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Separation of Glucose and Pentose Sugars by Selective Enzyme Hydrolysis of AFEX-Treated Corn Fiber

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Abstract

A process was developed to fractionate corn fiber into glucose- and pentose-rich fractions. Corn fiber was ammonia fiber explosion treated at 90°C, using 1 g anhydrous ammonia per gram of dry biomass, 60% moisture, and 30-min residence time. Twenty four hour hydrolysis of ammonia fiber explosion-treated corn fiber with cellulase converted 83% of available glucan-to-glucose. In this hydrolysis the hemicellulose was partially broken down with 81% of the xylan and 68% of the arabinan being contained in the hydrolysate after filtration to remove lignin and other insoluble material. Addition of ethanol was used to precipitate and recover the solubilized hemicellulose from the hydrolysate, followed by hydrolysis with 2% (v/v) sulfuric acid to convert the recovered xylan and arabinan to monomeric sugars. Using this method, 57% of xylose and 54% of arabinose available in corn fiber were recovered in a pentose-rich stream. The carbohydrate composition of the pentose-enriched stream was 5% glucose, 57% xylose, 27% arabinose, and 11% galactose. The carbohydrate composition of the glucose-enriched stream was 87% glucose, 5% xylose, 6% arabinose, and 1% galactose, and contained 83% of glucose available from the corn fiber.

Index Entries: Ammonia fiber explosion; arabinose; lignocellulose; sugar separation; xylose; glucose.

Introduction

According to the Renewable Fuels Association (2006), US ethanol capacity was 4.3×10^9 US gal in 2005, an increase of 1.4×10^9 US gall over the production level in 2002. The demand for and production of ethanol is expected to dramatically increase over the next decade to reduce US dependency on foreign oil. The majority of current ethanol processes use glucose derived from corn-starch as the primary feedstock; however, this will not be able to supply all the ethanol needs. An abundant natural and

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renewable resource such as lignocellulosic biomass must be utilized to supply our long-term needs for ethanol as a renewable bio-based fuel. In addition to ethanol, forty chemicals and chemical feedstocks have been identified as potential products from renewable plant biomass *(1–4)*.

Lignocellulosic biomass consists of three major components: cellulose, hemicellulose, and lignin. The complex structure of lignocellulosic biomass, the crystalline structure of cellulose, and the physical protection provided by hemicellulose and lignin, prevent efficient hydrolysis and subsequent release of fermentable sugars by hydrolytic enzymes. Therefore, pretreatment is required to alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars.

A number of pretreatment technologies have been proposed and investigated *(5)*. Among these, the ammonia fiber explosion (AFEX) is one of the most effective pretreatments. AFEX disrupts cell wall physical barriers as well as cellulose crystallinity and association with lignin and hemicellulose so that the hydrolytic enzymes can access the biomass macrostructure *(6,7)*. The AFEX process is one of the most environmentally friendly pretreatment processes with several distinguishing features. These features are:

1. Energy requirements are low and reaction temperatures are mild.
2. Ammonia is an abundant and widely available chemical, which can be contained and recycled with residual ammonia serving as a nitrogen source for subsequent fermentation or animal feeding operations.
3. Minimal waste is generated as less than 2% of the ammonia used in the treatment is retained by biomass, therefore, no neutralizing waste or wash streams are created in the process *(6,8,9)*.
4. Dry matter recovery following the AFEX treatment is essentially 100% and is stable for long periods and can be fed at high-solids loadings in fermentation processes *(10)*.
5. Cellulose and hemicellulose are well preserved in the AFEX process, with little or no degradation *(11)*.
6. There is no formation of furfural or furfural derivatives, which are known to have inhibitory effects on the metabolism of the yeast *Saccharomyces cerevisiae* in production of ethanol *(12,13)*.

It has been shown that AFEX treatment of biomass, such as corn stover, corn fiber, and switchgrass followed by enzymatic hydrolysis yields a stream of fermentable sugars containing both C5 sugars (arabinose and xylose) and C6 sugars (glucose), which can be used for production of industrial products such as ethanol and succinic acid *(6,8,14,15)*. However, the individual sugars could be used to produce more highly valued products if the C5 and C6 sugars could be separated from each other and from the lignin. For example, xylose could be

reduced to xylitol, a sweetener that prevents dental caries; arabinose to pharmaceutical intermediates; and glucose could be routed into existing starch-based processes, such as high-fructose corn syrup. An overall process that incorporates the separation of the C5 and C6 streams, would realize increased economic return plus operational and market flexibility.

Corn fiber was selected as the lignocellulosic biomass for this study. The current availability of corn fiber in concentrated amounts at wet mills allows us to model its possibilities as a biomass source without speculating on the gathering and storage costs associated with most other biomass sources. In addition, a limiting factor in the cost effectiveness of current ethanol production processes is the value obtained from the byproducts. The profitability of ethanol production is expected to be enhanced by applying a biorefinery concept and by modifying existing plants to produce value added products *(16)*. A biorefinery, in principle, will process most, if not all, of the incoming feedstocks and byproducts into valuable products while at the same time producing ethanol. Corn fiber is a high cellulose/hemicellulose lignocellulosic biomass with a low market value currently produced in large quantities at corn wet mills and is included in distiller's grains at dry mill ethanol plants. The fractionation of corn fiber into a higher value component would improve the cost effectiveness of current ethanol production processes *(8)*.

Previous studies *(11,15)* have shown the applicability of AFEX to corn fiber. AFEX-treated corn fiber showed significant improvement of glucose production during enzyme hydrolysis relative to untreated material and the generated sugars were readily fermented to ethanol. Unlike cellulose, only a small fraction of the xylan in the AFEX-treated corn fiber (4–6%) was converted to xylose and more than 81% of that was solubilized in the hydrolysate as oligomers during enzyme hydrolysis. Corn fiber xylan is one of the most substituted and complex xylans and is thus highly recalcitrant to enzyme hydrolysis. Available commercial cellulases and xylanases are not able to convert corn fiber xylan into xylose efficiently. In order to have an economically attractive process, all the available polymeric sugars must be utilized. The main objective of this study was to develop a process for maximum hydrolyzation of all the available sugars in corn fiber and to separate C5 and C6 sugars from each other.

Materials and Methods

Material: Corn fiber with 5% moisture content was provided by Bunge Milling (Danville, IL). The composition is listed in Table 1.

Cellulase: Spezyme CP (Genencor, Rochester, NY) lot No. 301-01320-216.

β-glucosidase, Novozyme 188 (Sigma, St. Louis, MO) batch No. DCN00206.

Glucoamylase: Glucostar L-400 (Dyadic International Inc., Jupiter, FL) lot No. P01311.

Table 1
Corn Fiber Composition (based on dry weight)

Starch (%)	Cellulose (%)	Xylan (%)	Arabinan (%)	Galactan (%)	Mannan (%)	Lignin (%)
10.61	19.71	28.5	13.7	3.8	0.39	12.41

Xylanase: X1-274, X2-275, X3-276, and X4-277 (Dyadic International Inc.), NS50014 and NS50030 (Novozyme, Franklinton, NC).

Feed enzyme: Rovabio Excel LC (Adisseo, Alpharetta, GA) lot No. B-04154-01.

Anhydrous ammonia: Linde Gas LLC (Lansing, MI). All other chemicals were purchased from Sigma.

Analytical Methods

Composition (glucan, xylan, galactan, arabinan, lignin, and ash) of the biomass was determined by following the National Renewable Energy Laboratory (NREL) procedure LAP-002: determination of carbohydrates in biomass by high-performance liquid chromatography (HPLC) (NREL LAP-002). NREL procedures are available at http://www.ott.doe.gov/biofuels/analytical_methods.html. The starch analyses were performed by Servi-tech Laboratories (Hastings, NE). Total sugars in the liquid fractions were determined by following the NREL LAP-014: dilute acid hydrolysis procedure for determination of total sugar in the liquid fraction of process samples.

AFEX Treatment

Corn fiber (500 g) was treated by the AFEX process in a one-gallon pressure reactor (PARR Instrument Co, Moline, IL). The AFEX process parameters were varied, biomass moisture content (20, 40, 60, and 70%), reaction time (10, 15, and 30 min), temperature (80, 90, 100, and 110°C), and ammonia loading (0.7 : 1 and 1 : 1, g ammonia : g dry biomass) to determine conditions that gave the highest glucose yield. Biomass with the desired moisture content level was added to the reactor. To ensure uniform distribution of heat and ammonia, the reactor was equipped with an agitator that mixed the biomass at 100 rpm during the process. The reactor was heated by an electrical heating mantel to approx 10°C lower than the target temperature before addition of the liquid ammonia. While the reactor was heating, the desired amount of ammonia was pumped to the reactor. The reaction timer was started when the reactor was within 5°C of the set-point. Temperature and pressure of the process was recorded every 3 min. When the desired reaction time (30 min) was complete, ammonia was quickly evacuated through a manual ball

valve. The treated biomass was removed from the reactor and left in a fume hood for 1 d to evaporate the residual ammonia. The treated biomass was stored at 4°C until used. All AFEX runs were performed in duplicate and the data for the conditions selected are presented as the average of the duplicates.

Small-Scale Enzyme Hydrolysis

The efficiency of AFEX treatment was evaluated through small-scale enzyme hydrolysis. The hydrolysis was performed in shake flask following NREL's LAP-009: enzymatic saccharification of lignocellulosic biomass procedure with the following modifications. Hydrolyses were performed with 5% solid loading and a mixture of 15 filter paper units of Spezyme CP and 42 cellobiose units of NOVO 188 per gram of cellulose and 0.0032 g of Glucostar L-400/g starch. The hydrolyses were carried out at 50°C and pH 4.8 for 72 h. Samples were taken at 0, 24, 48, and 72 h for sugar analysis.

Large-Scale Enzyme Hydrolysis

The 10 L enzyme hydrolyses were carried out in a New Brunswick Scientific Microferm 14-L fermentor (New Brunswick Scientific, Edison, NJ) equipped with a mechanical stirrer. There were three impellers on the agitation drive shaft, from top to the bottom, two six-blade Rushton impellers 3 in. apart and one lightnin A310 impeller at the bottom. The hydrolyses were mixed at 250 rpm. The hydrolysis used a 10% solids loading of AFEX-treated corn fiber. Fifteen filter paper units of Spezyme CP/g cellulose, 150 U of β-glucosidase/g of cellulose and 0.12 g of Glucostar (glucoamylase)/g of starch were added to the hydrolysis. To minimize potential contamination, all equipment and materials except the biomass were sterilized. Hydrolysis was performed at 50°C and 4.8 pH (active pH control) for 24 h with mixing. Samples were taken for sugar analysis at several time-points. At 24 h, the hydrolysate was removed from the fermentor and centrifuged for 20 min at 5°C at 7500 rpm (9500*g*) to separate the liquid and the solid residuals. Samples were taken from both supernatant and pellet portions for sugar and composition analysis.

Ethanol Precipitation

The supernatant from the enzyme hydrolysis was slowly added to 3X (v : v) cold ethanol (200 proof) to precipitate the solubilized polymeric sugars (mainly hemicellulose). The generated pellet was dried at 45°C in a vacuum oven and was analyzed for carbohydrate composition. The pellet was dissolved in water at 15% solid loading and then concentrated H_2SO_4 was added to a final concentration of 2% (v/v). The hydrolysis was carried out at 80°C for 24 h. The hydrolysate was analyzed for sugar content by HPLC. In this procedure, all liquid samples

were analyzed for both monomeric (HPLC) and polymeric (NREL-LAP-014) sugars content. All hydrolyses were performed in duplicate and the data were reported as average. The final hydrolysate was maintained at 4°C until used.

Evaluation of Acid Hydrolysis

Enzyme hydrolysate from corn fiber was prepared as described for the large-scale enzyme hydrolysis above. To a 500-mL flask 100 mL of the enzyme hydrolysate, without filtration, was transferred. Concentrated sulfuric acid was added to achieve the desired acid concentration (2, 1, and 0.5%). With stirring, the reaction was maintained at the appropriate temperature (80 and 100°C). The hydrolysate was periodically sampled and the saccharide concentrations determined following the NREL LAP-014 procedure.

Results and Discussion

Corn fiber treated under different AFEX conditions was enzyme hydrolyzed (small-scale) to identify the most effective set of AFEX conditions for treatment of corn fiber. The 72 h hydrolysis results showed that corn fiber treated at 90°C, with 60% moisture content for 30 min, with 1 g ammonia per gram of dry biomass gave the highest glucose yield (91 $\pm$ 2% based on the available glucose).

The process developed to fractionate AFEX-treated corn fiber to glucose and pentose-rich fractions is shown in Fig. 1. AFEX-treated corn fiber is first hydrolyzed with a cellulase enzyme, converting cellulose to glucose and solubilizing the xylan and arabinan-containing hemicellulose; presumably by partially hydrolyzing it to arabinoxylan oligomers. The insoluble lignins are removed by filtration. The solubilized hemicellulose is precipitated, by addition of ethanol, and collected yielding a glucose-enriched aqueous solution and a filter cake containing the arabinoxylan oligomers. Acid hydrolysis of this filter cake corn fiber gum (CFG) generates the monomeric arabinose and xylose.

Effect of Enzyme Hydrolysis Time on Hemicellulose Recovery

A series of experiments in small-scale (250-mL shake flask) were performed to determine the effect of enzyme hydrolysis time on the recovery of C5 oligomers by ethanol precipitation. Enzyme hydrolysis times of 16, 24, and 48 h were tested with the CFG being precipitated immediately following the hydrolysis. The best yield was obtained from the 24 h hydrolysis where 74% of the xylose and 76% of the arabinose contained in the original AFEX-treated corn fiber was recovered in the CFG. However, statistical analysis showed that the yields from the 16-h and 48-h hydrolyses were not significantly different. The 24-h enzyme hydrolysis was selected for our larger scale (14 L) experiments.

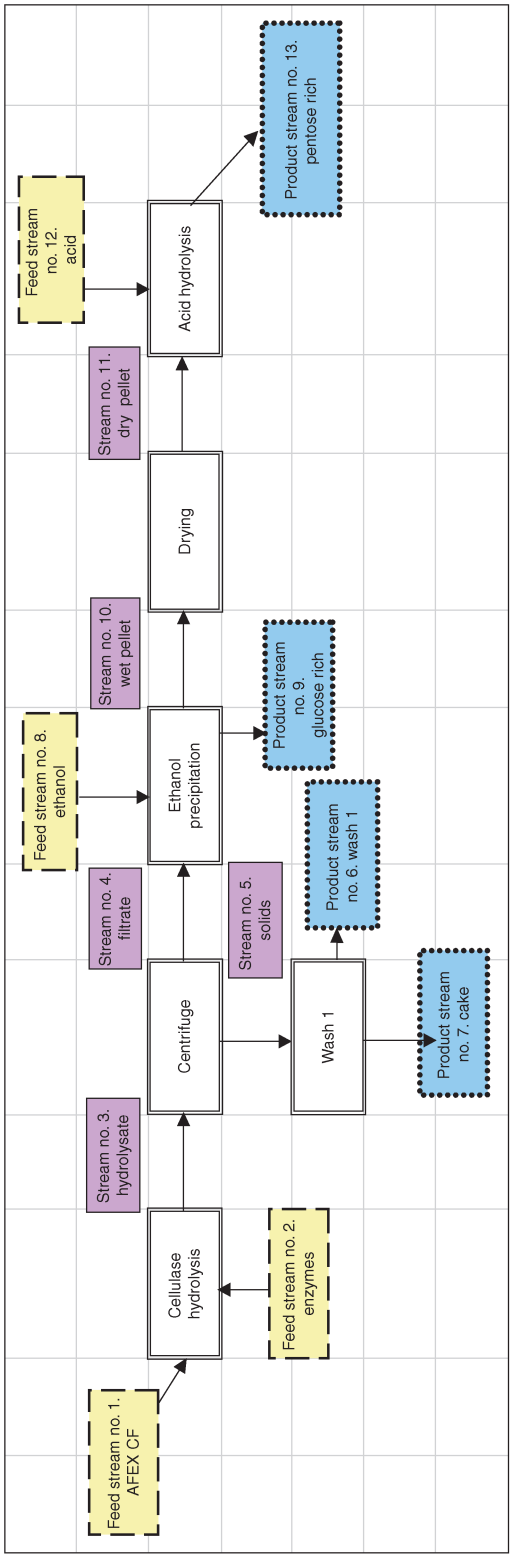

Fig. 1. Process flow diagram for separation of arabino/xylan and glucose from AFEX-treated corn fiber.

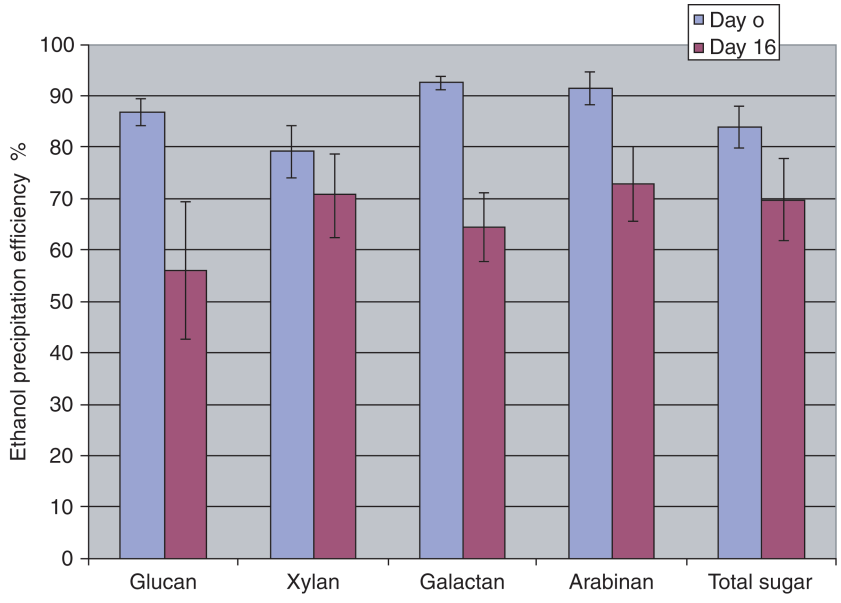

Fig. 2. Efficiency of ethanol precipitation of solubilized polysaccharides in enzyme hydrolysate of AFEX-treated corn fiber. Ethanol precipitation was performed immediately after finishing the enzyme hydrolysis (day 0) or after the hydrolysate had been stored at 4°C for 16 d (day 16). (Efficiency of ethanol precipitation was calculated by dividing the amount of sugar recovered in pellet by the total polysaccharides available in the hydrolysate.)

We also investigated the effect that extended storage time of the enzyme hydrolysate had on the efficiency of CFG precipitation. The ethanol precipitation of the arabinoxylan pellet was performed immediately after completing the enzyme hydrolysis (day 0) and after the hydrolysate had been stored at 4°C for 16 d (day 16). After drying, the weight and sugar composition of each pellet was determined. Results of these experiments are presented in Fig. 2. There was a reduction in yield of all the sugars after 16 d and the overall recovery dropped from an average of 83% to an average of 70% (decrease from 4.0 g sugar/10.0 g biomass on day 0, to 3.2 g sugar/10.0 g on day 16). As no attempt was made to denature the enzyme at the end of the hydrolysis, the loss of sugar yield in the precipitation is presumably because of continued enzyme hydrolysis when the hydrolysate is being stored, even though it was maintained at 4°C. This conclusion is also consistent with the mechanism by which the precipitation works; polysaccharides are precipitated, whereas monosaccharides and presumably low molecular weight oligomers are maintained in solution. Extended hydrolysis time should give a higher percent of monosaccharides in the hydrolysate. Based on these observations, it was decided to perform the ethanol precipitation stage within 24 h following completion of the enzyme hydrolysis.

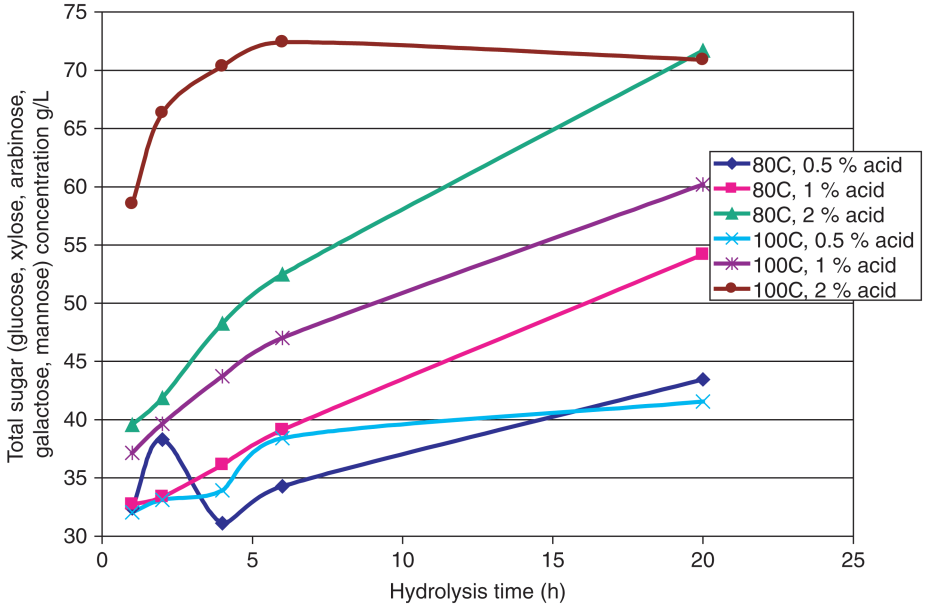

Fig. 3. Total sugar (glucose, xylose, arabinose, galactose, and mannose) concentration produced from CFG with different acid hydrolysis conditions.

Optimization of Acid Hydrolysis

To identify the acid hydrolysis conditions that gave the maximum conversion of the enzyme solubilized polysaccharides from corn fiber to monomeric sugars; sulfuric acid concentrations of 0.5, 1, and 2% (v/v) were evaluated at 80 and 100°C. These experiments were performed by adding concentrated sulfuric acid to the unfiltered enzyme hydrolysate. The results from these experiments are summarized in Fig. 3. Data showed that among the tested conditions the maximum sugar conversion was obtained with 2% (v/v) acid, at 100°C for 6 h or 80°C for 20 h.

We also investigated the use of 0.5 and 1% sulfuric acid over a longer period of time to explore the possibility of using lower acid concentration. A temperature of 80°C using 1% acid gave 71% yield after 285 h, and 0.5% acid gave 55% yield after 285 h. Even though the hydrolysis rate was higher at 100°C compared with 80°C, because of equipment limitations (difficult to maintain the 14-L fermentors at 100°C for 6 h) hydrolysis with 2% sulfuric acid at 80°C for 20 h was chosen for acid hydrolysis of CFG.

Enzyme Hydrolysis Followed by Ethanol Precipitation of Solubilized Hemicellulose

Three 10 L hydrolyses of AFEX-treated corn fiber showed that in 24 h 83% of available glucan was converted to glucose. The hemicellulose fraction was partially broken down, with 81% of the xylan and 68% of the arabinan

Table 2
Carbohydrate Composition of Generated CFG (based on dry weight)

Glucose (%)	Xylose (%)	Galactose (%)	Arabinose (%)	Mannose (%)
3.40	43.83	7.53	20.60	0

being contained in the hydrolysate after filtration to remove lignin and other insoluble material. An additional 5% of the xylan and 4% of the arabinan were recovered in a water-wash of the filter cake but this material was not added back to the hydrolysate. The xylan and arabinan contained in the hydrolysate was precipitated and collected as CFG by addition of the hydrolysate to three volumes of ethanol. The efficiency of the ethanol precipitation for xylan and for arabinan was 85 and 92.3%, respectively (calculated by dividing the amount of sugar recovered in pellet by the total polysaccharides available in the hydrolysate). This indicates that some of the polysaccharides, probably low-molecular weight oligomers, are not precipitated by the addition of the ethanol. The collected CFG was washed with de-ionized water and dried before acid hydrolysis. The carbohydrate composition of the obtained CFG is presented in Table 2.

Acid hydrolysis (2% H_2SO_4 at 80°C for 20 h) of CFG gave the C5-enriched sugar stream. The acid hydrolysis proceeded with 83% yield for the xylose and 86% yield for the arabinose from available oligomers in CFG. The final carbohydrate composition of the C5-enriched sugar stream was 5% glucose, 57.1% xylose, 26.9% arabinose, and 11% galactose, or 84% C5 sugars, and 16% C6 sugars.

Yield for Overall Process (Enzyme Hydrolysis, Ethanol Precipitation, and Acid Hydrolysis of CFG)

For the overall process, we were able to recover 83.2% of available glucose in the C6-rich stream; the C5-rich stream contained 57.1% of the available xylose, 54.1% of the available arabinose, and 58% of the available galactose. The sugar compositions of the product streams are summarized in Table 3 and the overall sugar recovery is provided in Table 4. Analyses of the waste streams from the process were consistent with losses observed in each step, giving a good overall mass balance. The mass balance was 111% for glucose, 94% for xylose, 105% for arabinose, and 90% for galactose (mass balance higher than 100 may be because of analytical error).

Enzyme Hydrolysis of CFG

Enzyme hydrolysis was investigated as an alternative to acid hydrolysis of CFG to produce monomeric sugars. Several different xylanases (X1-274, X2-275, X3-276, X4-277, and Rovabio Excel LC) were tested at the pH and temperature recommended by the manufacturer. Hydrolyses were performed for 72 h at enzyme loadings of 1, 10, 20, and 40% (w/w).

Table 3
Sugar Composition of Product Streams[a] Total sugars recovered (expressed as monomeric)

Product streams								
Stream	No. 6 (wash)		No. 7 (cake)		No. 9 (glucose rich)		No. 13 (pentose rich)	
Components	Weight (g)	Percentage	Weight (g)	Percentage	Weight (g)	Percentage	Weight (g)	Percentage
Glucan	0.12	0.38	2.16	7.13	3.15	10.38	–	–
Xylan	1.36	4.92	3.52	12.73	3.72	13.46	–	–
Arabinan	0.57	4.22	2.46	18.10	1.31	9.62	–	–
Galactan	0.21	3.98	0.72	13.58	0.40	7.53	–	–
Glucose	1.97	5.85	–	–	27.90	82.84	1.55	4.60
Xylose	0.13	0.41	–	–	1.71	5.44	17.92	57.03
Arabinose	0.13	0.83	–	–	1.96	12.66	8.35	54.09
Galactose	0.03	0.49	–	–	0.37	6.29	3.40	57.79

Expressed as grams per 100 g of starting material (composition is available in Table 1).
[a]Stream numbers are from Fig. 1.

Table 4
Overall Sugar Recovery for Separation of C5 and C6 from AFEX-Treated Corn Fiber[a]

	Recovered (g)	Recovered (%)
Glucose	37.44	111.17
Xylose	29.53	93.99
Arabinose	16.34	105.81
Galactose	5.28	89.67
Total	88.58	101.77

[a]Expressed as monomeric sugars.

None of the enzymes showed significant activity toward hemicellulose of CFG, presumable because of the complex and highly substituted nature of the xylan contained in corn fiber. Hydrolysis with 40% loading of Rovabio Excel LC resulted in the highest xylose yield (30.2%). Maximum observed xylose yield for the rest of these enzymes was 2.1–4.5%.

Conclusions

In this study, we demonstrated that 24 h enzyme hydrolysis of AFEX-treated corn fiber hydrolyzed 83% of the cellulose to glucose and solubilized 81 and 68% of the xylan and the arabinan, respectively. It was also demonstrated that 87% of the solubilized hemicellulose (85% of xylan and 92% of arabinan) could be precipitated and collected as CFG by addition of ethanol to the aqueous solution. The collected CFG was hydrolyzed to monomeric sugars with dilute acid, giving a sugar solution made up of 84% C5 sugars and 16% C6 sugars. Attempts were made to identify a xylanase that could hydrolyze the CFG to avoid the acid hydrolysis; however, the best enzyme tested, yielded only 30% xylose at high enzyme loading (40% [w/w]).

The use of AFEX followed by cellulose hydrolysis, ethanol precipitation of CFG, and acid hydrolysis of the precipitated CFG, provides a complete procedure for partially separating the C5 and C6 sugars contained in corn fiber into C5-enriched and C6-enriched sugar streams. Because of the incomplete separation and material losses in the process the recovery of xylose and arabinose in the C5-enriched sugar stream was 57% and 54%, respectively, and recovery of glucose in the C6-enriched stream was 83%. Future work will focus on developing improved methods to fully utilize all available sugars and enhance the purity and yields of glucose and pentose fractions.

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