydrolysis reaction was carried out at three pH values in a range of 4.5−6.5. The results showed a faster hydrolysis having higher

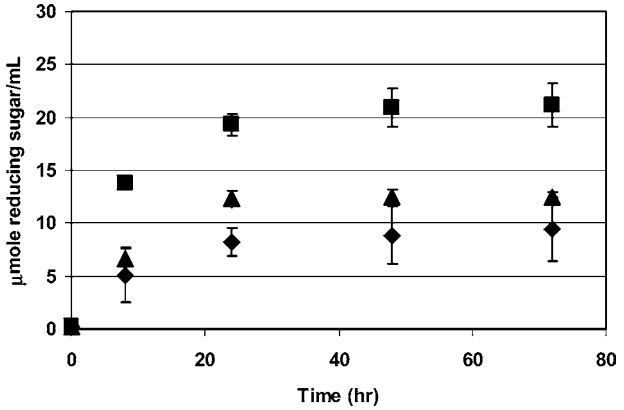

Figure 2. Effect of pH on XO production from cotton stalk xylan with xylanase: pH 4.5, ◆; pH 5.4, ■; pH 6.5, ▲. Each data point is the average of four replicate determinations, and the error bars show the data ranges.

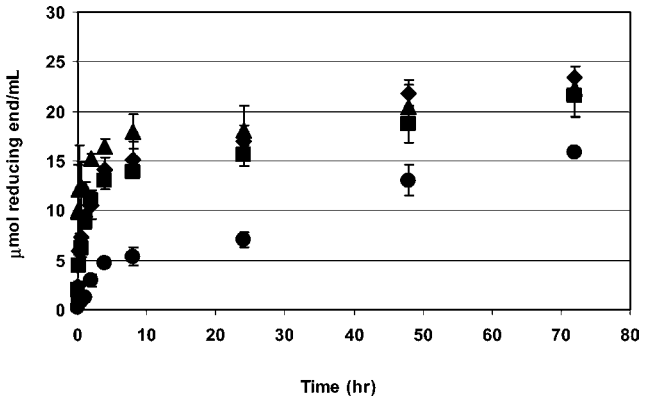

Figure 3. Effect of temperature on XO production from cotton stalk xylan with xylanase: 30 °C, ●; 40 °C, ■; 45 °C, ◆; 50 °C, ▲. Each data point is the average of four replicate determinations, and the error bars show the data ranges.

yields at pH 5.4. The rate of reducing sugar production decreased considerably after 24 h.

The effect of temperature on hydrolysis is given in **Figure 3**. As the temperature of the hydrolysis reaction was increased, the hydrolysis rate and yield also increased, as expected (**Figure 3**). However, the differences between 40, 45, and 50 °C were not very significant, most probably due to thermal instability of the enzyme.

The effect of enzyme concentration on 2% xylan hydrolysis at 40 °C is given in **Figure 4**. As the enzyme concentration was increased, both the xylan hydrolysis yield and rate also increased (**Figure 4A**). As observed from **Figure 4B**, there was no significant effect of enzyme dosage on product DP. Because the production rate of the XOs at the lowest enzyme concentration was too slow as observed from **Figure 4A**,**B**, a suitable enzyme concentration should be selected depending on economic analysis of the process.

The time course of hydrolysis of 10 different concentrations of xylan (1−10%) with 1.1 units/mL xylanase was studied (**Figure 5A**). Analysis of the products showed that the hydrolysis was characterized by a rapid increase in the reducing sugars depending on the substrate concentration. The hydrolysis products of CSX by the xylanase were examined by TLC, and the data for 1−5% xylan are given in **Figure 5B**. The XO DP was not affected by the substrate concentration. The amount of reducing sugar per milligram of substrate was highest when the

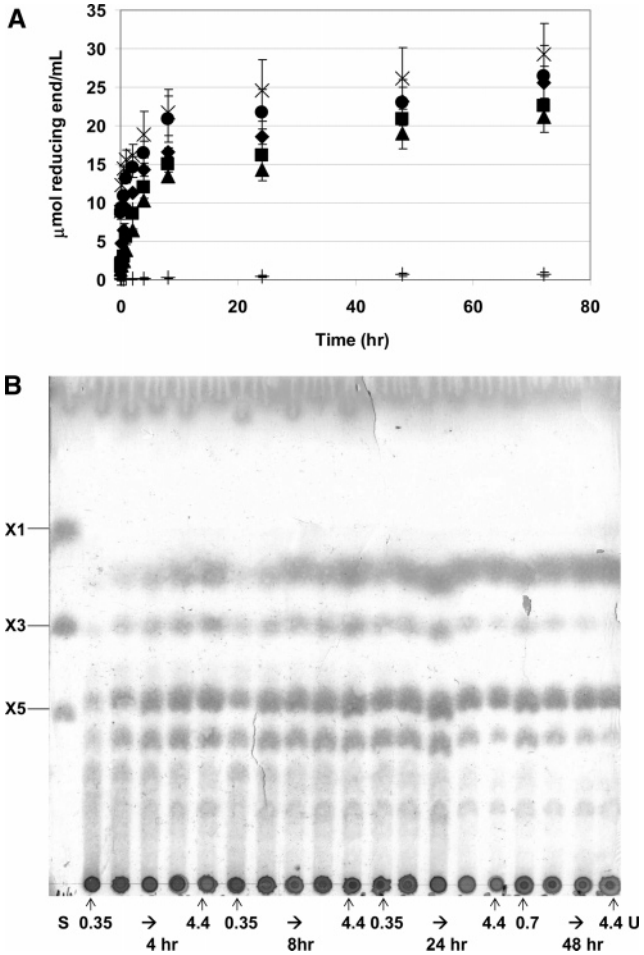

Figure 4. (**A**) Time course of effect of enzyme dosage on XO production from cotton stalk xylan: 0 units/mL, +; 0.35 unit/mL, ▲; 0.7 unit/mL, ■; 1.1 units/mL, ◆; 2.2 units/mL, ●; 4.4 units/mL, ×. Each data point is the average of four replicate determinations, and the error bars show the data ranges. (**B**) Thin layer chromatogram of XOs from cotton stalk xylan produced by xylanase. The solvent system was ethyl acetate/acetic acid/water (2:2:1), with detection with ethanol/sulfuric acid (18:2).

xylan concentration was %2 (**Figure 5C**). Utilization of xylan at concentrations >2% decreased XO yield, most possibly due to enzyme inhibition by the impurities present and/or increases in the viscosity and density of the reaction mixture. Yoon et al. (*17*) found that the production of pentose yield from corn cobs and corn husks was adversely affected by a high concentration of substrate, and they speculated that a high concentration of substrate might result in the reduction of water content in the reaction mixture, which lowered pentose yield.

To determine whether the depletion of the substrate or the deactivation of the enzyme was effective for the hydrolysis reaction, two control experiments were performed with the addition of either fresh substrate or enzyme. Enzyme hydrolysis was maintained by the addition of 0.25 mL of fresh substrate to 72 h hydrolysis reaction medium, indicating the persistence of the enzyme activity (**Figure 6**). However, hydrolysis could not be prolonged with the addition of 0.25 mL of fresh enzyme (1.1 units/mL), suggesting the formation of xylanase-resistant xylan fragments, which might be overcome by the addition of other types of xylanase having different substrate selectivities or xylan debranching enzymes.

The time course of XO production by 1.1 units/mL xylanase dosage from 2% CSX substrate at 40 °C is given **Figure 7**. Although on TLC plates (**Figures 4B** and **5B**) some oligosac-

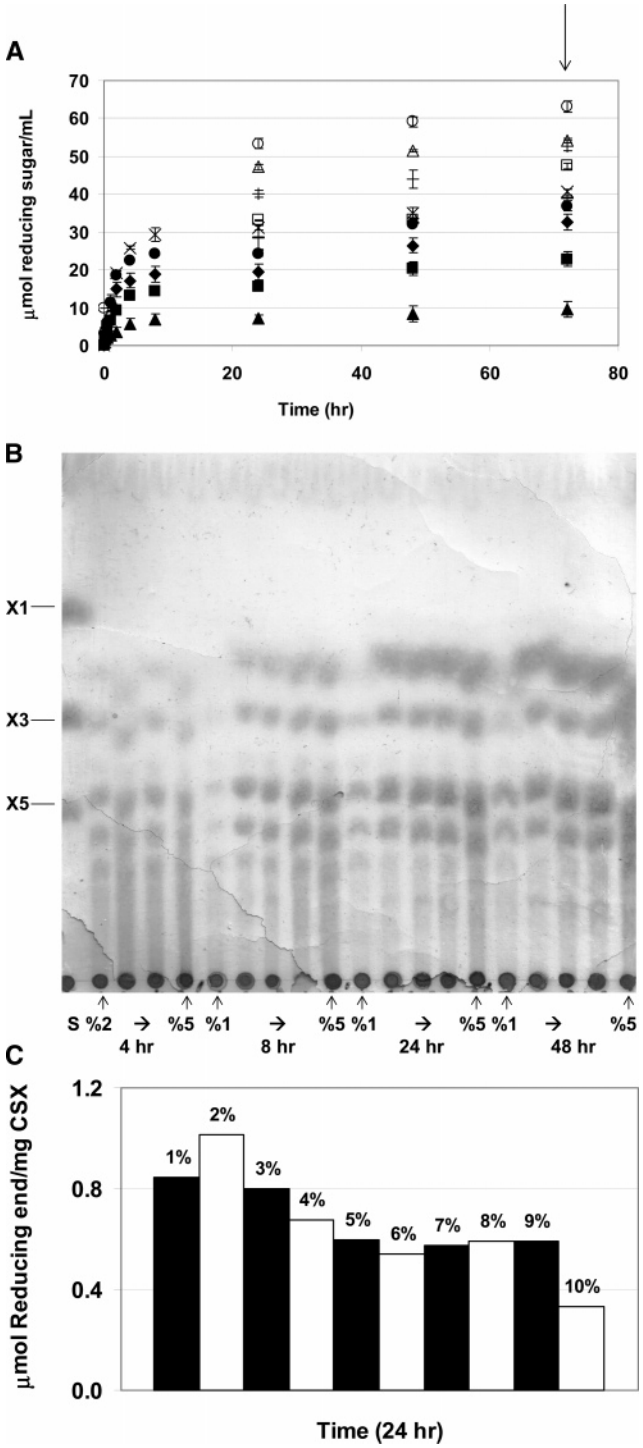

Figure 5. (**A**) Time course of effect of cotton stalk xylan concentration on XO production: 1%, ▲; 2%, ■; 3%, ◆; 4%, ●; 5%, ×; 6%, –; 7%, +; 8%, △; 9%, ○; 10%, □. Each data point is average of four replicate determinations, and the error bars show the data ranges. Arrow indicates where fresh enzyme and fresh substrate were added. (**B**) Thin layer chromatogram of XOs from cotton stalk xylan produced by xylanase. The solvent system was ethyl acetate/acetic acid/water (2:2:1), with detection with ethanol/sulfuric acid (18:2). (**C**) Amount of reducing sugar per milligram of substrate after 24 h of hydrolysis.

charides larger than X5 could be seen, only X1, X2, X3, and X5 could be quantified due to the limitation of the HPLC column. It was found that XO yield within a DP range from 1 to 5 is 53% after 24 h of the reaction at 40 °C, and xylopentaose, xylotriose, and xylobiose yields were 41, 4.0, and 6%, respec-

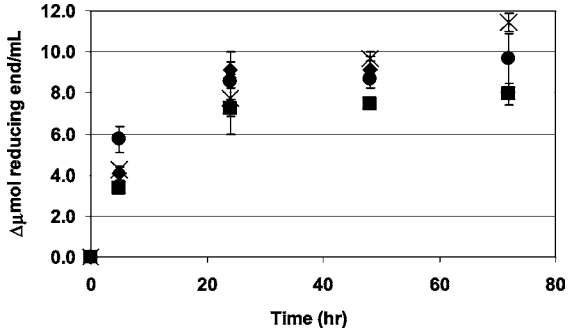

Figure 6. Time course of effect of cotton stalk xylan concentration on XO production, after fresh substrate added to 72 h: 2%, ■; 3%, ◆; 4%, ●; 5%, ×. Each data point is the average of four replicate determinations, and the error bars show the data ranges.

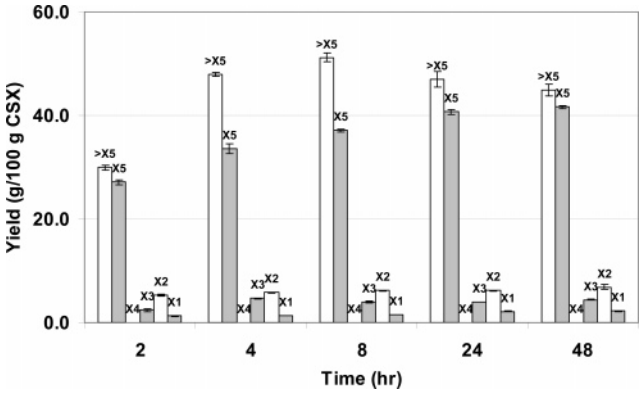

Figure 7. HPLC analysis of XOs from 2% CSX with 1 mL from 1.1 units/mL xylanase concentration at 40 °C. Each data point is the average of two replicate determinations, and the error bars show the data ranges.

tively, whereas xylose yield was about 2% in the hydrolysate. The relative content of X4 remained at a very low level. This means that, even if X4 was released, it immediately hydrolyzed into smaller oligosaccharides. Extending the reaction beyond 24 h did not result in significant increase in the XO yield from CSX. Whereas the TLC results did not show any xylose production, the HPLC result (**Figure 7**) showed that a small amount of xylose exists in the hydrolysate, showing a low exo-xylanase or β-xylosidase activity of the xylanase preparation (Veron 191). Enzyme preparations having high exo-xylanase or β-xylosidase activities produce high amounts of xylose, which may cause inhibition effects, for XO production (*37*). Yuan et al. *(6)* speculated that the alkaline-extracted xylan would be more resistant to the enzymatic hydrolysis due to substituents in xylans and the pretreatment such as steaming could increase the XO yield. However, these types of treatments increase the monosaccharide yield (*6*, *7*).

As observed from all of the hydrolysis progress curves (**Figures 2**, **3**, **4A**, **5A**, **6**, and **7**), the hydrolysis rate of xylan was very fast up to 8 h and leveled off after 24 h. On the other hand, product DP, especially >5, decreases when the hydrolysis period increases. Therefore, an optimum hydrolysis period should be determined depending on the required DP of the product. However, in general, a hydrolysis period of between 8 and 24 h seems to be reasonable.

For fractionation of enzymatically produced XOs, ultrafiltration experiments with membranes having different molecular weight cutoff values were carried out. Concentration and composition of oligosaccharides in the initial feed, the final retentate, and the total permeate were monitored by HPLC and total sugar assay.

Table 3. Recovery of Oligosaccharide in the Final Permeate of 3000 and 1000 MWCO Membrane

oligosaccharide	initial feed amount[a] (mg)	MWCO 3000 recovered (%)	MWCO 1000 recovered (%)
total	88 ± 7	78	72
>X5	42 ± 2	62	57
X5	28 ± 3	93	84
X4	0.0	0.0	0.0
X3	2.8 ± 0.8	96	93
X2	4.4 ± 0.7	89	89
X1	1.5 ± 0.1	93	93

[a] Mean ± standard deviation of two replicate determinations.

Mass balances through the two ultrafiltration processes are shown in **Figure 8**. A total of 88 mg of reaction hydrolysate, determined by total sugar assay, in 10 mL of reaction medium was applied to the first ultrafiltration with the membrane of 10 kDa MWCO. This membrane successfully removed the high molecular weight polysaccharides and proteins from the oligosaccharides. The HPLC profile of the permeate fraction showed the presence of XOs ranging from X1 to longer than X5, whereas the retentate contained mainly high molecular weight polysaccharides and the enzyme (**Figure 8**). There was no significant loss of XOs between DP1 and DP5 in the retentate of the membrane of the 10 kDa MWCO (**Figure 8A**). Therefore, if desired, the amount of >X5 removed can be increased, by increasing the retentate volume or by using cascade ultrafiltration system, without the loss of any oligosaccharides.

The oligosaccharide syrup permeate obtained from the first UF, composed of 43.9% >X5, 42.7% X5, 4.3% X3, 6.8% X2, and 2.3% X1, was processed by a second UF having a 3 kDa MWCO membrane (**Figure 8A**). The analysis of the final permeate (**Figure 8A**) showed high concentrations of XOs having a DP between 2 and 5, longer than five and xylose. The recoveries of >X5, X5, X3, X2, and X1 were 62, 93, 96, 89, and 93% in the permeate, respectively (**Table 3**). The retentate had the XOs having DP 5 and >5. The final retentate of the 3 kDa MWCO membrane contained 20% X5, whereas that of the 10 kDa MWCO membrane contained 0.3% X5 (**Figure 8A**). 78% of reaction hydrolyzate was refined as XOs with DP between 2 and 5 and >5 at the end of the two step ultrafiltration process (**Table 3**).

When the second ultrafiltration process was repeated with the membrane of MWCO 1 kDa, the retention of X5 and >X5 were higher than that with MWCO 3 kDa (**Figure 8**). Changing the membrane from 3 to 1 kDa MWCO did not change the composition in the final permeate, drastically, but decreased total oligosaccharide recovery (**Table 3**). The recoveries of longer chain oligosaccharides (>X5) in the permeate of 3 and 1 kDa MWCO were 62 and 57%, respectively. With 10 and 1 kDa MWCO ultrafiltration membranes, 72% of reaction hydrolysate was refined as XOs with DP between 2 and 5 and >5.

The main difference between 3 and 1 kDa membranes from 10 kDa membrane is that their retention abilities for X5 were higher (**Figure 8**). The main function of 3 and 1 kDa membranes in the process was to separate longer chain oligosaccharide >X5 from the rest. Therefore, both membranes can be considered as good candidates for refining of XOs. However, to separate smaller oligosaccharides (X5, X3, X2, and X1) from each other, the nanofiltration membrane should be used.

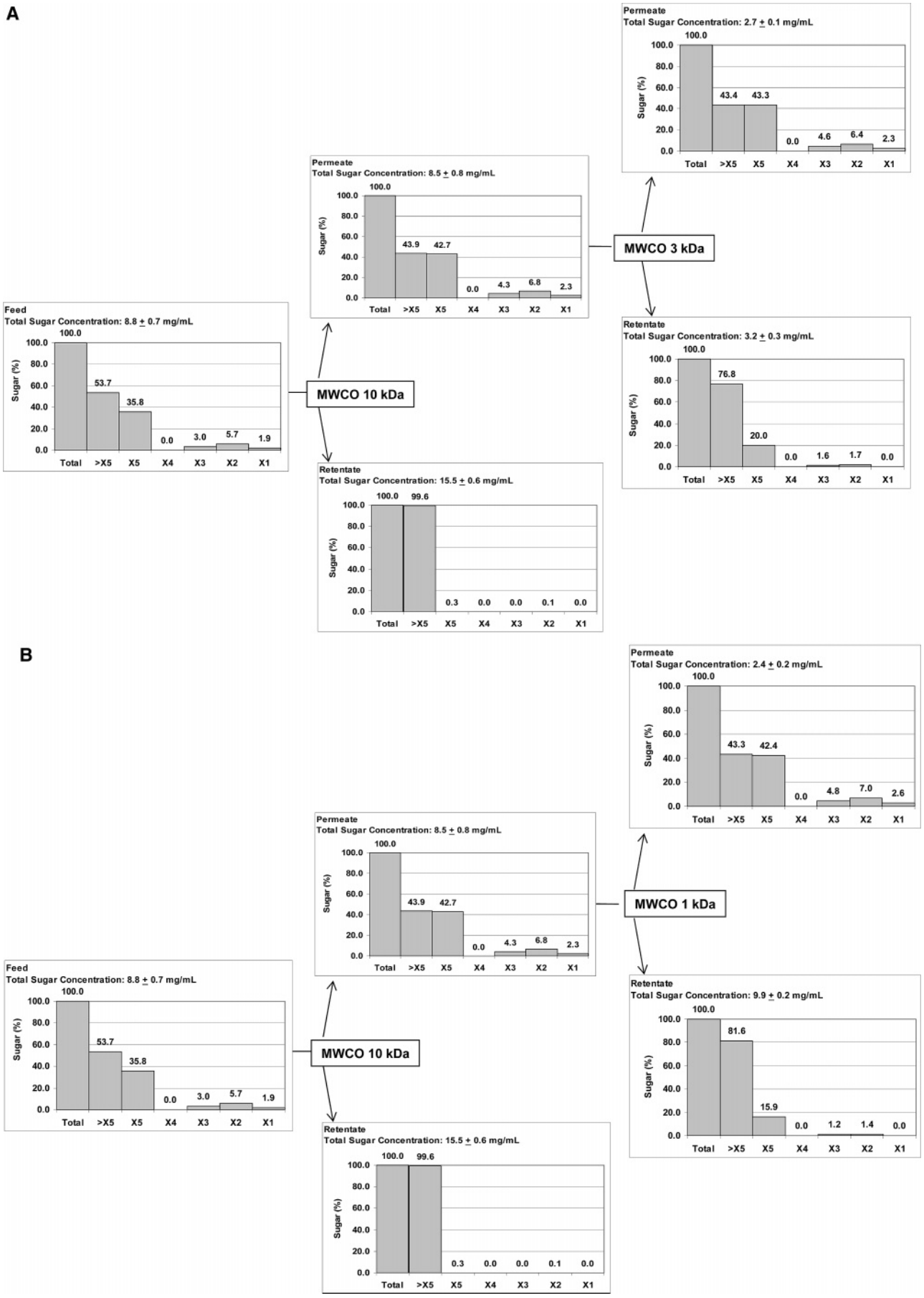

Figure 8. Mass balance through two-step ultrafiltration processes: (**A**) with 10000 Da MWCO membrane and 3000 MWCO membrane; (**B**) with 10000 Da MWCO membrane and 1000 MWCO membrane.

Swennen et al. (*19*) used ultrafiltration with 5, 10, and 30 kDa MWCO membranes for the isolation of enzymatically produced arabinoxylooligosaccharides from wheat xylan. They found that the ultrafiltered fractions were more heterogeneous and polydisperse and less strictly separated than the fraction obtained with the 1 and 3 kDa MWCO membranes used in this study. The ultrafiltration membrane with a narrow size distribution results in more selective separation. Yuan et al. (*6*) used different sizes of nanofiltration membranes for the separation of xylose and other monosaccharides from the XOs, produced by steam and enzyme-treated corncob. They removed only 78.8% of xylose from the remaining XOs with nanofiltration. Because the production of xylose in our study remained at a low level (**Figure 8**), the nanofiltration membrane was not used.

This research demonstrated that cotton stalk, which had no economical value, could be converted by enzymatic hydrolysis to a more valuable XO product without production of significant quantities of xylose after extraction of its xylan. A two-step ultrafiltration process, using membranes of 10 and 3 or 1 kDa cutoff, fractionated XO syrup without much loss.

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Received for review December 11, 2006. Revised manuscript received April 6, 2007. Accepted April 19, 2007. This work was supported by The Scientific and Technological Research Council of Turkey (104O359).

JF063580D