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Xylo-Oligosaccharides Production from Corn Fiber and In-Vitro Evaluation for Prebiotic Effect

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Xylo-oligosaccharides production from corn fiber and *in-vitro* evaluation for prebiotic effect

By

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Xylooligosaccharides (XOS) are considered to be prebiotics. Prebiotics are defined as non-digestible food ingredients that benefit the host by stimulating the growth and activity of a limited number of bacteria, such as the *Bifidobacterium* genus, in the colon. Corn fiber separated from distillers dried grains with solubles (DDGS) could be a valuable feedstock for XOS production. The objective of the first chapter was to determine the efficacy for autohydrolysis to produce XOS using fiber separated from DDGS. Fiber was treated with deionized water in a Parr-reactor, at temperatures ranging from 140 to 220 ^{o}C to produce XOS. The maximum total yield of XOS in the solution was 18.6 wt% of the corn fiber at 180 ^{o}C.

The objective of the second chapter was to evaluate and compare the prebiotic effect of XOS produced by autohydrolysis of DDGS fiber (XOS-D) with other substrates (FOS, commercial XOS (XOS-C), xylose, glucose and inulin) on intestinal bacteria, B. *adolescentis*, B. *breve* and *Lactobacillus brevis*. Bacterial growth on XOS-C was comparable with growth on FOS and inulin. XOS-D promoted bacterial growth more than that of control. Prebiotic potential of XOS produced from corn fiber was confirmed.

The objective of third chapter is to determine the yield of XOS from corn fiber separated from ground corn flour (FC) and DDGS (FD) at different autohydrolysis temperatures and hold-times. The conditions for maximum XOS production for FD and FC were 180 ºC with 20 min hold-time and 190 ºC with 10 min hold-time, respectively.

The fourth chapter focuses on production of XOS by enzymatic hydrolysis method for XOS production. Endo-1-4-xylanase enzyme was ineffective for corn fiber as well as corn fiber gum (CFG), despite evaluating a multitude of pretreatment methods and processing conditions. We have proposed use of Multifect Pectinase PE and Multifect Xylanase enzymes, based on work from other researchers.

For commercial applications such as food industries, XOS would need to be isolated from liquor. The fifth chapter of this study focuses on literature review of purification methods used in XOS purification.

DEDICATION

I sincerely dedicate to my Mentor and Advisor Dr Radhakrishnan Srinivasan.

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CHAPTER I

XYLO-OLIGOSACCHARIDES PRODUCTION BY AUTOHYDROLYSIS OF CORN FIBER SEPARATED FROM DDGS

1.1 Introduction

The need for renewable energy sources has led to a rapid increase in the production of fuel ethanol, as well as its co-product, distillers dried grains with solubles (DDGS). DDGS consists of non-fermentable components of the original grain, such as protein, lipids, and fiber. The effective utilization of DDGS has become important with respect to maintaining the economic viability of the renewable bio-fuel industry. For every 1 kg of corn utilized, nearly 1/3 kg of each of the products ethanol, DDGS, and CO_2 are produced. In 2006, nearly 10 million metric tons of DDGS was produced from this industry. DDGS, which has high protein and fiber contents, is used as livestock feed, mainly as ruminant (cattle) feed. However, with an increase in supply of distillers grains, innovative uses for DDGS are needed to increase its value. Corn fiber separated from distillers dried grains with solubles (DDGS) could be a valuable feedstock for production of xylo-oligosaccharides (XOS), which can be used as a functional food ingredient.

Recently, fiber has been separated from DDGS using a combination of sieving and air classification (the Elusieve process) to produce two beneficial co-products: (1) enhanced DDGS with reduced fiber, increased fat, and protein contents and (2) fiber (Srinivasan *et al.* 2006). Enhanced DDGS from Elusieve processing has lower fiber

content and hence, has potential to be used at higher inclusion levels in non-ruminant animal diets. Currently, fiber separated from DDGS is believed to have limited use as feed for ruminant animals (dairy and beef cattle). So, XOS production from corn fiber could increase its value.

Polymerized monosaccharides having a degree of polymerization (DP) between 2 and 10 are defined as oligosaccharides (Nakakuki 1993). Oligosaccharides that cannot be digested in the human stomach and small intestine are considered as functional food ingredients with potential to reduce the risk and possibility of heart diseases, bacterial/ viral infections, cancer, diabetes, hepatitis, emphysema, and cranial and muscular neurological diseases (Hakamori and Kannagi 1993; Faissner *et al.* 1994; Gibson 2004; Rivas *et al.* 2002; Chu and Whittaker 2004; Kawakubo *et al.* 2004; Ohtsubo and Marth 2006). Due to their various health benefits, oligosaccharides are used in pharmaceutical and food industries. Commonly used oligosaccharides are fructo-oligosaccharides, malto-oligosaccharides, galacto-oligosaccharides, and XOS.

XOS are xylose-based oligomers linked by β-1,4 bonds and they contain variable amounts of substituted groups such as acetyl, phenolic, and uronic acid. XOS are considered to be prebiotics. Prebiotics are defined as non-digestible food ingredients that benefit the host by stimulating the growth and activity of a limited number of bacteria, such as *Bifidobacterium* species, in the colon (Gibson and Roberfroid 1995). Prebiotics have applications in pet foods, human foods, and animal feeds.

Different methods used for the production of xylo-oligosaccharides are: enzymatic hydrolysis, alkali/acid hydrolysis, and autoydrolysis of carbohydrate polymer. In enzymatic hydrolysis, enzymes such as endoxylanases, β-xylosidases, and arabino-

furanosidases are used to break the xylan linkages to produce XOS. Enzymatic hydrolysis typically takes a longer time for completion than other methods. In acid/alkali hydrolysis methods, a dilute solution of acid or base is used to treat the substrates, typically at ambient temperature, to produce XOS. In the autohydrolysis method, water is added to the substrate and the mixture is heated to the range 100 to 250 ºC in an enclosed vessel to produce XOS. No prior work has been conducted on autohydrolysis of fiber separated from DDGS. Even though XOS production by autohydrolysis has been evaluated for some other agricultural materials such as corn cobs and wood, those results are not sufficient to determine the optimum conditions required for autohydrolysis of fiber separated from DDGS, because this is a different material in terms of composition and origin (Carvalheiro *et al.* 2004; Nabralatz *et al.* 2007). The objective of this study was to determine the efficacy for autohydrolysis to produce XOS using fiber separated from DDGS and determine the optimum temperature for production of XOS.

1.2 Experimental

1.2.1 Fiber Separation from DDGS

DDGS was procured from a local feed mill and processed to separate fiber using the Elusieve pilot-plant at Mississippi State University (Srinivasan *et al.* 2009). Quantity of fiber separated was 4% of the weight of DDGS. The fiber used in this study was the large size fiber fraction (size > 868 μm). Quantity of large size fiber fraction was 2% of the weight of DDGS. The fiber material was stored in vacuum-sealed bags in a refrigerator at 5 ºC until used.

1.2.2 Determination of Corn Fiber Composition

Three replicates of the corn fiber material were sent to Integrated Paper Services, Inc., Appleton, WI for determination of corn fiber composition. The samples were milled to approximately 40-mesh. Prior to carbohydrate and lignin analysis, samples were extracted with dichloromethane (DCM) in a soxhlet apparatus to remove substances such as waxes, fats, resins, phytosterols, and non-volatile hydrocarbons. The percent of extractives of each sample was calculated basedon its oven-dried weight. Extractives content for corn fiber material was 6.7%. The carbohydrate and lignin content determination of three dichloromethane extracted samples was done in duplicate. Lignin content was determined according to method described in Effland (1977). Approximately 300 mg of sample was hydrolyzed with acid and filtered. The acid-insoluble residue was oven-dried and weighed to calculate the percent of lignin content. The acid-soluble portion of each sample was neutralized, reduced, acetylated, and their carbohydrate composition was determined according to TAPPI Test Method T249 Cm-00 using a Flame Ionization Detector-Gas Chromatograph (FID-GC).

1.2.3 Determination of Carbohydrate Composition of Residue from Autohydrolysis

Three replicates of residue materials were analyzed. Samples were extracted with hexane in a soxhlet apparatus. The extractives comprised 15.4% of the residue. Carbohydrate content (glucan, xylan, galactan, and arabinan) of the remaining residue was determined using procedure similar to that used for corn fiber (previous section 1.2.2) at the ERRC, ARS USDA in Wyndmoor, PA.

1.2.4 Auto-Hydrolysis of Fiber

The autohydrolysis of fiber was conducted in a 750 mL Parr reactor (model 4843, Parr Instruments Co., Moline, Illinois, USA) (Fig. 1.1). The reactor (fitted with a six bolt metal cover) was heated with temperature control. In each batch, the Parr reactor was filled and loaded with 10 grams of corn fiber and 90 mL of deionized water. The holding time after the desired temperature was reached was 15 min. The treatment of fiber separated from DDGS samples was carried out at a desired set of temperatures between 140 and 220 ^{o}C. The time required to reach 140 to 220 °C was 14 to 45 min. The reaction mixture was not stirred during the heating period. There was some charring of corn fiber on the surface of the reactor cylinder at higher temperatures (200 and 220°C). Auto-hydrolysis was carried out in three replicates at each temperature, except for 200 ^{o}C and 220 ^{o}C.

The reaction mixture was filtered by gravity filtration using filter paper (Fisherbrand, USA, catalogue no. 09-801E, particle retention 5 to10 μm) and a size P5 funnel. The filtrate was further filtered by a vacuum filtration system using a glass fiber prefilter (Millipore, USA) on a Buchner funnel. The reaction mixture was filtered twice to obtain particle-free solution for HPLC analysis. The solid product was thoroughly washed with deionized water ranging from 100 mL to 120 mL to ensure complete oligosaccharides removal from residue and dried at room temperature. The washing was collected in a bottle, labeled as original liquor, and stored in the refrigerator at 0 ^{o}C.

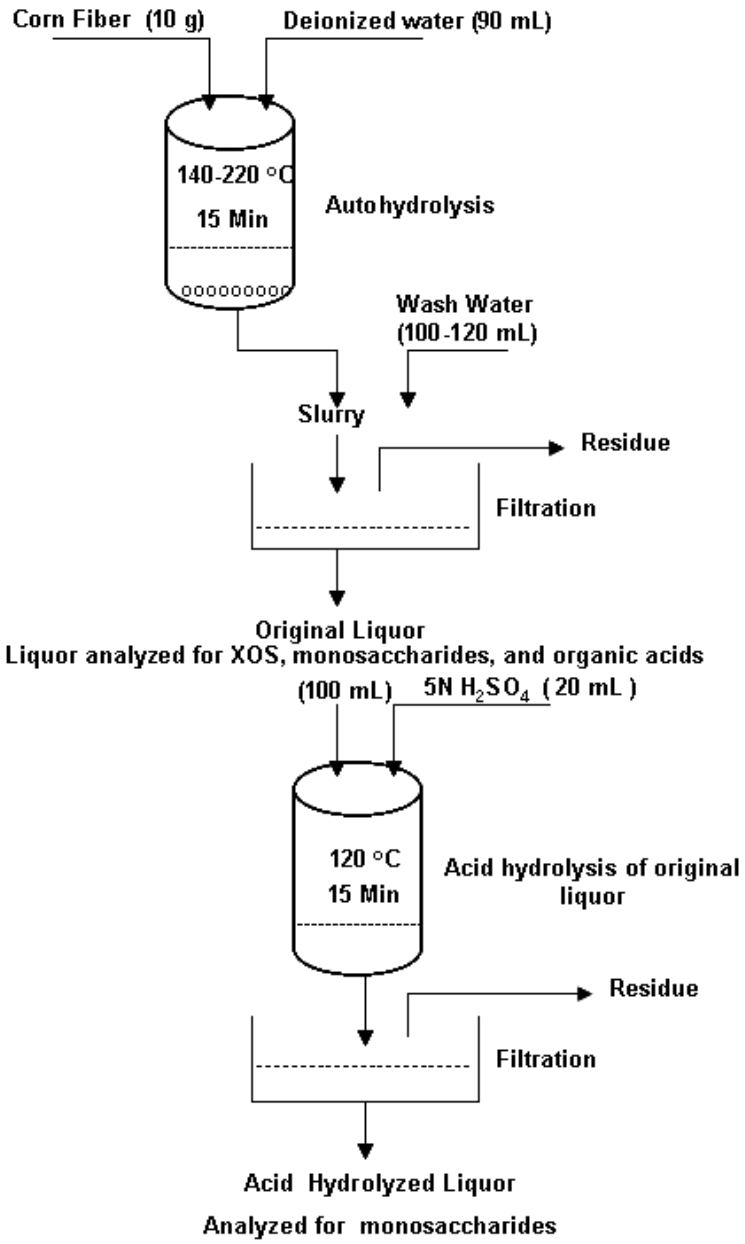

Figure 1.1 Schematic for autohydrolysis of corn fiber to produce XOS and acid hydrolysis of liquor

1.2.5 Acid Hydrolysis of Liquor from Autohydrolysis

The original liquor (100 mL) obtained after autohydrolysis reaction of fiber was mixed with 20 mL of 5N H_2SO_4 and heated at 120 °C for 45 minutes to hydrolyze XOS to

their monomeric sugars (Fig. 1.1) using NREL (National Renewable Energy Laboratory) procedure NREL/TP-510-42623. The acid hydrolyzed solution was filtered by vacuum filtration on a Buchner funnel to remove insoluble materials. Acid-hydrolyzed liquor was quantified for monosugars content to provide an indirect means to verify results of autohydrolysis. Monosugars content was determined using an HPLC method (Section 1.2.6) at Mississippi State University.

1.2.6 Quantification of XOS, Monosaccharides, and Acids using HPLC

The procedure used for quantification of XOS was similar to that used by earlier researchers (Carvalheiro *et al.* 2004; Nabralatz *et al.* 2007). An aliquot from the acid-hydrolyzed sample solution was further filtered using 0.22 μm syringe filters into a 2 mL vial (Agilent, USA) for sugar analysis. Sugars were analyzed by high-performance liquid chromatography (HPLC) using an Agilent 1200 series HPLC System device (Agilent, USA) equipped with a refractive index detector. The monosaccharide content of both the original liquor as well as acid hydrolyzed liquor was determined by the HPLC unit equipped with a Bio-Rad HPX 87 P (300 X 7.8 mm) column at 80 ^{o}C and a guard column (Bio-Rad Laboratories, USA) by injecting 20 μL of the sample solution and eluting the column with HPLC grade water (Sigma Aldrich, USA). The standard sugars used for identification and quantification were glucose, xylose, arabinose, galactose, and mannose (Sigma Aldrich, USA).

The XOS in the original liquor were analyzed by the HPLC device equipped with a Bio-Rad HPX 42 A column at 80 ^{o}C and a guard column (Bio-Rad Laboratories, USA) by eluting the column with HPLC grade water (Sigma Aldrich, USA) at a flow-rate of 0.6 mL/min. The XOS standards used were xylobiose, xylotriose, xylotetrose, xylopentose,

and xylohexose along with a monomeric xylose (Megazymes, Ireland). The acidic components and sugar degradation products present in the original liquid were analyzed by HPLC equipped with a Bio-Rad HPX 87 H (300 X 7.8 mm) column at 80 ^{o}C and a guard column (Bio-Rad Laboratories, USA) by eluting with 0.005M H_2SO_4 at a flow rate of 0.6 mL/min. Standard acids used were acetic acid, formic acid, and levulinic acid. Degradation compounds used were hydroxymethyl furfural (HMF) and furfural (Sigma Aldrich, USA).

1.3 Results and discussion

Moisture content of the corn fiber was 12.4% on a wet basis. The carbohydratecomposition of the corn fiber was glucan 18.0%, xylan 16.8%, arabinan 8.8%, mannan 0.8%, galactan 3.0%, and lignin content 1.3%, on wet basis. Protein content of corn fiber separated from DDGS was 12.4% on wet basis. Thus, the glucan content represented 18.0% and hemicelluloses content, comprising xylan, arabinan, galactan, and mannan chains, represented 29.4.% in the corn fiber. Based on the composition of corn fiber, the maximum expected amounts of glucose, xylose, arabinose, galactose, and mannose monosugars were 2.0 g, 1.9 g, 1.0 g, 0.3 g, and 0.0 g, respectively. Thus the total expected maximum amount of monosugars based on corn fiber composition was 5.2 g.

The liquor obtained after autohydrolysis of fiber in the Parr-reactor at temperatures ranging from 140 to 220 ^{o}C consisted mostly of a mixture of xylose oligomers with some free arabinose and glucose (Tables 1.1 and 1.2). Representative chromatograms for quantification of monosugars in the original liquor, XOS in the

original liquor, and XOS standards are shown in Fig. 1.2, Fig. 1.3, and Fig. 1.4, respectively.

Similar to observations of Aoyama (1996), Garrote *et al.* (1999), and Carvalheiro *et al.* (2004), production of XOS depended upon temperature. As the temperature was increased from 180 to 220 ^{o}C, the XOS production decreased, but the production of acetic acid and degradation compounds (HMF, furfural) increased. The maximum amount of XOS from the original liquor was obtained at temperature 170 to 180 ^{o}C (Table 1.1). The amount of XOS in the original liquor increased with increasing temperature up to 180 ^{o}C, but it decreased on further increase of temperature. The estimated xylose content in the acid hydrolyzed liquor based on hydrolysis of measured XOS, was higher than measured xylose content until a temperature of 150 ^{o}C. This may be due to co-elution of other sugar-oligosaccharides with XOS in the Aminex 42-A column, which was also observed when analyzing standard samples; cellobiose co-eluted with xylotriose. As the temperature was increased, there was an increase in the breakdown of other sugar-polymers (glucan, arabinan, and galactan) into monomers, giving rise to higher levels of monomers (arabinose, galactose, and glucose) at higher temperatures up to 180 ^{o}C (Table 1.2). This degradation of carbohydrate polymers into monomers would result in a decrease of other-sugar oligosaccharides, which probably led to lesser co-elution of XOS at higher temperature. Lesser co-elution at higher temperatures can be inferred from xylose content in acid hydrolyzed liquor being higher than the minimum xylose content expected from hydrolysis of measured XOS in the original liquor at higher temperatures (Table 1.2). Thus, the measured XOS values at temperatures higher than 160 ^{o}C are expected to be closer to actual values. In further studies that are underway, we are

pursuing preparative HPLC method to purify and quantify the exact amounts of individual oligomer compounds of xylan, arabinan, galactan, and cellulose.

In order to verify results, a component-wise mass balance was carried out for the autohydrolysis process at a single temperature (180 ^{o}C) (Table 1.3).

Table 1.1 XOS, Acetic Acid and Degradation Products Contents (mg) of Original Liquor

Temp (ºC)	XOS					Acetic Acid	Degradation compounds	
	Xylotriose	Xylotetrose	Xylopentose	Xylohexose	Total XOS		HMF	Furfural
140	1154	87	72	0	1314	47	0	0
150	1365	105	55	0	1524	50	0	0
160	1215	104	97	0	1415	72	0	0
170	1483	142	146	20	1790	534	0	0
180	1645	0	221	0	1865	640	0	0
200*	535	0	0	0	535	1355	524	1632
220*	190	0	0	0	190	1723	839	1705

Results are means of three replicates; HMF- Hydroxymethylfurfural
* Results are means of only one replicate
The range of coefficients of variation for xylotriose, xylotetrose, xylopentose, xylohexose and acetic acid were 6.9 - 13.3%, 86.6 - 99.3%, 15.6 – 86.6%, 0.0 – 173.2%, 6.1 – 73.3%, respectively.

Table 1.2 Monosaccharide Content (mg) of Acid Hydrolyzed Liquor and Original Liquor

Temp (ºC)	Monosaccharides measured in acid hydrolyzed liquor				Monosaccharides measured in original liquor				Minimum Xyl Expected in Acid Hydrolyzed Liquor Based on XOS values from oligosaccharide column
	Xyl	Glu	Gal	Ara	Xyl	Glu	Gal	Ara	
140	438	1446	299	433	366	656	0	435	1746
150	930	1477	238	647	436	661	115	1002	2104
160	2729	1525	456	1018	636	1023	213	1398	2442
170	3778	1269	0	1152	1011	1275	447	1647	3199
180	5096	1291	0	1066	1312	1424	700	1578	4096
200*	1385	1262	0	0	1072	1294	639	875	1156
220*	479	403	0	99	0	405	0	0	206

Results are means of three replicates; Xyl – Xylose, Glu – Glucose, Gal – Galactose, Ara – Arabinose; The range of coefficients of variation for glucose, glucose, xylose, arabinose, galactose were 1.4 – 9.9%, 3.6 - 9.3%, 5.2 - 60.9%, 13.0 – 69.7%, respectively.
* Results are means of only one replicate.
§ Calculated using values from table 1 as (xylose + xylobiose * 300/282 + xylotriose * 450/414 + xylotetrose * 600/546 + xylopentose * 750/678 + xylohexose * 900/810)

Table 1.3 Component-Wise Mass Balance for Autohydrolysis Process at 180 ºC

Material	Glucose (g)	Xylose (g)	Galactose (g)	Arabinose (g)	Total (g)
Corn fiber (Input)	2.00	1.91	0.34	1.00	5.25
Original liquor (sugars)	1.42	1.31	0.70	1.58	5.01
Original liquor (sugars from oligosaccharides)	0.00	1.86	0.00	0.00	1.86
Residue	0.00	0.00	0.00	0.00	0.00
Total output	1.42	3.17	0.70	1.58	6.87
% Difference between input and output	-29%	66%	106%	58%	31%

Compositional analysis showed that residue from autohydrosis did not contain any carbohydrates (glucan, xylan, galactan, and arabinan). It was found that the overall material balance error was 31% and the component wise error was -29% to 66% for all sugars except galactose. The mass balance error was higher (106%) for galactose perhaps because of the low quantities of galactose in the materials.

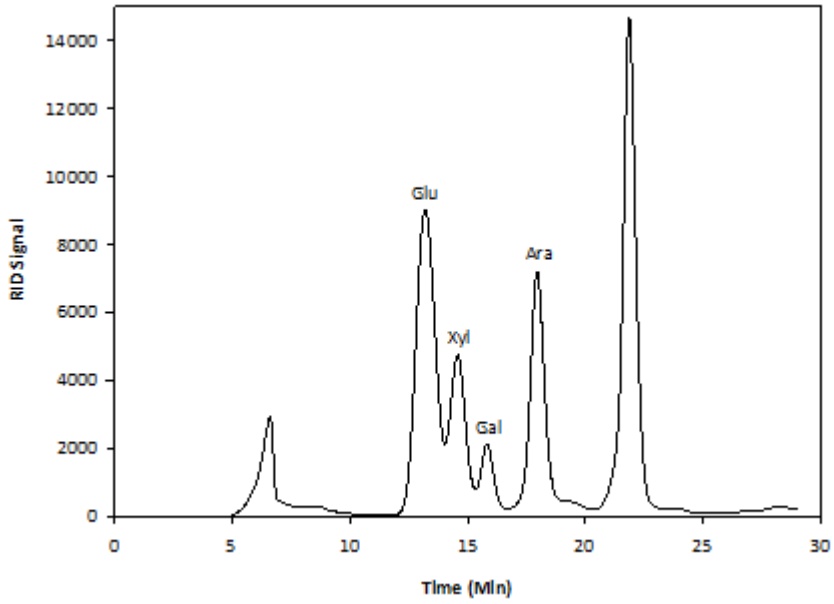

Figure 1.2 HPLC analysis of original liquor obtained by autohydrolysis of corn fiber at 170 ˚C for quantification of monosaccharide.

Using Bio-Rad HPX 87 P (300 X 7.8 mm) column at 80 oC by eluting with HPLC grade water. Actual run time was 60 min, but the chromatogram is truncated in the figure to improve clarity. Glu – glucose, Xyl – xylose, Gal – Galactose Ara – arabinose. Retention times for Glu, Xyl, Gal and Ara were 13.15, 14.52, 15.57 and 17.45 min, respectively.

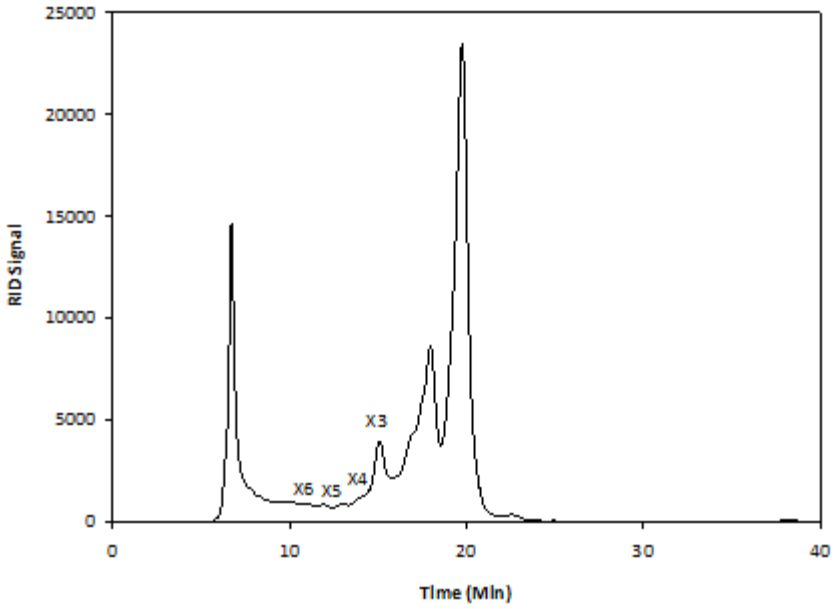

Figure 1.3 HPLC analysis of original liquor obtained by autohydrolysis of corn fiber at 170 °C for quantification of xylooligosaccharides (XOS).

Using Bio-Rad HPX 42 A column at 80 °C by eluting with HPLC grade water. Actual run time was 60 min, but the chromatogram is truncated in the figure to improve clarity. X3: Xylotriose, X4: Xylotetrose, X5: Xylopentose, X6: Xylohexose. Retention times for X3, X4, X5 and X6 were 14.98, 12.88, 11.56 and 11.01 min, respectively

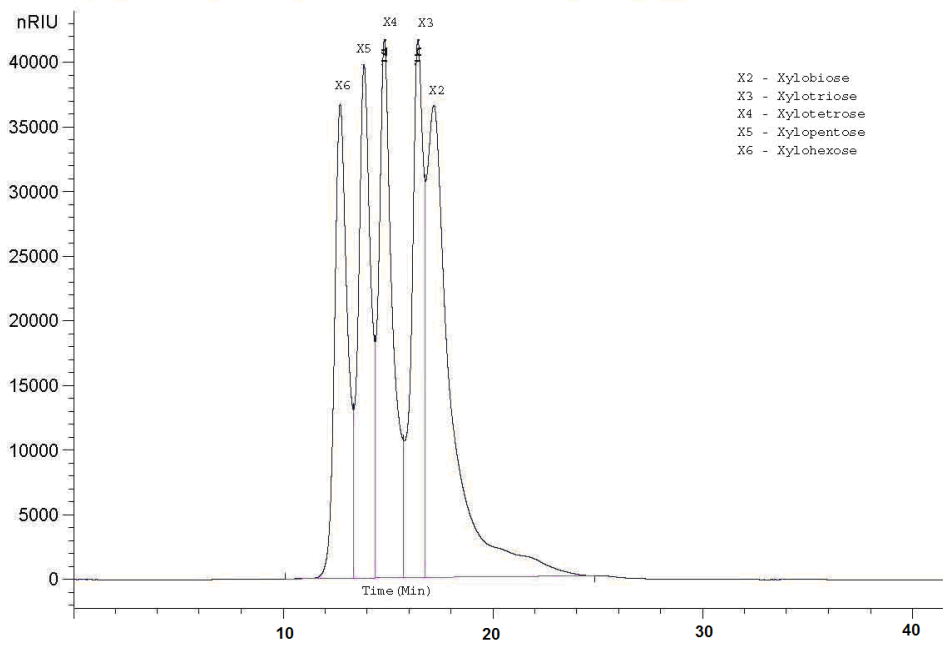

Figure 1.4 HPLC analysis of XOS standards for quantification of xylooligosaccharides (XOS) using Bio-Rad HPX 42 A column at 80 ^{o}C by eluting with HPLC grade water.

Actual run time was 60 min, but the chromatogram is truncated in the figure to improve clarity. X2: Xybiose, X3: Xylotriose, X4: Xylotetrose, X5: Xylopentose, X6: Xylohexose

As temperature was increased, the breakdown of xylan polymer increased. There was an increase in the xylose content from 316 to 2064 mg in the original liquor as temperature was increased from 140 to 180 ^{o}C (Table 1.1). The maximum yield of XOS was 18.6% of corn fiber at 180 ^{o}C, containing mainly xylotriose (1645 mg) and xylopentose (221 mg). The XOS produced at 170 ^{o}C contained mainly a mixture of xylotriose (1483 mg), xylotetrose (142 mg), xylopentose (146 mg), and xylohexose (20 mg). At 170 ^{o}C the original liquor showed the presence of a mixture of xylotriose, xylotetrose, and xylopentose. But at 180 ^{o}C the original liquor showed a mixture of only xylotriose and xylopentose, which may be due to co-elution of xylotetrose and xylohexose with xylotriose/xylopentose. Xylobiose was not detected in any of the

original liquors, which also is perhaps due to co-elution. It is evident from data presented in Table 1.1 that hemicellulose certainly was hydrolyzed on heating to form xylo-oligosaccharides. Results of the steam explosion work performed by Nunes and Pourquie (1996) were in agreement with this study in terms of decreasing oligomer/monomer concentrations at high temperatures and pressures due to thermal degradation.

The breakdown of arabinan into monomer seems to be complete at 170 ºC,as indicated by the highest arabinose content (1647 mg) in the original liquor at 170 ºC. At higher temperatures, the arabinose content in original liquor decreased because of its conversion into other compounds such as furfural (Kootstra *et al.* 2009; Table 1.2). The breakdown of galactan and cellulose to their respective monomers seemed to be maximized at 180 ºC, as indicated by the highest galactose and glucose contents (700 and 1424 mg, respectively) in the original liquor at 180 ºC. Contents of arabinose, galactose, and glucose monomers in acid hydrolyzed liquor were lower at some conditions, especially at higher temperatures, than respective monomer contents in original liquor. The loss of these sugars during acid hydrolysis at high temperature is more likely due to their conversion into known compounds such as furfural, hydroxymethyl furfural, and some other un-characterized degradation products (Table 1.2). For example, degradation of arabinose at higher temperatures has been reported by Kootstra *et al.* (2009). The HPLC analysis results of XOS and monosaccharides at 170/180 ºC (temperatures at which non-xylan sugars break down completely into monomers) were comparable to that expected based on composition of the original fiber. The validity of HPLC analysis results was verified by comparing the total monosugars content in the original liquor with the maximum expected monosugars content based on the carbohydrate content of the

corn fiber. Total monosugars content in original liquor at 140, 150, 160, 170, and 180 ºC were 1.5, 2.4, 3.3, 4.4, and 5.0 g, respectively, which were less than the maximum expected monosugars content of 5.2 g based on corn fiber composition. Thus, the HPLC results were in agreement with the original corn fiber composition.

The maximum XOS yield obtained in this study (18.6% of feedstock) was comparable to maximum XOS yield observed by earlier autohydrolysis studies. The maximum XOS yield from brewery's spent grain (BSG) was 14.1% (Carvalheiro *et al.* 2004). The maximum XOS yield from almond shells was 15.7% (Nabarlatz *et al.* 2007).

The original liquor had no traces of formic acid and levulinic acid, which are formed on the degradation of HMF and furfural compounds (Dunlop 1948; Ulbricht *et al.* 1984). As temperature increased, the acetic acid content in original liquor also increased, perhaps due to de-acetylation of arabinoxylan hemicellulose (Table 1.1).

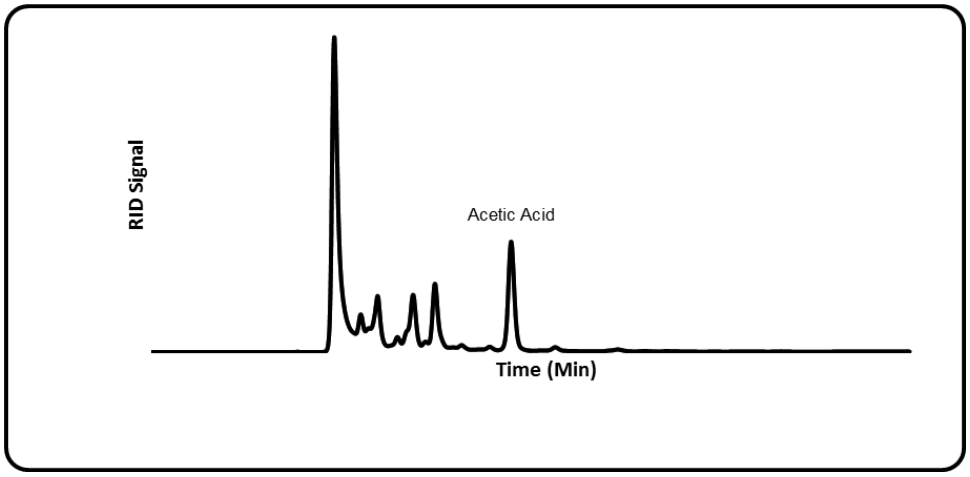

Figure 1.5 HPLC analysis of original liquor obtained by autohydrolysis of corn fiber at 170 ˚C for quantifications of sugar degradation and acidic components.

Using Bio-Rad HPX 87 H (300 X 7.8 mm) column at 80 ˚C by eluting with 0.005M H_2SO_4. Furfural and HMF were not present at 170 °C. Furfural and HMF were present at temperatures above 200 °C. Retention times for HMF and furfural were 30.44 and 46.70 min, respectively. Actual run time was 60 min, but the chromatogram is truncated in the figure to improve clarity

A representative chromatogram showing quantification of acetic acid and degradation products is given in Fig. 5. Acetic acid production increased from 47 mg to 1723 mg as the temperature increased from 140 to 220 ^{o}C, and the formation of HMF and furfural started at 200 ^{o}C. It is beneficial that HMF and furfural were not present at 180 ^{o}C, when the XOS production was at its maximum. In industrial scale production of XOS, original liquor from autohydrolysis would need to be purified by separating the monosugars using membranes and decolorized by adsorption methods.

1.4 Conclusions

1. Xylo-oligosaccharides (XOS) can be produced by autohydrolysis of fiber separated from distillers dried grains with solubles (DDGS).
2. The production of XOS increased as the temperature increased from 140 to 180 ^{o}C, and XOS production was lower at temperatures of 200 to 220 ^{o}C.
3. The maximum total XOS yield was 18.6 % of corn fiber, which was obtained at 180 ^{o}C. The original liquor had no traces of formic acid and levulinic acid.
4. As temperature increased, the acetic acid content in original liquor increased. Formation of HMF and furfural started at 200 ^{o}C.

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CHAPTER II

IN VITRO PREBIOTIC BACTERIAL ANALYSIS OF XYLOOLIGOSACCHARIDES PRODUCED BY AUTOHYDROLYSIS OF CORN FIBER

2.1 Introduction

Consumers have become concerned about the consequences of feeding antibiotics to livestock for both human and animal health. The overuse of antibiotics could lead to drug resistance in pathogens of significance to humans. The nature of the problem has been addressed by the Health Council of Netherlands, 1998; and many researchers in their reviews (Sou, 1997). The need to substitute antibiotic growth promoters has led researchers and producers to investigate alternatives. Several alternatives such as probiotics, prebiotics (non digestible oligosaccharides), enzymes and other modifiers of microbial activity are being considered by the livestock industry.

Prebiotics are defined as non-digestible food ingredients that benefit the host by stimulating the growth and activity of a limited number of commensal bacteria in the colon (Gibson and Roberfroid 1995). The gastrointestinal tract (GIT) of animals contains up to 500 different types of commensals, of which lactic acid bacteria constitute approximately 10%. Commensals contribute to numerous biological functions, such as maintaining a well-balanced flora (Kontula et al. 2000). Like soluble dietary fiber, oligosaccharides such as xylooligosaccharides (XOS), inulin, fructooligosaccharides (FOS), and oligofructose are not digested in the small intestine, but digested in the large

intestine. They are selectively fermented by intestinal flora, resulting in promotion of optimal intestinal function.

Bifidobacterium are the most studied reference genus for prebiotic effectiveness. Along with FOS, oligofructose and inulin, XOS are considered to be emerging prebiotics. Sugar molecules with a degree of polymerization ranging from 2 to 10 are defined as oligosaccharides (Nakakuki .T, 1993). XOS are xylose based oligomers derived from xylan rich hemicelluloses. Xylan are β-1,4 linked polymers of D-xylose with D-glucuronic acid, L-arabinose, acetyl or phenolic substituents substituted at positions C2 and C3 (Ebringerova. A and Heinze, 2000). Previous researchers, Holzapfel and Schillinger (2002) demonstrated that the intestinal microbiota plays pivotal role in the maintenance of health and in the prevention of disease. Modler (1994) reported that the presence of bifidobacteria in the GIT has been associated with beneficial health effects; various studies have demonstrated the bifidogenic nature of prebiotics (Gibson et al., 1995). Bifidobacteria differ from other colonic genera in their fermentation of carbohydrates. Previous investigations have reported that XOS were preferentially fermented by bifidobacteria, through in vitro and in vivo studies (Okazaki et al., 1990; Campbell et al., 1997). FOS and XOS showed a prebiotic effect both in “in vivo” and “in vitro” (Gibson, 1999) and XOS (Vazquez et al., 2000).

Corn fiber could be a good source for producing XOS. Corn fiber is a rich source of branched polymer hemicellulose, which is the second most abundant polysaccharide after cellulose in the plant cell wall (Schädel et al., 2010). The hemicelluloses constitute an important group of polysaccharides, linked to microfibrils of cellulose and pectins, the most important are: xylans, arabinoxylans, mannans, galactomannans, glucomannans,

arabinogalactan II, beta-1,3-glucan and beta-1,3-beta-1,4-glucans.[13] XOS production from plant hemicelluloses generally involves breakdown of xylans using acid treatment, alkaline treatment, autohydrolysis, and enzymatic hydrolysis.

Recently, we demonstrated the production of XOS from corn fiber using an autohydrolysis method, wherein corn fiber was treated with water at high temperatures in an enclosed vessel (Samala et al. 2012). Corn fiber was obtained from distillers dried grains with solubles (DDGS) by using the Elusieve process, a combination of sieving and air classification. DDGS is a by- product of fuel ethanol production from corn. Samala et al. (2012) showed that autohydrolysis performed at 180 °C with 15 minutes hold time were the optimum conditions for XOS production.

The objective of this study was to evaluate and compare the prebiotic effect of XOS produced from DDGS fiber (XOS-D) with other substrates such as FOS, commercial XOS (XOS-C), xylose, glucose and inulin, on intestinal bacteria, B. *adolescentis*, B. *breve* and *Lactobacillus brevis*.

2.2 Materials and methods

2.2.1 Corn Fiber

DDGS was procured from a local feed mill and processed to separate fiber using the Elusieve pilot-plant at Mississippi State University (Srinivasan et al. 2009). The large size fiber fraction (size > 868 μm) was used in this study and was stored in vacuum sealed bags at 5 ^{o}C until use.

2.2.2 Xylooligosaccharide Production by Auto-Hydrolysis of Fiber at 180 °C

The autohydrolysis of fiber was conducted in a 750 mL Parr reactor (model 4843, Parr Instruments Co., Moline, Illinois, USA) (Fig. 2.1) heated with temperature control. In each batch, the Parr reactor was filled and loaded with 10 grams of corn fiber and 90 mL of deionized water. The mixture was carried out at 180 °C and held for 15 min. The reaction mixture was filtered by gravity filtration using filter paper (Fisherbrand, USA, catalogue no. 09-801E, particle retention 5-10 μm) and a size P5 funnel. The filtrate was further filtered by a vacuum filtration system using a glass fiber prefilter (Millipore, USA) on a Buchner funnel. The reaction mixture was filtered twice to obtain a particle-free solution for HPLC analysis. The solid residue was thoroughly washed with deionized water ranging from 100 - 120 mL, to ensure complete oligosaccharides removal, and dried at room temperature. The washing was collected in a bottle, labeled as original liquor and stored at 0 °C. The solid residue was dried at 50 °C for 48 h.

2.2.3 Quantification of XOS and degradation products in the liquor

The XOS in the liquor were analyzed by HPLC equipped with a Bio-Rad HPX 42 A column at 80 °C and a guard column (Bio-Rad Laboratories, USA) by eluting the column with HPLC grade water (Sigma Aldrich, USA) at a flow-rate of 0.6 mL/min. The XOS standards used were xylobiose, xylotriose, xylotetrose, xylopentose, and xylohexose and a monomeric xylose (Megazymes, Ireland). The acidic components and sugar degradation products present in the original liquid were analyzed by HPLC equipped with a Bio-Rad HPX 87 H (300 X 7.8 mm) column at 80 °C and a guard column (Bio-Rad Laboratories, USA) by eluting with 0.005M H_2SO_4 at a flow rate of 0.6 mL/min. Standard acids used were: acetic acid, formic acid, and levulinic acid. Degradation

compounds used were hydroxymethyl furfural (HMF) and furfural (Sigma Aldrich, USA).

2.2.4 Lignocellulosic composition of corn fiber

Ligonocellulosic composition was based on procedure used by Wang et al. (2011) of NREL method "Determination of Structural Carbohydrates and Lignin in Biomass" for the determination of lignocellulosic composition and monosaccharides such as glucose, xylose, arabinose, galactose, and mannose (Sluiter et al. 2008). Residue (0.3 g) was treated with 72% H2SO4 for 1 h; the mixture was hydrolyzed with 84 mL of water and autoclaved at 121°C for one hour. The hydrolysis solution was filtered through crucibles with a filtering disk for separation of filtrate and residue.

Cellulose, hemicellulose, and acid-soluble lignin were determined using filtrate while acid-insoluble lignin was determined using residue. The filtrate was analyzed for glucose, xylose, arabinose, galactose, and mannose by HPLC analysis. Conversion factors were used to convert monomer sugar concentrations into polymeric compositions in the starting materials. Spectrophotometry was used to determine acid-soluble lignin. The residue was dried at 105° C for 16 h and the weight of the residue comprised ash and acid-insoluble lignin. Ash content was determined as the quantity of residual material remaining after heating biomass at 600 °C. Lignocellulosic compositional determination was performed for all samples, and the values reported are the mean value of three replicates.

2.2.5 Lyophilization of liquor solution

The autohydrolysis liquor, obtained from corn fiber separated from DDGS at 180 °C, was lyophilized using Labanco Lyophilization Equipment (Kansas City, MO) equipped with a high vacuum pump JAVAC (Brook Crompton Betts Pty.) to obtain the xylo-oligosaccharides in powdered form. The conditions were as follows: vacuum pressure with 0.5 mbar and temperature maintained at -40°C.

2.2.6 Nutrient Broth Media Preparation

Three different broths were used in this study: Haas Bushnell Broth (HB), Tryptone Polypeptone Broth (TP) and Polypeptone Soybean Meal Broth (PS). HB Broth was prepared as follows: 15 g Noble agar; 1 g KH_2PO_4; 1 g K_2HPO_4; 1 g NH_4NO_3; 0.2 g M $MgSO_4$ 7 H_2O; 0.05 g $FeCl_3$; 0.02 g $CaCl_2$ $2H_2O$ and glucose (dextrose) 10 g suspended in one liter dH_2O.

TP Broth preparation was modified from Wang et al. (2010) and consisted of: tryptone, 10 g ; polypeptone, 5 g; glucose, 5 g; Tween 80, 1g; yeast extract,2.5 g; cysteine, 0.5 g; dipotassium phosphate, 2 g; magnesium chloride hexahydrate, 0.5 g; zinc sulphate heptahydrate, 0.25 g; calcium chloride,0.15 g; ferric chloride traces suspended in one liter dH_2O.

PS Broth nutrient base medium used in the experiment was modified from Moura et al. (2007)[19] and contained: polypeptone, 22 g; soybean meal papaic digest, 3 g; sodium chloride, 5 g; yeast nitrogen base, 5 g; cysteine hydrochloride, 0.5 g; dipotassium phosphate, 2.5 g; ammonium chloride, 0.16 g; calcium chloride, 0.08 g; magnesium chloride hexahydrate, 0.08 g; ferric chloride traces suspended in one liter dH_2O. Prepared media were autoclaved at 121 °C and 15 psi for 30 minutes and were cooled to

approximately 50 °C in a water bath. XOS-C, XOS-D, Inulin and FOS were added at 10 g per L of prepared broth after cooling.

2.2.7 Organism and culture conditions

Three different organisms were used in this study: B. adolescentis, B. breve, and Lactobacillus brevis. The selection of species (B.adolescentis, B.breve and L.brevis) was based on previous research performed by Crittenden et al. (2002) which showed their ability to grow and utilize XOS.21,22 B. adolescentis ATCC 15703, B. breve ATCC 15700, and Lactobacillus brevis ATCC 8287 were obtained from American Type Culture Collection (ATCC; Manassas, VA). The bacteria were cultured at 37 °C under anaerobic conditions established by an Anoxomat Anaerobic Culture System using the default anaerobic gas mixture (MART Microbiology, Drachten, Netherlands). Cultures were prepared in Clostridrial broth (Oxoid) two days prior to performing experiments. Cultures were brought to stationary state at approximately 108 cfu ml-1 titer (colony forming unit).

The prebiotic effect of XOS-C, XOS-D, Inulin and FOS was evaluated through separate in vitro incubations using B. adolescentis, B. breve, and Lactobacillus brevis. Each strain was anaerobically grown in separate incubations using XOS-C, XOS-D, Inulin, FOS, glucose, xylose and control in triplicate wells. Each experiment was performed in flat bottom polystyrene 96-well plates in which 150 µL media with XOS-C, XOS-D, Inulin, glucose, xylose and FOS was inoculated with 25 µL of bacterial culture. Nutrient base medium with no bacteria was used as a negative control for microplate readings. A TECAN GENios Automated Microplate reader was run with Magellan v. 2.0 software (Tecan; Durham, NC). Optical density readings were measured at 0-7 days post-

inoculation at 550 nm. Bacterial growth was recorded in triplicate. 96-well plates were incubated anaerobically with moisture in an Anoxomat Anaerobic Culture System using the default anaerobic gas mixture.

In summary, seven different substrates (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) were evaluated for growth of three different organisms (Bifidobacterium adolescentis, breve and L. brevis) on three different growth broths (HB, TS and PS). Growth was reported in terms of maximum optical density at 550 nm and specific growth achieved during a period of 6 days. Optical density at 550 nm (OD550) was normalized by taking logarithm of OD at day 6 divided by OD at day 0), for each organism/substrate/media combination. Normalized optical density is referred to as specific growth.

2.2.8 Statistical Analysis

Analysis of variance (ANOVA) and Duncan's test (SAS Institute, Cary, NC) were used to compare maximum optical density means of *B.adolescentis, L.brevis, and B.breve* using substrates (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) on the broths (TP, PS and HB) and for ranking of the substrates. Statistical significance level was 5% ($p < 0.05$).

Standard error is calculated as standard deviation of replicates by the square root of number of replicates.

2.3 Results

2.3.1 Physicochemical properties

Moisture content of corn fiber separated from DDGS was 12.4% (wet basis). Carbohydrate composition of fiber was: glucan 18.0%, xylan 16.8%, arabinan 8.8%, mannan 0.8%, galactan 3.0%, and lignin content 1.3%. Cellulose content represented 18.0% and hemicelluloses content, comprising of xylan, arabinan, galactan, and mannan chains, represented 29.4.% in the corn fiber.

The total XOS production from corn fiber was 18.6%. Degradation products (furfural and HMF) which act as microbial inhibitors, such as that observed by Carvalheiro et al. (2005) were not present in the liquor solution. The liquor contained mainly xylotriose (16.4% yield) and xylopentose (2.2% yield). Compositional analysis showed that residue from autohydrolysis did not contain any carbohydrates (glucan, xylan, galactan and arabinan). The maximum XOS yield obtained in this study (18.6% of feedstock) was comparable to maximum XOS yields observed by earlier autohydrolysis studies. The maximum XOS yield from brewery's spent grain (BSG) was 14.1% (Carvalheiro *et al.* 2004). The original liquor had no traces of formic acid and levulinic acid, which are formed on the degradation of HMF and furfural compounds (Dunlop 1948; Ulbticht *et al.* 1984). XOS-C composition was: xylobiose (26.9%), xylotriose (40.0%), xylotetrose (19.6%), xylopentose (7.6%) and xylohexose (5.7%).

2.3.2 Prebiotic Properties – Three Media Types

Figures 2.1, 2.2 and 2.3 are representative growth curves for three different combinations of broth and microbe on all seven substrates. Most microbial growth reached a maximum by day 2; growth thereafter was stationary (Figures 2.1, 2.2 and 2.3).

The figures demonstrate that growth does occur on XOS-D. Among the three broths, HB broth resulted in the lowest growth for all tested microbes (Table 2.1). This can be attributed to the lack of supportive nutrients in HB broth, though; any growth noted in this media could be attributed to the addition of substrate. PS broth and TP broth contain additional nutrients in the form of glucose or soybean meal, thus there was no clear trend in terms of performance between PS broth and TP broth. PS broth was favorable for monosugars compared to other broths. Among the three organisms, *L. brevis* performed best (Table 2.1). There was no clear trend in terms of performance between *B. breve* and *B. adolescentis*. Moura et al. (2007) also reported that among the tested strains, *L. brevis* displayed the highest growth and XOS consumption. *B. breve* grew more effectively on monomers (xylose and glucose) in all three broths (Table 2.1).

2.3.3 Prebiotic Properties – Effect of bacterium and substrate

B.adolescentis displayed a high preference for FOS followed by XOS-C over xylooligosaccharides produced from XOS-D (Table-2.1). *B.breve* grew better on glucose followed by FOS and XOS-C, while *B. Breve* demonstrated significant growth on xylooligosaccharides produced from XOS-D. *L. brevis* displayed highest growth on the XOS-C and similar growth on both XOS-D and FOS. For XOS-D, highest growth recorded was for *L. brevis* (0.461) followed by *B.breve* (0.267) and *B.adolescentis* (0.263).

The results of ranking for specific growth rates of *B.adolescentis, L.brevis, and B.breve* using substrates (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) in the broths (TP, PS and HB) are summarized in Table 2.2. Among the seven substrates, all performed better than or equal to the control for all tested bacteria in all three broths,

except for *B. adolescentis* in HB Broth (Table 2.2). Thus, bacteria growth was negligible in the absence of substrate. Monosugars (xylose and glucose) typically enhanced growth over all other substrates for *B. breve* and *L. brevis*; the exception was *B. breve* in HB broth (Table 2.2). Monosugars were not among the top three performing substrates for *B. adolescentis* (in all three broths).

Commercial oligosaccharides (FOS, XOS-C and inulin) outperformed other substrates in *B. adolescentis*; however they did not show consistently good performance for *B. breve* and *L. brevis*. The exception was FOS, which performed well for *B. breve*. The comparable performance of XOS-C with other prebiotics (FOS and inulin) confirms the prebiotic potential of XOS. XOS-C performed better than XOS-D for all tested bacteria.

2.4 Discussion

XOS-D showed better performance than the control for all tested bacteria/broth combinations except for *B. breve* in PB broth and *B. adolescentis* in HB broth. XOS-D showed better or comparable performance when compared to FOS and inulin for *L. brevis*, which outperformed other bacteria when grown on XOS-D (Table 2.1). This indicates that there was no substantial inhibitory effect in XOS-D. As stated earlier, monosugars performed better than most other substrates. Since monosugars are present in XOS-D, we cannot conclude that growth of microorganisms with XOS-D was associated with the monosugar or due to the oligosaccharides. That being said, the performance of XOS-C coupled with the performance of XOS-D substrate is an indication that the oligosaccharides in XOS-D would promote growth (Table 2.2). Similar to XOS-C, Inulin and FOS also performed better than XOS-D for *B. adolescentis* and *B. breve*. The reason

for lower performance of XOS-D is perhaps due to the lower concentration of oligosaccharides in XOS-D compared to that in XOS-C. Purification of oligosaccharide compounds from XOS-D is required to quantify the performance of oligosaccharides present in XOS-D.

Crittenden et al. (2002) assessed the prebiotic potential of pure XOS in complex cultures and reported that bacteria from the bifidobacterial strains have ability to utilize XOS. Similar bacterial growth performance on commercial XOS (XOS-C) confirms the validity of this study.

Walter et al. (2011) discussed vertebrate symbiotic associations with vast and complex microbial communities of the large intestine, which suggests the complexities of *in vivo* interactions can't be approached *in vitro*, and thus few studies can simulate the many anticipated ecological interactions. An approach for future work would consist of mixed populations mimicking *in vivo* conditions.

2.5 Conclusions

The trend for growth observed on XOS-D was that XOS-D promoted bacterial growth more than that of control. XOS-D showed better or comparable performance than FOS and inulin for *L. brevis*. The bacterial growth performance of commercial XOS (XOS-C) coupled with the performance of XOS-D substrate confirmed prebiotic potential of oligosaccharides in XOS-D. Corn fiber separated from a low value coproduct (DDGS) could be a raw material for producing prebiotics.

Table 2.1 Maximum specific growth of *B.adolescentis, L.brevis, and B.breve* using substrates (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) in the broths (TP, PS and HB)

A	Microorganism	Control	Glucose	Xylose	Inulin	FOS	XOS-C	XOS-D
TP	*L.brevis*	0.204 [a]	0.730 [b]	0.675 [a]	0.177 [b]	0.465 [b]	0.974 [a]	0.461 [a]
	B. brevis	0.093 [b]	0.916 [a]	0.471 [a]	0.341 [a]	0.805 [a]	0.762 [b]	0.267 [b]
	B. adoloscentis	0.007 [c]	0.573 [c]	0.596 [a]	0.362 [a]	0.856 [a]	0.718 [b]	0.263 [b]
PS	*L.brevis*	0.210 [b]	0.751 [a]	0.814 [a]	0.233 [b]	0.315 [c]	0.607 [a]	0.416 [a]
	B. brevis	0.388 [a]	0.771 [a]	0.502 [b]	0.224 [b]	0.584 [b]	0.460 [b]	0.186 [b]
	B. adoloscentis	0.207 [b]	0.328 [b]	0.244 [c]	0.502 [a]	0.787 [a]	0.718 [a]	0.415 [a]
HB	*L.brevis*	0.010 [c]	0.512 [a]	0.608 [a]	0.330 [a]	0.207 [a]	0.631 [a]	0.250 [a]
	B. brevis	0.108 [b]	0.326 [b]	0.091 [b]	0.310 [a]	0.213 [a]	0.196 [b]	0.128 [b]
	B. adoloscentis	0.238 [a]	0.083 [c]	0.062 [b]	0.310 [a]	0.208 [a]	0.208 [b]	0.082 [b]

Results are means of three replicates. Means in the same column for each broth followed by same letter are not significantly different ($p < 0.05$).
Optical Density at 550nm (OD_{550}) was normalized by taking Log10(OD at day 6/OD at day 0), for each organism/substrate/media combination

Table 2.2 Substrate (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) ranking using maximum specific growth for *B.adolescentis, L.brevis, and B.brevis* in HB broth, PS Broth and TP Broth

Microbe	**L. b*revis***			**B. a*dolescentis***			**B. *breve***		
Rank	**TP Broth**	**PS Broth**	**HB Broth**	**TP Broth**	**PS Broth**	**HB Broth**	**TP Broth**	**PS Broth**	**HB Broth**
1	XOS-C 0.974 [a]	Xylose 0.814 [a]	XOS-C 0.631 [a]	FOS 0.856 [a]	FOS 0.787 [a]	Inulin 0.310 [a]	Glucose 0.916 [a]	Glucose 0.771 [a]	Glucose 0.326 [a]
2	Glucose 0.730 [ab]	Glucose 0.751 [a]	Xylose 0.608 [ab]	XOS-C 0.718 [b]	XOS-C 0.718 [a]	Control 0.238 [ab]	FOS 0.805 [a]	FOS 0.584 [b]	Inulin 0.310 [a]
3	Xylose 0.675 [ab]	XOS-C 0.607 [b]	Glucose 0.512 [b]	Xylose 0.596 [c]	Inulin 0.502 [b]	XOS-C 0.208 [abc]	XOS-C 0.762 [a]	Xylose 0.502 [bc]	FOS 0.213 [ab]
4	FOS 0.465 [bc]	**XOS-D** 0.416 [c]	Inulin 0.330 [c]	Glucose 0.573 [c]	**XOS-D** 0.415 [c]	FOS 0.208 [abc]	Xylose 0.471 [b]	XOS-C 0.460 [cd]	XOS-C 0.196 [ab]
5	**XOS-D** 0.461 [bc]	FOS 0.315 [d]	**XOS-D** 0.250 [cd]	Inulin 0.362 [d]	Glucose 0.328 [cd]	Glucose 0.083 [d]	Inulin 0.341 [b]	Control 0.388 [d]	**XOS-D** 0.128 [b]
6	Control 0.204 [d]	Inulin 0.233 [d]	FOS 0.207 [d]	**XOS-D** 0.263 [d]	Xylose 0.244 [de]	**XOS-D** 0.082 [d]	**XOS-D** 0.267 [bc]	Inulin 0.224 [e]	Control 0.108 [b]
7	Inulin 0.177 [d]	Control 0.210 [d]	Control 0.010 [e]	Control 0.007 [e]	Control 0.207 [e]	Xylose 0.062 [d]	Control 0.093 [c]	**XOS-D** 0.186 [e]	Xylose 0.091 [b]

Values are means of three replicates.

[a-e]Means within a column with no common superscript differ significantly ($P < 0.05$).

Means in the same column followed by same letter are not significantly different ($p < 0.05$).

The values obtained were mean value of three replicates.

Optical Density at 550nm (OD_{550}) was normalized by taking Log10(OD at day 6/OD at day 0), for each organism/substrate/media combination

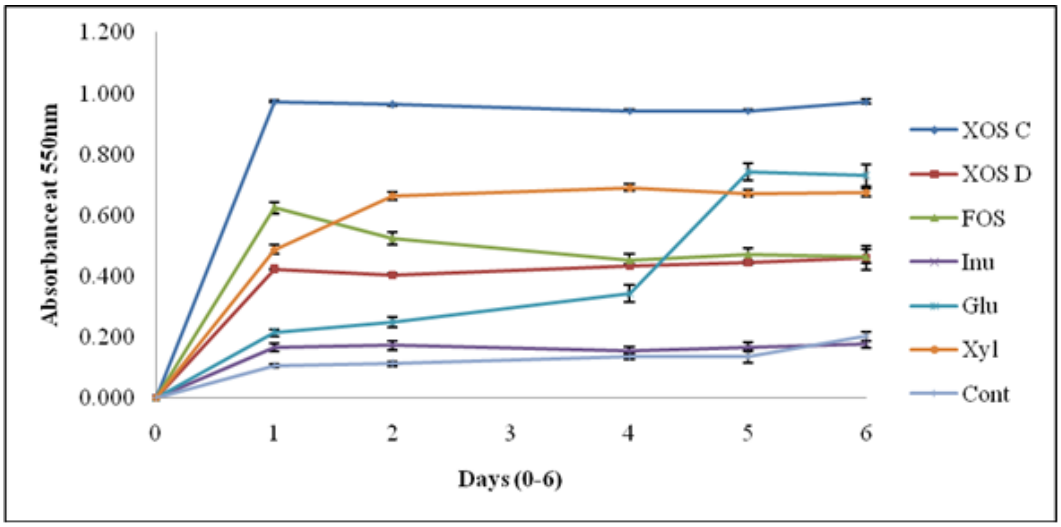

Figure 2.1 Lactobacillus brevis growth on commercial XOS, XOS-D, FOS, Inulin, Glucose, Xylose and Control (without sugars) in TP broth as measured with an absorbance at 550nm

For a period of 7 days starting from (0-6) (data were the mean values of three replicates). Standard error is calculated as standard deviation of replicates by the square root of number of replicates

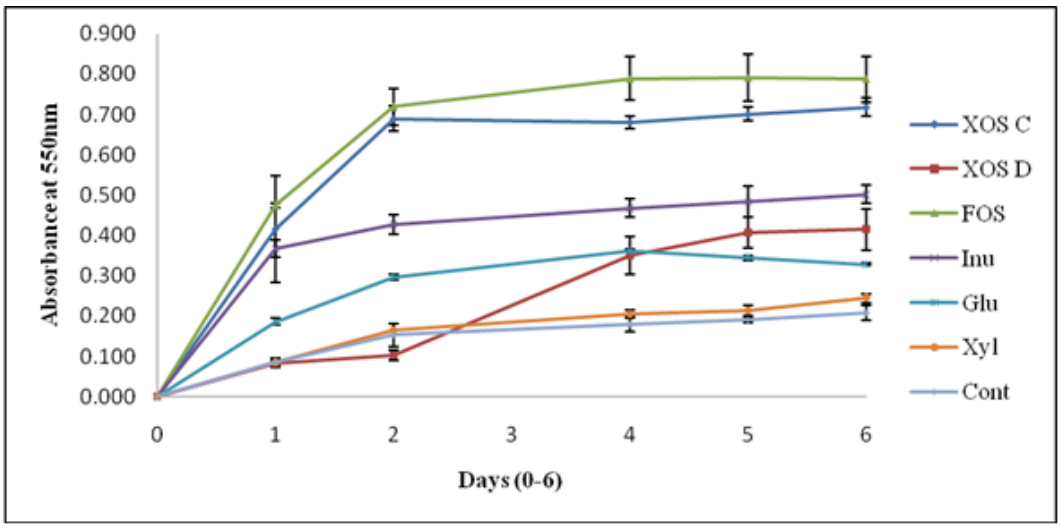

Figure 2.2 Bifidobacterium adolescentis growth on commercial XOS, XOS-D, FOS, Inulin, Glucose, Xylose and Control (without sugars) in PS broth with absorbance at 550nm

For a period of 7 days starting from (0-6) (data were the mean values of three replicates). Standard error is calculated as standard deviation of replicates by the square root of number of replicates

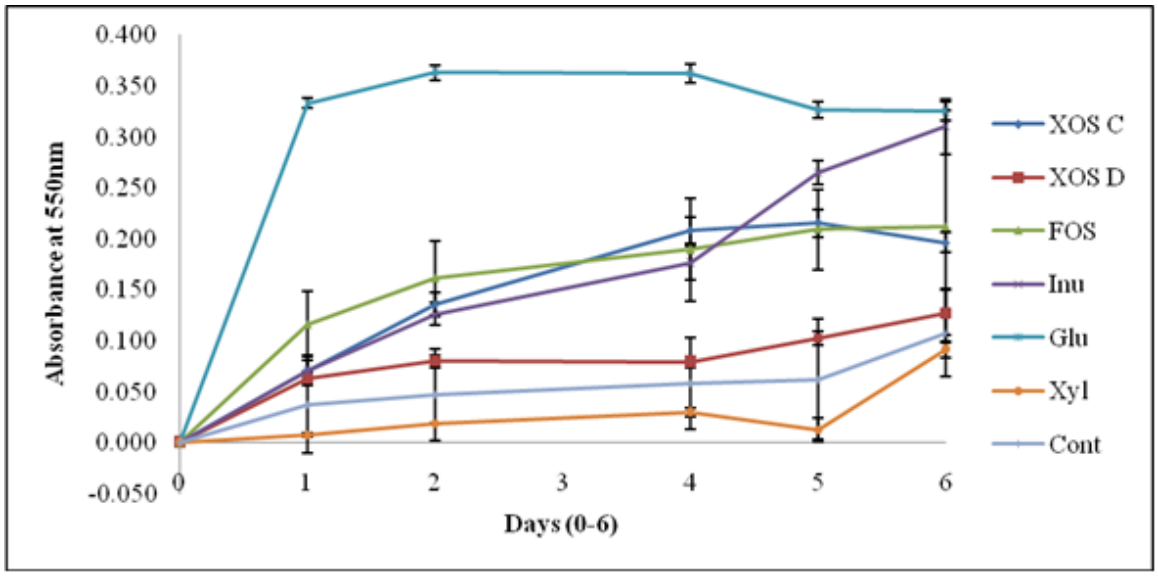

Figure 2.3 Bifidobacterium breve growth on commercial XOS, XOS-D, FOS, Inulin, Glucose, Xylose and Control (without sugars) in Hashbushnel broth with absorbance at 550nm.

For a period of 7 days starting from (0-6) (data were the mean values of three replicates. Standard error is calculated as standard deviation of replicates by the square root of number of replicates

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CHAPTER III

COMPARISON OF XYLO-OLIGOSACCHARIDES PRODUCTION BY AUTOHYDROLYSIS OF FIBERS SEPARATED FROM GROUND CORN FLOUR AND DDGS

3.1 Introduction

Corn is the most widely produced grain in the United States, with 52% used as animal feed, 37% for ethanol production (fuel and beverage) and the remaining 11% for the production of food products.(ERS, 2012). In the dry grind process, corn processing results in fuel ethanol, distillers dried grains with solubles (DDGS) and carbon dioxide (CO2). Accompanying the growth in ethanol production, DDGS production has expanded substantially. Fiber was separated from DDGS and ground corn flour (GCF), to increase the nutritional value for non-ruminants, using the Elusieve process, which is a combination of sieving and elutriation (air classification) (Srinivasan et al. 2009).

Ground corn and DDGS are major ingredients in swine and poultry diets. Ground corn inclusion level in poultry and swine diets is typically 65% and 50%, respectively. DDGS inclusion level in poultry and swine diets is typically 8% and 30%, respectively. Removal of fiber from feed ingredients increases nutritional value for non-ruminants (swine and a poultry), which do not digest fiber well. It is envisaged that Elusieve process will be used in feed mills to separate fiber from ground corn flour as well as DDGS. Elusieve process removes 10% by weight of corn and 15% by weight of DDGS

as fiber. Thus, there will be a significant quantity of fiber separated from corn, in addition to the fiber separated from DDGS.

Fiber is rich in hemicellulose and cellulose, which can be further broken down into oligosaccharides by hydrolysis methods. This work deals with xylo-oligosaccharides (XOS) from corn fiber for the purpose of using XOS as prebiotic. Oligosaccharides are sugar molecules with degree of polymerization (DP) between 2 to10 and can be classified as digestible or non-digestible based on the physiological properties (Nakakuki 1993). Mussatto and Mancilha (2007), reported that non-digestible oligosaccharides are fermentable substances in large intestine that benefit human health and are termed as prebiotics. Prebiotics are defined as non-digestible food ingredients that benefit the host by stimulating the growth and activity of a limited number of bacteria, such as Bifidobacterium species, in the colon (Gibson and Roberfroid 1995). Prebiotics have applications in pet foods, human foods, and animal feeds.

XOS are xylose based oligomers linked by β-1, 4 bonds; they contain variable amounts of substituted groups such as acetyl, phenolic, and uronic acid. XOS are sugar oligomers produced by the hydrolysis of xylan, the major component of plant hemicelluloses. XOS has been produced from corncobs, almond shells, olive stones, wheat straw, barley straw (Nabarlatz et al. 2004) and other materials by hydrolysis methods and XOS are considered to be prebiotics (Gibson and Roberfroid 1995).

Fiber separated from DDGS is a good feedstock for producing XOS (Samala et al 2012). XOS production would be influenced by feedstock. Pandya et al (2012) separated corn fiber from ground corn flour before the dry grind process for production of ethanol or for non-ruminant feed.There is a need to determine the suitability of fiber separated

from ground corn flour for XOS production and determine the optimum process parameters for autohydrolysis. Utilization of corn fiber in the production of higher value food components such as corn fiber oil, corn fiber gum and XOS would increase value of corn fiber.

In the autohydrolysis method, water is added to the substrate and the mixture is heated to the range 150 to 220 ºC in an enclosed vessel to produce XOS subjected to different reaction times 5, 10, 20 and 30 minutes. The objective of this study is to determine the yield of XOS from corn fiber separated from ground corn flour and DDGS at each temperature and holdtime, determine the optimum conditions (temperature and holdtime) for authoydrolysis and characterize the produced liquor for oligomers, monosugars, acids and degradation compounds. The results of XOS yield from authohydrolysis of fiber separated from ground corn flour will be compared with that for fiber separated from DDGS.

3.2 Experimental

3.2.1 Fiber Separation from DDGS and Ground Corn Flour

Similar to the work performed by Samala et al (2012), DDGS was procured from a local feed mill and processed to separate fiber using the Elusieve pilot-plant at Mississippi State University (Srinivasan et al. 2009). Quantity of fiber separated was 4% of the weight of DDGS. The fiber used in this study was the large size fiber fraction (size > 868 μm). Quantity of large size fiber fraction was 2% of the weight of DDGS. Fiber separated from DDGS was referred to as "FD".

Yellow-dent corn grain procured from the farmers cooperative (Starkville, MS) was hammer milled using a 3.2 mm retainer screen (8/64 inch screen) in the hammer mill

as described by Pandya et al (2012). The Elusieving process was also similar to that used by Pandya et al (2012). Large sized fiber (> 1,532 μm) was used for this study. Fiber separated from ground corn flour was referred to as "FC".

The fiber materials were stored in vacuum-sealed bags in a refrigerator at 5 °C until used.

3.2.2 Determination of lignocellulosic content of fiber separated from DDGS

Procedure used was same as that described by Samala et al (2012). Three replicates of the FD material were sent to Integrated Paper Services, Inc., Appleton, WI for determination of corn fiber composition. Extractives content for FD was 6.7%. The carbohydrate and lignin content determination of three dichloromethane extracted samples was done in duplicate.

3.2.3 Determination of cellulose and hemicellulose contents of residue from authohydrolysis and fiber separated from ground corn flour

The cellulose and hemicellulose contents of samples other than FD were determined at Mississippi State University. Lignin contents were not determined for these samples. The samples were milled to approximately 40-mesh. Prior to carbohydrate and lignin analysis, samples were extracted with methylene chloride in a soxhlet apparatus to remove extractives. Extractives content for residues from FD authohydrolysis was 12.59 to 35.22%. Extractives content for residues from FC authohydrolysis was 6.58 to 17.79%.

The compositional determination of extracted samples was same as that described by Wang et al (2011) based on the NREL method for “Determination of Structural Carbohydrates and Lignin in Biomass” except that lignin was not measured in this work.

"Specimens (0.3 g) were treated with 72% H_2SO_4 for 1 hr. The mixture was hydrolyzed by adding 84 mL of water and autoclaving at 121 ^oC for 1 hr. The resultant hydrolysis solution was then filtered through the crucibles with a filtering disc to separate the filtrate and residue. The filtrate was used to determine the content of cellulose and hemicellulose. The filtrate was analyzed for glucose, xylose, arabinose, galactose and mannose by HPLC analysis. Conversion factors were used to convert monomer sugar concentrations into polymeric compositions in the starting materials" (Wang et al 2011). Composition for FC material is reported based on mean for eight replicates. Compositions for residue materials are reported based on mean for three replicates.

3.2.4 Auto-Hydrolysis of fibers separated from DDGS and GCF

The experiment procedure used in this study was modification of that by Samala et al. (2012). The autohydrolysis of fiber was conducted, in triplicates for each experimental condition, using a 750 mL Parr reactor (model 4843, Parr Instruments Co., Moline, Illinois, USA). Hold-times of 5, 10, 20, and 30 min at different temperatures ranging between 150 and 220 °C (in intervals of 10 °C) were used in this study. The time taken to reach 150 to 220 °C was 16 to 45 min.

The reaction mixture was filtered by gravity filtration and the filtrate was further filtered by a vacuum filtration system. The reaction mixture was filtered twice to obtain particle-free solution for HPLC analysis. The solid product was thoroughly washed with deionized water and dried at room temperature. The washing was collected in a bottle, labeled as original liquor. The residue sample was dried in oven at 100 ^oC for 3 hours. The residue samples from some of the treatments was used for the determination of

extractives, cellulose and hemicellulose content in order to verify mass balance and verify data integrity.

Characterization of autohydrolysis liquor for XOS components, monosugars, acids, furfural and hydfroxymethyl furfural (HMF) by HPLC analysis of liquor was same as that described by Samala et al (2012).

3.2.5 Statistical Analysis

Analysis of variance (ANOVA) and Duncan's test (SAS Institute, Cary, NC) were used to compare the means of xylooligomers (X_2-X_6), monosaccarides (glucose, xylose, galactose, and arabinose), acetic acid, degradation compounds (HMF and furfural) and yields at the highest XOS production from corn fiber separated from DDGS and GCF. Statistical significance level was 5% ($p < 0.05$).

3.3 Results and discussion

3.3.1 Raw material composition of fiber separated from DDGS and GCF

The moisture contents of FD and FC were 12.4% and 12.7%, respectively. The extractives content for FD and FC were 6.7% and 1.8%, respectively. FD composition was glucan 18.0%, xylan 16.8%, arabinan 8.8%, mannan 0.8%, galactan 3.0%, and lignin content 1.3%, on wet basis. FC composition was glucan 18.4 %, xylan 14.3 %, arabinan 6.4 % and galactan 2.0%, on wet basis. Glucan and xylan chains are the main polysaccharides in the raw materials used in the study. Hemicellulose content, comprising xylan, arabinan, galactan, and mannan chains, for FD and FC were 29.4% and 22.7%, respectively.

3.3.2 XOS production using fiber separated from DDGS and GCF

The formation of xylo-oligosaccharides mainly depended on the temperature and hold-time. The liquors obtained after autohydrolysis of FD and FC consisted mostly of a mixture of xylose oligomers with some free arabinose, glucose and galactose (Tables 3.1 and 3.2). Representative chromatograms for quantification of XOS, monosugars, acids and degradation products in the liquor, and chromatograms of the respective standards are shown in figures 1 to 6.

For the same hold-time, XOS production increased as temperature increased till a threshold temperature and then decreased as temperature was increased further (Tables 3.1 and 3.2). For example, for FD at 5 min hold-time, XOS production increased from 230 mg to 544 mg as temperature was increased from 150 ºC to 190 ºC and then decreased to 1 mg as temperature was increased to 220 ºC (Table 3.1). For example, for FC at 30 min hold-time, XOS production increased from 101 mg to 729 mg as temperature was increased from 150 ºC to 180 ºC and then decreased to 19 mg as temperature was increased to 220 ºC (Table 3.2). As temperature increased, the breakdown of xylan chain into XOS increased. As temperature increased beyond the optimum level, XOS breakdown into xylose monosugar and further conversion to degradation products could have occurred causing a decrease in XOS production.

The maximum production of XOS from FD was at 180 ºC and 20 min (803 mg) (Table 3.1). This was in agreement with previous works: 180 ºC with 15 min hold-time reported by Samala et al (2012), and190 ºC with 5 min hold-time reported by Carvelheiro et al. (2004). The maximum production for XOS for FC was 190 ºC and 10 min (892 mg). For FD's maximum XOS production condition (180 ºC and 20 min hold-time), the

liquor contained xylobiose (253 mg), xylotriose (344 mg), xylotetrose (90 mg), xylopentose (70 mg), and xylohexose (46 mg). For FC's maximum XOS production condition (190 ºC and 10 min hold-time), the liquor contained xylobiose (434 mg), xylotriose (255 mg), xylotetrose (130 mg), and xylohexose (67 mg).

The yields of XOS as proportion of the initial xylan content in FD and FC were 54.6% and 71.5%, respectively (Table 3.7). The yields were comparable to values reported by other researchers: 52% from miscanthus (Ligero et al. 2011), 43% from barley straw (Nabarlatz et al. 2007), and 55% from bamboo (Ayoma and Seki 1999). The maximum XOS yields obtained in this study of 10.9% of initial feedstock from FD and 10.2% of initial feedstock from FC were comparable to maximum XOS yield observed by earlier autohydrolysis studies (Table 3.7). The maximum XOS yield from brewery's spent grain (BSG) was 14.1% (Carvalheiro et al. 2004). The maximum XOS yield from almond shells was 15.7% (Nabarlatz et al. 2007).

3.3.3 Comparison of XOS production for fibers separated from DDGS and from GCF

The conditions for maximum XOS production from FD and FC were 180 ºC with 20 min hold-time and 190 ºC with 10 min hold-time, respectively (Table 3.7). XOS yield as proportion of total material was higher for FC compared to FD. XOS yield from FC, calculated as proportion of initial xylan used, was higher (71.5%) by an even larger extent than from FD (54.6%) because initial xylan content of FC was lower than for FD. Thus, fiber separated from GCF resulted in higher XOS yield than fiber separated from DDGS (Table 3.7).

There was no significant difference in total monosaccharides content in liquors from FC and FD at condition of maximum XOS production. Acetic acid content in liquor from FD was higher than for FC. HMF and furfural content were present in trace quantities (89 mg for both) in liquor from FC, while there was no presence of HMF and furfural in liquor from FD.

3.3.4 Monosaccharides, acids, furfural and hydroxymethylfurfural (HMF)

For same hold-time, as temperature increased, monosaccarides content in liquor increased till a threshold temperature and then decreased as temperature was increased further (Tables 3.3 and 3.4). For example, for FC at 10 min hold-time, total monosaccharides content increased from 506 mg to 2003 mg as temperature was increased from 150 ºC to 190 ºC and then decreased to 186 mg as temperature was increased to 220 ºC (Table 3.4). The increase in monosaccharides content with increase in temperature can be attributed to the breakdown of carbohydrates into monosaccharides and the decrease in monosaccharides content can be attributed to the conversion of the monosaccharides into other compounds. Pentose degradation leads to formation of furfural, whereas hexose degradation leads to formation of HMF. For example, degradation of arabinose at higher temperatures has been reported by Kootstra et al. (2009).

For same hold-time, acetic acid production increased as temperature increased (Tables 3.3 and 3.4). Acetic acid formation was due to the breakdown of acetyl groups attached to the xylan chain. At similar authohydrolysis conditions, acetic acid production was higher for FD than for FC (Tables 3.3, 3.4 and 3.8). Higher acetic acid production for FD can perhaps be attributed to acetyl groups in the xylan chain of DDGS being

partially loosened during the thermal treatments used in the jet cooking and DDGS drying stages of fuel ethanol production.

For same hold-time, as temperature increased, HMF and furfural contents in liquor increased till a threshold temperature and then stayed constant or increased as temperature was increased further (Tables 3.3 and 3.4). Formation of HMF and furfural is attributed to degradation of monosaccharides (Kootstra et al 2009, Carvalheiro et al. 2004).

3.3.5 Mass balance for verification of data integrity

Component-wise and overall mass balances at selected conditions are reported in tables 3.5 and 3.6. Mass balances were calculated in terms of the soluble sugars (monomers and oligomers as monomeric equivalents) in the liquor solution and residue obtained after the autohydrolysis.

It was found that the overall unaccounted material was 17% to 52% and the range of component-wise unaccounted material for glucose, xylose, galactose and arabinose were 2 to 56%, 12 to 73%, 3 to 99% and 23 to 73%. The mass balance error was higher for galactose perhaps because of the low quantities of galactose in the materials. Considering that mass balance errors were within acceptable limits in a majority of the conditions, it can be concluded that the data integrity was good.

3.4 Conclusions

For the same hold-time, XOS production increased as temperature increased till a threshold temperature and then decreased as temperature was increased further. For same hold-time, as temperature increased, monosaccarides content in liquor increased till a

threshold temperature and then decreased as temperature was increased further. For same hold-time, acetic acid production increased as temperature increased. At similar authohydrolysis conditions, acetic acid production was higher for fiber separated from DDGS than for fiber separated from GCF. For same hold-time, as temperature increased, HMF and furfural contents in liquor increased till a threshold temperature and then stayed constant or increased as temperature was increased further

The conditions for maximum XOS production for fibers separated from DDGS and GCF were 180 ºC with 20 min hold-time and 190 ºC with 10 min hold-time, respectively. Fiber separated from GCF (FC) resulted in higher XOS yield of 71.5% than fiber separated from DDGS (FD) reported 54.6% at maximum XOS production conditions, calculated based on initial xylan content.

Table 3.1 XOS contents in liquors obtained from the autohydrolysis of corn fiber separated from DDGS.

Conditions		Xylo-Oligosaccharides Quantification for DDGS Fiber					
Hold Time (min)	Temperature (°C)	Xylobiose (mg)	Xylotriose (mg)	Xylotetrose (mg)	Xylopentose (mg)	Xylohexose (mg)	Total XOS (mg)
5	150	32	124	19	28*	27*	230
	160	33	142	19	12	32	238
	170	34*	193	27*	37	22	313
	180	56*	118*	21*	16*	20	231
	190	119	235	97	60	34	544
	200	0	113	12	20	1	146
	210	0	7	0	1*	0	8
	220	0	1*	0	0	0	1
10	150	23	77	17	11	42	171
	160	30	134	13*	12	2*	192
	170	63	221	67	52	31	433
	180	128	220	53	40	22	463
	190	102	216	88	48	25*	478
	200	0	88	5	18	0	111
	210	0	5*	0	0	0	5
	220	0	0	0	0	0	0
20	150	34	139	20	15	61	269
	160	33	151	28	21	12	245
	170	106*	192	70	48	26	442
	180	254	344	90	70	46	803
	190	275	147	48	6	4*	481
	200	0	16	0	8	0	24
	210	0	0	0	0	0	0
	220	0	0	0	0	0	0
30	150	30	134	20	18	64	265
	160	18	197	43	32	23	314
	170	128	243*	86*	51*	25*	533
	180	67	217	66	46	27	424
	190	5	80	17	11*	0	113
	200	0	21	3	7	0	29
	210	0	0	0	0	0	0
	220	0	0	0	0	0	0

Results are means of three replicates

The range of coefficients of variation for xylobiose, xylotriose, xylotetrose, xylopentose, xylohexose are 2-63%, 1-63%, 5-52%, 7-55 %, 1-69 % respectively. *COV- ranging from 65-120%

Table 3.2 XOS contents in liquors obtained from the autohydrolysis of corn fiber separated from GCF.

Conditions		Xylo-Oligosaccharides Quantification for Corn Flour Fiber				
Hold Time (min)	Temperature (°C)	Xylobiose (mg)	Xylotriose (mg)	Xylotetrose (mg)	Xylopentose (mg)	Xylohexose (mg)
	150	32	13	6	0	51
	160	40	19	11	8	79
	170	111	44	63	42	260
5	180	66	104*	96	62	328
	190	460	197	90	43	790
	200	285	84*	31	10*	411
	210	67*	6*	15	0	87
	220	0	5	4	0	9
	150	24	8	3	0	36
	160	56	27	17	8	108
	170	93	38	75	54	260
10	180	76	58	141	102	377
	190	440	255	130	67	892
	200	100	11	19	0	129
	210	36	0	10	0	46
	220	0	7*	3	0	10
	150	57	26	14	0*	97
	160	57	32	20	12*	120
	170	94	43	76	53	266
20	180	126	54	148	98	425
	190	363	146	56	21*	586
	200	37	0	9	0	46
	210	33*	0	8*	0	41
	220	0	10	4*	0	14
	150	59	26	14	3*	101
	160	67	49*	34*	37	186
	170	127	55	109	80	371
30	180	204*	302	141	81	729
	190	248	90	18	1	357
	200	37	0	10	0	47
	210	12	0	2	0	15
	220	0	11	8*	0	19

Results are means of three replicates;
The range of coefficients of variation for xylobiose, xylotriose, xylotetrose, xylohexose are 4-67%, 3-59%, 1-61% and 3-65% respectively. *COV- ranging from 65-120%

Table 3.3 Monosaccharides, acid and degradation compounds contents in liquors obtained from autohydrolysis of corn fiber separated from DDGS

Conditions		Monosaccharides for DDGS Fiber					Degradation Products		Acids
Hold Time (min)	Temperature (°C)	Glucose (mg)	Xylose (mg)	Galactose (mg)	Arabinose (mg)	Total (mg)	HMF (mg)	Furfural (mg)	Acetic Acid (mg)
5	150	147	85	28	193	453	0	0	896
	160	182	121	52	270	626	0	0	901
	170	301	220	122	303	947	0	0	1396
	180	183*	133*	65*	241*	623	0	0	795
	190	264	349	168	266	1047	46	39	1412
	200	223	130	89	109	550	163	210	2029
	210	85*	12	21	16*	135	165	85	1408
	220	14	4*	10	21	49	239	102	2485
10	150	110	74	20	137	342	0	0	1011
	160	184	123	53	267	627	0	0	1021
	170	351	269	150	300	1069	0	0	1244
	180	348	253	135	378	1114	0	0	1386
	190	336	342	163	237	1078	48	23	1208
	200	152*	56*	55*	72	335	185	84	2091
	210	92*	33	35*	38	198	186	94	1591
	220	14	3*	9	18	43	278	144	2839
20	150	161	102	34	236	533	0	0	1216
	160	244	173	87	330	834	0	0	1081
	170	316	280	147	224	967	0	0	1502
	180	493	378	204	469	1544	0	0	1724
	190	266	191	118	108*	684	147	62	1883
	200	76	12	26	35	149	213	45	1745
	210	16	6	9	28	56	208	63	2083
	220	8	4	9	3	24	160	53	2403
30	150	185	122	44	269	621	0	0	1043
	160	310	229	130	374	1042	0	0	1290
	170	391	382	194	256	1223	0	0	1107
	180	318	254	138	270	979	0	0	1367
	190	196	98	79	29*	402	166	51	1830
	200	104	16	34	23	176	175	93	1365
	210	17	8	10	17*	51	202	46	2112
	220	7	4	8	16	35	128	56	2625

Results are means of three replicates. HMF- Hydroxymethylfurfural
The range of coefficients of variation for glucose, glucose, xylose, arabinose, galactose were 5-64%, 2-57%, 3-61%, 4-64%, respectively. *COV- 65-115%

Table 3.4 Monosaccharides, acid and degradation compounds contents in liquors obtained from the autohydrolysis of corn fiber separated from GCF.

Conditions		Monosaccharides for CFF Fiber					Degradation Products		Acids
Hold Time (min)	Temperature (°C)	Glucose (mg)	Xylose (mg)	Galactose (mg)	Arabinose (mg)	Total (mg)	HMF (mg)	Furfural (mg)	Acetic Acid (mg)
	150	132	98	34	241	506	0	0	0
	160	203	143	50	323	719	0	0	0
	170	395	315	131	485	1326	0	0	112
5	180	421	401	160	374	1356	7	18	214
	190	762	634	225	381	2003	112	97	370
	200	788	502	194	310	1793	388	198	259
	210	563	184	112	148	1007	318	136	271
	220	112	14	29	30	186	106	129	245
	150	112	63	22	163	360	0	0	0
	160	247	181	65	382	876	0	0	0
	170	418	343	145	483	1389	0	0	194
10	180	526	497	208	480	1711	24	12	189
	190	712	640	234	410	1996	89	89	361
	200	742	287	151	209	1389	298	176	227
	210	472	90	90	105	757	378	152	247
	220	54	18	19	24	115	310	135	361
	150	149	104	39	232	524	0	0	0
	160	269	199	73	406	946	0	0	0
	170	421	343	146	483	1393	0	0	210
20	180	541	526	214	475	1755	24	21	300
	190	776	561	206	338	1882	140	83	207
	200	471	113	92	116	792	227	117	301
	210	343	105*	70	89*	607	315	118	208
	220	27	14	17	21	80	427	146	472
	150	227	153	53	351	785	0	0	0
	160	291	229	90	398	1009	0	0	0
	170	438	394	166	433	1431	0	0	215
30	180	598	609	225	420	1852	54	47	308
	190	882	480	196	297	1855	180	91	303
	200	495	92	95	110	792	241	154	373
	210	105	13	25	24	167	422	109	257
	220	29	16	20	23	89	461	182	628

Results are means of three replicates. HMF- Hydroxymethylfurfural
The range of coefficients of variation for glucose, glucose, xylose, arabinose, galactose were 2-57%, 1-57%, 1-64%, 1-65%, respectively. *COV- ranging from 65-135%

Table 3.5 Mass balance of sugars for corn fiber separated from DDGS at a few selected conditions.

Conditions	Material	Glucose	Xylose	Galactose	Arabinose	Total
None	Input (mg)	1577	1472	263	771	4082
150 °C, 5 min	Liquor (mg)	129	305	25	169	627
	Residue (mg)	977	917	80	389	2362
	Total output (mg)	1105	1221	105	558	2989
	% Unaccounted	30	17	60	28	27
150 °C, 30 min	Liquor (mg)	162	373	39	235	809
	Residue (mg)	1124	533	44	177	1879
	Total output (mg)	1286	906	83	413	2688
	% Unaccounted	18	38	68	46	34
160 °C, 5 min	Liquor (mg)	160	344	46	237	786
	Residue (mg)	1189	621	48	201	2059
	Total output (mg)	1348	965	94	438	2845
	% Unaccounted	14	34	64	43	30
170 °C, 5 min	Liquor (mg)	264	505	107	266	1142
	Residue (mg)	1117	155	0	23	1295
	Total output (mg)	1381	660	107	289	2437
	% Unaccounted	12	55	59	63	40
180 C, 5 min	Liquor (mg)	161	348	57	211	776
	Residue (mg)	1117	46	0	0	1163
	Total output (mg)	1278	394	57	211	1940
	% Unaccounted	19	73	78	73	52
180 °C, 10 min	Liquor (mg)	305	685	118	331	1439
	Residue (mg)	1131	36	0	0	1167
	Total output (mg)	1436	721	118	331	2606
	% Unaccounted	9	51	55	57	36
180 °C, 20 min	Liquor (mg)	432	1134	179	411	2155
	Residue (mg)	1012	31	0	0	1043
	Total output (mg)	1444	1165	179	411	3198
	% Unaccounted	8	21	32	47	22
180 °C, 30 min	Liquor (mg)	278	646	121	237	1282
	Residue (mg)	1008	23	0	0	1032
	Total output (mg)	1287	670	121	237	2314
	% Unaccounted	18	55	54	69	43

Table 3.6 Mass balance of sugars for corn fiber separated from GCF at a few selected conditions

Conditions	Material	Glucose	Xylose	Galactose	Arabinose	Total
None	Input (mg)	1868	1432	192	646	4138
150 °C, 5 min	Liquor (mg)	115	137	29	211	493
	Residue (mg)	1508	872	100	476	2956
	Total output (mg)	1623	1010	130	686	3449
	% Unaccounted	13	29	33	-6	17
150 °C, 30 min	Liquor (mg)	198	235	47	306	786
	Residue (mg)	757	964	335	31	2087
	Total output (mg)	955	1199	382	338	2873
	% Unaccounted	49	16	-99	48	31
160 °C, 5 min	Liquor (mg)	177	204	44	282	707
	Residue (mg)	1342	494	39	217	2092
	Total output (mg)	1519	698	83	499	2799
	% Unaccounted	19	51	57	23	32
170 °C, 5 min	Liquor (mg)	345	535	115	423	1417
	Residue (mg)	473	1043	123	0	1640
	Total output (mg)	818	1578	238	423	3057
	% Unaccounted	56	-10	-24	34	26
180 C, 5 min	Liquor (mg)	368	678	139	327	1512
	Residue (mg)	1321	59	0	9	1389
	Total output (mg)	1689	737	139	335	2901
	% Unaccounted	10	49	27	48	30
180 °C, 10 min	Liquor (mg)	459	811	181	419	1870
	Residue (mg)	668	116	0	0	784
	Total output (mg)	1127	927	181	419	2654
	% Unaccounted	40	35	6	35	36
180 °C, 20 min	Liquor (mg)	472	884	187	415	1958
	Residue (mg)	1258	57	0	0	1316
	Total output (mg)	1730	941	187	415	3274
	% Unaccounted	7	34	3	36	21
180 °C, 30 min	Liquor (mg)	522	1260	196	367	2346
	Residue (mg)	1302	0	0	0	1302
	Total output (mg)	1824	1260	196	367	3647
	% Unaccounted	2	12	-2	43	12

Table 3.7 Comparison of xylooligomers content (mg) obtained from the autohydrolysis of corn fiber separated from DDGS and GCF at maximum production condition.

Raw Material	Condition	Xylobiose (mg)	Xylotriose (mg)	Xylotetrose (mg)	Xylopentose (mg)	Xylohexose (mg)	Total XOS (mg)	Yield % of Total	Yield % of xylan
FD	180 °C, 20 min	254[b]	344[a]	90[b]	70[a]	46[a]	803[b]	10.9[a]	54.6[b]
FC	190 °C, 10 min	440[a]	255[b]	130[a]	0[b]	67[a]	892[a]	10.2[a]	71.5[a]

Results are means of three replicates. Means in the same column for each xylooligomer followed by same letter are not significantly different ($p < 0.05$).

Table 3.8 Comparison of monosugar, degradation compound, and acid contents (mg) in liquors obtained from the autohydrolysis of corn fiber separated from DDGS and GCF at maximum XOS production condition.

Raw Material	Condition	Glu (mg)	Xyl (mg)	Gal (mg)	Ara (mg)	HMF (mg)	Furfural (mg)	Acetic Acid (mg)	Total Mono (mg)
FD	180 °C, 20 min	493[b]	378[b]	204[a]	469[a]	0[b]	0[a]	1724[a]	1544[a]
FC	190 °C, 10 min	712[a]	640[a]	234[a]	410[a]	89[a]	89[a]	361[b]	1996[a]

Results are means of three replicates. Means in the same column for each monosugar, degradation compound, acid followed by same letter are not significantly different ($p < 0.05$).
Glu, Xyl, Gal, Ara –Glucose, Xylose, Galactose, Arabinose respectively

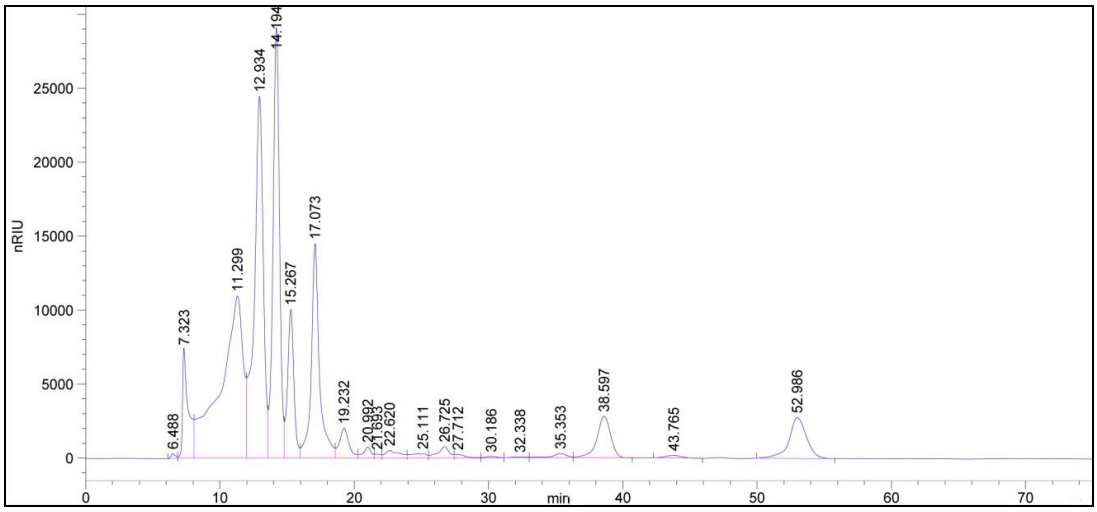

Figure 3.1 HPLC chromatogram for quantification of monosaccharides in original liquor obtained by autohydrolysis of corn fiber separated from GCF at 190 °C and 10 minutes hold-time.

Using Bio-Rad HPX 87 P (300 X 7.8 mm) column at 80 oC by eluting with HPLC grade water. Retention times for glucose, xylose, galactose and arabinose were 12.93, 14.19, 15.26 and 17.07 min, respectively

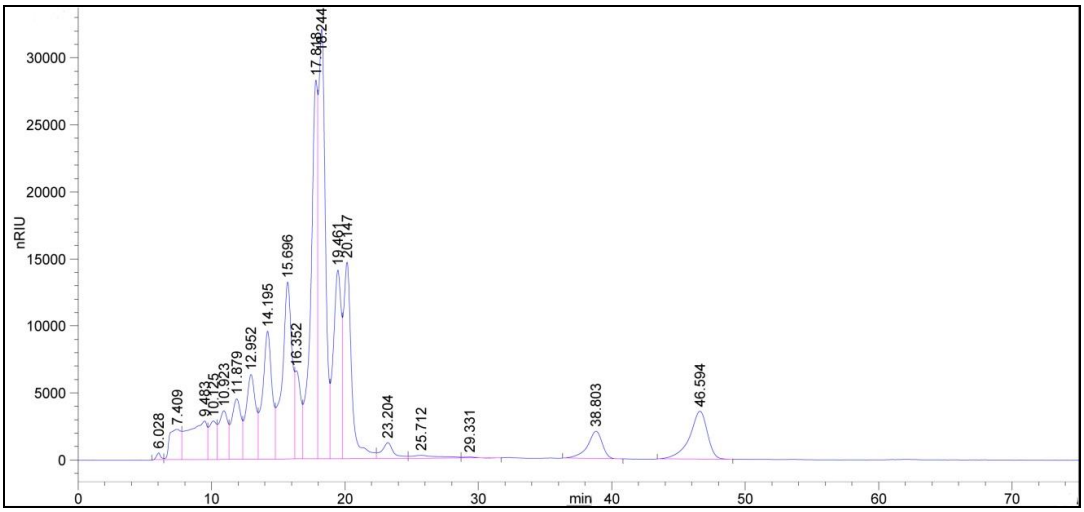

Figure 3.2 HPLC chromatogram for quantification of xylo-oligosaccharides (XOS) in original liquor obtained by autohydrolysis of corn fiber separated from GCF at 190 ˚C and 10 minutes hold-time.

Using Bio-Rad HPX 42 A column at 80 oC by eluting with HPLC grade water. Retention times for xylobiose, xylotriose, xylotetrose, and xylohexose were 14.19, 12.95, 11.97 and 10.92 min, respectively

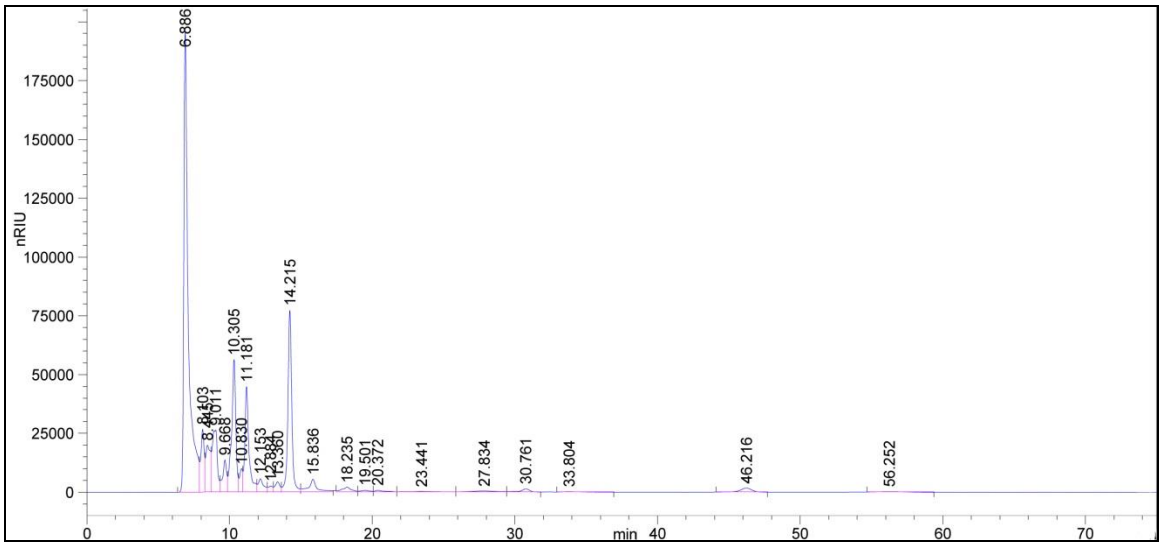

Figure 3.3 HPLC chromatogram for quantification of sugar degradation and acidic components in original liquor obtained by autohydrolysis of corn fiber separated from DDGS at 180 °C and 20 min hold-time.

Using Bio-Rad HPX 87 H (300 X 7.8 mm) column at 80 °C by eluting with 0.005M H2SO4 . Retention times for Acetic acid, HMF and furfural were 15.85, 30.66 and 46.22 min, respectively.

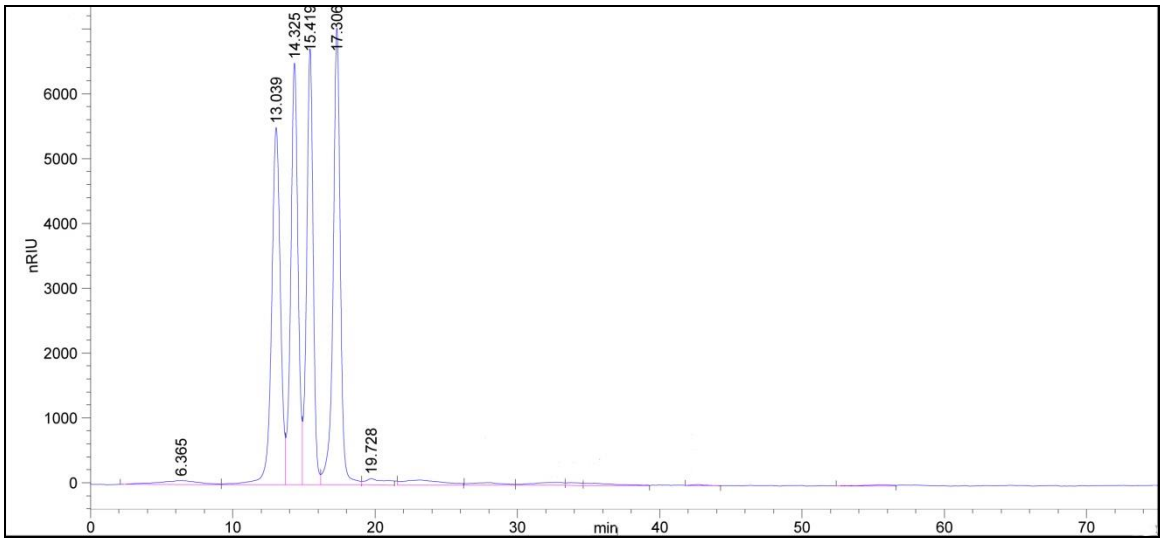

Figure 3.4 HPLC analysis of standards for quantification of monosaccharides using Bio-Rad HPX 87 P column at 80 °C by eluting with HPLC grade water.

Retention times for glucose, xylose, galactose and arabinose were 13.03, 14.32, 15.41 and 17.30 min, respectively

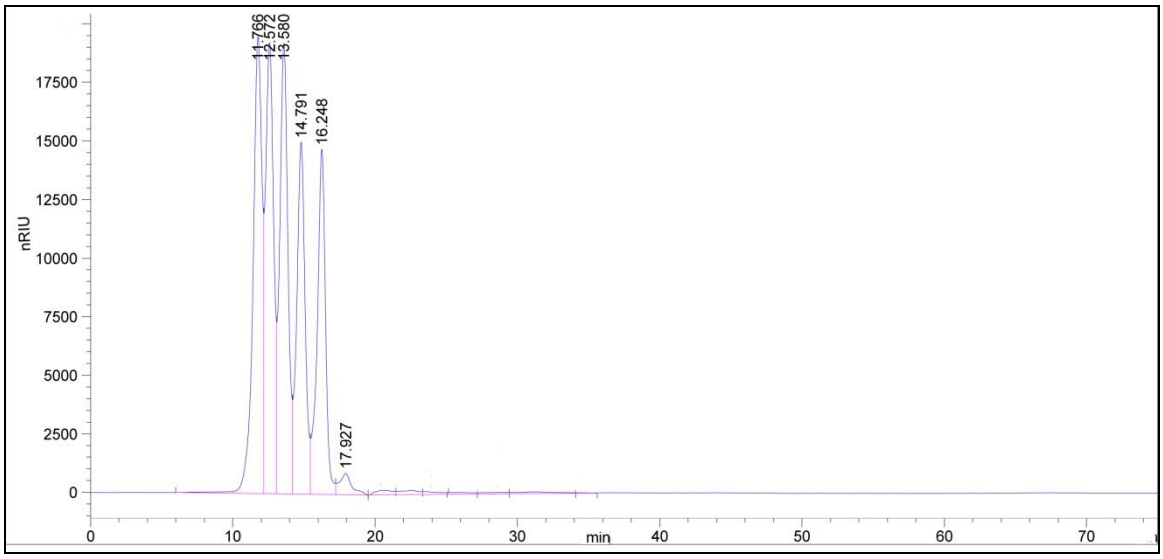

Figure 3.5 HPLC analysis of standards for quantification of xylooligosaccharides (XOS) using Bio-Rad HPX 42 A column at 80 °C by eluting with HPLC grade water.

Retention times for xylobiose, xylotriose, xylotetrose and xylohexose were 16.24, 14.79, 13.58, 12.57 and 11.76 min, respectively

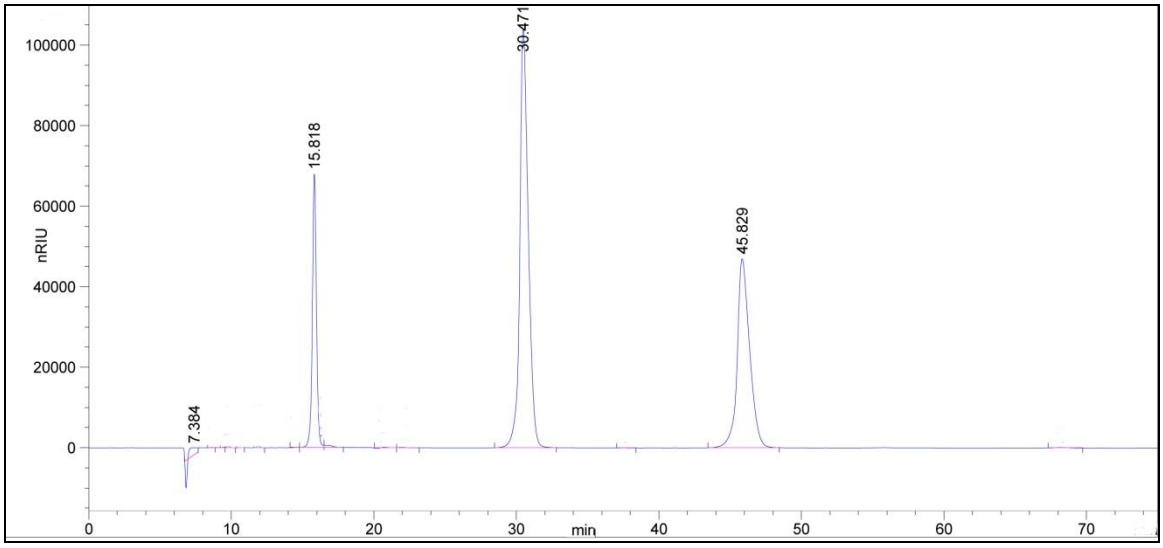

Figure 3.6 HPLC analysis of standards for quantification of acids (acetic acid) and degradation products (HMF, furfural) using Bio-Rad HPX 87 H (300 X 7.8 mm) column at 80 °C by eluting with 0.005M H_2SO_4.

Retention times for acetic acid, HMF and furfural were 15.81, 30.47 and 45.82 min, respectively.

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CHAPTER IV

ENZYMATIC HYDROLYSIS METHOD FOR PRODUCTION OF XYLO-OLIGOSACCHARIDES USING CORN FIBER SEPARATED FROM DDGS

4.1 Introduction

Corn fiber separated from DDGS by eluseive process contains good amounts of hemi-cellulose which constitute xylans, arabinans, galactans and mananns. Hemi-Cellulose based materials can be broken down into oligomers and monomers. Breaking down the hemicellulose into sugars, predominantly oligomers, can be done by enzymatic hydrolysis. This uses enzymes, biological catalysts, to break down the hemicellulose polymers into oligomeric sugars. Hemi-cellulose which contain xylan has complex heteroxylans containing β-(1, 4)-linked xylose residues (Saha, 2000). Saulnier et al. in 1995 reported that xylan backbone is substituted with monomeric side chains of arabinose or glucoronic acid linked to O-2 and/or O-3 of xylose residues.

Break down of xylan into XOS can be obtained by hydrolysis/ autohydrolysis, acid/ alkali or by enzymes. Samala et al., 2012 reported that treatment of xylan containing ligocellulosic biomass with temperatures ranging from 140-220 °C at 15 minutes hold time using autohydrolysis process resulted in xylooligosaccharides, monosugars, with deacetylation of xylans to acetic acid and degradation products HMF and furfural from 190-220°C. Acid hydrolysis has been given a limited amount of

attention because of the potential problems associated with the process. The yield of XOS is minimal with acid hydrolysis because acid prefers to cleave xylan into individual xylose units rather than XOS, and it yields several toxic compounds including furfural and HMF.

High content of carbohydrate and low cost of corn fiber separated from distillers dried grains with solubles (DDGS) made an alternative for feed industry and food industry. Enzymatic hydrolysis with xylanases would not produce toxic by-products, but it still produces considerable amounts of xylose. Aside from lacking prebiotic properties, xylose is also undesirable because it can inhibit the continued production of XOS. Since corn fiber DDGS a by-product of ethanol production represent an abundant and cheap renewable resource for production of XOS, the present investigation deals with XOS production from corn fiber DDGS by enzymatic hydrolysis. The process would be considered effective and successful if yield of XOS is higher than yield of XOS from autohydrolysis. The current study predominantly focuses on production of XOS by enzymatic hydrolyis methods for XOS production, and comparing of % yield of XOS with autohydrolysis method.

4.2 Materials and methods

4.2.1 Materials

4.2.1.1 Corn fiber separated from DDGS (FD)

The raw material used for enzymatic hydrolysis was the same corn fiber samples used in the study in production of XOS from autohydrolysis (Samala et al 2012). The moisture content was 12.4%. Corn fiber separated from DDGS (FD) was ground into powder form and stored in vacuum-sealed bags in a refrigerator at 5 ˚C until used.

4.2.1.2 Corn Fiber Gum (CFG)

FD did not result in good yields of XOS in the enzymatic method (details are in results and discussion section). Hence, we decided to use hemicellulosic material isolated from FD as a raw material. Corn fiber gum (CFG) is a hemicellulosic material produced from corn fiber using an alkali treatment method. CFG produced using FD by Srinivasan et al. (2008) was obtained from storage in USDA, Wyndmoore, PA and used in this study. Total CFG yields from fiber varied from 35.8% to 44.2%. CFG production method was explained by Srinivasan et al. (2008). CFG composition was xylose 64.0%, arabinose 24.8%, galactose 6.8%, glucuronic acid 3.9%, and glucose 0.5%. The CFG samples were stored a refrigerator at 0 ˚C until used.

4.2.1.3 Buffer Solution Preparation

Buffer solutions with different pH (4.5, 5.0, and 6.0) were prepared for the experiment. Buffer solution with pH of 4.5was prepared by dissolving 5.22 g of citric acid and 7.39 g of sodium citrate in 1000 mL of deionized water. Magnetic stirrer was used for mixing. Solutions with pH of 5.0 and 6.0 were prepared similarly by using calculated quantities of citric acid and sodium citrate. Prepared buffer solution was stored in refrigerator at 4°C until ready to use for the experiment.

4.2.2 Enzymes

4.2.2.1 Endo-1-4-Xylanase

Endo-1-4-Xylanase, extracted from Trichoderma longibrachiatum, enzyme was purchased from Sigma-Aldrich (Product # X2629, Lot # 089k1701v). Enzyme activity of Endo-1-4-Xylanase was 9.1 units/mg reported by the manufacturer.

4.2.3 Enzymatic Hydrolysis

4.2.3.1 Enzymatic Hydrolysis without Pretreatment method

Commercial enzymes concentrates were assayed. 250-mL Erlenmeyer flasks were used for the enzymatic hydrolysis experiment. Solid substrate (FD or CFG) was mixed with 100 mL of buffer solution. The solid substrate mixed with buffer solution was heated to 40, 50 or 60 °C before addition of enzyme. Addition of enzyme (endo-1-4-xylanase) was done on the basis of xylan content present in the substrate on dry basis. Enzyme loadings used were 5.45, 27.25, 54.5 and 109 mg. The 250-mL Erlenmeyer flasks were placed in the laboratory shaking incubator at 150 rpm at 45°C. The experiment was carried out for 48 hrs to observe the maximal XOS yield. An aliquot of the supernatant (1.0 mL) was drawn using a pipette from the flasks at times of 15 min, 30 min, 60 min, 1 hr, 2 hr, 12 hr, 24hr and 48 hr. Enzymatic hydrolysis was done in three replicates for all combinations of following conditions: solid substrate (FD or CFG) pH (4.5, 5.0 or 6.0), temperature (40, 50 or 60 °C) and enzyme loadings (5.45, 27.25, 54.5 or 109 mg). For CFG, enzyme loading of 5.45 mg was not used. Thus, 189 flasks were used for enzymatic hydrolysis and 1512 samples were drawn at different times. Quantification of XOS components was performed by HPLC described by Samala et al (2012).

4.2.3.2 Enzymatic Hydrolysis with Pretreatment method

The experimental procedure described is exactly same as the previous section 4.2.3.1, except the mixture of buffer solution and substrate (FD or CFG) was heated to 121 °C for 30 min instead of temperature (40, 50, 60 °C), pH at 4.5 and enzyme loadings (54.5, 109 mgs). The samples were collected in triplicates for analysis of XOS.

4.3 Results and discussion

4.3.1 Raw material composition of FD and CFG

The moisture and extractive contents of FD were 12.4% and 6.7%, respectively. Composition of FD was glucan 18.0%, xylan 16.8%, arabinan 8.8%, mannan 0.8%, galactan 3.0%, and lignin content 1.3%, on wet basis (Samala et al 2012). CFG composition was xylose 64.0%, arabinose 24.8%, galactose 6.8%, glucuronic acid 3.9%, and glucose 0.5%

4.3.2 Enzymatic hydrolysis

With FD as substrate, the maximum XOS yield as % of starting xylan was 9.9% to 16% (Table 4.1). Thus, endo-1-4-xylanase was not effective in producing XOS from FD. Hence, we decided to evaluate CFG as a substrate that might be broken down better by endo-1-4-xylanase.

With CFG as substrate, the maximum XOS yield as % of starting xylan was 7.6% to 15% (Table 4.2). Thus, endo-1-4-xylanase was not effective in producing XOS from CFG also.

Autohydrolysis resulted in XOS yield as high as 54.6%. Considering that endo-1-4-xylanase enzyme resulted in low yields (7.6 to 16%) from FD as well as CFG, we can conclude that enzymatic hydrolysis using endo-1-4-xylanase enzyme was ineffective. Hence, we communicated with Dr. Dien in ARS, USDA, Peoria, IL in order to establish an effective enzymatic method. He pointed us to one of their group's recent works: Dien et al. (2008). Dien et al. (2008) reported that endo-xylanase is ineffective at saccharifying the xylan in fiber portion of DDGS. They postulated that "the chemically complex structure and ferulic cross bridges may be responsible for much of the xylan's

resistance to enzymes because the side chains protect xylan back bone from the action of endo-xylanase (Grabber et al. 1998a; Saulnier et al. 2001)".

Dien et al. (2008) found that commercial grade (e. g. impure) pectinase and xylanase were effective in breaking down the xylan in DDGS. "Even though DDGS does not contain pectin, commercial pectinase preparations contain multiple side activities that may aid the cellulose in hydrolyzing DDGS. As such, the pectinase preparation was used solely for hemicelluloses relevant activities. Therefore, the pectinase mixture was added based upon its xylanase acticity as opposed to pectinase activity. Adding Multifect Pectinase PE significantly improves the yields of both xylose and arabinose" (Dien et al. 2008). Based on this, we have ordered commercial Multifect Pectinase PE and Multifect Xylanase enzymes and we are developing the procedure for enzymatic XOS production from corn fiber.

Table 4.1 XOS (mg) and XOS Yield% from Enzymatic Hydrolysis of FD by pretreatment and non-pretreatment methods of Liquor obtained.

Raw Material	Temp (°C)	Parameters			Pretreatment	XOS (mg)	XOS Yield %
		Enzyme Loading (mg)	pH	Time (Hrs)			
FD	60	54.5	4.5	24	No	115.1	13.7
		109	4.5	24	No	119.0	14.2
		54.5	5	24	No	122.6	14.6
		109	5	12	No	118.0	14.0
		54.5	6	24	No	134.2	16.0
		109	6	12	No	130.6	15.5
		54.5	4.5	24	121°C/30 Min	144.5	10.8
		109	4.5	24	121°C /30 Min	168.5	13.7
FD	50	54.5	4.5	24	No	95.1	11.3
		109	4.5	24	No	103.0	12.3
		54.5	5	24	No	102.6	12.2
		109	5	24	No	108.0	12.8
		54.5	6	24	No	107.9	12.9
		109	6	24	No	110.6	13.1
FD	40	54.5	4.5	24	No	123.0	14.6
		109	4.5	24	No	116.0	13.8
		54.5	5	24	No	83.5	9.9
		109	5	24	No	126.0	15.0
		54.5	6	24	No	84.5	10.1
		109	6	24	No	127.0	15.1

XOS-xylooligosaccharides. Results are means of three replicates. The range of coefficients of variation for xos is1.3-97.5%, respectively.

Table 4.2 XOS (mg) and XOS Yield% from Enzymatic Hydrolysis of CFG by pretreatment and non-pretreatment methods of Liquor obtained.

Raw Material	Temp (°C)	Parameters			Pretreatment	XOS (mg)	XOS Yield %
		Enzyme Loading (mg)	pH	Time (Hrs)			
CFG	60	54.5	4.5	24	No	200.3	12.3
		109	4.5	24	No	226.4	13.9
		54.5	5	24	No	181.6	11.2
		109	5	24	No	194.5	12.0
		54.5	6	24	No	214.7	13.2
		109	6	24	No	214.4	13.2
		54.5	4.5	24	121°C/30 Min	155.7	11.7
		109	4.5	24	121°C /30 Min	190.3	15.0
CFG	50	54.5	4.5	24	No	184.8	11.3
		109	4.5	24	No	197.3	12.1
		54.5	5	24	No	152.7	9.4
		109	5	24	No	158.9	9.8
		54.5	6	24	No	190.0	11.7
		109	6	24	No	187.7	11.6
CFG	40	54.5	4.5	24	No	153.7	9.4
		109	4.5	24	No	165.7	10.2
		54.5	5	24	No	123.6	7.6
		109	5	24	No	134.2	8.3
		54.5	6	24	No	163.0	10.0
		109	6	24	No	157.7	9.7

XOS-xylooligosaccharides. Results are means of three replicates. The range of coefficients of variation for xos is7.3-79.3%, respectively.

4.4 Conclusions

Enzymatic hydrolysis using endo-1-4-xylanase enzyme was ineffective for FD as well as CFG. Previous researchers also have reported that endo-xylanase is ineffective at saccharifying the xylan in fiber portion of DDGS. They have also reported that adding commercial Multifect Pectinase PE enzyme significantly aids the breaking down of xylan. Based on this, we have ordered commercial Multifect Pectinase PE and Multifect

Xylanase enzymes and we are developing the procedure for enzymatic XOS production from corn fiber.

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CHAPTER V

METHODS FOR PURIFICATION OF XYLO-OLIGOSACCHARIDES FOR COMMERCIAL USE

5.1 Overview

Xylo-oligosaccharides (XOS) with different degrees of polymerization (DP) can be obtained by autohydrolysis performed at 180°C with 15 minute hold time from corn fiber DDGS (Samala et al., 2012). Different researchers demonstrated that xylan containing raw materials, such as corn fiber, DDGS fiber, corncobs, rice husk, etc., when treated in aqueous media at temperatures ranging from 150-220°C at different reaction times, the hemicellulosic polymers are broken down and solubilized resulting in liquors containing monosaccharides, oligomers, polysaccharides, acetic acid, furfural, HMF and other degradation products (Parajo et al., 2004; Garrotte et al., 2007; Gullon et al., 2011). For commercial applications such as food industries, XOS would need to be isolated from liquor and the remaining compounds (monosaccharides, oligomers, polysaccharides, acetic acid, furfural and HMF) need to be separated. Thus, a purification procedure is required to obtain food grade xylo-oligosaccharides.

Multiple purification methods are used to refine the xylo-oligosaccharides containing crude liquor (Figure 1). Depending on the degree/grade of purity required, a combination of treatments can also be employed to obtain high grade XOS (Yuan et al.,

2004; Vegas et al., 2005; Rivas et al., 2006). The aim of this study is to do a literature review of purification methods used for XOS purification.

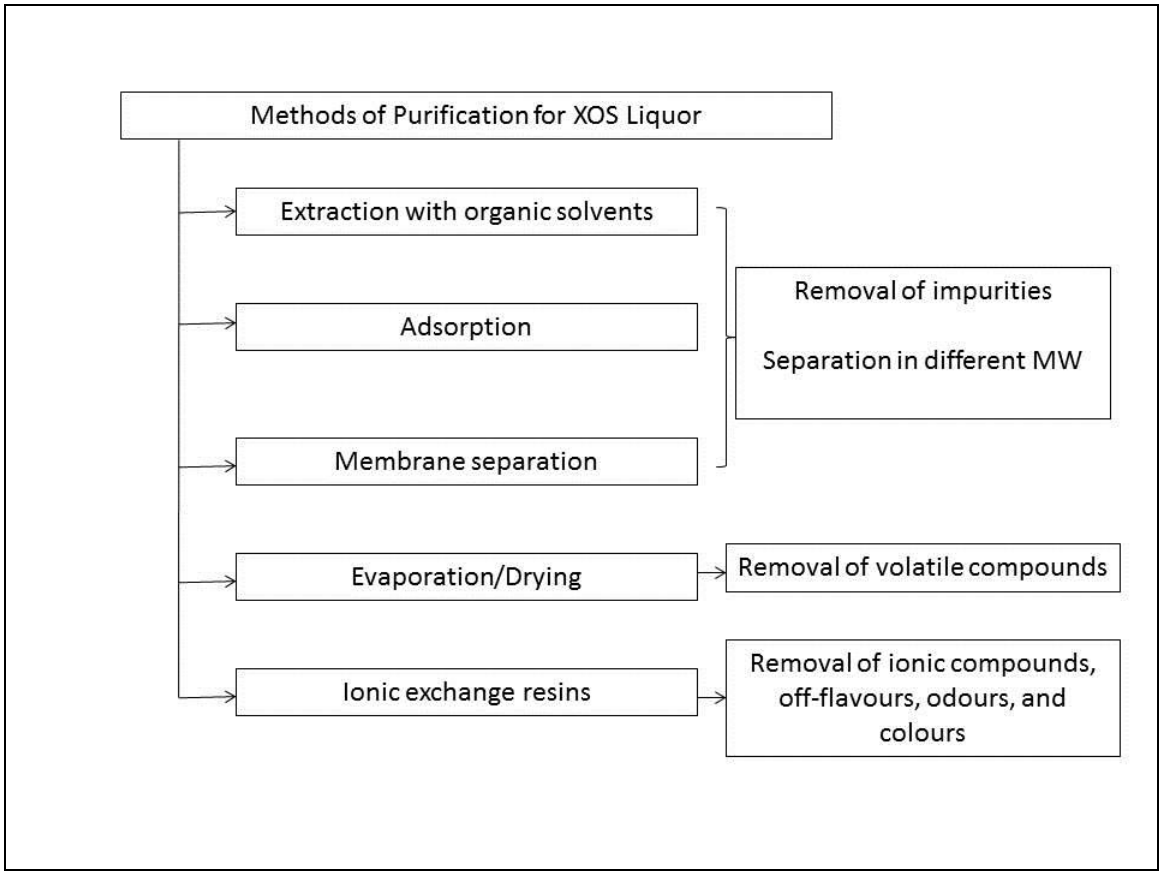

Figure 5.1 Purification strategies to obtain Xylo-oligosaccharides from crude liquor adapted from Nabarlatz, et al. 2007.

5.2 Use of Organic Solvents

Solvent extraction is widely used to remove non-saccharide components from autohydrolysis crude liquors, yielding both a selectively refined aqueous phase and a solvent-soluble fraction mainly made up of phenolics and extractive-derived compounds (Vazquez et al., 2005; Moure et al., 2006). Organic solvents, due to their low boiling

point and easy recovery, are mainly employed for refining hydrolysates. While apolar solvents like ethers and ketones are widely used organic solvents to extract phenolics from water (Garrote et al., 2003; Vegas et al., 2005), solvents such as alcohols and acetone have been employed for the recovery of hemicelluloses degradation products (Vazquez et al., 2005; Swennen et al., 2005). Considering that the autohydrolysis liquor in our work contains hemicellulose degradation products, solvents such as alcohols and acetone would be used.

Garrote et al. (2003) used ethyl acetate as a solvent to extract the liquors (liquor to ethyl acetate ration of 1:3) obtained by non-isothermal autohydrolysis of Eucalyptus globulus wood and corncobs in order to remove non-saccharide components. Garrote et al. (2003) indicated that the autohydrolysis conditions have a strong effect on the yield of ethyl acetate extracts. Higher severities in reaction conditions such as reaction time and reaction temperature resulted in improved yields of ethyl acetate soluble dissolved solids and ethyl acetate soluble phenolics.

Solvent precipitation of liquors has been employed for refining XOS using ethanol, acetone and 2-propanol (Vegas et al., 2004, 2005; Vazquez et al., 2005; Rivas et al., 2006). The recovery yields and the extent of purification is a function of the starting lignocellulose material and type of solvent employed (Vazquez et al., 2005). Presence of water in the solvent extraction system limits the precipitation of hemicellulose-derived degradation products into the solvent phase (Vazquez et al., 2005). Vegas et al (2005) and Vazquez et al., 2005 reported that to minimize the adverse effect of water, solvent extraction of freeze-dried autohydrolysis liquors has to be carried out using the similar kind of solvents employed in the solvent precipitation method. Hence, it is preferable

that freeze-drying of autohydrolysis liquor be carried out prior to solvent extraction of XOS. Ethanol showed the best refining effect in terms of maximum recovery of hemicellulose derived non-volatiles, but resulted in limited solid recovery yields (56.2%) compared to 2-propanol (93.8%) and acetone (89.2%) (Vasquez et al., 2005).

Swennen et al. (2005) studied ethanol precipitation for purification of enzymatically produced arabinoxylan (AX) hydrolysate liquors to obtain mixtures of AX poly- and oligosaccharides. Degree of polymerization (DP) and degree of substitution (DS) of AX decreased with increasing ethanol concentrations, indicating that the solubility of enzymatically degraded AX depends on the DP and, only to a lesser extent, on the DS of these compounds. Swennen et al. (2005) studied ultrafiltration as a possible alternative to ethanol precipitation. More heterogeneous and polydisperse fractions were obtained using ultrafiltration procedures, resulting in less strict separation and more overlap between the different ultrafiltration fractions. Swennen et al. (2005) proposed ultrafiltration as a more economically viable process over solvent extraction method by attributing to cost, handling, and the environmental impact of the solvents as negative factors.

5.3 Membrane Separation

Ultrafiltration using membranes has been employed in the recent studies and was shown to be effective for the purification of crude liquor containing xylo-oligosaccharides (Swennen et al., 2005; Vegas et al., 2006; Akpinar et al., 2007, 2010; Nabarlatz et al., 2007; Egues et al., 2012). In ultrafiltration, the retentate retains higher molecular weight compounds and permeate comprises lower molecular weight compounds.

Akpinar et al. (2007) used 10, 3, and 1 kDa molecular weight cut-off (MWCO) ultrafiltration disc membranes (Millipore-Amicon, Bedford, MA) for the purification of XOS containing liquor obtained by enzymatic hydrolysis of cotton stalk. Using 10 kDa membrane, complete removal of xylanase enzyme and unhydrolyzed xylan was achieved without losing any oligosaccharides (OS) having DP 5 or smaller. After a two-step membrane processing, permeate containing mostly oligosaccharides was obtained. When the membrane of MWCO 1 kDa was used in the second ultrafiltration process, the retention of OS with DP=5 and >5 were higher than when membrane with MWCO 3 kDa was used. Changing the membrane from 3 to 1 kDa MWCO decreased total oligosaccharide recovery, while no noticeable change in the final permeate composition was observed. The retention abilities for DP 5 were higher for 3 and 1 kDa membranes compared to 10 kDa membrane.

Vegas et al. (2006) purified rice hulk autohydrolysis liquors by nanofiltration using a TiO2/ZrO2 Kerasep Nano membrane (Orelis, Miribel, France), with 0.25 m2 and a molecular weight cutoff value of 1 KDa. The membrane can withstand a transmembrane pressure of 20 bar, operating temperatures of up to 100 °C, and pH values in the range 1-14. The nanofiltration step was followed by either ion-exchange or ethyl acetate solvent extraction step inorder to achieve higher purification effects, which were measured by the increase in the xylooligosaccharides proportion as well as by the decrease of the contents of non-saccharide non-volatile compounds and monosaccharides in the refined products. The TiO2/ZrO2 Kerasep Nano membrane allowed purification by the preferential removal of monosaccharides and nonsaccharide compounds from rice hulk crude liquors. Moreover, stronger purification effects were noticed when

nanofiltration of liquors was followed by ethyl acetate solvent extraction before double ion-exchange processing.

Nabarlatz et al. (2007) studied ultrafiltration of crude liquor obtained by autohydrolysis of dry almond shells in a bench scale plant using flat-sheet samples of the G-series thin-film ultrafiltration membranes (Osmonics, USA). Four different membranes with different molecular weight cut-off (MWCO), GE (1.0 kDa), GH (2.5 kDa), GK (3.5 kDa) and GM (8.0 kDa), were used in their study. The trans-membrane differential pressure was varied from 2.6 to 9 bar, and the experiments were performed at a temperature of 25 ± 1°C. Membrane fouling was assessed by measuring water permeability before and after the experiments. Nabarlatz's work demonstrated that the selectivity towards the permeation of lignin related products over oligosaccharide related product is better at low fluxes of permeate and with membranes of low MWCO. While the flux of lignin-related impurities was shown to vary between 10 g/h m2 for the l kDa membrane at 2.7 bar and 264 g/h m2 for the 8 kDa membrane at 8.6 bar, the flux of xylo-oligosaccharides varied between 19 to 579 m2 at the similar conditions. Regarding MWCO, the 1 kDa membrane showed cut-off of 2.75 ± 0.70 kDa, while the 8 kDa membrane's was 10.9 ±0.5 kDa. Moreover the polymeric membranes were found to possess a good resistance to fouling indicated by the water permeability of the used membranes (close to 93% of that of an unused membrane). Nabarlatz's work concludes that it is feasible to design a separation process using membranes with different MWCO to refine xylo-oligosaccharides, and to fractionate them into different molar mass distribution products.

A recent study by Egues et al. (2012) demonstrated the potential of membrane separation technology for the purification as well as fractionation of hemicelluloses in corn waste autohydrolysis liquor. The ceramic membranes (with cutoffs of 1, 5, and 10 kDa; TAMI Industries) used for ultrafiltration are multi-channeled, and consist of seven channels with 2 mm ID, 10 mm external 10 mm, and a surface of 110 cm2. The length of the membranes was 250 mm, while the breaking pressure was about 80 bar. The hemicellulose purification and fractionation was performed by successive filtration of corn waste autohydrolysis liquor by increasing the ceramic membrane cutoff (1, 5, and 10 kDa). The 10 kDa retentate fraction possessed the highest hemicellulosic sugars (mainly arabinoxylan-type polysaccharides) and organic matter content. Moreover, this fraction consisted of defined molecular weight molecules while other fractions (including the liquor) showed a wide range of different molecular weight components. The precipitated samples of 10 kDa retentate fraction had higher hemicellulose content and lower residue and sulfur content than other ultrafiltrated fractions. The lower molecular weight sugars and salts were retained by smaller cutoff (1 and 5 kDa) membranes.

5.4 Adsorption

Purification of XOS containing crude liquors by adsorption method was performed mostly using charcoal/ activated carbon (Sanz et al., 2005; Rivas et al., 2006; Yang et al., 2007; Shen et al., 2013). Adsorption method is usually accompanied by one or more purification methods such as solvent extraction or solvent precipitation (Sanz et al., 2005; Rivas et al., 2006) or chromatography method (Yang et al., 2007) or ion-exchange and membrane concentration method (Shen et al., 2013). Sanz et al., employed activated charcoal for the extraction of oligosaccharides from a honey sample. The

oligosaccharides adsorbed on charcoal were later obtained by solvent (ethanol) extraction and the residual charcoal was separated using filtration. Yang et al. (2007) used activated charcoal combined with chromatography to purify the XOS containing broth obtained by enzymatic treatment of lignocellulolytic agricultural waste (Bagasse, corncob, wheat bran, and peanut shell). The accumulated XOS concentration in the broth was 23.7%. Yang et al., reported that one gram of activated charcoal could adsorb 35.1 mg of XOS. The adsorbed xylose and XOS (on the activated charcoal) was eluted by water and 30% ethanol, respectively. The recovery of XOS was reported to be about 95% and the purity was about 71.4%.

5.5 Ion Exchange Resins

Extraneous components (such as acetic acid, uronic acid, non-saccharides, etc.) present in the lignocellulosic liquors obtained by autohydrolysis can be extracted using ion exchange resins. Vasquez et al., (2007) employed two different ion exchange resins, Amberlite IRA 400 and Amberlite IRA 96, to decrease the content of non-saccharide components present in the liquors obtained by hydrothermal treatment of Eucalyptus globulus wood samples. The highest degree of purification was obtained using Amberlite IRA 400, with final isolate consisting of 92.2 wt. % of saccharides and 7.76 wt. % of non-saccharide components (mainly phenolic compounds). In a separate study conducted by DeMancilha and Karim (2003), seven ion-exchange resins (Table 1) were tested in order to detoxify corn stover hydrolyzate. Purolite A 103 S resin was found to be the most effective resin for purification, with 100% removal efficiency for HMF and acetic acid.

Table 5.1 Evaluation of Ion Exchange Resins for Removal of Inhibitory Compounds

Resin	HMF removal	Furfural removal	Color removal	Xylose removal	Acetic acid removal
Purolite					
A 103 S	100	99	95	94	100
A 860 S	49	97	41	97	0
A 500 P	65	99	74	96	0
C 155 S	37	52	83	92	0
Finex					
CS 11 GC	59	76	84	98	0
CS 13 GC	50	75	79	98	0
CS 14 GC	57	82	75	100	0

Adapted from DeMancilha and Karim (2003). Evaluation of Ion Exchange Resins for Removal of Inhibitory Compounds from Corn Stover Hydrolyzate for Xylitol Fermentation, *Biotechnol Prog.*, **2003,** 19*,* 1837-1841.

Larsson et al. (1999) employed 12 different detoxification methods, including two ion-exchange methods, to improve both cell growth and ethanol production by Baker's yeast, Saccharomyces cerevisiae, using a dilute-acid hydrolyzate of spruce. Other detoxification methods include treatment with alkali (sodium hydroxide or calcium hydroxide), treatment with sulfite (0.1% [w/v] or 1% [w/v] at pH 5.5 or 10), evaporation of 10% or 90% of the initial volume, anion exchange (at pH 5.5 or 10), enzymatic detoxification with the phenoloxidase laccase; and detoxification with the filamentous fungus Trichoderma reesei. The efficacy of the detoxification methods was analyzed by examining the changes in the concentrations of fermentable sugars and three groups of inhibitory compounds—aliphatic acids, furan derivatives, and phenolic compounds; the fermentability of the detoxified hydrolyzate was also assayed. While anion exchange at pH 5.5 or 10, treatment with laccase, treatment with calcium hydroxide, and treatment with T. reesei were the most efficient detoxification methods, evaporation of 10% of the

initial volume and treatment with 0.1% sulfite were the least efficient detoxification methods. Laccase treatment was the only detoxification method that specifically removed phenolic compounds. Anion exchange at pH 10 was the most efficient method in removing all three major groups of inhibitory compounds, but resulted in loss of 26% of fermentable sugars attributing to the hydrophobic interaction with the polystyrenedivinylbenzene based resin matrix.

5.6 Drying/ Evaporation

Vacuum evaporation and low temperature drying methods are used to increase the xylo-oligosaccharides concentration while removing the undesired volatile compounds in the autohydrolysis liquors (Larsson et al., 1999; Moure et al., 2006). In addition, the evaporation stage also corresponds to ease of experimental handling and limited solvent utilization (Rivas et al., 2006). Vacuum evaporation, besides removing water, also aids in the removal of acetic acid and other low molecular weight acid contents of crude liquors. Drying/evaporation technique is not very effective refining method when used alone (Larsson et al., 1999), and therefore employed as an initial step prior to the application of other refining methods such as solvent precipitation, ion-exchange or charcoal adsorption.

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