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Pretreatment of Corn Stover by Soaking in Aqueous Ammonia

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Abstract

Soaking in aqueous ammonia (SAA) was investigated as a pretreatment method for corn stover. In this method, the feedstock was soaked in aqueous ammonia over an extended period (10–60 d) at room temperature. It was done without agitation at atmospheric pressure. SAA treatment removed 55–74% of the lignin, but retained nearly 100% of the glucan and 85% of the xylan. The xylan remaining in the corn stover after SAA treatment was hydrolyzed along with the glucan by xylanase present in the Spezyme CP enzyme. In the simultaneous saccharification and fermentation (SSF) test of SAA-treated corn stover, using *S. cerevisiae* (D_5A), an ethanol yield of 73% of theoretical maximum was obtained on the basis of the glucan content in the treated corn stover. The accumulation of xylose in the SSF appears to inhibit the cellulase activity on glucan hydrolysis, which limits the yield of ethanol. In the simultaneous saccharification and co-fermentation (SSCF) test, using recombinant *E. coli* (KO11), both the glucan and xylose were effectively utilized, resulting in on overall ethanol yield of 77% based on the glucan and xylan content of the substrate. When the SSCF process is used, the fact that the xylan fraction is retained during pretreatment is a desirable feature since the overall bioconversion can be carried out in a single step without separate recovery of xylose from the pretreatment liquid.

Index Entries: Corn stover; pretreatment; soaking; ammonia; simultaneous saccharification and fermentation (SSF); SSCF.

Introduction

Most pretreatment methods designed to improve enzymatic digestibility generate hydrolysates containing a mixture of sugars and lignin. Soluble lignin present in the pretreatment liquid is known to inhibit the enzymatic hydrolysis and bioconversion processes *(1–6)*. Hydrolysates of common pretreatment processes also contain various other toxic components that create inhibitory environment in which microorganisms cannot

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sustain their viability required for efficient bioconversion *(7–13)*. In order to utilize these soluble sugars, the contaminated hydrolyzates must be cleaned and detoxified before they are subjected to bioconversion. This is an untested troublesome unit process and undoubtedly a significant cost factor. The underlying reason for this is that many of the pretreatment methods developed to this point are carried out under acidic and/or high-temperature conditions severe enough to make the biomass susceptible to enzymatic hydrolysis.

We have investigated a pretreatment method based on the use of aqueous ammonia *(14–16)*. In this method, aqueous ammonia is used in a flow-through reactor at relatively high temperatures (160–180°C). While this process results in a high degree of delignification, it also requires high-energy input because of the high temperature and relatively high liquid throughput. In this process, about half of the xylan is removed along with the lignin, which complicates xylan recovery in the downstream processing. The primary intent of this work was to seek an alternative pretreatment process that can alleviate these problems. The approach we have taken is to apply reaction conditions mild enough to prevent formation of toxic byproducts. One approach that might be feasible is to use a low-temperature alkaline treatment. To this end, a room-temperature treatment with aqueous ammonia was attempted because of the reduced heat input needed during the treatment phase and also to reduce the interaction of ammonia with the hemicellulose component of the substrate. Retention of xylan is a desirable factor in pretreatment because it can usually be hydrolyzed by the xylanase activity normally present in most commercial "cellulase" mixture. Room-temperature treatments with various alkaline reagents have previously been attempted for pretreatment of lignocellulosic biomass with varying degree of success *(17–19)*.

In this study, a pretreatment method based on aqueous ammonia with a longer reaction time at room temperature was tested. It was carried out in a closed vessel without agitation under atmospheric pressure. It is thus termed as soaking in aqueous ammonia (SAA) process. The focus of this work is to evaluate the overall effectiveness of the SAA as an alternative pretreatment process. The effects of reaction parameters on the composition and the digestibility of the remaining glucan and xylan were investigated. The reaction parameters of interest were solid-to-liquid ratio, reaction time, and ammonia concentration. As a method of evaluation for the SAA, the simultaneous saccharification and fermentation (SSF) was included. In the case of the SAA, utilization of xylan fraction is important because most of it is retained in the solid after treatment. A proper test of the SAA should therefore include a bioconversion process where both the glucan and xylan are utilized. As such we have assessed, a simultaneous saccharification and co-fermentation (SSCF) using recombinant *Escherichia coli* (KO11), one of the most effective ethanologenic microorganisms currently available for the fermentation of mixed sugars (*9,21*).

Materials and Methods

Materials

Air-dried ground corn stover was supplied by 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 (cat. no. C-8200, lot no. 11K0246).

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

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

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

Experimental Setup and Operation

Corn stover was treated with 29.5 wt.% of aqueous ammonia in screw-capped laboratory bottles at room temperature for 1–60 d. Solid-to-liquid ratios ranging from 1:2 to 1:15 were applied. After soaking, the solids were separated by filtering, washed with DI water until its pH was around 7.0, and subjected to the enzymatic digestibility tests. Klason lignin, carbohydrate content, and digestibility were determined by NREL Chemical Analysis and Testing Standard Procedure *(22).*

Digestibility Test

The enzymatic digestibility of corn stover was determined in duplicate according to the NREL Chemical Analysis and Testing Standard Procedure *(22)*. The conditions of the enzymatic digestibility tests are 50°C and pH 4.8 (0.05 *M* sodium citrate buffer) on a shaker bath agitated at 150 rpm. Enzyme loadings of 15 and 60 FPU of Spezyme CP/g-glucan supplemented with 30 CBU of β-glycosidase (Novozyme 188)/g-glucan were used. The initial glucan concentration was 1% (w/v) based in 100 mL of total liquid. The 250 mL screw-capped Erlenmeyer flasks containing the enzyme hydrolysis preparations were placed in an incubator shaker

(New Brunswick Scientific, Innova-4080). Samples were taken periodically and analyzed for glucose, xylose, and cellobiose content using HPLC. Total released glucose after 72 h of hydrolysis was used to calculate the enzymatic digestibility. α-Cellulose and untreated corn stover were put through the same procedure as a reference and control, respectively.

Simultaneous Saccharification and Fermentation (SSF)/Co-Fermentation (SSCF)

A 250 mL Erlenmeyer flask was used as the bioreactor. It was shaken in the incubator shaker (New Brunswick Scientific, Innova-4080) at 38°C with 150 rpm. Into a 100 mL working volume of liquid, treated corn stover samples were added and achieve 3% w/v glucan content. α-Cellulose was put through the same procedure as the control. The SSF/SSCF runs were performed with buffer without external pH control, starting at pH 5.0/7.0 at the beginning of the fermentation and gradually decreasing to pH 4.5/6.0 at the end. The loading of cellulase enzyme (Spezyme CP) was 15 FPU/g-glucan, and that of β-glucosidase (Novozyme 188) was 30 CBU/g-glucan.

The ethanol yield in SSF/SSCF test was calculated as follows:

$$\text{Theoretical maximum ethanol yield}(\%) = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial sugar (g) in reactor} \times 0.511} \times 100$$

Note. Sugar is interpreted as glucose in the SSF results or glucose plus xylose in the SSCF work.

Analytical Methods

The solid samples, such as treated/untreated corn stover, α-cellulose, etc., were analyzed for sugar and Klason lignin following NREL Chemical Analysis and Testing Standard Procedures *(22)*. Each sample was analyzed in duplicate. Sugars were determined by HPLC using a Bio-Rad Aminex HPX-87P column. For the SSF/SSCF tests, an HPX-87P and 87H column were used to measure the sugar content and ethanol, respectively. An YSI 2300 Glucose/Lactate analyzer was used for rapid analysis of glucose during inoculum preparation. A refractive index detector was used for HPLC analysis.

Results and Discussion

Compositional Changes and Enzymatic Digestibility

Figure 1 summarizes the change of composition with soaking time. The pretreatment conditions were 1:12 of solid-to-liquid ratio, 1–60 d, room temperature, and 29.5 wt% ammonia. The major composition change was in lignin. Approximately half of the lignin was removed within 4 d (Fig. 1).

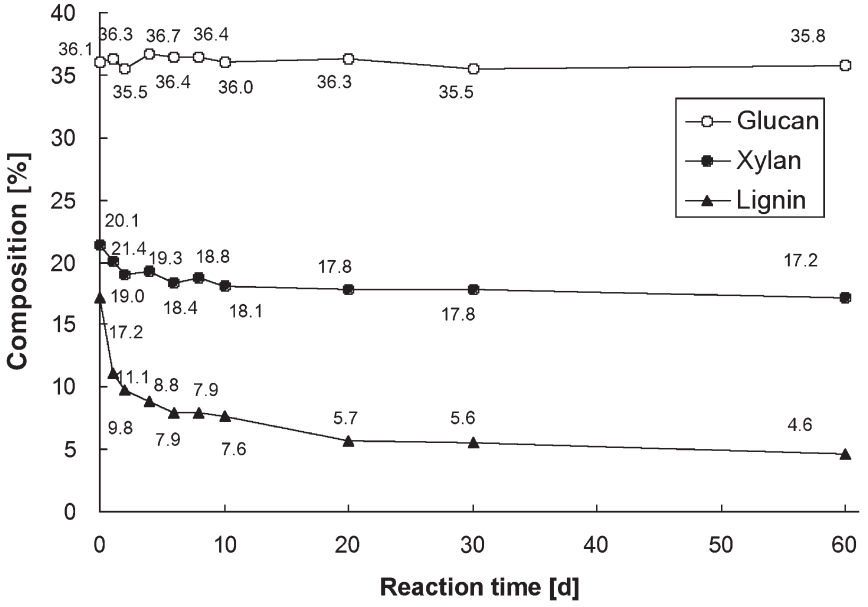

Fig. 1. Variation of solid composition with soaking time. All sugar and lignin content based on the oven-dry untreated biomass. The data in the figure show the mean value ($n = 2$; SD<0.4 for K-lignin, SD<0.3 for glucan and xylan, SD: standard deviation).

Delignification reached 55.8% after 10 d and 73.5% after 60 d. Xylan dissolution was 10% after 4 d, 11.3% after 10 d, and 13% after 60 d. The glucan content was well preserved showing no significant changes over the entire treatment period.

The enzymatic digestibilities of the SAA-treated corn stover for various soaking times and solid-to-liquid ratios are shown in Fig. 2. The data indicate that delignification and digestibility increase as the treatment time increases. However, the increase beyond 10 d was relatively insignificant. The 72-h digestibilities of the samples treated for 10–60 d were 92–97% (not shown in the figure) with an enzyme loading of 60 FPU/g glucan. With 15 FPU/g glucan, the digestibilities were considerably lower, being in the range of 86–89%. The 10-d SAA-treated corn stover showed digestibility of 86.3%, whereas the 1-d and 4-d sample showed 65% and 79% with 15 FPU/g-glucan, respectively. Increasing the soaking time beyond 10 days had no significant effect on the digestibility (Fig. 2A).

The enzymatic digestibilities as affected by solid-to-liquid ratios are summarized in Fig. 2B. The enzymatic digestibility of the 1:4 sample is 86.1% with 15 FPU/g glucan after 60 d soaking. Digestibility of this sample is equivalent to that of the 1:12 10-d sample. Data in this figure indicate that the solid-to-liquid ratio can be reduced to 1:4 with no significant adverse effects on the digestibility.

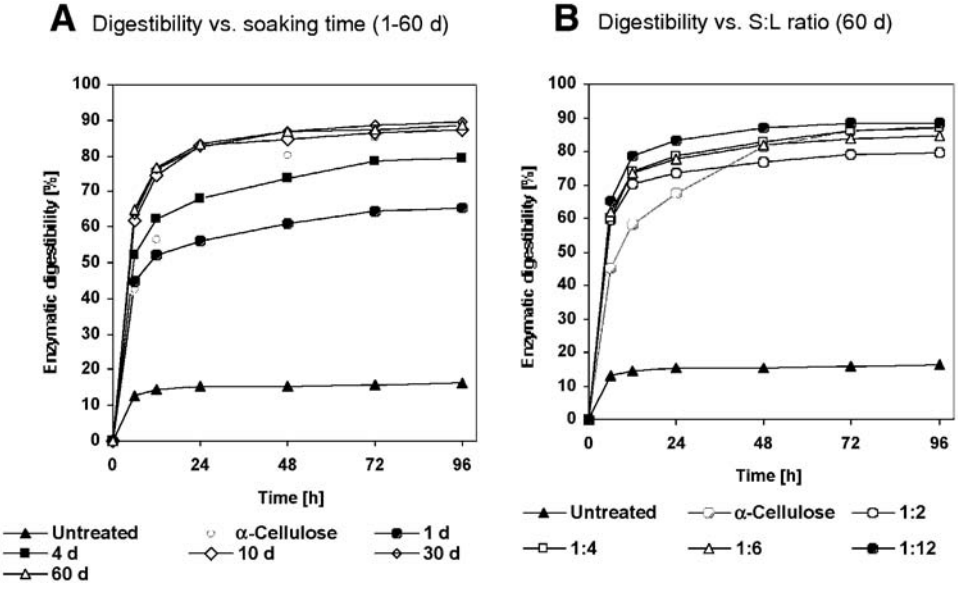

Fig. 2. Effect of soaking time and solid-to-liquid ratio on digestibility: (A) solid: liquid ratio=1:12, 1–60 d treatment; (B) solid:liquid ratio=1:2–1:12, 60 d of treatment. The data in the figure show the mean value (n=2; standard deviation<2.5).

The enzymatic digestibility results for xylan in SAA-treated corn stover (solid:liquid = 1:12) are shown in Fig. 3. The xylan digestibility was in the range of 84–85% with 60 FPU/g glucan and 72–75% with 15 FPU/g glucan for the samples treated for 10 d. As seen in this figure, there was no improvement in xylan digestibility with increase of the soaking time beyond 10 d. The observed xylan digestibilities of 72–75% correspond to 86–89% for glucan digestibilities. The xylanase activity in Spezyme CP, while it is quite substantial, does not match the cellulase activity on glucan.

Tests with varying L/S ratios indicate that lignin removal and enzymatic digestibility increased slightly when the L/S ratio was increased from 8 to 15 (Fig. 4). Delignification increased from 54 to 59%, and digestibility with 15 FPU/g glucan increased from 85 to 87%. Xylan content decreased only by 1%. This indicates that the presence of excess ammonia does not increase digestibility, nor affect xylan removal significantly.

Simultaneous Saccharification and Fermentation/Co-Fermentation of SAA-Treated Corn Stover

Simultaneous saccharification and fermentation (SSF) of SAA-treated corn stover and α-cellulose was performed using *Saccharomyces cerevisiae* ATCC® 200062 (NREL-D_5A). The samples treated for 10 d with 1:8 of solid:liquid ratio were used. The ethanol and glucose data for SAA-treated corn stover and α-cellulose are presented in Fig. 5. The theoretical maximum

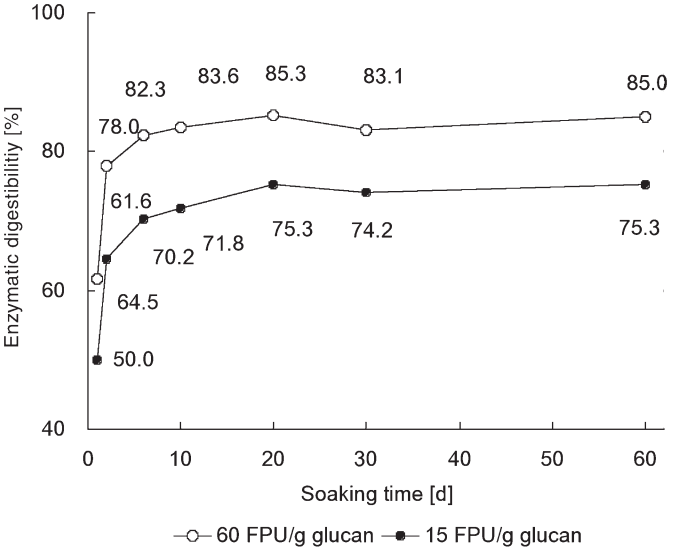

Fig. 3. Effect of soaking time on digestibility of xylan. Solid-to-liquid ratio is 1:12 (based on wt.). The data in the figure show the mean value ($n = 2$; standard deviation < 2.5).

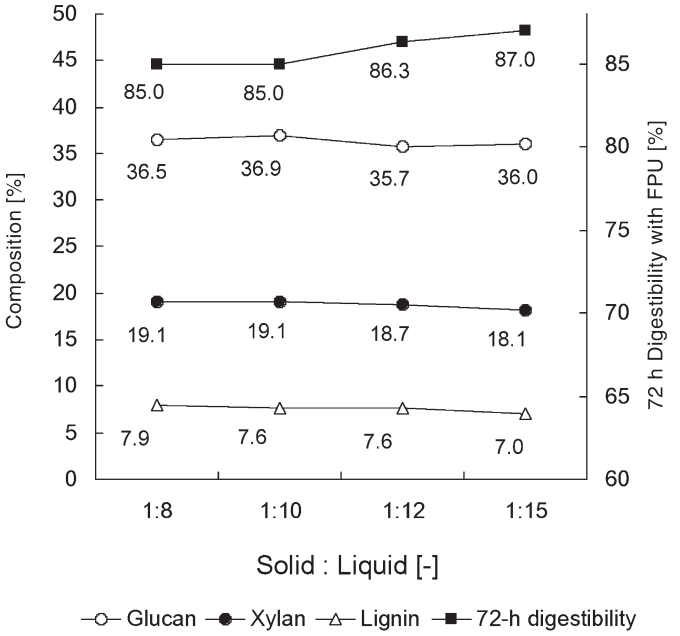

Fig. 4. Effect of solid-to-liquid ratio on solid composition and digestibility. Reaction time: 10 d. All sugar and lignin content based on the oven-dry untreated biomass. The data in the figure show the mean value ($n = 2$; SD<0.5 for K-lignin, SD < 0.2 for glucan and xylan, SD < 2.5 for digestibilities. SD: standard deviation).

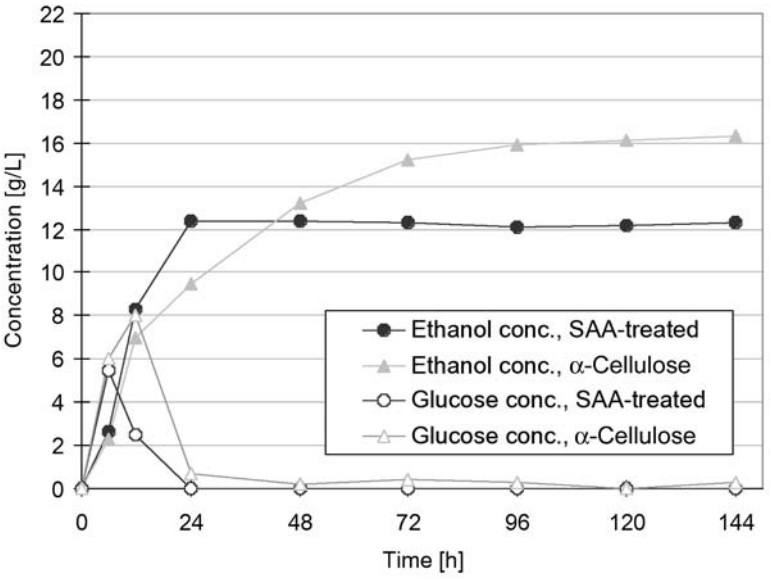

Fig. 5. Simultaneous saccharification and fermentation (SSF) of SAA-treated corn stover by D_5A yeast. 1:8 of solid-to-liquid ratio and 10 d SAA-treated corn stover. The data in the figure show the mean value ($n = 3$; standard deviation < 0.7).

ethanol yield (100%) corresponds to 17.0 g/L with 3% w/v glucan loading. At the 24 h point, the ethanol yield of the SAA-treated sample reached 73% (12.4 g/L) of the theoretical maximum. At this point, the ethanol yield from the treated corn stover is substantially higher than that from α-cellulose (56%, 9.5 g/L).

Table 1 summarizes the compositional changes after the SAA treatment (10 d, 29.5 wt% ammonia; solid:liquid =1:10) and the ammonia treatment at high temperature and high pressure (low-liquid ARP 170°C, 2.5 MPa, 3.3 mL of 15 wt% NH3 per g of corn stover).

The major difference in the compositions is for lignin and xylan. In order to verify the possibility of interaction between released lignin/sugars and ethanol during the reaction, the enzymatic digestibility tests were repeated with addition of 5% v/v of ethanol to the two differently treated samples (see the data in Table 1). However, the digestibilities and ethanol concentrations were unaffected in this test.

According to the digestibility test, 72-h digestibility (85%) of SAA-treated corn stover was slightly lower than that of the ARP-treated corn stover (90%). The xylan content of 18.7% in the SAA-treated corn stover is much higher than 9.9% of the ARP-treated corn stover. This translates to 85% xylan retention for SSA treatment compared to 48% for ARP. The digestibility test using α-cellulose was performed to verify the effect of xylose on the enzyme activity (Fig. 6). One reactor contained 3% of glucan as a control, while the other reactor contained 3% glucan plus 3% of xylose.

Table 1
Compositions of Two Different Pretreatment Methods.

Compositions	Unit	Untreated	Low-liquid ARP	Soaking in aqueous ammonia
S.R.	[%]	100	57.5 (0.6)	75.9 (2.3)
Glucan	[%]	36.1	36.4 (0.5)	36.6 (0.3)
Xylan	[%]	21.4	9.9 (0.4)	18.7 (0.7)
Other sugar	[%]	7.8	1.3 (0.2)	2.5 (0.8)
Acetyl group	[%]	2.2	0.3(0.0)	0.4(0.0)
Klason lignin	[%]	17.2	5.1 (0.0)	7.5 (0.4)
Enzymatic digestibility	[%]	15.1	90.0	85.0

[a] S.R.: solid remaining % based on untreated corn stover.
[b] All data in table are based on oven-dry original untreated corn stover.
[c] Values are expressed as mean and standard deviation (n=3 for low-liquid ARP, n=5 for soaking in aqueous ammonia).

In Fig. 6, the 72 h-digestibility decreased by 12% after supplementation with 3% of xylose. This result indicated that xylose has a direct inhibitory effect on the glucan hydrolysis by cellulase enzymes, a finding that agrees with previous studies *(23–25)*. We speculate that the low ethanol yield observed from the SSF of SAA-treated sample was due to cellulase inhibition by xylose. Xylose accumulation during the SSF is shown in Fig. 7.

Simultaneous saccharification and co-fermentation (SSCF) of SAA-treated corn stover (10 d and 1:8 of solid:liquid ratio) and α-cellulose was performed using the recombinant *E. coli* ATCC® 55124. The main advantage of the SSCF is that microorganisms can utilize hexose and pentose concurrently in a single reactor.

Xylose and glucose concentration profiles of SSF and SSCF are presented in Fig. 7. In the early phase of SSF and SSCF (Fig. 7), where the cells are growing, glucose accumulation was noticeable (Fig. 7). After 24 h, however, glucose was undetectable in both of the SSF and SSCF tests, indicating that the process proceeds under glucose-limited conditions. The SSF/SSCF process is therefore controlled by the hydrolysis reaction rather than the microbial action. The advantage of the SSF/SSCF that it eliminates the glucose inhibition on cellulase enzyme is therefore reaffirmed here. It is interesting to note the contrasting xylose profiles between SSF and SSCF: increasing buildup of xylose throughout the SSF and initial buildup and depletion of xylose in the SSCF (Fig. 7). This reflects the difference between the two different microorganisms. When fermenting a treated corn stover by SSCF, the microorganism used glucose preferentially, followed by xylose (Figs. 7 and 8) when they both exist in appreciable

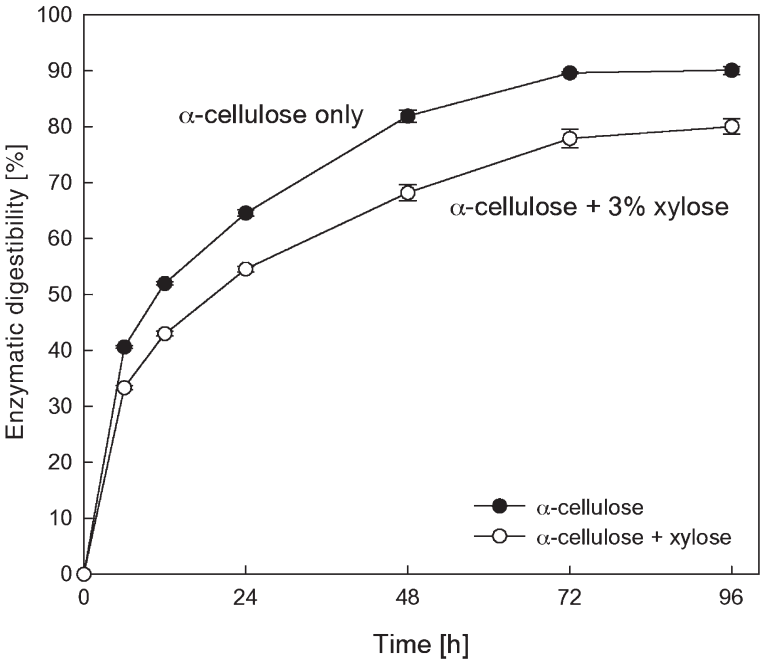

Fig. 6. Xylose inhibition on enzyme activity in the cellulose hydrolysis: Substrate: α-cellulose. The data in the figure show the mean value (n = 2).

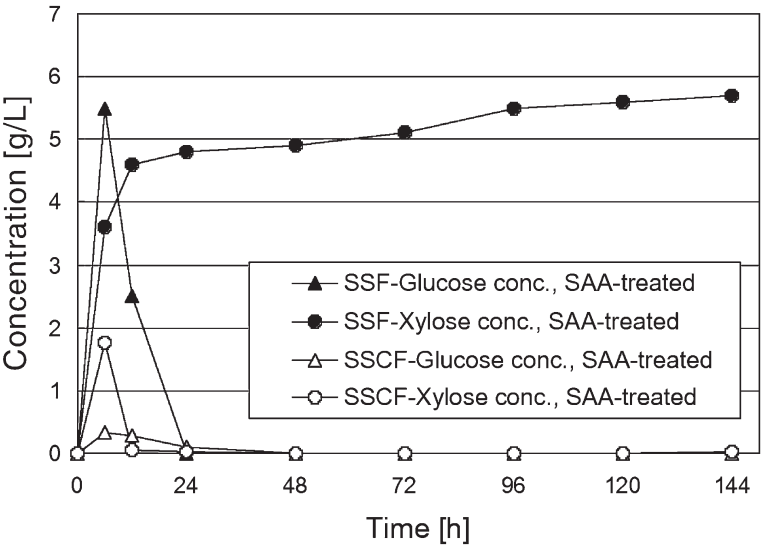

Fig. 7. Xylose accumulation and consumption in SSF/SSCF. The data in the figure show the mean value (n = 3; standard deviation < 0.5).

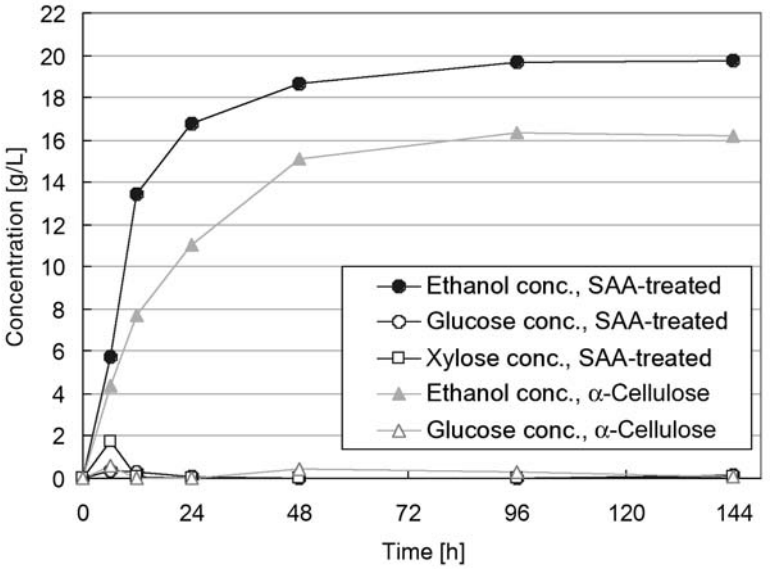

Fig. 8. Simultaneous saccharification and co-fermentation (SSCF) of SAA-treated corn stover by recombinant *E coli* (KO11): substrate: 1:8 of solid-to-liquid ratio and 10 d SAA-treated corn stover. The data in the figure show the mean value (n=3; standard deviation <0.8).

quantities. This agrees with a previous study that the recombinant *E. coli* does not metabolize xylose until the glucose is completely consumed (*20*). However, the sugar profiles in Fig. 7 indicate that when the SSCF proceeds under sugar-limited condition where the glucose and xylose concentrations are extremely low, the recombinant *E. coli* consumes both sugars concurrently.

Figure 8 presents the ethanol and sugar profiles in the SSCF. The SAA-treated corn stover had a composition of 49% of glucan and 24% of xylan. With 3% w/v glucan loading, the total carbohydrate concentration in the SSCF reactor is 44.7 g/L (30 g glucan + 14.7 g xylan). The maximum observed ethanol yield in the SSCF of SAA treated corn stover was 77% (of the theoretical maximum) and the corresponding ethanol concentrate was 19.8 g/L at 96 h. The yield of ethanol on the basis of glucan content alone was 116% of theoretical maximum, which proves that the xylan fraction was utilized in the SSCF by KO11. For comparison purpose, a separate SSCF test was conducted for α-cellulose under identical experimental conditions including the glucan loading. As shown in Fig. 8, the SSCF of pretreated corn stover gave higher overall ethanol yield and the conversion process was faster. In a spot check at 24 point, we found that the ethanol concentration of the SAA-treated corn stover reached 16.8 g/L, substantially higher than 11.0 g/L observed for α-cellulose. The results of the SSCF experiments indicate that it is a process technically feasible for bioconversion of

corn stover to ethanol. The main advantages of this process are that it is done in a single step and that both glucan and xylan are utilized. It also reveals that there are rooms for further improvements especially in ethanol yield and concentration.

Conclusions

SAA at room temperature is a pretreatment method technically feasible for corn stover. The process is simple, less capital intensive, and retains a higher fraction of xylan than the high-temperature process (ARP). Consumption of ammonia is expected to be low due to reduced acetate generation.

The major effects of SAA at room temperature are swelling of cellulose and delignification, both factors contributing to improved digestibility. SAA treatment beyond 10 d has only a marginal effect on delignification and digestibility. However, it is beneficial in reducing the solid-to-liquid ratio.

The ethanol yield for the SAA treated corn stover in the SSF test using *S. cerevisiae* (D_5A) was 73% of theoretical maximum. The unused xylose appears to inhibit the cellulase activity. In the SSCF using recombinant *E. coli* (KO11), xylose was effectively utilized. The ethanol yield reached 77% of the theoretical maximum (116% based on glucose alone).

Acknowledgments

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References

1. Chang, V.S. and Holtzapple, M.T. (2000) *Appl. Biochem. Biotechnol.* **84/86,** 5–37.
2. Cowling, E.B. and Kirk, T.K. (1976) *Biotechnol. Bioeng. Symp.* **6,** 95–123.
3. Dulap, C.E., Thomson, J., and Chiang, L.C. (1976) *AIChE. Symp. Ser.* **72, 158,** 58.
4. Lee, D., Yu, A.H.C., and Saddler, J.N. (1995) *Biotechnol. Bioeng.* **45**, 328–336.
5. Mooney, C.A., Mansfield, S.D., Touhy, M.G., and Saddler, J.N. (1998) *Bioresou. Technol.* **64**, 113–119.
6. Schwald, W., Brownell, H.H., and Saddler, J.N. (1988) *J. Wood Chem. Tech.* **8,** 543–560.
7. Björling, T. and Lindman, B. (1989) *Enzyme Microb. Technol.* **11,** 240–246.
8. Fein, J.E., Tallim, S.R., and Lawford, G.R. (2004) *Can. J. Microbiol.* **30,** 682–690.
9. Hahn-Hägerdal, B., Jeppsson, H., Olsson, L., and Mohagheghi, A. (1994) *Appl. Microbiol. Biotechnol.* **41,** 62–72.
10. Sanchez, B. and Bautista, J. (1988) *Enzyme Microb. Technol.* **10,** 315–318.
11. Tran, A.V. and Chambers, R.P. (1986) *Enzyme Microb. Technol.* **8,** 439–444.
12. Van Zyl, C., Prior, B.A., and du Preez, J.C. (1991) *Enzyme Microb. Technol.* **13,** 82–86.
13. Watson, N.E., Prior, B.A., Lategan, P.M., and Lussi, M. (1984) *Enzyme Microb. Technol.* **6,** 451–456.
14. Kim, T.H., Kim, J.S., Sunwoo, C., and Lee, Y.Y. (2003) *Bioresou. Technol.* **90,** 39–47.
15. Iyer, P.V., Wu, Z.W., Kim, S.B., and Lee, Y.Y. (1996) *Appl. Biochem. Biotechnol.* **57/58,** 121–132.

16. Kim, S.B. and Lee, Y.Y. (1996) *Appl. Biochem. Biotechnol.* **57/58,** 147–156.
17. Morris, P.J. and Mowat, D.N. (1980) *Can. J. Animal Sci.* **60,** 327–336.
18. Oji, U.I., Mowat, D.N., and Winch, J.E. (1977) *J. Animal Sci.* **44,** 798–802.
19. Streeter, C.L. and Horn, G.W. (1982) *Animal Feed Sci. Technol.* **7**, 325–329.
20. Dien, B.S., Hespell, R.B., Wyckoff, H.A., and Bothast, R.J. (1998) *Enzyme Microb. Technol.* **23,** 366–371.
21. Ohta, K., Beall, D.S., Mejia, J.P., Shanmugam, K.T., and Ingram, L.O. (2004) *Appl. Environ. Microbiol.* **57,** 893–900.
22. NREL (1996) *Chemical Analysis and Testing Laboratory Analytical Procedures (CAT)*, National Renewable Energy Laboratory, Golden, CO.,
23. Xial, Z., Zhang, X., Gregg, D.J., and Saddler, J.N. (2004) *Appl. Biochem. Biotechnol.* **113/116,** 1115–1126.
24. Nigam, P. and Prabhu, K.A. (1991) *J. Basic Microb*. **31,** 279–283.
25. Todorovic, R. and Grujic, S. (1987) *Microbios Lett.*, **34,** 71–78.