0273-2289/06/129–132/586–598/$30.00

Enzymatic Production of Xylooligosaccharides From Corn Stover and Corn Cobs Treated With Aqueous Ammonia

YONGMING ZHU,[1] TAE HYUN KIM,[1] Y. Y. LEE,*[,1]
RONGFU CHEN,[2] AND RICHARD T. ELANDER[3]

[1]Department of Chemical Engineering, Auburn University, AL 36849, E-mail: yylee@eng.auburn.edu; [2]SNC Lavalin GDS, Inc. Houston, TX 77096; and [3]National Bioenergy Center, National Renewable Energy Laboratory, Golden, CO 80401

Abstract

A novel method of producing food-grade xylooligosaccharides from corn stover and corn cobs was investigated. The process starts with pretreatment of feedstock in aqueous ammonia, which results delignified and xylan-rich substrate. The pretreated substrates are subjected to enzymatic hydrolysis of xylan using endoxylanase for production of xylooligosaccharides. The conventional enzyme-based method involves extraction of xylan with a strong alkaline solution to form a liquid intermediate containing soluble xylan. This intermediate is heavily contaminated with various extraneous components. A costly purification step is therefore required before enzymatic hydrolysis. In the present method, xylan is obtained in solid form after pretreatment. Water-washing is all that is required for enzymatic hydrolysis of this material. The complex step of purifying soluble xylan from contaminant is essentially eliminated.

Refining of xylooligosaccharides to food-grade is accomplished by charcoal adsorption followed by ethanol elution. Xylanlytic hydrolysis of the pretreated corn stover yielded glucan-rich residue that is easily digestible by cellulase enzyme. The digestibility of the residue reached 86% with enzyme loading of 10 filter paper units/g-glucan. As a feedstock for xylooligosaccharides production, corn cobs are superior to corn stover because of high xylan content and high packing density. The high packing density of corn cobs reduces water input and eventually raises the product concentration.

Index Entries: Corn stover; corn cobs; xylooligosaccharides; xylan; aqueous ammonia; pretreatment; endoxylanase.

Introduction

Xylooligosaccharides (XOS) with a low degree of polymerization (DP) have been proven to promote proliferation of bifidobacteria, beneficial microorganisms in human intestine *(1–3)*. The demand of XOS as a food additive has shown rapid growth over the last two decades *(4,5)*. XOS are

*Author to whom all correspondence and reprint requests should be addressed.

not foreign for human consumption as they exist in natural plants and food substances including bamboo shoots, fruits, vegetables, milk, and honey. XOS can be produced by autohydrolysis of hemicellulose in lignocellulosic biomass *(5–7)*. In this case, relatively mild conditions are applied because XOS can easily be converted to monomer (xylose). The hydrolyzates from those processes contain a variety of undesirable components, such as soluble lignin, lignin- and sugar-degradation products, organic acids, and ash. Extensive downstream purification is, therefore, required *(8)*.

Alternatively, XOS can be produced by enzymatic hydrolysis. Of the three major types of xylanases (endoxylanase, exoxylanase, and xylosidase), endoxylanase and exoxylanase are the ones responsible for production of XOS. Certain natural plant materials can be digested directly by endoxylanase *(9)*, but such feedstock is scarce, not available in large enough quantities for commercial production. In most natural lignocellulosic biomass, xylan exists mainly as xylan–lignin complex *(10–12)*, and becomes resistant to enzyme attack. For this reason, current commercial processes are carried out in two stages: alkaline extraction of xylan from lignocellulosic biomass followed by enzymatic hydrolysis of the dissolved xylan *(2,3)*. Because the alkaline extract is heavily contaminated, a complex purification process must be applied to the crude xylan before the enzymatic conversion. The situation is about the same as for the xylan produced by autohydrolysis. Recently, wood pulp has been investigated as feedstock for enzymatic production of XOS *(13)*. In this process, the washed wood pulp was hydrolyzed with hemicellulase or xylanase to give a XOS-lignin complex, which is further treated with acid or heat to generate XOS.

A pretreatment method based on soaking in aqueous ammonia (SAA) under moderate severity reaction condition for extended period (e.g., 10–15% NH_3, 65–90°C, 12–24 h) has been investigated in our laboratory. This method was found to be very efficient as a pretreatment for corn stover. The unique features of the SAA are that most of the lignin is removed and all of the cellulose and most of the xylan is retained in solid after treatment. A considerable portion of the ash is also dissolved in this treatment. The SAA-treated corn stover is therefore clean, carbohydrate-rich, and amenable for enzymatic digestion. It is well suitable for enzymatic production of XOS. The primary goal of this research is to assess the feasibility of producing low-DP XOS by enzymatic conversion of the SAA-treated corn stover and corn cobs. It was also of our interest to evaluate the reacted glucan-rich residue, a byproduct, as a feedstock for enzymatic saccharification.

Experimental Methods

Materials

Corn stover supplied by NREL was stored at 5°C. The moisture content was 9–14%. Corn cob was a kind gift of Andersons, Inc, Maumee, Ohio. It has a supplier's coding of 2040 WC, which is "milled woody portion

of corn cobs." It has a moisture content of 8–10 %. The composition of 2040 WC as determined by the supplier is: 47.1% cellulose, 37.3% hemicellulose, 6.8% lignin, and 1.2% ash. Endoxylanase, extracted from *Thermomyces lanuginosus*, was purchased from Sigma-Aldrich (cat. no. X2753, lot no. 100K1359). It has manufacturer's nominal activity of approx 2500 xylanase units/g. The cellulase Spezyme CP (Genencor) was supplied by NREL. The cellulolytic activity as determined by NRELwas 30 filter paper units/mL. Activated carbon powder was purchased from Sigma-Aldrich (cat. no. C7606, batch no. 073K0037).

Aqueous Ammonia Treatment

The treatment was conducted in a 250-mL autoclave. Heating and temperature controls were done in a GC oven (Varian Model 3700). Twenty grams of dry corn stover and 200 g of 15% ammonia solution (or in the case of corn cobs, 28 g dry corn cob particles and 75 g of 15% ammonia solution) were placed into autoclave for each treatment. The treated materials were washed with deionized water until the pH became neutral. The washed corn stover and corn cobs were then subjected to composition analysis and used as the substrate for XOS production.

Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted in 250-mL Erlenmeyer flasks, which were placed in a laboratory shaking incubator (150 rpm) with temperature control. Solid substrates (treated or untreated corn stover/corn cobs and α-cellulose) were mixed with citrate buffer to reach a total volume of 100 mL. The substrates and buffer were sterilized at 121°C for 30 min before being placed in the incubator. Enzyme was added after the flask content reached the desired temperature. Addition of cellulase enzyme (Spezyme CP) was done on the basis of the glucan content of the substrate, whereas the addition of endoxylanase enzyme was done on the basis of dry weight of substrate.

Purification of XOS

The hydrolyzate from the xylanolytic hydrolysis was centrifuged at 3800*g* for 10 min. Activated carbon powder was added into supernatant liquid with loadings varying in the range of 1–10% of the liquid weight. The flask was placed in a room-temperature incubator shaker at 200 rpm for 30 min to stabilize the carbohydrate-carbon adsorption. The mixture was suction filtered with a 50-mL Pyrex crucible filter and washed with 4×50 mL distilled and deionized water. The XOS-enriched carbon cake thus obtained was eluted by ethanol twice in succession: 2×50 mL 15% ethanol and 2×50 mL 30% ethanol. Where necessary, the third elution was applied with 50% ethanol. The eluates were concentrated by vacuum rotoevaporation and freeze-dried to recover the product in solid form.

Table 1
Composition of Untreated and SAA-Treated Corn Stover[a] (% [w/w], Dry Basis of Samples) and Recovery of Sugars and Solids After Washing

	Untreated corn stover	Ammonia-treated corn stover	Recovery (%)
Glucan	36.8	54.4	96.1
Xylan	22	24.9	73.6
Galactan	0.68	1	95.6
Arabinan	3.5	3.1	57.6
Mannan	0.7	0.6	55.7
Insoluble lignin	17.2	6.1	23
Acid-soluble lignin	3.2	1.6	32.5
Acetyl	3.2	0	0
Ash	7.5	7.9	68.5
Total	94.8	99.6	65[b]

[a]SAA-treatment conditions: 15% ammonia, L/S = 10, 90°C, 24 h.
[b]Solid remaining.

Analysis

The liquid samples were analyzed for sugars by HPLC operated with Bio-Rad Aminex HPX-87P column and a refractive index detector. The HPLC was operated at 85°C with a flow rate of 0.55 mL/min. Sugar oligomers in the hydrolyzate were determined by the increase of sugar monomers after secondary hydrolysis (4% H_2SO_4, 121°C, 1 h) following the NREL Standard Analytical Procedure No. 014 *(14)*. The sugar, acetyl, and lignin contents in the solids were measured by NREL Standard Analytical Procedure No. 002 *(14)*. The cellulose digestibility of the treated biomass was determined by NREL Standard Analytical Procedure No. 009 *(14)*.

Results and Discussion

Aqueous Ammonia Treatment

The details of the SAA-treatment procedure and the results on corn stover were reported by Kim and Lee *(15)*. Table 1 shows the effect of SAA treatment on the composition of corn stover. It is clearly seen that the lignin content (both acid-soluble and -insoluble lignin) decreased significantly after treatment, whereas the sugars were largely retained (96.1% of glucan and 73.6% of xylan being recovered in treated and washed solids). These data support the idea of using the SAA-treated corn stover as a substrate for enzymatic production of XOS.

Optimal Conditions for Enzyme Activity and Hydrolysis

A series of experiments were carried out to determine the optimal pH and temperature for the enzymatic activity. The SAA-treated corn stover

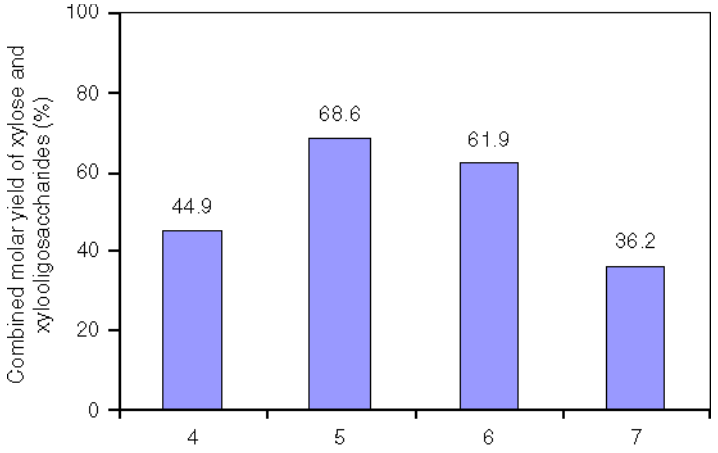

Fig. 1. Effect of pH on the combined molar yield of xylose and XOS from hydrolysis of SAA-treated corn stover at 50°C after 96 h. Enzyme complex: endoxylanase X2753; enzyme loading: 0.04 g/g solids; SAA-treatment conditions: 15% ammonia, 90°C, L/S = 10, 24 h.

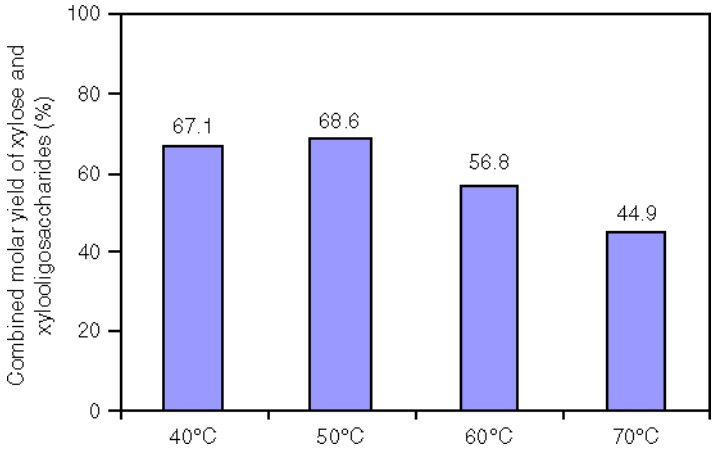

Fig. 2. Effect of temperature on the combined yield of xylose and XOS from hydrolysis of SAA-treated corn stover at pH = 5.0 after 96 h. Enzyme complex: endoxylanase X2753; enzyme loading: 0.04 g/g solids; SAA-treatment conditions: 15% ammonia, 90°C, L/S = 10, 24 h.

used as the substrate. The enzyme activity was expressed as the combined molar yields of xylose and XOS. From the results shown in Figs. 1 and 2, pH 5.0 and 40–50°C were the optimal range applicable for the endoxylanase enzyme (X2753). These findings are in agreement with the optimal ranges reported for endo- and exo-xylanase enzyme complexes *(6,16–18)*.

In order to evaluate the efficiency of the enzyme complex for the digestion of xylan, three enzyme loadings (w/w) were applied at pH 5.0 and 50°C (Fig. 3). The combined molar yield of xylose and XOS increased with the increase of enzyme loading within the range of 0.01–0.16. However, the

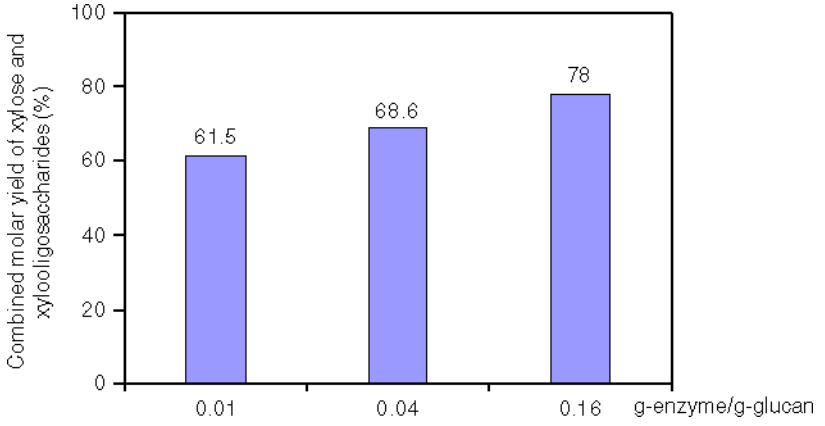

Fig. 3. Effect of enzyme loading on the combine molar yield of xylose and XOS from hydrolysis of SAA-treated corn stover at pH = 5.0 and 50°C after 96 h. Enzyme complex: endoxylanase X2753; SAA-treatment conditions: 15% ammonia, 90°C, L/S = 10, 24 h.

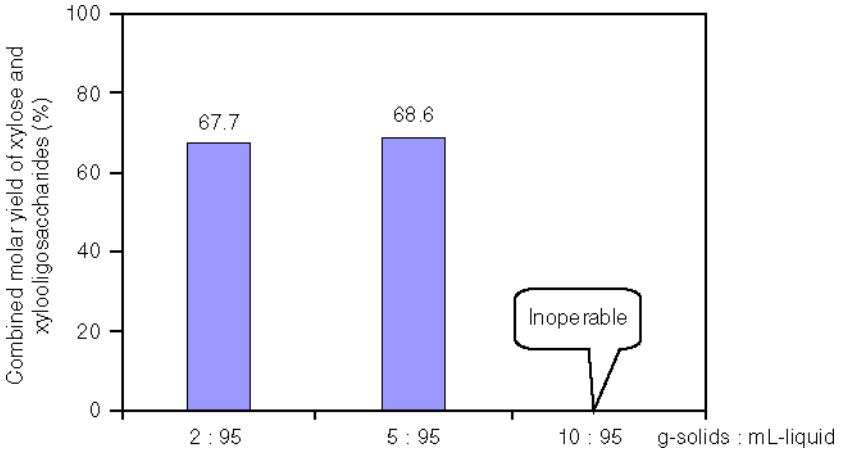

Fig. 4. Effect of substrate loading on the combined molar yield of xylose and XOS from hydrolysis of SAA-treated corn stover at pH = 5.0 and 50°C after 96 h. Enzyme complex: endoxylanase X2753; enzyme loading: 0.04g enzyme/g solids; SAA-treatment conditions: 15% ammonia, 90°C, L/S = 10, 24 h.

efficiency of the enzyme (sugar yield/enzyme loading) decreased with the increase of enzyme loading. Considering the cost of enzyme, we have selected the enzyme/solids ratio of 0.04 (w/w) in subsequent experiments.

Figure 4 presents the effect of substrate concentration on the total molar yield of xylose and XOS. We found no significant change of yield within the solids concentration of 2–5% (w/w). Attempts to further increase the solid concentration to 10% (w/w) failed because of the difficulty of agitating the medium. Obviously the solids concentration appropriate for digestion of SAA-treated corn stover with xylanase is less than 10% (w/w). Usually a fed-batch operation is a solution for this type of

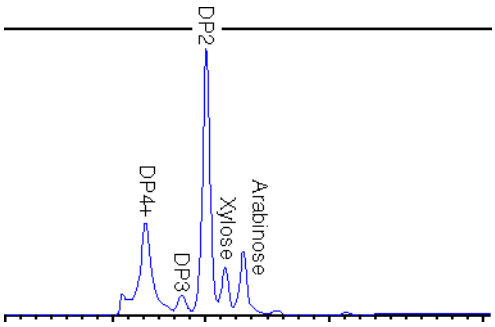

Fig. 5. XOS from enzymatic hydrolysis of SAA-treated corn stover. Hydrolysi conditions: 5% (w/w) solids, pH = 5.0, 50°C, 150 rpm, 72 h. Enzyme complex: Endoxylanase X2753; Enzyme loading: 0.04 g/g-solids; SAA-treatment conditions: 15% ammonia, 90°C, L/S = 10, 24 h.

Table 2
Composition of Xylanlytic Hydrolyzate From SAA-Treated Corn Stover (g/L)

	Monosaccharide	Oligosaccharides	Total
Glucose	–	–	1.488
Xylose	1.147	8.754	9.901
Arabinose	0.078	1.072	1.15

Hydrolysis conditions: 5% (w/w) solids, pH = 5.0, 50°C, and 72 h. Enzyme complex: Endoxylanase X2753; Enzyme loading: 0.04 g/g-solids; SAA-treatment conditions: 15% ammonia, 90°C, L/S = 10, 24 h.

problem. However, it is questionable whether it will work in this case because hydrolysis of xylan does not alter the structure of cellulose fibers, which constitute the framework of the lignocellulosic biomass. As a proof, we noticed that the shape and rigidity of the SAA-treated corn stover solids were not changed significantly after 96 h of digestion. In subsequent experiments, the enzymatic hydrolysis of SAA-treated corn stover for XOS production was conducted under pH = 5.0, 50°C, 0.04 g X2753 endoxylanase/g-solids, solids loading 5% (w/w).

Characterization of XOS

Hydrolysis of xylan in SAA-treated corn stover with endoxylanase generated a liquid product containing XOS with a wide distribution of DP. As shown in the chromatogram of Fig. 5, the hydrolyzate is made mainly of XOS with DP2 and DP4+. Small amount of xylose and arabinose was also detected. It appears that portion of the XOS of DP4+ exists in the form of heteropolysaccharides (i.e., arabinoxylan), as indicated by appearance of sugars other than xylose in the secondary hydrolyzates (Table 2). Nonetheless, no negative effects of these heteropolysaccharides were reported pertaining to their use as food additives.

Table 3
Xylan Digestibility of Untreated and SAA-Treated Corn Stover

	Untreated corn stover	SAA-treated corn stover
Xylan digestibility (%)	14	66.1

Note: Xylan digestibility is expressed as the formation of xylose and XOS. Hydrolysis conditions: 5% (w/w) solids, pH = 5.0, 50°C, and 72 h. Enzyme complex: Endoxylanase X2753; Enzyme loading: 0.04 g/g-solids; SAA-treatment conditions: 15% ammonia, 90°C, L/S = 10, 24 h.

Improvement of Xylan Digestibility by SAA Treatment

The positive effect of delignification on the cellulose digestibility is well known. However, very little study, if any, was focused on the influence of delignification on xylan digestibility. This is primarily because few of the previous pretreatment methods lead to the concurrence of extensive lignin removal and large xylan retention. In other words, the selectivity toward delignification was usually very low in most pretreatment methods. However, the SAA pretreatment provided a unique substrate for this study. As shown in Table 1, when the corn stover was treated with 15% ammonia at 90°C for 24 h, the Klason lignin was reduced from 17.2% to 8.2%, whereas the percentage of xylan increased slightly from 22% to 24.96%. The increase in percent xylan is owing to the weight decrease after pretreatment.

Table 3 compares the digestibilities of corn stover xylan with and without delignification. The SAA treatment increased the xylan digestibility from 14% to 66.1%. This improvement is in line with the increase of cellulose digestibility on delignification. However, the fates of cellulose and xylan in the SAA treatment are different. In lignocellulosic substrates, cellulose exists as homopolymers, which are surrounded by a complex of lignin and hemicellulose. The removal of lignin facilitates the transport of cellulase enzyme to the cellulose surface, but leaves the cellulose chains intact. However, in the ammonia treatment, some of the hemicellulose-lignin linkages are disrupted, and some of the side chains attached to the xylose backbone are removed. Ammonia treatment brings about significant changes to the hemicellulose structure. Despite these differences, the increased substrate accessibility by enzymes remains as the common factor responsible for increased digestibility in both cases.

Refining and Fractionation of XOS

Hydrolysis of SAA-pretreated corn stover with endoxylanase produces hydrolyzates made mainly of XOS (Table 2). It is necessary to remove the impurities such as soluble lignin and ash in the hydrolyzate to bring it to food-grade. For this purpose, the XOS was treated with charcoal (carbon) adsorption and ethanol elution. The carbon adsorption takes advantage of the different interactions between the XOS and carbon *(19)*. The higher the

Table 4
Fates of XOS in Carbon-Ethanol Refining[a]

Carbon : Hydrolyzate (w/w)	0.01	0.05	0.1
XOS loss by water washing	Significant	Moderate	Small
XOS yield from 15% ethanol elution	5.9%	21.3%	34.5%
XOS yield from 30% ethanol elution	2.5%	15.6%	15.9%
XOS yield from 50% ethanol elution	Not done	Not done	4.4%
Total XOS yield from ethanol elution	8.4%	36.9%	54.8%

[a]Yields are molar yields on the basis of xylan in SAA-treated corn stover.

DP the stronger the adsorption to charcoal. For example, xylose (DP1) has little interaction with carbon showing no adsorption on it. It is therefore removed from the XOS-carbon complex by water washing. Small amount of XOS are also lost in water-washing stage before the ethanol elution, the actual amount depending on DP and carbon loading. As shown in Table 4, significant loss of XOS occurs with carbon-to-hydrolyzate ratio of 0.01 (w/w). Increasing the carbon to hydrolyzate ratio to 0.05 (w/w) markedly reduced the XOS loss thus increasing the yield of it. Further increase of the carbon-to-hydrolyzate ratio to 0.1 (w/w) essentially eliminated XOS loss as evidenced by negligible amount of XOS being detected in the water eluate. Most of the XOS adsorbed on charcoal were recovered with 15% ethanol elution. The remaining XOS were recovered by 30% ethanol elution. Although very low in quantity, the products recovered from the 50% ethanol elution included only high-DP XOS. In all practical sense, 30% ethanol elution is sufficient for recovery of the XOS of interest. Knowing that the xylan digestibility is 66.1% (Table 3), the recovery of XOS from the solubilized xylan is estimated to be more than 70% when 30% ethanol elution is applied with carbon-to-hydrolyzate ratio 0.1 (w/w). These data also indicate that successive elution with different ethanol levels may be used as a tool, if needed, to further fractionate the XOS into products of different DP. The final XOS retained pure-white color after freeze-drying.

Glucan Digestibility After Xylanase Treatment

Corn stover, after the SAA treatment, turned into a carbohydrate-rich product. Subsequent hydrolysis of the SAA-treated corn stover by xylanase generated a glucan-rich substrate. As shown in Table 5, the glucan content in the corn stover rose to 69.6% after hydrolysis by endoxylanase, which is almost twice that of untreated corn stover. The residue remaining after XOS production is a byproduct suitable for further bioconversion. We have measured the enzymatic digestibility of this substrate (Fig. 6). For the digestibility test, the moisture of the treated solids was reduced (to 79.2%) by squeezing. The solids, however, were not sterilized so that endoxylanase enzyme remains active along with the cellulase enzyme. It is conceivable that the combined action of cellulase and xylanase may not deteriorate the

Table 5
Composition of SAA-Treated Corn Stover After Xylanolytic Hydrolysis (% [w/w], Dry Basis of Sample)

	SAA-treated corn cobs
Glucan	69.6
Xylan	11.9
Arabinan	1.4
Mannan	0.7
Acid-insoluble lignin	6.4
Acid-soluble lignin	1.3
Acetyl	0

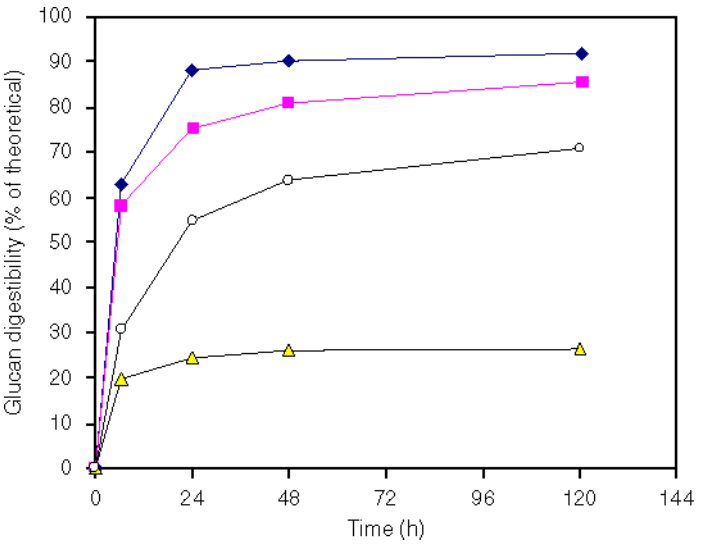

Fig. 6. Glucan digestibility of untreated, SAA-treated and SAA-treated xylanase-digested corn stover and α-cellulose. All the substrates except the xylanase-digested SAA-treated corn stover were sterilized at 121°C for 30 min before enzyme addition. Digestibility test conditions: 1 wt% glucan loading, 10 FPU/g-glucan, pH = 4.8, 50°C, 150 rpm. ▲, untreated corn stover; ♦, SAA-treated corn stover; ■, SAA-treated xylanase-digested corn stover; ○, α-cellulose.

digestibility of glucan. As shown by the curves in Fig. 6, the glucan digestibility of the SAA-treated corn stover maintained at a considerably high level (86% after 120 h) after xylan removal by endoxylanase. This means that the xylanase-treated corn stover can continue to serve as an excellent substrate for further saccharification.

XOS Production From Corn Cobs

Corn cobs were also evaluated as a substrate for XOS production. The procedures were almost identical to those of corn stover; treated with aqueous ammonia and hydrolyzed by endoxylanase. The SAA-treatment conditions

Table 6
Composition of SAA-Treated Corn Cobs[a]

	Dry basis (%)
Glucan	50.4
Xylan	37
Galactan	1.2
Arabinan	3.9
Total sugars	92.5
Acetyl	0
Acid-insoluble lignin	3.6
Acid-soluble lignin	2
Ash	1
Total components	99.1

[a]SAA-treatment conditions: 15% aqueous ammonia, L/S = 2.8, 60°C, 48 h.

Table 7
XOS Production Using Untreated and SAA-Treated Corn Cobs

	Untreated	Treated
Concentration in hydrolyzate (g/L)	4.11	15.74
Molar yield (% of xylan)	21.1	80.5

Note: Xylanlytic hydrolysis conditions: 5% solids loading, 0.04 g-xylanase/g-solids, pH = 5.0, 50°C, 150 rpm, 72 h. SAA-treatment conditions: 15% aqueous ammonia, L/S = 2.8, 60°C, 48 h.

were 15% ammonia, 60°C, L/S 2.8, and 48 h. One noticeable difference was that a significantly lower liquid-to-solid ratio was applied for the corn cobs (only 2.8) because it has a much higher bulk density. High solid loading is beneficial in that it lowers energy input as well as wastewater.

SAA treatment of corn cobs yielded a superior substrate for saccharification. As shown in Table 6, the total sugar content reached 92.5%, the total lignin level went down to 5.6%, and the ash content was reduced to 1%. The following hydrolysis of the treated and washed corn cobs using endoxylanase gave a fairly clear hydrolyzate. Table 7 shows the concentrations and yields of XOS in the hydrolyzate for both untreated and SAA-treated corn cobs. The yield increased by a factor of four after SAA treatment and the XOS concentration reached 1.57%. The SAA-treated corn cobs can be loaded more densely into the hydrolysis reactor than corn stover (Table 8). The XOS concentration rose to 4.7% with 15% solids loading. The product inhibition by XOS to the xylanase appears to be insignificant as the XOS yield decreased slightly with increase of solid loading from 5% to 15%. The HPLC chromatogram of XOS from the hydrolysis of SAA-treated corn cobs is very similar to that from the SAA-treated corn stover (data not shown). Xylose accounted for approx 10% (w/w) of the hydrolyzed xylan, the rest being XOS.

Table 8
Enzymatic Production of XOS From SAA-Treated Corn Cobs Using Different Solids Loadings

Solids loading	5%	10%	15%
Concentration in liquor (g/L)	15.74	30.02	47.18
Molar yield (%)	80.5	72.7	71.8

Note: Enzymatic digestion conditions: 0.04 g-xylanase/g-solids, pH = 5.0, 50°C, 150 rpm, 72 h. SAA-treatment conditions: 15% aqueous ammonia, L/S = 2.8, 60°C, 48 h.

Conclusions

SAA treatment of corn stover and corn cobs resulted in delignified and xylan-enriched substrates. They are highly susceptible to enzymatic hydrolysis by endoxylanase. The hydrolyzates contained predominantly XOS with a small amount of xylose and other components. Refining of the XOS can be accomplished by carbon adsorption of oligomers followed by ethanol elution. Under optimal-treatment conditions, xylose was totally removed and the XOS were obtained in good yield. Removal of xylan from the SAA-treated corn stover caused a slight decrease in the digestibility of the remaining glucan, but still retained a level above 85% with 10 FPU/g-glucan. For XOS production, corn cobs are a more suitable feedstock than corn stover because corn cobs possess higher xylan content and greater bulk density.

Acknowledgments

The financial support for this research was provided by US Environmental Protection Agency through a grant (Technology for a Sustaining Environment-Grant No. EPA-RD-83164501). A cost-share was provided to this project by Auburn University.

References

1 Modler, H. W. (1994), *Int. Dairy J.* **4,** 383–407.
2 Suwa, Y., Koga, K., Fujikawa, S., Okazaki, M., Irie, T., and Nakada, T. (1999), US 5939309.
3 Yu, S., Yong, Q., and Xu, Y. (2002), *Faming Zhuanli Shengqing Gongkai Shuomingshu*, CN 1364911 A, 7.
4 Crittenden, R. G. and Playne, M. J. (1996), *Trends Food Sci. Technol.* **7,** 353–361.
5 Vazquez, M. J., Alonso, J. L., Dongminguez, H., and Parajo, J. C. (2000), *Trends Food Sci.Technol.* **11,** 387–393.
6 Vazquez, M. J., Alonso, J. L., Dongminguez, H., and Parajo, J. C. (2001), *World J. Microbiol. Biotechnol.* **17,** 817–822.
7 Kabel, M. A., Carvalheiro, F., Garrote, G., et al. (2002), *Carbohydrate Polymers* **50,** 47–56.
8 Carvalheiro, F., Esteves, M. P., Parajo, J. C., Pereira, H., and Girio, F. M. (2003), *Bioresource Technol.* **91,** 93–100.
9 Takao, Y. and Yoshio, I. (1996), *Japanese Patent JP* 5194241.
10 Watanabe, T., Karina, M., Sudiyani, Y., Koshijima, T., and Kuwahara, M., (1993), *Wood Res.* **79,** 13–22.

11 Gubitz, G. M., Csom, G., Johansson, C. I., and Saddler, J. N. (1998), *International Conference on Biotechnology in the Pulp and Paper Industry, 7th, Vancouver, B. C., June 16–19, CC167-C170*. Canadian Pulp and Paper Association, Technical Section, Montreal, Que.
12 Yang, R., Xu, S., and Wang, Z. (2001), *Zhongguo Liangyou Xuebao* **16(6),** 43–46.
13 Izumi, Y., Sugiura, J., Kagawa, H., and Azumi, N. (2002), *US patent application* 2002195213 A1, 16 pp.
14 Laboratory Analytical Procedures (2004), NREL, Golden, CO.
15 Kim, T. H. and Lee, Y. Y. (2004), *App. Biochem. Biotechnol.* **121–124,** 1119–1131.
16 Frederick, M. M., Frederick, J. R., Fratzke, A. R., and Reilly, P. J. (1981), *Carbohydrate Res.* **97(1),** 87–103.
17 Cai, J. M., Wu, K., Pan, R. R., et al. (2000), *Chinese patent* CN 1266633.
18 Mao, L. -S., Xu, Y., Song, X. -Y., Yong, Q.,Yu, and S. -Y. (2001), *Linchan Huaxue Yu Gongye* **21(4),** 33–38.
19 Pellerin, P., Gosselin, M., Lepoutre, J. -P., Samain, E., and Debeire, P. (1991), *Enzyme Microb. Technol.* **13,** 617–621.