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Pretreatment of Corn Stover by Low-Liquid Ammonia Recycle Percolation Process

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Abstract

A pretreatment method using aqueous ammonia was investigated with the intent of minimizing the liquid throughput. This process uses a flow-through packed column reactor (or percolation reactor). In comparison to the ammonia recycle percolation (ARP) process developed previously in our laboratory, this process significantly reduces the liquid throughput to one reactor void volume in packed bed (2.0–4.7 mL of liquid/g of corn stover) and, thus, is termed low-liquid ARP (LLARP). In addition to attaining short residence time and reduced energy input, this process achieves 59–70% of lignin removal and 48–57% of xylan retention. With optimum operation of the LLARP to corn stover, enzymatic digestibilities of 95, 90, and 86% were achieved with 60, 15, and 7.5 filter paper units/g of glucan, respectively. In the simultaneous saccharification and fermentation test of the LLARP samples using *Saccharomyces cerevisiae* (NREL-D_5A), an ethanol yield of 84% of the theoretical maximum was achieved with 6% (w/v) glucan loading. In the simultaneous saccharification and cofermentation (SSCF) test using recombinant *Escherichia coli* (KO11), both the glucan and xylan in the solid were effectively utilized, giving an overall ethanol yield of 109% of the theoretical maximum based on glucan, a clear indication that the xylan content was converted into ethanol. The xylooligomers existing in the LLARP effluent were not effectively hydrolyzed by cellulase enzyme, achieving only 60% of digestibility. SSCF of the treated corn stover was severely hampered when the substrate was supplemented with the LLARP effluent, giving only 56% the overall yield of ethanol. The effluent appears to significantly inhibit cellulase and microbial activities.

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Index Entries: Corn stover; pretreatment; aqueous ammonia; bioenergy; simultaneous saccharification and cofermentation.

Introduction

Corn stover is one of the prime renewable feedstocks being considered for conversion into fuels and chemicals. It is abundant and widely available in the United States. A recent study indicates that about 80–100 million dry t/yr of corn stover can be utilized for this purpose *(1)*. Production of ethanol from lignocellulosic biomass is quite different from the process used for corn, because the carbohydrates are much more difficult to solubilize than starch in grain *(2)*. Lignocellulosic material is very resistant to enzymatic breakdown. Pretreatment thus becomes an essential element in the overall conversion of the lignocellulosic substrates. It is necessary to enhance the susceptibility of the biomass to the enzyme action. The research effort in pretreatment has been extensive. Various methods have been applied for different substrates with varying degrees of success. Among the well-recognized methods are steam/steam explosion *(3–6)*, grinding/milling *(7–11)*, hot water/autohydrolysis *(12–15)*, acid treatment *(16–20)*, and alkali treatment *(21–26)*.

Our laboratory has been working on pretreatment methods using aqueous ammonia. As a pretreatment reagent, ammonia has several of desirable characteristics: swelling of cellulosic materials, highly selective delignification reaction, low interaction with carbohydrates, and high volatility (ease of separation). The ammonia recycle percolation (ARP) process has been investigated in our laboratory for pretreatment of hardwood and agricultural residues including corn stover *(22,23,26,27)*. Figure 1 shows the projected schematics of the ARP process. The liquid throughput, reaction time, ammonia concentration, and reaction temperature are the primary factors influencing the reactions occurring in the ARP. An economic analysis of this process indicates that the amount of liquid throughput is a major cost item, because it is directly related to the process energy. In this research, a modification of the ARP process was sought specifically to minimize the amount of liquid input and the reaction time. The modified process was termed low-liquid ARP (LLARP). The ARP is a semibatch research reactor. It is a fixed-bed reactor operated in a flow-through mode. From a process viewpoint, a continuous reactor is highly desirable. The ARP can be operated continuously with proper reactor design. One such option is a cocurrent screw-fed reactor. To achieve a continuous operation, however, it is necessary to limit the reaction time within the range of 10–20 min. Short reaction time and low liquid input represent substantial modification of the conventional ARP.

The primary objective of the present study was to determine whether a modified ARP that can meet these requirements for continuous operation could be constructed and, if so, to assess the performance of the modified

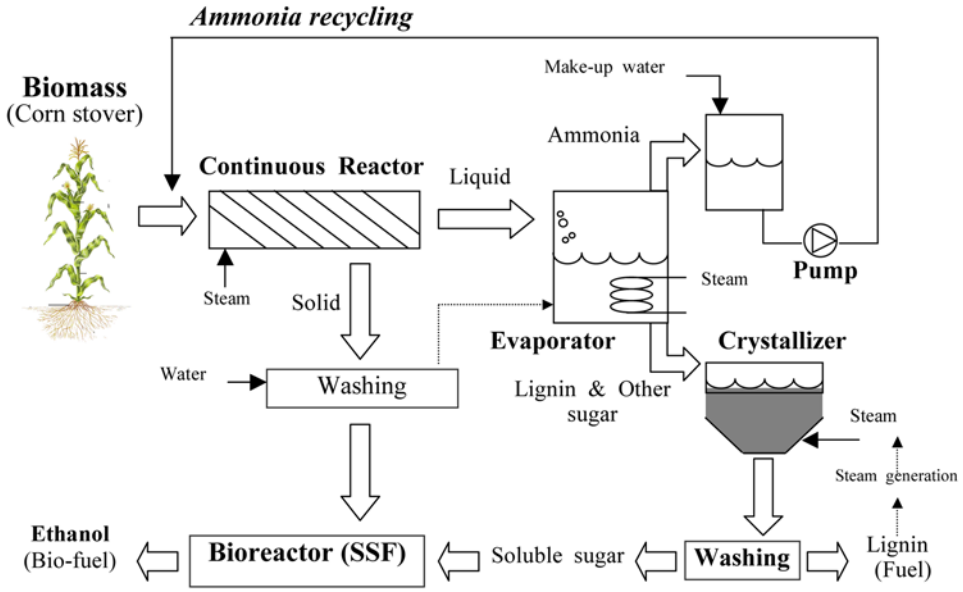

Fig. 1. Projected scheme of ARP process.

process. Attempts were made to optimize the pretreatment conditions within the range of LLARP. The optimization criteria included the digestibility of the pretreated substrates and the ultimate ethanol yields from simultaneous saccharification and fermentation (SSF) and from simultaneous saccharification and cofermentation (SSCF).

Materials and Methods

Corn Stover and Enzymes

Air-dried ground corn stover was a kind gift of the Biofuels Research Center of the National Renewable Energy Laboratory (NREL) (Golden, CO). The corn stover was screened to a nominal size of 9–35 mesh. The initial composition of the corn stover, as determined by NREL, was 36.1 wt% glucan, 21.4 wt% xylan, 3.5 wt% arabinan, 1.8 wt% mannan, 2.5 wt% galactan, 17.2 wt% Klason lignin, 7.1 wt% ash, 3.2 wt% acetyl group, 4.0 wt% protein, and 3.6 wt% uronic acid. α-Cellulose was purchased from Sigma (St. Louis, MO) (cat. no. C-8200, lot no. 11K0246).

Cellulase enzyme, Spezyme CP (lot no. 301-00348-257; Genencor), was obtained from NREL. The average activity and the protein content of the enzyme, as determined by NREL, were 31.2 filter paper units (FPU)/mL and 106.6 mg/mL, respectively. The activity of β-glucosidase (Novozyme 188 from Novo; Sigma cat. no. C-6150, lot no. 11K1088) was 750 cellobiase unit (CBU)/mL.

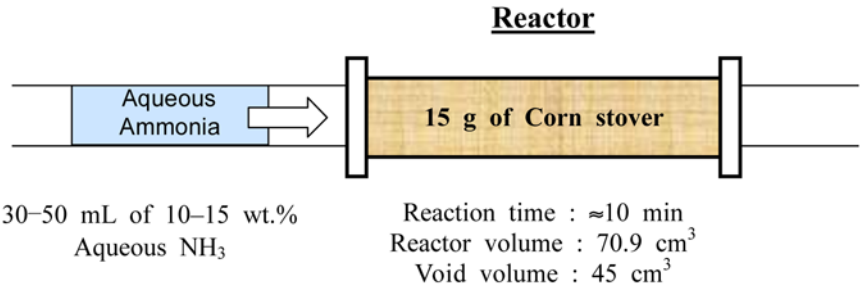

Fig. 2. Schematics of LLARP experiment.

Simultaneous Saccharification and Fermentation

The microorganism used for SSF was *Saccharomyces cerevisiae* ATCC® 200062 (NREL-D_5A). The growth medium was YP medium containing 1% yeast extract (cat. no. Y-0500; Sigma) and 2% peptone (cat. no. P-6588; Sigma).

Simultaneous Saccharification and Cofermentation

Recombinant *Escherichia coli* ATCC® 55124 (KO11) was employed for SSCF tests. Luria-Bertani (LB) medium (cat. no. L-3152; Sigma) was used for KO11, which contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L of chloramphenicol.

ARP Experiments

Figure 2 presents a schematic diagram and the reaction/operating conditions of the LLARP experiment. The experimental setup consisted of stock solution reservoirs, a pump, a temperature-controlled oven, and a liquid holding tank. The reactor was constructed of SS-316 tubing with a 0.9-in. id and a 10-in. length (70.9 cm^3 of internal volume). A 1-L cylinder (SS-304) was used as the receiver tank. In the LLARP experiment, 15 g of dry biomass was packed into the reactor with no presoaking step. The oven was preheated for 25 min, and 2.5 MPa of N_2-backpressure was applied to the reactor system at all times to prevent boil-up of ammonia solution. A specified volume of aqueous ammonia was pumped through the system, followed by wash water. A quick quenching of the reactor was applied after completion of the reaction.

Digestibility Test

The enzymatic digestibility of corn stover was determined in duplicates according to the NREL Chemical Analysis and Testing Standard Procedure *(28)*. The conditions of the enzymatic digestibility tests were 50°C and pH 4.8 (0.05 *M* sodium citrate buffer) in a shaker bath agitated at 150 rpm. Enzyme loadings of 15 and 60 FPU of Spezyme CP/g of glucan

supplemented with 30 CBU of β-glycosidase (Novozyme 188; Sigma cat. no. C-6150)/g of glucan were used. The initial glucan concentration was 1% (w/v) in 100 mL of total liquid. The 250-mL screw-capped Erlenmeyer flasks containing the enzyme hydrolysis preparations were placed in an incubator shaker (Innova-4080; New Brunswick Scientific). Samples were taken intermittently over a 72-h period and analyzed for glucose, xylose, and cellobiose content using high-performance liquid chromatography (HPLC). Total released glucose after 72 h of hydrolysis was used to calculate the enzymatic digestibility. α-Cellulose and untreated corn stover were subjected to the same procedure as a reference and control, respectively.

Simultaneous Saccharification and Fermentation/Cofermentation

Spezyme CP (lot no. 301-00348-257; Genencor) was used as cellulase enzyme. It was supplemented with β-glucosidase, Novozyme 188 (Novo; Sigma cat. no. C6150, lot no. 11K1088) at a level of 2 CBU/FPU. A 250-mL Erlenmeyer flask was used as the bioreactor. It was shaken in an incubator shaker (Innova-4080; New Brunswick Scientific) at 38°C and 150 rpm. Into a 100-mL working volume of liquid, treated corn stover samples were added so that the glucan content became 3% (w/v). (For a treated corn stover that contains 60% glucan and 10% moisture content, the actual amount of sample to be loaded is calculated as 3.0 g of glucan/[0.6 × (100 × 10)/100] = 5.56 g of treated corn stover. A total working volume of 100 mL of liquid includes 5.56 g of treated corn stover, 10 mL of concentrated medium, 10 mL of cell inoculum, enzymes, antibiotics, and deionized water). α-Cellulose was put through the same procedure as a control. The SSF and SSCF runs were performed with buffer without external pH control, starting at pH 5.0 and 7.0 at the beginning of the fermentation and gradually decreasing to pH 4.5 and 6.0 at the end, respectively. The loading of cellulase enzyme was 15 FPU/g of glucan, and that of β-glucosidase was 30 CBU/g of glucan.

The ethanol yield was calculated as follows:

$$\text{Ethanol yield [\% of theoretical maximum]} = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial sugar (g) in reactor} \quad \times 0.511} \times 100$$

Note That sugar is interpreted as glucose in the SSF or glucose plus xylose in the SSCF. The theoretical ethanol yield based on the stoichiometry is 0.511 g of ethanol/g of glucose and 0.511 g of ethanol/g of xylose, or 2 mol of ethanol/mol of glucose and 1.67 mol of ethanol/mol of xylose, respectively.

Analytical Methods

The solid samples were analyzed for sugars and Klason lignin following the NREL *Chemical Analysis and Testing Laboratory Analytical Procedures (28)*. Each sample was analyzed in duplicates. The moisture content was measured with an infrared moisture balance (IR-30; Denver Instrument). The sugars in the liquid samples were determined after secondary acid

hydrolysis to account for the oligomer contents. The conditions of the secondary hydrolysis were 4 wt% sulfuric acid and 121°C for 1 h. Sugars were determined by HPLC using a Bio-Rad Aminex HPX-87P column. For the SSF test, a Bio-Rad HPX-87H column was used to measure the ethanol and glucose content in the broth. A YSI 2300 Glucose/Lactate analyzer was used for rapid analysis of glucose in the inoculua (according to the YSI manual, the linearity is up to ±5%). A refractive index detector was used in the HPLC.

Statistical Analysis

A mean value and a standard deviation were calculated using JMP software version 5.0 (SAS Institute, Cary, NC). SigmaPlot Version 8.0 (SPSS, Chicago, IL) was used to plot the results.

Results and Discussion

Effect of Ammonia Concentration and Flow Rate

Two levels of ammonia concentration (10 and 15 wt%) were applied for the ARP pretreatment. Table 1 summarizes the compositional changes in the solid and liquid samples, and their effects on enzymatic hydrolysis. The results indicate that the LLARP removed basically lignin but also induced solubilization of about half of the xylan. The ARP removed 62–70% of the total lignin. Table 1 also shows that the accountability of carbohydrate (total glucan and xylan in the solid plus liquid) was above 95% for glucan and xylan. The carbohydrates are thus well preserved in the ARP treatment. The digestibilities of the treated samples were in the range of 85–90% with 15 FPU/g of glucan. The digestibilities of 15 wt% NH_3-treated samples were slightly higher than those of 10 wt% NH_3-treated samples. The enzymatic hydrolysis results presented in Fig. 3A show glucose profiles with an enzyme loading of 15 FPU/g of glucan. Enzymatic hydrolysis occurred rapidly, attaining most of the hydrolysis (80–90% of the 72-h digestibilities) within the first 12 h. For all conditions tested, the hydrolysis rates of ARP-treated samples were much higher than those of α-cellulose.

Effect of Ammonia Throughput

The amount of liquid throughput is one of the major cost factors in the ARP. To verify the effects of liquid throughput, three different levels were applied to the ARP operation, all within the very low range: 2.0, 3.3, and 4.7 mL of 15 wt% ammonia/g of corn stover; Table 2 summarizes the results. The data show that the lignin removal was significantly affected by the liquid throughput. The extent of delignification was reduced from 70 to 59% as the liquid throughput was reduced from 3.3 to 2.0/g of solid. The glucan and xylan contents were unaffected by the liquid throughput. The 72-h digestibilities remained relatively constant with respect to the

Table 1
Effect of Flow Rate and Ammonia Concentration on Composition and Digestibility[a]

	Solid				Liquid		Total		Digestibility[d]	
Flow rate (mL/min)	SR[b] (%)	Lignin[c] (%)	Glucan (%)	Xylan (%)	Glucan (%)	Xylan (%)	Glucan (%)	Xylan (%)	60 FPU (%)	15 FPU (%)
Untreated	100	17.2	36.1	21.4	—	—	36.1	21.4	21.2	15.7
Ammonia (10 wt%)[a]										
2.5	59.8	6.6	36.1	10.9	0.5	10.2	36.6	21.1	94.7	85.1
5.0	58.1	5.1	35.0	9.6	0.5	11.0	35.5	20.6	99.7	87.7
7.5	59.2	5.1	35.9	10.9	0.5	9.7	36.4	20.6	94.1	86.8
Ammonia (15 wt%)										
2.5	58.5	6.4	35.9	10.7	0.5	10.6	36.4	21.3	95.2	86.2
5.0	57.5	5.1	35.6	10.3	0.5	10.1	36.1	20.4	95.3	90.1
7.5	57.0	5.6	35.7	10.1	0.8	10.5	36.5	20.6	97.2	90.0

[a]The data presented are based on the oven-dried untreated biomass. Pretreatment conditions were 170°C and 3.3 mL of 10 or 15 wt% NH_3 liquid throughput/g of corn stover. All experiments were done in duplicates. Data represent the mean values.

[b]SR, solid remaining after reaction.

[c]Klason lignin.

[d]Digestibility at 72 h. Enzymatic hydrolysis conditions were 60 or 15 FPU/g of glucan, pH 4.8, 50°C, and 150 rpm.

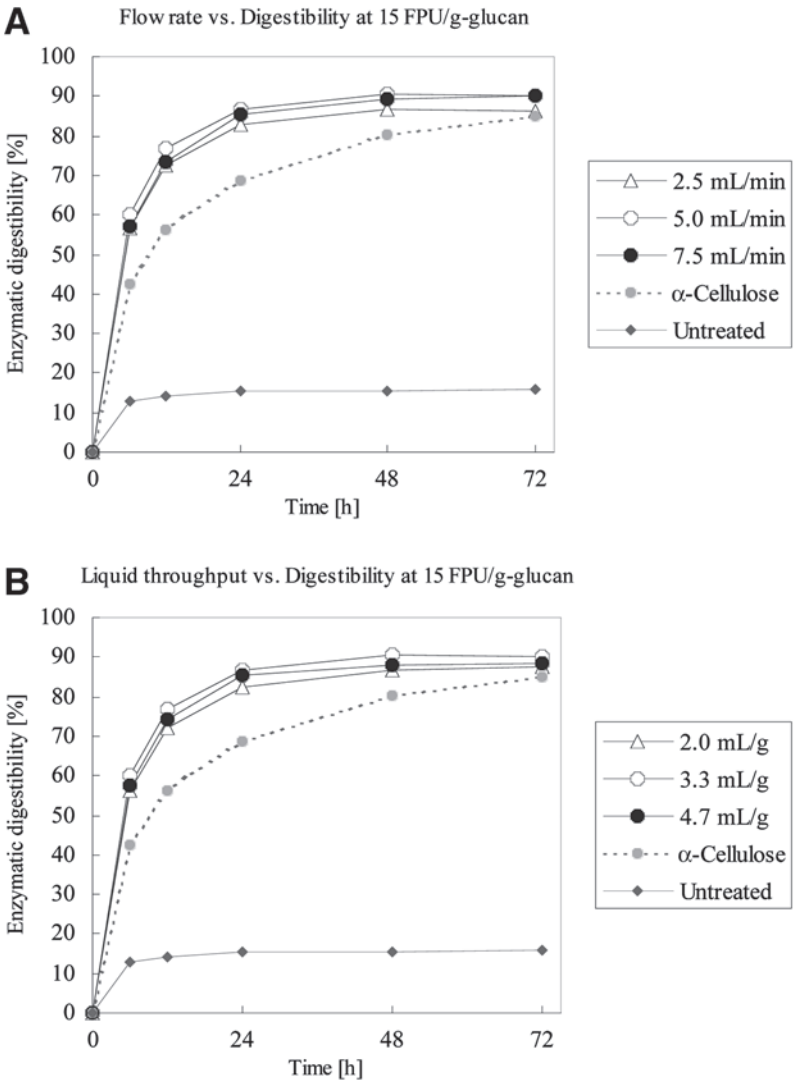

Fig. 3. Enzymatic digestibility of LLARP-treated samples at different enzyme loadings. Enzymatic hydrolysis conditions were 72 h, 15 FPU/g of glucan, pH 4.8, 50°C, and 150 rpm. Pretreatment conditions were 170°C, **(A)** 2.5–7.5 mL/min flow rate and 3.3 mL of 15 wt% NH_3 throughput/g of corn stover and **(B)** 5 mL/min flow rate, 2.0–4.7 mL of 15wt% NH_3 throughput per gram of corn stover. The data indicate the mean values (SD ≤ 2.5%; n = 2).

liquid throughput. Figure 3B presents the enzymatic hydrolysis profiles. With 15 FPU/g of glucan all three cases were in the range of 87–90%. These data collectively indicate that the liquid input and residence time could be reduced to 3.3 mL/g of biomass and 10 min without adversely affecting the overall performance of the ARP.

Table 2
Effect of Aqueous Ammonia (15 wt%) Throughput on Composition and Digestibility[a]

Liquid throughput (mL/g solid)	Solid				Liquid		Total		Digestibility[d]	
	SR[b] (%)	Lignin[c] (%)	Glucan (%)	Xylan (%)	Glucan (%)	Xylan (%)	Glucan (%)	Xylan (%)	60 FPU (%)	15 FPU (%)
Untreated	100	17.2	36.1	21.4	—	—	36.1	21.4	21.2	15.7
2.0	62.1	7.1	36.1	11.4	0.8	9.2	36.9	20.6	93.8	87.5
3.3	57.5	5.1	35.6	10.3	0.5	10.1	36.1	20.4	95.3	90.1
4.7	56.9	5.1	35.8	10.0	0.5	9.8	36.3	19.8	97.8	88.4

[a]Data are based on the oven-dried untreated biomass. Pretreatment conditions were 170°C and 2.0–3.3 mL of 15 wt% NH_3 liquid throughput/g of corn stover. All experiments were done in duplicates. Data represent the mean values.

[b]SR, solid remaining after reaction.

[c]Klason lignin.

[d]Digestibility at 72 h. Enzymatic hydrolysis conditions were 60 or 15 FPU/g of glucan, pH 4.8, 50°C, and 150 rpm.

Effect of Temperature on LLARP

The effect of temperature on the LLARP was investigated at a range of 110–170°C. Four different temperatures (110, 130, 150, and 170°C) were applied, maintaining the liquid throughput at 3.3 mL of 15 wt% NH_3/g of corn stover. Table 3 summarizes the compositional changes in both the solid and the liquid, and the digestibilities with three different enzyme loadings (60, 15, and 7.5 FPU/g of glucan). Both xylan and lignin removal increased as the temperature was increased. With treatment at 170°C, about 50% of xylan was solubilized. Higher lignin and xylan removal appear to be the primary reason for the increased enzymatic digestibility.

Corn stover treated by LLARP operated at 150°C attained 88% digestibility, which is quite close to the 90% digestibility obtained from the sample treated at 170°C. In both cases, the enzyme loading was 15 FPU/g of glucan. However, when the enzyme loading was lowered to 7.5 FPU/g of glucan, the digestibility of the 150°C-treated sample (77%) was significantly lower than that of the 170°C-treated sample (86%). Attempts at other temperatures (110 and 130°C) resulted in poor digestibilities, generally lower than 50%. Although the digestibility of the 150°C sample was lower than that of the 170°C sample, low-temperature treatment may still be a viable option because high-temperature treatment raises equipment and operation costs.

Figure 4A,B presents additional enzymatic hydrolysis results representing enzyme loadings of 15 and 7.5 FPU/g of glucan, respectively. Of note from Table 3 and Fig. 4 is that the digestibilities for the residual xylan in the substrates treated at 110–170°C were in the range of 58–78% with an enzyme loading of 15 FPU/g of glucan. It is obvious that there is a substantial xylanase activity in the "cellulose," Spezyme CP. However, the xylanase activity was not high enough to bring the digestibility of xylan to the same level of glucan.

SSF of LLARP-Treated Corn Stover

Corn stover samples were prepared by treating them under the optimum conditions: 170°C, 3.3 mL of 15 wt% ammonia/g of corn stover, 5-mL/min flow rate, and 10-min reaction time. The glucan content of the pretreated corn stover was 62%. The treated corn stover and α-cellulose (a reference substrate) were subjected to the SSF tests following the NREL *Chemical Analysis and Testing Laboratory Analytical Procedures (28)*. Two different glucan loadings (3 or 6% [w/v] glucan) were applied. The ethanol yield and glucose concentration in the SSF operation were monitored.

Figure 5 presents the ethanol yields from the treated corn stover and α-cellulose. With an initial loading of 3% (w/v) glucan, the ethanol yield of treated corn stover reached 84% of the theoretical maximum (14.3 g/L), which occurred at 96 h (Fig. 5A). At the 24-h point, the ethanol yield of the pretreated sample reached 78%. At this point, the ethanol yield of corn stover was substantially higher than that of α-cellulose (56%) (Fig. 5A). Figure 5B presents the ethanol yield with a loading of 6% (w/v) glucan.

Table 3
Effect of Temperature on Composition and Digestibility[a]

Reaction temperature (°C)	Solid				Liquid		Total			Digestibility[d]		
	SR[b] (%)	Lignin[c] (%)	Glucan (%)	Xylan (%)	Glucan (%)	Xylan (%)	Glucan (%)	Xylan (%)		60 FPU (%)	15 FPU (%)	7.5 FPU (%)
Untreated	100	17.2	36.1	21.4	—	—	36.1	21.4		21.2	15.7	13.8
170	57.5	5.1	35.6	10.3	0.5	10.1	36.1	20.4	G	95.3	90.1	85.9
									X	85.5	77.9	71.2
150	60.4	6.3	35.8	12.3	0.5	8.5	36.3	20.8	G	96.9	88.0	77.1
									X	86.0	73.6	61.8
130	66.2	6.4	36.1	15.1	0.4	6.5	36.5	21.6	G	95.5	79.6	64.7
									X	87.6	67.2	58.2
110	76.1	9.2	36.2	18.5	0.3	3.1	36.5	21.6	G	82.4	69.8	53.4
									X	72.2	57.9	46.4

[a]Data are based on the oven-dried untreated biomass. Pretreatment conditions were 110–170°C and 3.3 mL of 15 wt% NH_3 liquid throughput/g of corn stover. All experiments were done in duplicates. Data represent the mean values.

[b]SR, solid remaining after reaction.

[c]Klason lignin.

[d]Digestibility at 72 h. Enzymatic hydrolysis conditions were 60, 15, or 7.5 FPU/g of glucan; pH 4.8; 50°C; and 150 rpm. G represents digestibility for glucan, and X represents digestibility for xylan.

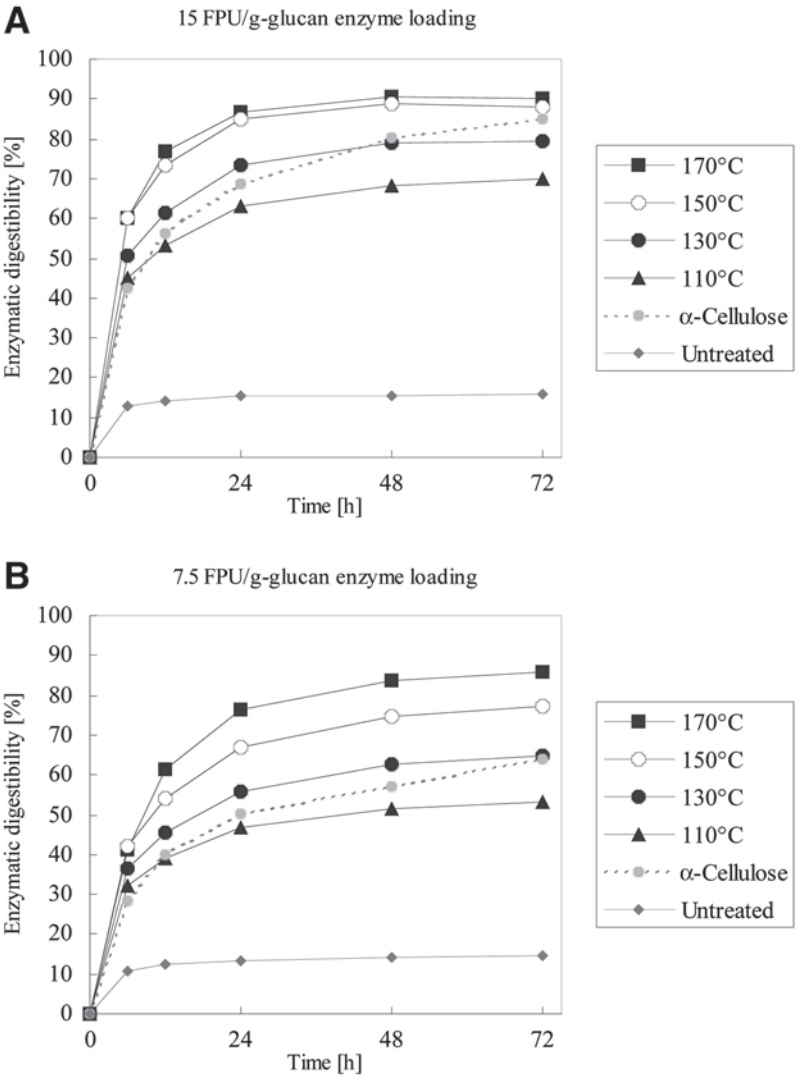

Fig. 4. Enzymatic digestibility of LLARP-treated samples at different enzyme loadings. Pretreatment conditions were 110–170°C, 3.3 mL of 15 wt% NH_3 throughput/g of corn stover, and 5.0 mL/min flow rate. Enzymatic hydrolysis conditions were 72 h, **(A)** 15 or **(B)** 7.5 FPU/g of glucan, pH 4.8, 50°C, and 150 rpm. The data indicate the mean values (SD ≤ 2.9%; $n = 2$).

The yield was about the same as that of 3% glucan loading. The glucose level in the broth quickly dropped to almost zero after 12 h for a loading of 3% (w/v) glucan and 24 h for 6% (w/v) glucan. The bioconversion process thus occurs mostly under glucose-limited condition. Unlike with the 3% glucan loading, the ethanol profile was similar to that of α-cellulose in the 6% run. The percentage of conversion of ethanol within the 24-h period was faster with 3% glucan loading. The 12- and 24-h ethanol yields of a loading

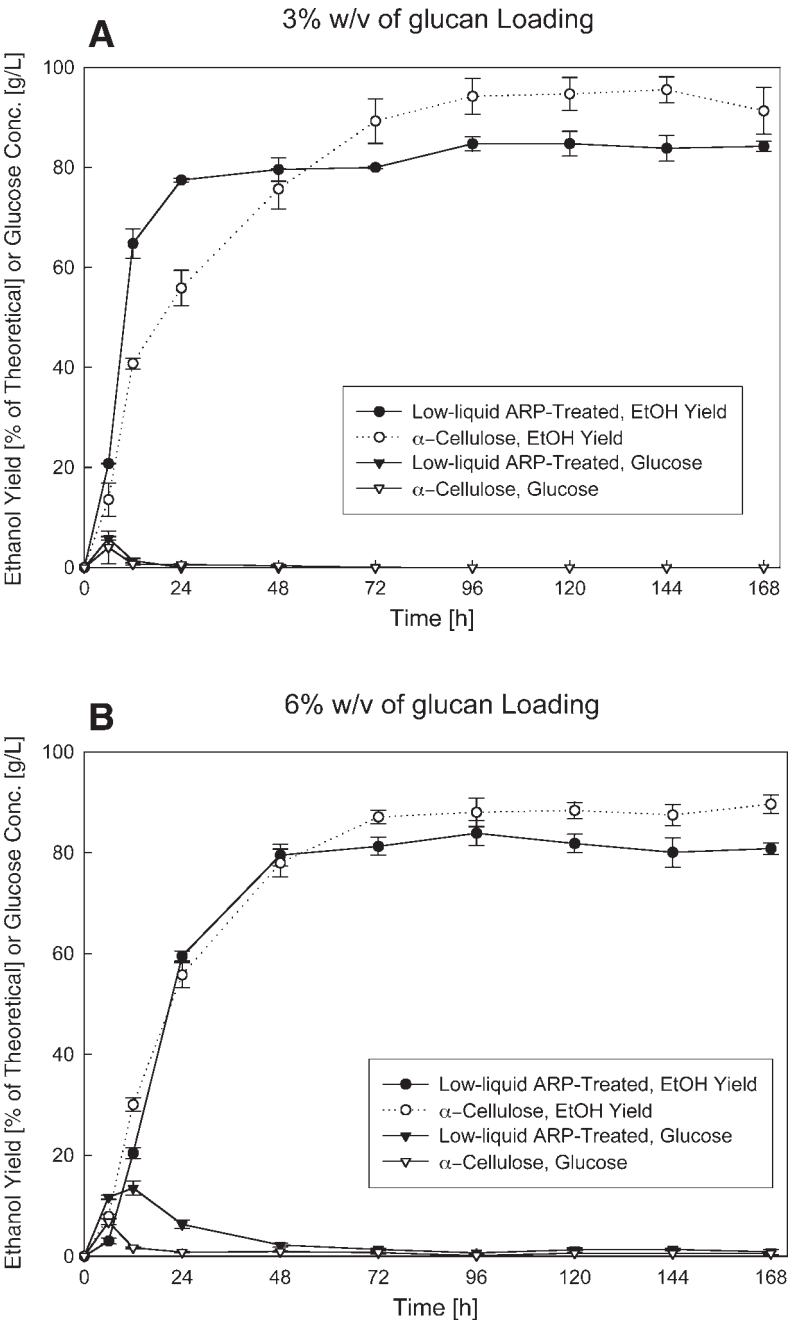

Fig. 5. Ethanol yield of LLARP-treated samples using SSF. Pretreatment conditions were 170°C, 3.3 mL of 15 wt% NH_3 throughput/g of corn stover, and 5.0 mL/min flow rate. SSF test conditions were *S. cerevisiae* ATCC 200062 (D_5A); substrate: 3 or 6% (w/v) glucan loading/100-mL reactor, 15 FPU of cellulase/g of glucan; 30 CBU of β-glucosidase/g of glucan; YP medium (1% yeast extract, 2% peptone); anaerobic condition: 38°C, 150 rpm. **(A)** n = 4, SD ≤ 4.6% for LLARP, n = 4 for α-cellulose; **(B)** n = 4, SD ≤ 2.8% for LLARP, n = 2 for α-cellulose. The data indicate the mean values.

of 3% (w/v) glucan were 65 and 78%, respectively, whereas those of 6% (w/v) glucan were 21 and 60%, respectively. The 12- and 24-h glucose concentrations of a loading of 3% (w/v) glucan were 7.7 and 4.9 g/L, respectively, whereas those of 6% (w/v) glucan were 11.7 and 13.5 g/L, respectively. Although the percentage of conversion into ethanol was slower at the beginning with higher glucan loading, the terminal ethanol yields were identical (84%) for the two different glucan loadings. The advantage of SSF as a bioprocess—that glucose inhibition is essentially eliminated—is proven here. The yield data are based only on glucan content, because the yeast (D_5A) is not capable of converting xylose.

SSCF of LLARP-Treated Corn Stover and Hydrolysates

SSCF of treated corn stover and α-cellulose was performed using the recombinant *E. coli* ATCC 55124 (KO11). LLARP-treated corn stover contains 61% glucan and 18% xylan. The total carbohydrate content of a loading of 3% (w/v) glucan is therefore 38 g/L. One needs to pay attention to both carbohydrates in the SSCF because the recombinant *E. coli* is capable of fermenting hexose and pentose into ethanol.

Figure 6 shows the ethanol yield from the SSCF for various substrates. Maximum ethanol concentration was 19.4 g/L, which corresponds to 89% of the theoretical maximum based on the total amount of glucan and xylan. This was substantially higher than the 16.6 g/L obtained from the SSCF of α-cellulose. For utilization of sugar in the hydrolysate, the liquor from the LLARP was collected and rota-evaporated to remove ammonia. The pH was adjusted to 2.0 and the liquor was left at room temperature for 72 h to precipitate lignin. Precipitated lignin was separated by centrifugation, which removed approx 80% of the original soluble lignin in the ARP liquor. During the lignin precipitation, about 20–30% of xylan was also precipitated along with lignin. LLARP treatment dissolves about half of xylan in the hydrolysate but preserves all of glucan in the treated solid. Thus, the hydrolysate contains mainly xylan among sugars. SSCF was repeated with the addition of the treated liquor as well as LLARP-treated corn stover. The maximum ethanol in this run was only 9.5 g/L, which is 56% of the theoretical maximum based on glucan and xylan. The yield of SSCF with the addition of liquor was significantly lower than that without the addition of liquor. The lower yield with hydrolysate can be attributed to toxic components that exist in the LLARP liquor. No information is available as to what specific components and what level of them are released during the LLARP at this time. Obviously these toxins are sufficiently inhibitory to the cellulase and to the microorganism to suppress ethanol production, as evidenced in similar previous work *(29,30)*.

Hydrolysis of xylose oligomers by cellulase enzyme (Spezyme CP) was also investigated using the ARP effluent containing 2.1 g/L of xylose equivalent. Figure 7 summarizes the digestibility test results. The digestibility of soluble xylooligomer was about 60% with an enzyme loading of 30 FPU/g of xylan. Digestibility rapidly reached the maximum in 6 h. In the

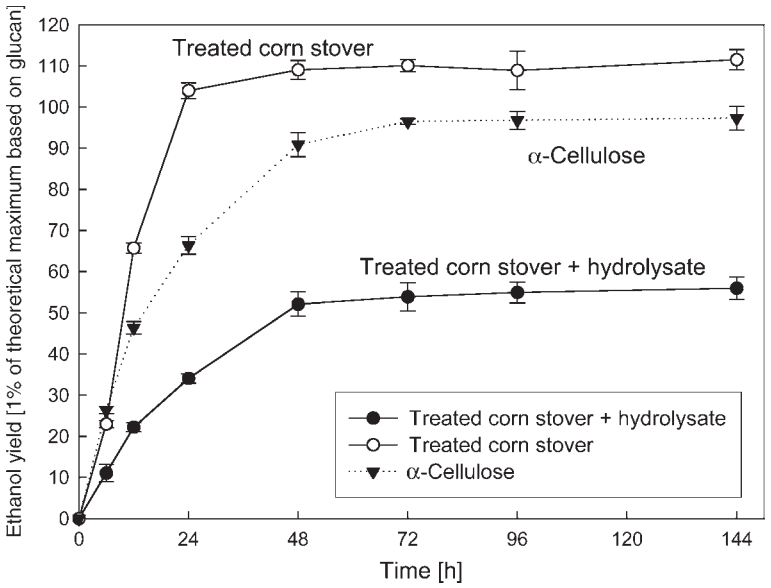

Fig. 6. Ethanol yield of LLARP-treated samples using SSCF. Pretreatment conditions were 170°C, 3.3 mL of 15 wt% NH_3 throughput/g of corn stover, 5.0 mL/min flow rate. SSF test conditions were *E.* ATCC 55124; substrate: 3% (w/v) glucan loading/100-mL reactor, 15 FPU of cellulase/g of glucan; 30 CBU of β-glucosidase/g of glucan; LB medium (0.5% yeast extract, 1% tryptone); anaerobic condition: 38°C, pH 7.0, 150 rpm; n = 3 for LLARP-treated corn stover and α-cellulose and n = 2 for LLARP-treated corn stover + hydrolysate. The data indicate the mean values (n = 3, SD ≤ 3.4% for treated corn stover + hydrolysate; n = 3, SD ≤3.0% for α-cellulose; n = 3, SD ≤ 4.6% for treated corn stover).

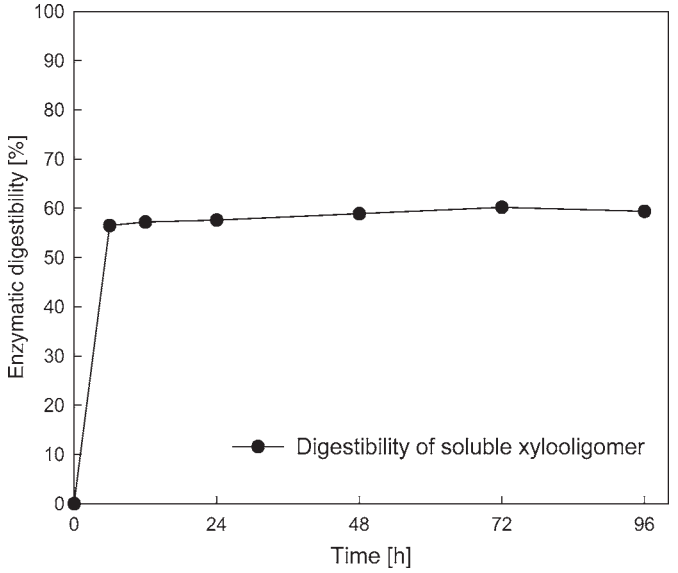

Fig. 7. Enzymatic digestibility of soluble xylooligomers in LLARP hydrolysate. Reaction conditions were 170°C, 3.3 mL of 15 wt% NH_3 throughput/g of corn stover, and 5.0 mL/min flow rate. Enzymatic hydrolysis conditions were 72 h, 60 FPU/g of xylooligomer, pH 4.8, 50°C, and 150 rpm. To treat the hydrolysate for lignin precipitation the pH was adjusted to 1.8 for >72 h and then it was centrifuged. The data indicate the mean values (SD ≤ 2.5%; n = 2).

previous digestibility test for xylan in the solid sample, about 78% of digestibility was achieved by cellulase (Table 3). The soluble xylooligomers were no better substrate than the xylan in solid. We speculate that the limited hydrolysis has to do with the structure of the oligomers, wherein a part of it is not hydrolyzed by the xylanase contained in the Spezyme CP. The low enzymatic digestibility of soluble xylooligomers plays a large role in lowering the overall ethanol yield in SSCF.

Conclusion

Ammonia throughput in the ARP could be reduced to the level of 3.3 mL/g of solid without adversely affecting the enzymatic digestibility or the SSF conversion efficiency of corn stover. The amount of liquid throughput was such that about one reactor void-volume was passed through the packed-bed reactor for the entire operation of the percolation reactor. The LLARP also reduced the reaction time and the process energy. The short residence time of the liquid required in this process (3.3 mL of 15 wt% ammonia/g of corn stover over 10 min) makes it feasible to construct a continuous process. The LLARP removed 70% of the original lignin and 47% of xylan under the optimum conditions (170°C, 10-min reaction time, and 3.3 mL of 15 wt% ammonia/g of corn stover). The glucan contents, however, remained intact.

The enzymatic digestibility generally increased with reaction temperature and ammonia concentration within the range of the LLARP applied. The digestibilities of corn stover treated under optimum conditions were 95, 90.1, and 86% with enzyme loadings of 60, 15, and 7.5 FPU/g of glucan, respectively. The residual xylan in the treated samples was also hydrolyzed by the "cellulase" (Spezyme CP), attaining 86, 78, and 71% of digestibilities with the same enzyme loadings.

In the standard SSF test (3% [w/v] glucan loading) using yeast, the ethanol yield from the LLARP-treated corn stover reached 85% of the theoretical maximum at 96 h. About the same level of ethanol yield (84%) was obtained with the use of higher glucan loading (6% [w/v]). In the SSCF test using recombinant *E. coli* (KO11), both the glucan and xylan in the treated solid were effectively converted into ethanol, giving 89% (19.4 g/L) of the theoretical maximum based on total glucan and xylan contents in the treated corn stover. The addition of the pretreatment reactor effluent containing xylooligomers into the bioreactor significantly hindered the bioprocess, lowering the enzymatic digestibility to 60% and ethanol yield of SSCF to 56%. The contaminants in the ARP liquor appear to inhibit the enzymatic reaction as well as the microorganism activity in SSCF.

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