



Production of xylooligosaccharides by controlled acid hydrolysis of lignocellulosic materials

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ABSTRACT

Different agricultural wastes, namely tobacco stalk (TS), cotton stalk (CS), sunflower stalk (SS), and wheat straw (WS), were used for the production of xylooligosaccharide (XO). XO production was performed by acid hydrolysis of xylan, which was obtained by alkali extraction from these agricultural wastes. The major component of these agricultural wastes was determined as cellulose (30–42%), followed by xylan (20%) and lignin (20–27%). Xylans from these wastes had mainly xylose (85–96%) with small amount of glucose, while wheat straw xylan contained also arabinose. The best xylan conversion into XOs was achieved with 0.25 M H₂SO₄ with 30-min reaction time. Under these conditions, the XO yield was between 8% and 13%. The yield of XOs depends on both acid concentration and hydrolysis time, but the yield of monosaccharide depends on the structure and composition of xylan besides acid concentration and the time. The more branched xylan, WSX, gave the highest monosaccharide (~16%) and furfural (~49 mg/100 g xylan) yield. This research showed that all xylans from selected agricultural wastes generated XOs with similar profiles, and these oligosaccharides could be used as functional food ingredients or soluble substrates for xylanases.

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1. Introduction

Xylooligosaccharides (XOs) are sugar oligomers produced during the hydrolysis of xylan, the major component of plant hemicelluloses.¹ Since they affect the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon and thus improve health, they have been used as prebiotics, which are important ingredients of functional food.^{2–5} They have various physiological importances such as: (i) reducing cholesterol level, (ii) maintaining the gastrointestinal health, (iii) improving the biological availability of calcium, (iv) reducing the risk of colon cancer, (v) having cytotoxic effects on human leukemia cells, and (vi) having beneficial effect on type 2 diabetes mellitus.^{6–9} XOs can be used as a source of xylose for the production of xylitol¹⁰ or for the preparation of ethers and esters which can be used as thermoplastic compounds for water-soluble films, coatings, and capsules.¹¹ Besides, β -(1→4)-linked XOs are especially important substrates that serve as model compounds for xylanase (1,4- β -D-xylan xylohydrolases, EC 3.2.1.8), which is the key enzyme for hydrolysis of β -(1→4) glycosidic linkages in xylan.¹² Although xylan is the natural substrate for xylanolytic enzymes, the structural complexity of xylan limits its usefulness for detailed mechanistic studies that are aimed at understanding the mode of action of

the enzymes. Therefore, XOs are used to gain better understanding of reaction mechanism of xylanolytic enzymes.

The composition and structure of the XO depend on the source and the production process. XOs can be prepared by autohydrolysis of lignocellulosic biomass, or can be prepared from partial acid or enzymatic hydrolyzates of xylans from lignocellulosic materials, such as birchwood, beechwood, corncob, and oat spelts. In autohydrolysis process, lignocellulosic material is heated in an aqueous media, and the hydronium ions coming from water autoionization cause 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).^{13–15} However, the hydrolyzates from this process contain a variety of undesirable components, such as soluble lignin and a large amount of monosaccharides and their dehydration product.¹⁶ Therefore, extensive purification is necessary. In addition, the molecular weight distribution of XOs produced through autohydrolysis shows the presence of significant proportion of high DP compounds (MW 1000–3000 g/mol) and very low DP compounds (<MW 300 g/mol).¹¹ Alternatively, XOs can be produced by enzymatic or acid hydrolysis of xylan. Since, in lignocellulosic biomass, xylan exists as a xylan–lignin complex and becomes resistant to hydrolysis,¹⁷ currently XOs production is carried out in two stages: alkaline extraction of xylan from lignocellulosic biomass followed by enzymatic^{18–20} or acid hydrolysis^{21,22} of the xylan. In enzymatic hydrolysis, xylanases with different substrate specificities produce different

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hydrolysis end products. In addition, the profile of XOs is dependent not only on the enzyme but also on the xylan source.²³ Depending on the source of xylan, enzymatic production of XO can afford branched heteroxylooligosaccharides²⁴, and such heterooligosaccharides made it difficult to separate individual XO by chromatographic fractionation.²² However, the fragmentation of xylan by acid hydrolysis can be easily accomplished by partial acid hydrolysis. Acids hydrolyze the glycosidic bonds, randomly between adjacent xylose units. This method is practical for the preparation of XOs.²²

Food industries and laboratories working on xylanolytic enzymes spend considerable amounts of time and/or money to obtain XOs. The fast growth of the functional food market and the increasing number of other industrial applications are forcing to find renewable and cheap xylan sources instead of the hardwood xylan for the preparation of XOs. Agricultural wastes of lignocellulosic nature are widely available in Turkey, and they contain 15–25% of xylan. Some of the widely distributed and abundant agricultural wastes found in Turkey are cotton stalk, tobacco stalk, sunflower stalk, and wheat straw.²⁵ These wastes could potentially be appropriate substrates for production of XOs. In addition, since they are usually left to rot or burned in the field after harvesting,²⁶ utilization of these materials for industrial purposes does not only solve the proper disposal of these wastes, but also provides additional income for farmers and generates employment.

The aim of this study was to prepare a series XOs from agricultural wastes. For this purpose, the chemical composition, production and yield of xylan from selected agricultural wastes: tobacco stalk (TS), cotton stalk (CS), sunflower stalk (SS), and wheat straw (WS) were explored. The suitability of xylan produced from these materials was tested for production of series of XOs, where acid hydrolysis and XO production conditions from selected agricultural wastes were optimized.

2. Results and discussion

Characterization of four xylan-rich agricultural wastes, TS, WS, SS, and CS, shows that the composition of these raw materials differs among the plant type (Table 1). Like most of the non-wood fibers, ash content of TS, CS, SS, and WS was markedly higher than that of the wood species.²⁶ Among them, the lowest amount of ash (around 3%) was determined in SS, which is in agreement with the literature.²⁷ The major component was determined as cellulose (30–42%), followed by xylan (19–21%) and lignin (20–27%). Alkali-extracted xylans of TS (TSX), CS (CSX), SS (SSX), and WS (WSX) were around 20% of the total dry weight. Hot water extracts including tannins, gums, sugars, starches, and coloring matter were higher in CS (11.34%) than in WS, TS, and SS. Ethanol-benzene extracts showing the waxes and fats portions of the agricultural wastes were the lowest in SS (1.74%) and the highest in CS (4.97%).

Table 1

Chemical composition of tobacco stalk (TS), cotton stalk (CS), sunflower stalk (SS), and wheat straw (WS), expressed as weight percent of dry weight (mean \pm standard deviation of three replicate determinations)

Components	Content (%)			
	TS	CS	SS	WS
Ash	6.5 \pm 1.0 ^a	6.8 \pm 0.2 ^a	3.2 \pm 0.4 ^b	5.5 \pm 0.3 ^a
Alkali extractable xylan	20.0 \pm 1.6 ^a	19.8 \pm 0.3 ^a	19.1 \pm 3.8 ^a	20.9 \pm 0.7 ^a
Cellulose	40.4 \pm 1.8 ^a	30.5 \pm 0.3 ^b	42.3 \pm 3.0 ^a	40.0 \pm 1.9 ^a
Lignin	20.7 \pm 1.2 ^b	26.6 \pm 0.4 ^a	26.5 \pm 0.4 ^a	20.0 \pm 0.2 ^b
Extract with hot water	9.2 \pm 0.3 ^b	11.3 \pm 0.1 ^a	7.2 \pm 0.7 ^c	9.3 \pm 0.5 ^b
Extract with EtOH–benzene	3.2 \pm 0.1 ^c	5.0 \pm 0.2 ^a	1.7 \pm 0.2 ^d	4.3 \pm 0.5 ^b

The experimental values that have no common letter are significantly different ($p < 0.05$) according to Duncan's multiple range test.

Based on the sugar composition of xylans extracted from these agricultural residues, all xylans are shown to be heterogeneous polymers of pentose and hexose sugars and sugar acids (Table 2). Among the selected sources, CSX showed the highest glucose content, and WSX showed the highest arabinose content.²⁶ Xylose content was in the range of 85–96%. No acetyl group could be detected because xylan loses acetyl groups during the alkali extraction of xylan.²⁸

All extracted xylans contain uronic acids, although amount and occurrence depend on the plant source. Uronic acid content was higher in TSX, CSX, and SSX (10–11%) than in WSX (5%) (Table 2). The composition of CSX, TSX, and SSX is similar to common species of hardwood xylan, glucuronoxylan, while the composition of WSX is arabinoxylan.

The effect of different acid concentrations (0.125 M, 0.25 M, and 0.5 M H₂SO₄) on XOs production from TSX, CSX, SSX, and WSX was evaluated through measurement of reducing sugars (Fig. 1). Reducing sugar production rates increased with time and acid concentration. The analysis of the composition of XOs produced from TSX, CSX, SSX, and WSX showed that 0.5 M sulfuric acid produced mainly monosaccharides rather than oligosaccharides (Fig. 2). Since the production rate of the XOs with 0.125 M sulfuric acid was too slow as observed from Figure 1 and 0.25 M H₂SO₄ was used in all further experiments.

The amount of reducing sugar per milligram substrate during different times of hydrolysis is shown in Figure 3. The hydrolysis rate of xylan was very fast initially and slowed down after a 60-min period. Increase in the reducing sugar per milligram substrate was similar at three different concentrations of the substrate. Therefore, unlike for enzymatic hydrolysis of xylan, in acid hydrolysis, xylan concentration higher than 2% can be used without worrying about the impurities present, and/or increase in the viscosity and density of the reaction mixture, which lowers the oligosaccharide yield.²⁹

XO productions after 30 and 60 min by 0.25 M H₂SO₄ concentration from 2% TSX, CSX, SSX, and WSX are given in Figure 4. The xylans were hydrolyzed to a variety of oligosaccharides ranging from X1 to X6 and >X6. Although on HPLC chromatogram (Fig. 5), some oligosaccharides larger than X6 such as X7 and X8 could be seen, these oligosaccharides were shown as >X6 due to the lack of standard. Since a reaction time longer than 30 min resulted in hydrolysis of the substrate to mainly monosaccharides including xylose, glucose, or arabinose depending on the source of xylan, reaction times longer than 60 min were not included in this figure. When the products from four kinds of xylans are compared, it is clear that the reaction time and acid concentration are the major factors which influence the resulting products. For all xy-

Table 2

The content of neutral sugars (relative% xylan, w/w) and uronic acids (% xylan, w/w) in the extracted xylan (mean \pm standard deviation of three replicate determinations) (mean \pm standard deviation of three replicate determinations) and substitution degree of each alkali-extracted xylan

Components	Content (g/100 g)			
	TSX	CSX	SSX	WSX
Arabinose	0 ^b	0 ^b	0 ^b	11.47 \pm 0.17 ^a
Xylose	93.46 \pm 1.18 ^a	85.12 \pm 7.28 ^b	95.73 \pm 3.72 ^a	85.17 \pm 0.107 ^b
Glucose	6.54 \pm 0.99 ^b	14.88 \pm 7.28 ^a	4.27 \pm 3.72 ^b	3.36 \pm 0.07 ^b
Uronic acid	10.00 \pm 1.42 ^a	11.16 \pm 0.19 ^a	11.27 \pm 1.57 ^a	4.72 \pm 1.11 ^b
Glucose/xylose (mol/mol)	0.058	0.15	0.038	0.033
Arabinose/xylose (mol/mol)	0	0	0	0.13

The experimental values that have no common letter are significantly different ($p < 0.05$) according to Duncan's multiple range test.

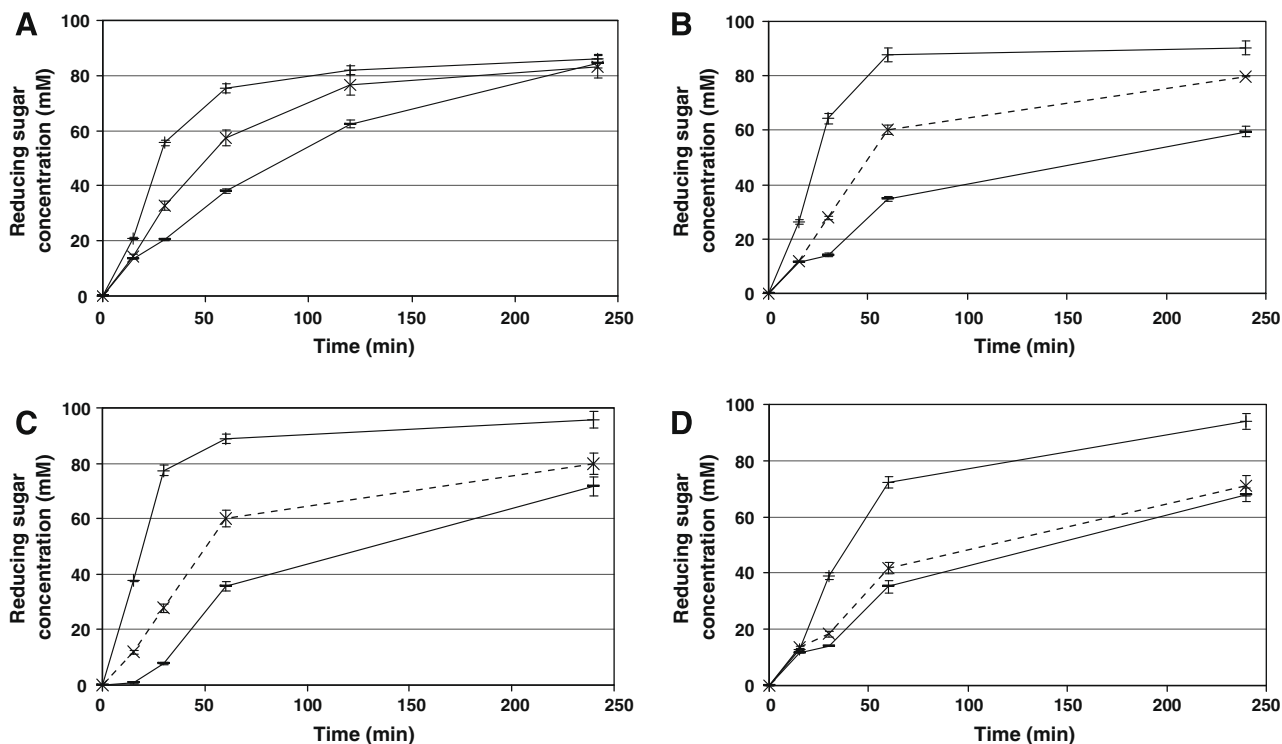


Figure 1. Effect of acid concentration on XO production from 2% xylan. (A) TSX, (B) CSX, (C) WSX, (D) SSX (each data point was the average of four replicate determinations, and the error bars show the data ranges) (0.125 M: —, 0.25 M: X, 0.5 M: +).

lans, the amount of longer chain XOs is higher for 30-min reaction than for 60-min reaction. When the reaction time increased from 30 min to 60 min, the amount of monosaccharide increased drastically depending on the source of xylan and the amount of XOs (X2, X3, X4, X5, X6, and >X6) decreased. The substituent groups and sugar composition of xylan influenced the amount of monosaccharide production. With 30-min reaction time, the monosaccharide production (xylose + arabinose) was the highest in XOs from

WSX. It was speculated that since WSX had a considerably high content of arabinose, this monosaccharide was easily split off during the hydrolysis process resulting in an increase in the monosaccharide level. Since desalting with Amberlite MB20 removed the acidic XOs, regardless of the structural differences of the xylan types presented in this paper, all xylans generated linear and neutral XO with different degrees of polymerization (DP). HPLC chromatograms of XOs from SSX, WSX, CSX, and TSX with 0.25 H₂SO₄

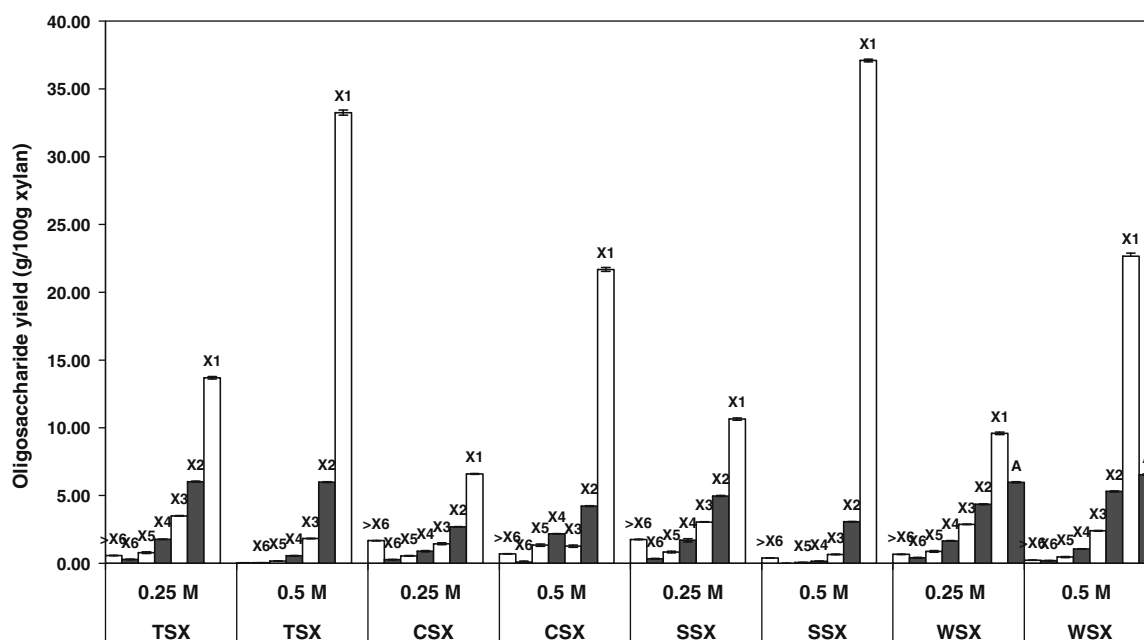


Figure 2. HPLC analysis of XOs from 2% TSX, CSX, SSX, and WSX with different acid concentrations (each data point was average of two replicate determinations, and the error bars show the data ranges). A: arabinose, X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose, X6: xylohexaose, >X6: longer chain oligosaccharide.

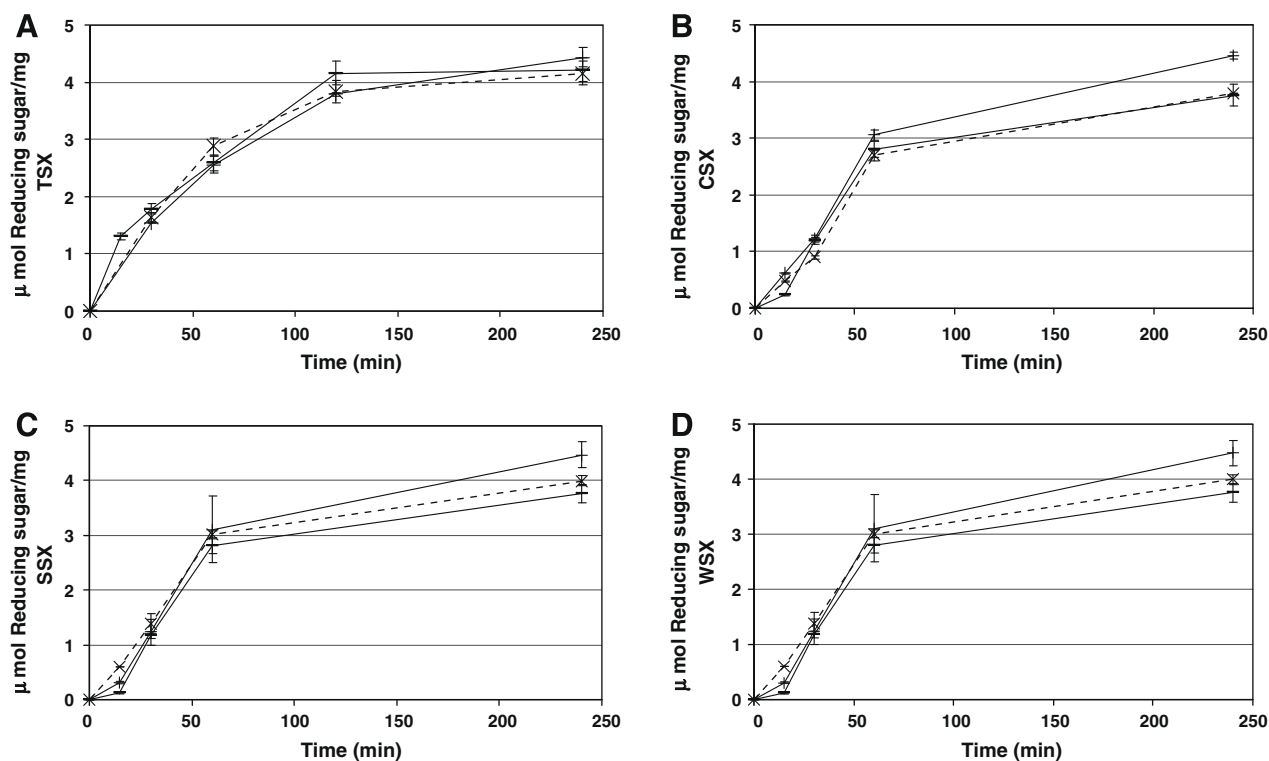


Figure 3. Amount of reducing sugar per milligram substrate (TSX, CSX, WSX, and SSX) (A) TSX, (B) CSX, (C) WSX, (D) SSX (each data point represents an average of four replicate determinations, and the error bars show the data ranges) (1% xylan: —, 2% xylan: X, 4% xylan: +).

after 30-min hydrolysis time were very similar. The HPLC chromatogram (Fig. 5) showed that DP profile of XOs from SSX, TSX, CSX, and WSX was similar to one another, and XOs in the DP range of 2–6 ($X_2 > X_3 > X_4 > X_5 > X_6$) were obtained from TSX, CSX, SSX, and WSX. Only, the HPLC chromatogram of WSX had an arabinose peak in addition to peaks of XOs, which was not surprising due to the arabinoxylan structure of WSX (Table 2).

Comparison of XO, monosaccharides, and furfural yields upon acid hydrolysis is shown in Table 3. XO, monosaccharide yields and furfural production were the lowest in CSX. SSX and TSX yielded the highest oligomer concentration among the four tested materials. Because of the high monosaccharide and furfural production, the dehydration product of pentose monosaccharides was also the highest in WSX. The present results suggest that

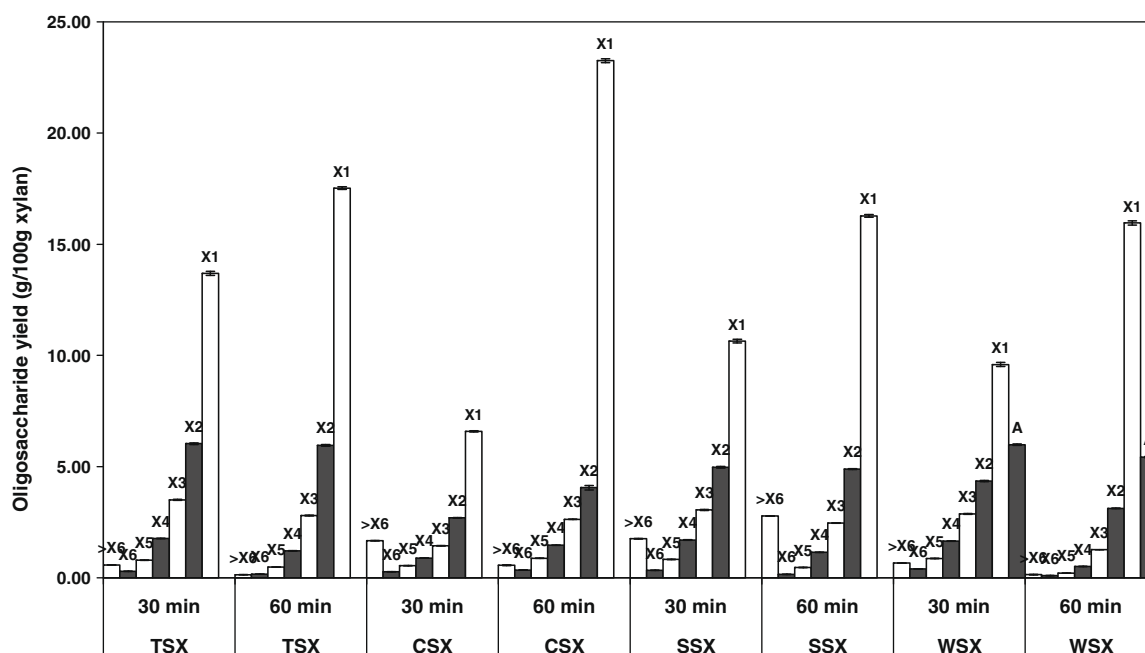


Figure 4. Yield of XOs from 2% TSX, CSX, SSX, and WSX with 0.25 M H_2SO_4 (each data point was the average of two replicate determinations, and the error bars show the data ranges). A: arabinose, X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentose, X6: xylohexaose, >X6: longer chain oligosaccharide.

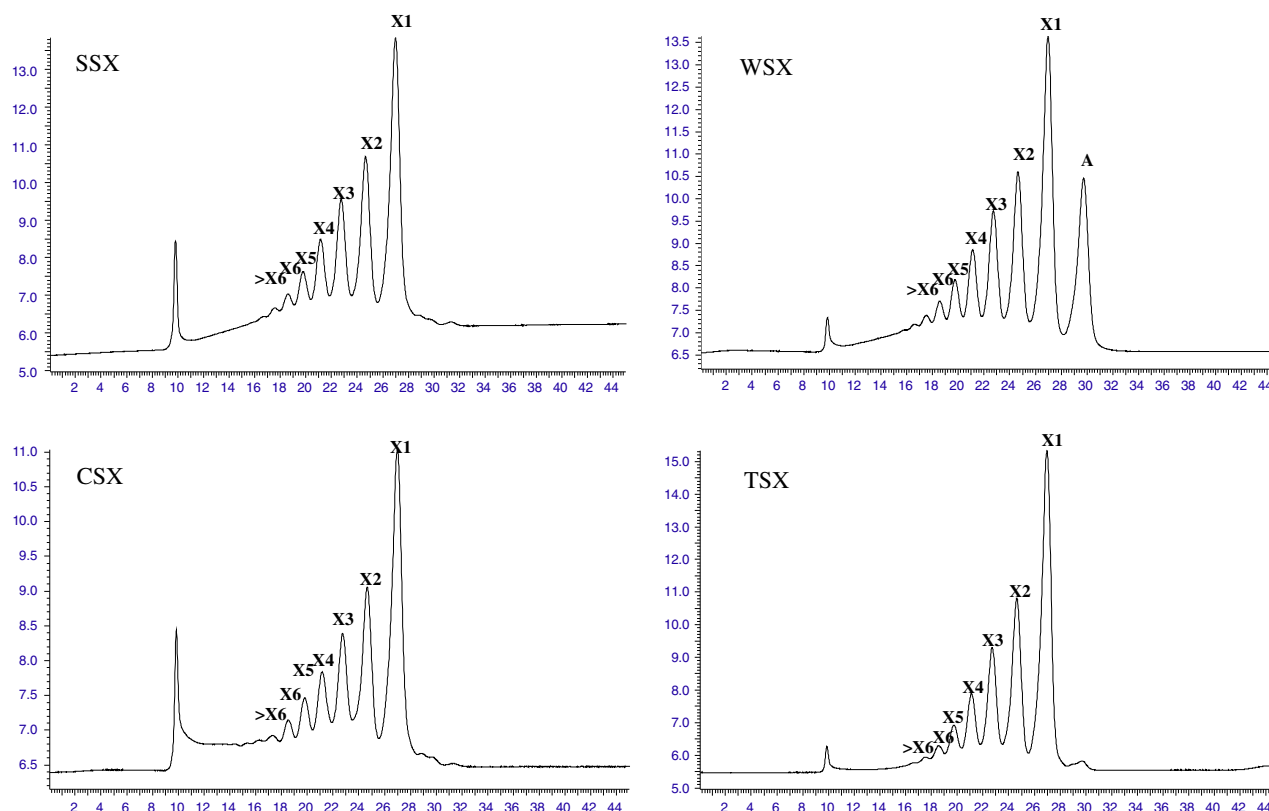


Figure 5. HPLC chromatogram of XOs, produced from SSX, WSX, CSX, and TSX with 0.25 M H_2SO_4 .

due to their simple structures, TSX and SSX can be more effectively converted by acid hydrolysis to XOs than CSX and WSX.

Generally, enzymatic production of XO is more preferable, because it requires mild conditions for hydrolysis and it does not produce undesirable by-products or high amounts of monosaccharides. However, simple and fast kinetics are the advantages of acid hydrolysis. In 30 min, 7–13 g of XO was produced from 100 g xylan, whereas several hours are required to produce the same amount of oligosaccharides through enzymatic hydrolysis.^{18,23} Previous studies done on *Aspergillus niger* xylanase²⁹ and *Trichoderma longibrachiatum* xylanase^{23,30} for production of XOs from different agricultural wastes showed that the DP of the XOs depended not only on the enzyme but also on the xylan source. Most of the xylanases are sterically hindered by the substituents in the xylan and prefer hydrolysis of unsubstituted xylan, while some endoxylanases prefer hydrolysis of more branched xyans.³¹ In the case of acid hydrolysis, the distribution of DPs of the products was only dependent on the concentration of acid and the reaction

time. One of the major disadvantages of the acid hydrolysis of xylan is production of a considerable amount of monosaccharides and then furfural. In the case of enzymatic production, monosaccharide production is very low, and furfural is not produced. However, production of monosaccharides and furfural in acid hydrolysis can be controlled by decreasing the reaction time or acid concentration, and besides it can be removed from the target product by chromatographic methods, such as adsorption chromatography and membrane separation. With controlled acid hydrolysis, the product DP can be easily controlled, and thus desired DPs and amounts of XOs can be produced.

The results showed that all the xyans from these wastes, independently on their structure, generated linear XOs that have similar profile, and these oligosaccharides could be used as functional food ingredients or soluble substrates for xylanase. In general, food-grade oligosaccharides are not pure products, but they are rather mixtures containing homo- or hetero-, branched or linear, substituted or unsubstituted oligosaccharides with different degrees of polymerization. However, xylanase from laboratories needs XOs with defined structure and degree of polymerization for enzymatic studies since the product with complex structure limits its usefulness for detailed enzymatic studies. XOs produced in this research can be used easily as a substrate for understanding the reaction mechanism and hydrolysis processes of xylanase.

3. Conclusion

The purpose of the current study was to prepare a series XOs from agricultural wastes. The chemical composition of four agricultural wastes and of xylan from these wastes is presented in this paper. Comparative assessment of the result shows that the composition of xylan, acid concentration, and reaction time determined the yield and the amount of each oligosaccharide in the

Table 3

Comparison of XO, monosaccharides and furfural yields upon acid hydrolysis of TSX, CSX, SSX, and WSX (mean \pm standard deviation of three replicate determinations)

	TSX	CSX	SSX	WSX
Conversion of xylan to XOs (g XOs/100 g xylan)	12.96 \pm 0.85 ^a	7.52 \pm 0.10 ^c	12.64 \pm 0.33 ^a	10.23 \pm 1.06 ^b
Xylose (g xylose/100 g xylan)	13.69 \pm 0.33 ^a	6.59 \pm 0.10 ^d	10.64 \pm 0.27 ^b	9.59 \pm 0.16 ^c
Arabinose (g arabinose/100 g xylan)	0	0	0	5.99 \pm 0.21 ^a
Furfural (mg furfural/100 g xylan)	18.53 \pm 4.61 ^b	8.13 \pm 0.96 ^c	13.93 \pm 2.53 ^b	48.70 \pm 1.15 ^a

The experimental values that have no common letter are significantly different ($p < 0.05$) according to Duncan's multiple range test.

hydrolyzate. The conversion of TSX, CSX, SSX, and WSX into XOs can be easily achieved best by 0.25 M H₂SO₄ in 30 min. Under these conditions, all the xylans were hydrolyzed to a variety of oligosaccharides ranging from X2 to X6 and >X6. However, the most important limitation of acid hydrolysis is the production of high amounts of monosaccharides and furfural. The following conclusions can be drawn from the present study. The yield of XOs depends on both acid concentration and hydrolysis time, but the yield of monosaccharide depends on the structure and composition of xylan besides acid concentration and the time. These four agricultural wastes, which had little economical value, appeared to be very suitable for the production of different series of XOs, and these oligosaccharides could be used for further applications.

4. Experimental

4.1. Materials

Aminex HPX 42A (300 × 7.8 mm; average particle size: 25 µm) and Aminex HPX 87H columns (dimension: 300 × 7.8 mm; average particle size: 25 µm) were purchased from Bio-Rad Laboratories, CA, USA. Xylooligosaccharide standards, xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) were obtained from Megazyme, Ireland. All the other chemicals were of analytical grade obtained either from Sigma Chemical Company, MO, USA, or from Merck KGaA, Germany.

Samples of tobacco stalk (TS), sunflower stalk (SS), cotton stalk (CS), and wheat straw (WS) were collected from a local farmer in Turkey, dried, and fractioned to a particle size of 5–10 mm.

4.2. Material characterization

Uronic acid was determined by the *m*-hydroxydiphenyl method.³² Reducing sugars were quantified with the DNS method³³ by using xylose as a standard. Neutral monosaccharides were determined by HPLC on Aminex HPX 87H column after hydrolysis with sulfuric acid.¹⁸ Moisture, ash, cellulose, xylan, lignin, hot water, and 1:2 EtOH–benzene extractives were determined according to the method provided by ASTM.^{34,35}

4.3. Extraction of xylan

Xylans from agricultural products were extracted according to Zilliox and Debeire³⁶ with slight modification. TS, CS, SS, and WS were swollen at 60 °C for 16 h in water. Xylan from 2 g of these materials was extracted for 3 h at 35 °C in 17 mL of 24% KOH including 1% (w/v) NaBH₄. The extract was centrifuged at 10,000g for 20 min followed by filtration on Whatman 1 Filter paper. The xylan was precipitated in 2 vol of cold EtOH and in 0.2 vol of AcOH, and filtered on coarse filter paper. After filtration, the solid was dried in a vacuum oven at 60 °C.

4.4. Xylan hydrolysis

Xylan (0.2 g) was suspended in 10 mL of 0.25 M H₂SO₄, and this suspension was incubated in a boiling water bath. Samples (10 mL) were taken at regular intervals, and 10 mL of water was added to the reaction mixture to cool down. After cooling down, the reaction mixture was neutralized with calcium carbonate, and the resulting precipitate was removed by centrifugation. The resulting supernatant was filtered through coarse filter paper and desalted with Amberlite MB 20. The results of hydrolysis reaction were monitored by measuring the reducing sugars formed with the dinitrosalicylic acid (DNS) method. The reaction products were further analyzed by HPLC as described below to determine the degree of polymerization (DP) of the XOs.

In order to optimize acid concentration on the production of oligosaccharides, the reaction was conducted using the method described above with different acid concentrations (0.125, 0.25, and 0.5 M). The effects of other variables on XO production were conducted in the same manner as described above except that the condition varied with respect to xylan concentration (1%, 2%, and 4%) and reaction time (0, 15, 30, 60, 120, and 240 min).

4.5. HPLC separation of XOs

XOs were chromatographed on a Perkin Elmer HPLC system equipped with a refractive index detector (Perkin Elmer Series 200) and column oven (Perkin Elmer Series 200). Sample (20 µL) was injected to the HPLC system. XOs were eluted using distilled-deionized water as the mobile phase from Biorad Aminex HPX 42A, and neutral sugars were eluted with 5 mM H₂SO₄ as the mobile phase from Biorad Aminex HPX 87H.¹⁸ The Aminex HPX 42A column (300 × 7.8 mm) was used at 50 °C and a flow rate of 0.4 mL/min. The Aminex HPX 87H column (300 × 7.8 mm) was used at 45 °C and a flow rate of 0.3 mL/min. A complete analysis of XOs and neutral sugar was carried out in 45 min. Concentration of an oligosaccharide was quantified using average peak areas compared with mixture of standard oligosaccharides (X1 through X6) and expressed as mg/mL oligosaccharide. Concentration of neutral sugar was quantified using average peak areas compared with mixture of standard sugars (xylose, glucose, arabinose, and galactose) and expressed as mg/mL sugar.

4.6. Statistical analysis

The experimental results are expressed as mean ± SD (standard deviation), and mean comparison was carried out by Duncan's multiple range test. The SPSS statistical computer package was used to analyze the experimental data (SPSS, Inc., Chicago, IL, USA).

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