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Enzymatic Hydrolysis of High-Moisture Corn Fiber Pretreated by AFEX and Recovery and Recycling of the Enzyme Complex

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ABSTRACT

Corn fiber is a grain-processing residue containing significant amounts of cellulose, hemicellulose, and starch, which is collected in facilities where fuel ethanol is currently manufactured. Preliminary research has shown that corn fiber (30% moisture dry weight basis [dwb]) responds well to ammonia-fiber explosion (AFEX) pretreatment. However, an important AFEX pretreatment variable that has not been adequately explored for corn fiber is sample moisture. In the present investigation, we determined the best AFEX operating conditions for pretreatment of corn fiber at high moisture content (150% moisture dwb). The optimized AFEX treatment conditions are defined in terms of the moisture content, particle size, ammonia to biomass ratio, temperature, and residence time using the response of the pretreated biomass to enzymatic hydrolysis as an indicator. Approximate optimal-pretreatment conditions for unground corn fiber containing 150% (dwb) moisture were found to be: temperature, 90°C; ammonia: dry corn fiber mass ratio, 1:1; and residence time 30 min (average reactor pressure under these conditions was 200 pounds per square inch [psig]). Enzymatic hydrolysis of the treated corn fiber was per-

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**Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

formed with three different enzyme combinations. More than 80% of the theoretical sugar yield was obtained during enzymatic hydrolysis using the best enzyme combination after pretreatment of corn fiber under the optimized conditions previously described. A simple process for enzyme recovery and reuse to hydrolyze multiple portions of AFEX-treated corn fiber by one portion of enzyme preparation is demonstrated. Using this process, five batches of fresh substrate (at a concentration of 5% w/v) were successfully hydrolyzed by repeated recovery and reuse of one portion of enzyme preparation, with the addition of a small portion of fresh enzyme in each subsequent recycling step.

Index Entries: Biomass; Fuel Alcohol; Corn Fiber; Ammonia Pretreatment.

INTRODUCTION

Currently, over one billion gallons of ethanol are produced per y in the United States, with approx 95% derived from corn starch *(1)*. In addition, millions of tons of crop and crop-processing residues are generated annually in the United States. Corn fiber represents a renewable lignocellulosic biomass resource that is available in sufficient quantities from the corn wet milling industry to serve as a low-cost feedstock for ethanol production. However, lignocellulose conversion to ethanol is a significantly different technology and is much less developed than corn starch conversion to ethanol. Technologies for lowering costs associated with ethanol production from lignocellulosic biomass can improve the competitiveness of ethanol as a fuel or fuel additive *(2)*. Over the past few years considerable progress has been made on the lignocellulose-to-ethanol processes. But two important bottlenecks remain: To develop an effective and economical pretreatment technique to provide adequate yields of fermentable sugars, and to reduce enzyme costs because the lignocellulose conversion process using enzymatic hydrolysis is hampered by the high cost of cellulases *(3)*. Either lower enzyme loadings must be used or enzymes must be recycled. It has been projected that the recycling of 60% of the cellulolytic enzymes could have a major impact on the contribution of the enzymes to overall process costs *(4)*.

Various pretreatments have been used to increase the rate and extent of lignocellulosic hydrolysis *(5,6)*. While many pretreatments are effective, few are potentially inexpensive enough to generate a product valued at about $0.05 per pound. Among the few effective pretreatments that may be economical, a recently developed AFEX process has attracted attention *(7–10)*.

Corn fiber in the wet-milling industry is now mechanically dewatered (an inexpensive process) to about 150% moisture on a dry-weight basis (60% on total weight basis). Pretreatment at this high moisture content (i.e., the substrate as it is without further size reduction or expensive dry-

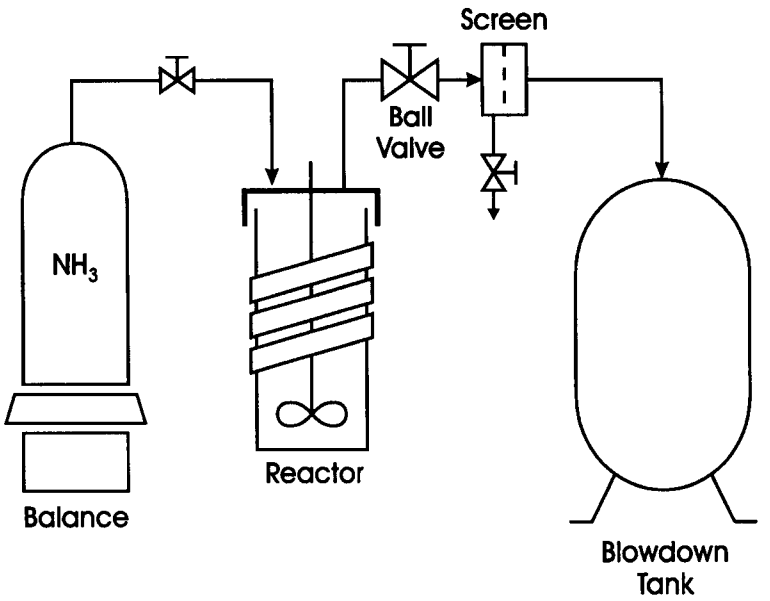

Fig. 1. Schematic diagram of AFEX apparatus.

ing) would eliminate further raw-material processing steps, and help reduce the costs of ethanol production from corn fiber. The objectives of this research were to determine the best AFEX operating conditions for the pretreatment of high-moisture unground corn fiber, and to demonstrate a simple process for recovery and recycling of the enzyme complex for hydrolysis of pretreated substrate.

MATERIALS AND METHODS

Substrate

Corn fiber (containing 150% moisture, dwb) was obtained from the Pekin Energy Company (Pekin, IL). This corn fiber was used directly (without further processing) as a raw material for AFEX treatment. However, for some experiments, corn fiber was sun-dried to approx 30% moisture content (dwb) and/or ground in a Wiley mill to pass a 40-mesh sieve prior to AFEX treatment.

AFEX Treatment

Figure 1 shows a schematic diagram of the AFEX apparatus. The reactor consisted of a 4-L pressure vessel. The vessel was charged with approx 100 g of corn fiber, and the lid was bolted shut. Liquid ammonia was added to the corn-fiber sample and the mixture was agitated for 30 min at experimental temperature. The high pressure was then quickly released to atmospheric

Table 1
Enzyme Combinations Used For the Hydrolysis of AFEX-Pretreated Corn Fiber

Enzyme	Source	Reported activity	Combination used per g of dry substrate		
			A	B	C
Spezyme™ AA 20 (alpha amylase)	Genencor International Inc., Cedar Rapids, IA	20,000 liquefon units (LU)/ml	240.0 LU	240.0 LU	240.0 LU
Spezyme™ GA 300 (gluco amylase)	Genencor International Inc., Cedar Rapids, IA	300 spezyme glucoamylase units (SGU)/ml	3.6 SGU	3.6 SGU	3.6 SGU
Mulitfect® PL enzyme (Pectinase concentrated liquid)	Genencor International Inc., Cedar Rapids, IA	6,600 apple pomace pectin viscosity units (APPV)/ml	105.6 APPV		105.6 APPV
Spezyme® CP (cellulase)	Genencor International Inc., Cedar Rapids, IA	90 Genencor cellulase units (GCU)/ml	6.8 GCU		
Cytolase™ 300 (cellulase)	Genencor International Inc., Cedar Rapids, IA	132 International units (IU)/g		10.0 IU	10.0 IU
Novozyme™ 188 (cellobiase)	Novo Laboratories Wilton, CT.	250 Cellobiase units (CBU)/ml		28.4 CBU	28.4 CBU

pressure by opening a large ball valve connected to a 230-L blowdown tank. The ammonia vapors were absorbed into the water. The pretreated corn fiber was then removed and air-dried overnight to remove residual ammonia.

Enzymatic Hydrolysis

The enzymatic hydrolysis of pretreated corn fiber was performed using a 500-mL screw-capped conical flask with 5% (w/v, solid/liquid) slurry of biomass in a 0.05 *M* citrate buffer, pH 4.8. The enzymes and their activities are listed in Table 1 and were used in three different combinations. Spezyme AA 20 was first added to the hydrolysis flasks, then heated at 90°C in a water bath for 1 h. The hydrolysis flasks were then cooled to 50°C and the rest of each enzyme combination was added and hydrolysis continued at 50°C in a 100 rpm shaker incubator for 48 h. To avoid microbial contamination, sodium azide (0.15%) was added. Samples of 1 mL were taken periodically, boiled in capped test tubes for 15 min to stop the hydrolysis and then filtered through a 0.22-μM nylon membrane.

Enzyme Recovery and Recycling

Figure 2 shows the enzyme recovery and recycle process. The enzymatic hydrolysis of each batch of fresh substrate (5% w/v) using enzyme combination C was performed as previously mentioned except that the fresh substrate for recycling was previously treated at 90°C with Spezyme AA 20 for 1 h, then freeze dried and stored in the refrigerator until use. Enzyme

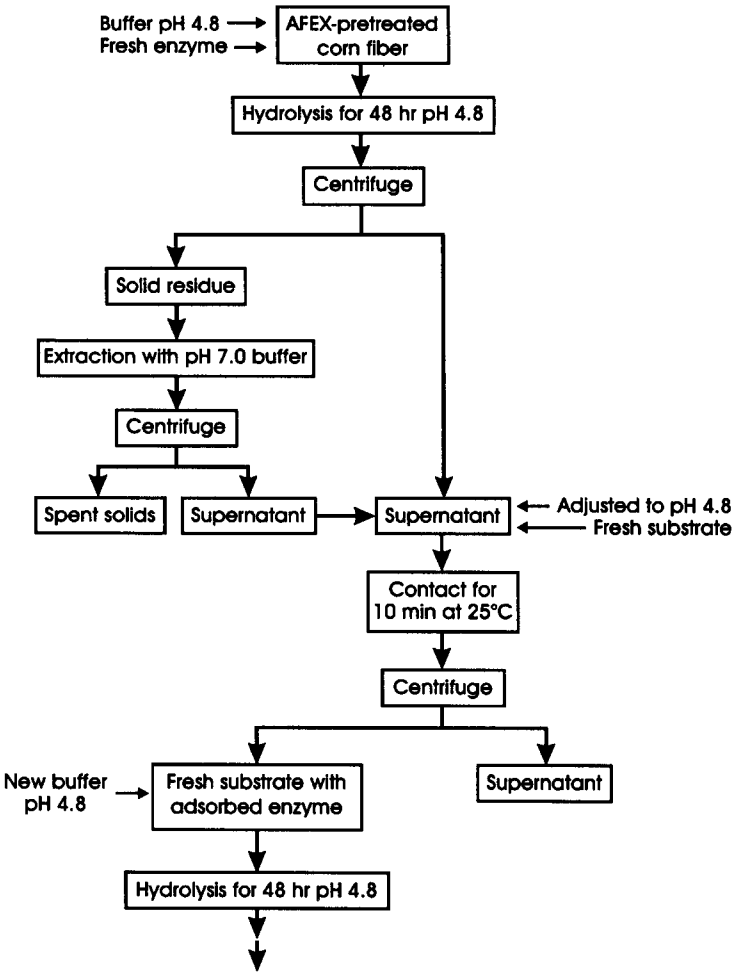

Fig. 2. Enzyme recovery and recycle process flowsheet.

combination C (except Spezyme AA 20) was added at the beginning of the experiment. After 48 h of hydrolysis for each run, the hydrolysis mixture was cooled in an ice bath. The unhydrolyzed residue was separated from the supernatant by centrifugation and then extracted with 25 mL phosphate buffer (pH 7.0) for 1 h. The centrifugation was repeated. The supernatant obtained from this step was combined with the former supernatant. Enzymes in the supernatant were adsorbed on the fresh substrate by mixing in a 500-mL flask and shaking for 10 min at 25°C. Thereafter the contents were centrifuged. The substrate with adsorbed enzymes was transferred to another flask and hydrolyzed at 50°C for 48 h. The unrecovered (lost) cellulase activity and all of the cellobiase activity (Novozyme 188, 28.4 CBU/g of dry substrate) were added to the reaction mixture at the beginning of each new hydrolysis step (i.e., each recycling step). Supplemental cellobiase was added because this enzyme was not expected

to be adsorbed efficiently to the cellulosic residue *(11,12)*. Duplicate experiments for each recycling step were carried out to measure reducing sugar concentration and enzyme activity.

Analytical Methods

Starch content was determined by the polarimetric method of the AACC *(13)*. Protein was estimated by the method of Lowry et al. *(14)*. Total reducing sugars were determined by the dinitrosalycilic acid (DNS) assay *(15)*. Glucose levels were confirmed using a YSI glucose analyzer. Glucose concentrations of some selected samples were also determined by high-pressure liquid chromatography (HPLC, Spectra-Physics, San Jose, CA) with an HPX-87C column (Bio-Rad Laboratories, Hercules, CA) at 85°C. Neutral sugars were determined as described previously *(16)*. The overall enzyme activity of enzyme combination C was measured as filter paper activity by the standard filter-paper assay procedure *(17)*. The supernatants obtained after each recycling (from duplicate experiments) were filtered using an ultrafiltration unit fitted with a 2000 molecular weight cut-off membrane (RG 03, Osmonics, MN). The enzyme solution remaining in the ultrafiltration device was recovered completely with a small amount of buffer, and then assayed for activity. The percent adsorption of filter paper activity by fresh substrate was determined by subtracting the remaining activity in the supernatant from the total activity present before addition of fresh substrate. Enzyme activity was expressed relative to original filter paper activity (100%) of enzyme combination C. All data presented are mean values from two independent experiments with duplicates.

RESULTS AND DISCUSSION

Effect of AFEX-Pretreatment on the Chemical Composition of Corn Fiber

Temperatures ranging from 60 to 110°C were used in AFEX treatment of corn fiber at an ammonia to dry biomass ratio of 1:1. Analysis of samples after treatment indicated no major changes in chemical composition. The major polysaccharide component was hemicellulose composed of arabinose, xylose, and galactose as determined by neutral sugar analysis. This xylan comprised about 31% of the corn fiber, whereas the starch content was 15–22%. Polysaccharide unaccounted for by neutral sugar analysis was calculated to be cellulose and was about 16% of the corn fiber, a value similar to that reported previously *(1)*. The only major difference noted in the composition of untreated corn-fiber and AFEX-treated corn-fiber was the increase in apparent protein content of the AFEX-treated material. The protein content increased from 11 to 20% based on Lowry assays of sodium hydroxide-

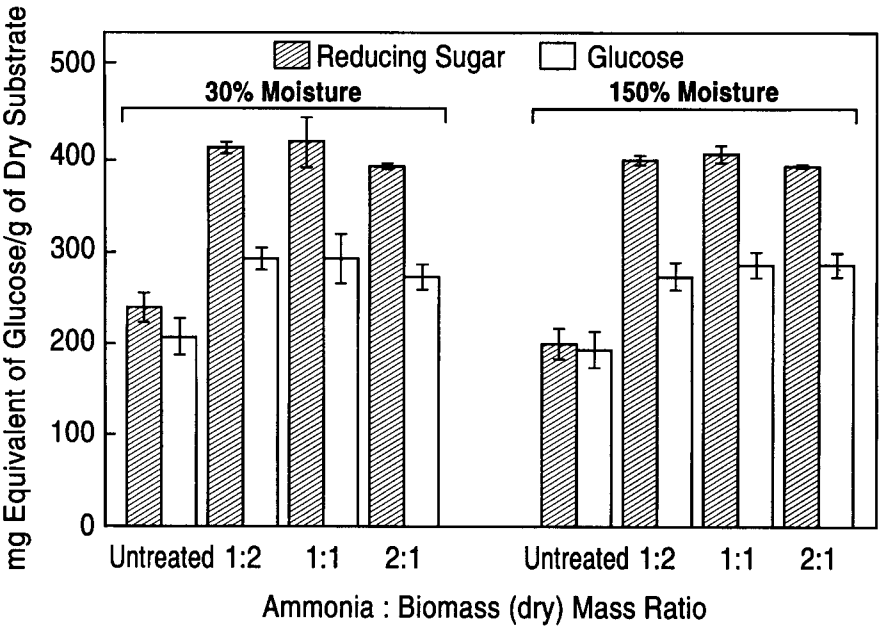

Fig. 3. Effect of moisture content on the enzymatic hydrolysis of AFEX-pretreated corn fiber. Ground (40-mesh) corn fiber was pretreated at 80°C with different ammonia to dry-biomass ratios. Enzymatic hydrolysis was carried out with enzyme combination A.

treated samples. However, in other experiments when corn fiber was treated for 60 min or longer rather than the standard 20 min to solubilize proteins for the Lowry assay, a similar large increase in apparent protein was observed.

Effect of Initial Moisture Content on the Enzymatic Hydrolysis of AFEX-Pretreated Corn Fiber

Figure 3 shows the effect of the initial moisture content in corn fiber on the subsequent enzymatic hydrolysis (for 48 h) after AFEX pretreatment. Increasing the initial moisture content of corn fiber from 30% (dwb) to 150% (dwb) apparently had no effect on the subsequent enzymatic hydrolysis. In other words, a moisture content as high as 150% (dwb), did not hamper the reactivity of ammonia on corn fiber. Apparently the affinity of ammonia for biomass components (cellulose, hemicellulose, and so on) is sufficiently strong so that the ammonia still reacts directly with these components, rather than being simply diluted by the increased moisture content. However, previous evidence also indicates that the moisture in the biomass allows formation of ammonium hydroxide which hydrolyzes hemicelluloses and thus promotes the overall effect of AFEX treatment *(8)*. Hemicellulose hydrolysis has been shown to increase the subsequent hydrolysis of cellulose *(18)*. These results demonstrate an

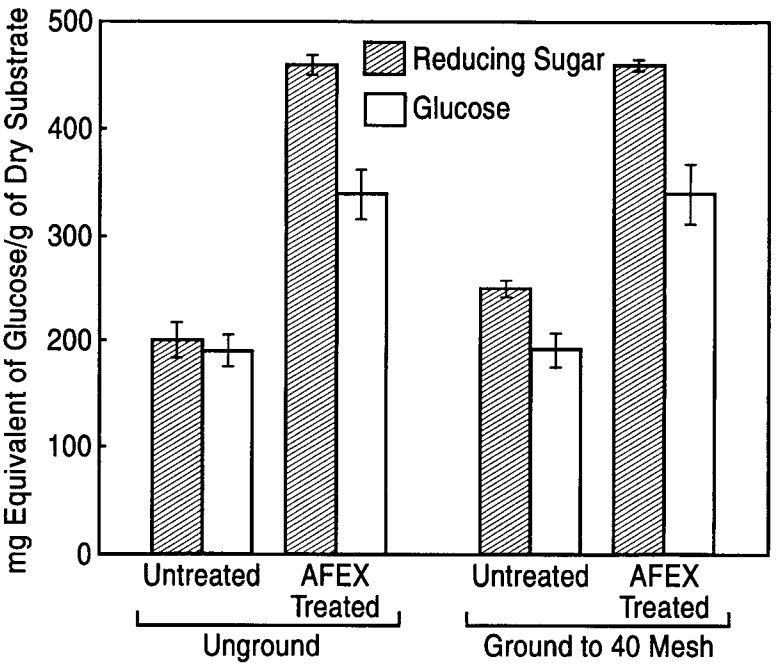

Fig. 4. Effect of particle size on the enzymatic hydrolysis of AFEX-pretreated corn fiber. Corn fiber (containing 150% moisture [dwb]) was pretreated at 90°C with an ammonia to dry-biomass ratio of 1:2. Enzymatic hydrolysis was carried out with enzyme combination A.

advantage of AFEX pretreatment over steam explosion because wet feedstocks require considerably more energy input because of the high heat capacity of water *(19)*. However, AFEX pretreatment of corn fiber at high moisture content (i.e., the substrate as it is) reduces the cost of the overall AFEX bioconversion process by eliminating drying.

Effect of Particle Size on the Enzymatic Hydrolysis of AFEX-Pretreated Corn Fiber

Figure 4 shows the effect of particle size on subsequent enzymatic hydrolysis. Both ground (to 40 mesh) and unground AFEX-pretreated corn fiber were hydrolyzed for 48 h. No difference in the reducing sugar yields and glucose concentrations were evident between these two samples. It is generally known that grinding of lignocellulosic biomass enhances enzymatic hydrolysis by creating more surface area *(20)*. However, for AFEX pretreatment it appears that ammonia effectively penetrated the biomass matrix and reacted with interior cellular components of the corn fiber. Thus, for this pretreatment technique, prior grinding of biomass to small particles did not seem necessary. Physically, the AFEX process seems to create most surface area by splitting fiber bundles axially *(8)*, i.e., across the fiber radius.

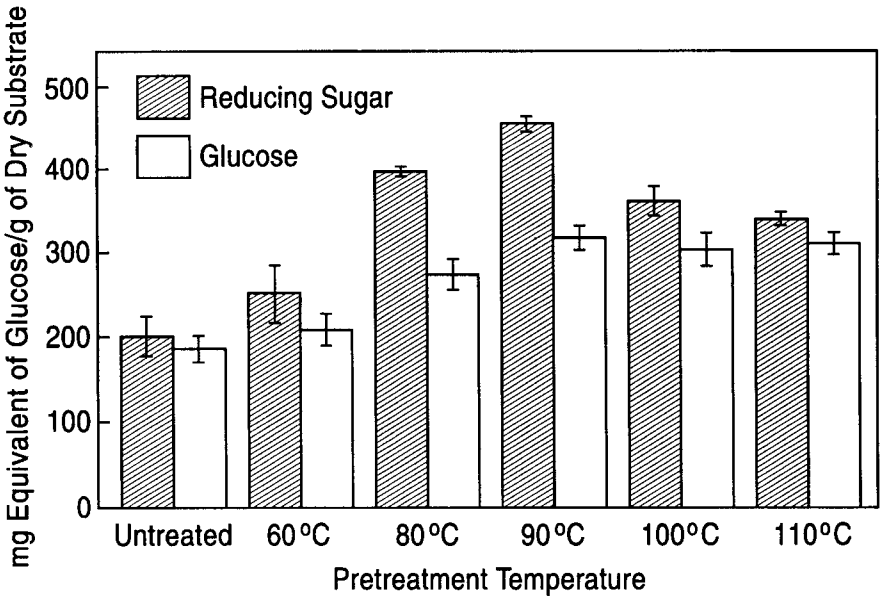

Fig. 5. Effect of pretreatment temperature on the enzymatic hydrolysis of AFEX-pretreated corn fiber. Unground corn fiber (containing 150% moisture [dwb]) was pretreated at different temperatures with an ammonia to dry biomass ratio of 1:2. Enzymatic hydrolysis was carried out with enzyme combination A.

In contrast, grinding techniques that repeatedly cut the ends of the fibers consume large amounts of energy to create relatively little new surface area.

Effect of Pretreatment Temperature on the Enzymatic Hydrolysis of AFEX-Pretreated Corn Fiber

Reactor temperature is an important variable, because it determines the amount of ammonia vaporized during the explosive flash and influences system pressure. More ammonia vapors flash at higher reactor temperatures, causing greater disruption of the fibrous structure. Also, chemical reactions, such as alkaline hydrolysis of hemicellulose, are accelerated at higher temperatures. Figure 5 shows the dramatic effect of increasing pretreatment temperature upon subsequent enzymatic hydrolysis. Enzymatic hydrolysis was carried out for 48 h. Sugar yields increased with increasing pretreatment temperature and attained their maximum value at 90°C. Therefore, a 90°C pretreatment temperature was used in subsequent experiments. Further increases in pretreatment temperature resulted in a decrease in sugar yields. This is an unexpected result and further experiments are required to explain this phenomenon. However, these results illustrate an additional advantage of the AFEX process compared to steam explosion. The steam explosion treatment can form degradation products owing to pyrolysis and acid-catalyzed sugar dehydration *(21)*. However, the AFEX

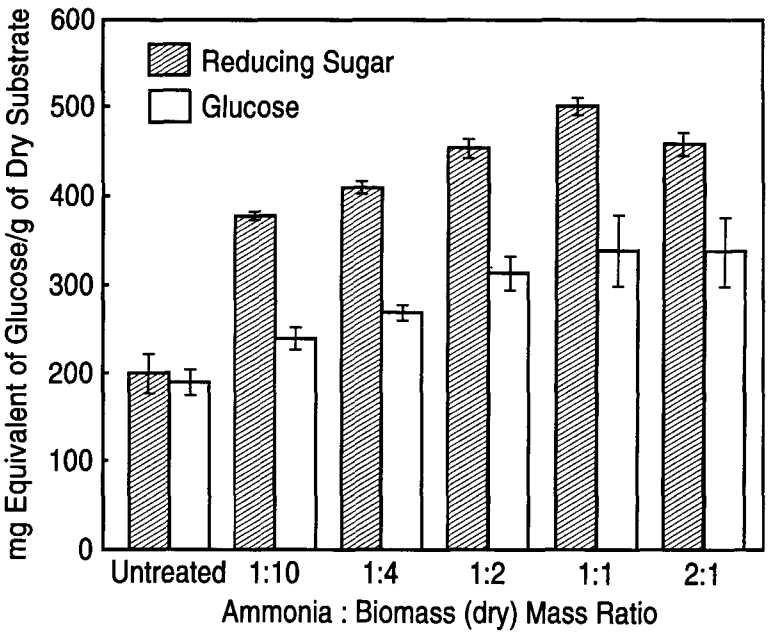

Fig. 6. Effect of ammonia to biomass ratio on the enzymatic hydrolysis of AFEX-pretreated corn fiber. Unground corn fiber (containing 150% moisture [dwb]) was pretreated at 90°C with various ammonia to dry-biomass ratios. Enzymatic hydrolysis was carried out with enzyme combination A.

process avoids base-catalyzed degradation because of its comparatively low temperature.

Effect of Ammonia to Biomass Ratio on the Enzymatic Hydrolysis of AFEX-Pretreated Corn Fiber

Figure 6 shows the effect of ammonia to biomass ratio on the subsequent enzymatic hydrolysis of AFEX-pretreated corn fiber. Enzymatic hydrolysis was carried out for 48 h. Sugar yields increased with increasing ammonia loading and attained a maximum value at a mass ratio of 1:1 (ammonia:biomass). Ammonia at this loading (i.e., 1 g NH_3/g of corn fiber [dwb]) provided maximum overall enhancement of reactivity (such as cellulose swelling and decrystallization, hemicellulose hydrolysis, lignin alterations [*9*], and so on) during pretreatment. It should be noted that liquid ammonia has long been known to be a decrystallizing agent for cellulose (*22*) and can effect a phase change in the cellulose-fiber structure from cellulose I to cellulose III (*23*). Ammonia can also react with lignocellulosics by ammonolysis of the ester crosslinks of some uronic acids with the xylan units (*24*), and cleaving the bond linkages between hemicellulose and lignin (*25,26*). However, it is also evident from Fig. 6 that further increases in ammonia loading decreased sugar yields. It is possible that

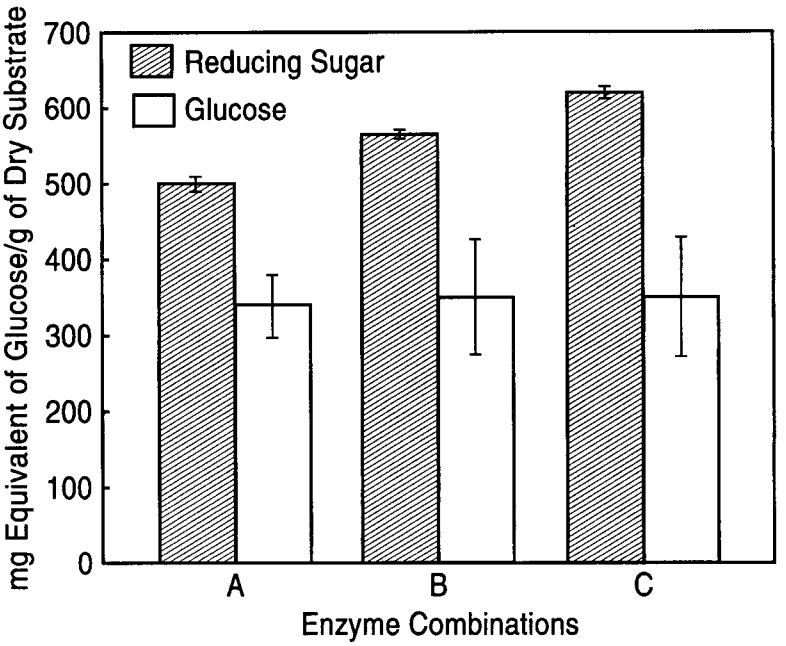

Fig. 7. Hydrolysis of AFEX-pretreated corn fiber with various combinations of enzymes. Unground corn fiber (containing 150% moisture [dwb]) was pretreated at 90°C with an ammonia to dry-biomass ratio of 1 : 1.

excess liquid ammonia plasticizes (27) the fiber and thereby reduces the disruptive effect of the pressure release.

The forgoing results demonstrate that the best AFEX operating conditions for unground corn fiber containing 150% moisture (dwb) are: temperature, 90°C; ammonia:biomass, 1:1 (mass ratio); and residence time 30 min. (The residence time includes heating time in this batch laboratory device and was selected based on previous work with other substrates.)

Hydrolysis of AFEX-Pretreated Corn Fiber with Various Combinations of Enzymes

The types and amounts of enzymes used in the hydrolysis of pretreated corn fiber strongly influence the sugar yields obtainable. A single enzyme will probably not be able to efficiently hydrolyze corn fiber because it contains significant amounts of hemicellulose, starch, and cellulose. However, from a process economics view point in fuel ethanol production, it is necessary to hydrolyze and ferment essentially all of the carbohydrates contained in the pretreated corn fiber. We used various commercial enzyme preparations (Table 1) in three different combinations (*see* Materials and Methods). Figure 7 shows the performance of each enzyme combination. It is apparent that enzyme combination C performed best in hydrolyzing AFEX-pretreated corn fiber. A maximum of 620 mg of reducing sugars containing 350 mg of glucose

Table 2
Hydrolysis of AFEX-Treated Corn Fiber with Recovered Enzyme[a]

Enzyme recovery and reuse step	Recovered FP activity after 48 h of hydrolysis					
	Reducing sugar concentration (mg equivalent of glucose/ g of dry substrate)	% in* supernatant	% in desorption buffer	Total	% FP activity adsorbed with fresh substrate	% FP activity added before initiating new hydrolysis
I	620	61	39	100	92	8
II	621	57	38	95	91	9
III	605	58	36	94	90	10
IV	602	50	36	86	80	20
V	605	48	21	69	-	-

[a] As a percent of the initial FP activity in the first hydrolysis step.

were produced per g of pretreated corn fiber during 48 h of hydrolysis. Therefore, enzyme combination C was used in further experiments.

Recovery and Recycling of the Enzyme Complex after Hydrolysis of AFEX-Pretreated Corn Fiber

The cost of enzymes used for saccharification of cellulosic residues is dominant in the overall bioconversion process. Therefore, it is not surprising that considerable attention has been focused on the use of recovered enzymes for the saccharification of pretreated lignocellulosic substrates *(28–30)*. We examined a simple method for effective recovery and recycling of the enzyme complex during hydrolyses of AFEX-pretreated corn fiber. Figure 2 shows the enzyme recovery and recycle process.

Initially, a batch of AFEX-pretreated corn fiber (5% w/v) was hydrolyzed for 48 h using enzyme combination C with a maximum cellulase enzyme dose of 10 IU/g of dry substrate. Because enzyme combination C contains multiple-enzyme activities, the filter paper activity (cellulase enzyme) was somewhat arbitrarily selected as the standard parameter to determine the overall enzyme activity of this enzyme complex. However, the enzymatic hydrolyzate obtained after 48 h was centrifuged to separate the unhydrolyzed solid residue from the supernatant. The enzyme activity in the supernatant (of the first batch) was found to be 61% of the original activity as measured in a duplicate sample (Table 2), indicating that the remaining enzyme activity (39%) was still with the solid residue. We applied a simple pH manipulation technique as described by Sinitsyn et al. *(31)*, to recover the remaining enzyme activity from the solid residue. Briefly, the unhydrolyzed solid residue was extracted with phosphate buffer (pH 7.0) for 1 h. Centrifugation was then repeated. This technique successfully recovered the

remaining enzyme from the spent solid of the first hydrolysis batch (Table 2). The supernatant obtained after extraction of the solid residue was added to the former supernatant. A batch of fresh substrate was then added to the supernatant and allowed to adsorb the enzymes by mixing in a 500-mL flask and shaking for 10 min at 25°C *(29)*. Most of the recovered enzymes (92%) were rapidly adsorbed onto the fresh substrate by this procedure, possibly owing to a strong affinity of cellulase enzymes for cellulosic biomass *(12)*. The substrate with adsorbed enzymes was then transferred to another flask (containing citrate buffer, pH 4.8) and hydrolyzed at 50°C for another 48 h. This step minimizes the end product (glucose in the supernatant from first hydrolysis batch) inhibition of enzyme activity. It is important to note that the requirement of a large amount of buffer solutions (which may be expensive) for this laboratory-scale experiment can be replaced by a pH-controlled hydrolysis procedure for large scale. However, before initiating the second batch hydrolysis, the remaining 8% enzyme activity and Novozyme (28.4 CBU/g of dry substrate) were added to make 100% as mentioned in the Materials and Methods section. Similarly, a total of five batches of fresh substrate were hydrolyzed in this way. An average of more than 600 mg of reducing sugar/g of dry substrate were produced from each step (Table 2).

Inspection of Table 2 indicates that during the early stages of enzyme recycling, most of the original enzyme activity could be recovered. However, a gradual decrease in the recovered enzyme activity was observed at later stages of recycling, probably owing to processes such as thermal or mechanical inactivation *(32)* rather than factors such as lignin interference (because corn fiber has essentially no lignin) *(33,34)*. However, it is apparent from Table 2 that a total of five batches of fresh substrate (5% w/v in each batch) were efficiently hydrolyzed by using the initial enzyme dose of 10 IU per g of dry substrate which was ultimately reduced to approx 3 IU per g of dry substrate by this recycling process. Although these results are yet to be verified at a large scale, it appears that recovery and recycling of cellulase enzymes could have significant practical value. According to Wright et al. *(35)*, recovering 50% of the original activity at an enzyme loading of 20 IU per g of solids reduces the cost of ethanol $0.36/gal. However, the benefits of enzyme recycle will be less at lower enzyme loadings.

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