0273-2289/01/90/0251/$12.25

Pretreatment with Ammonia Water for Enzymatic Hydrolysis of Corn Husk, Bagasse, and Switchgrass

Masahiro Kurakake,* Wataru Kisaka, Kazuya Ouchi, and Toshiaki Komaki

Department of Food Science and Technology, Fukuyama University, Sanzou, Gakuenchou 1 banchi, Fukuyama, Hiroshima 729-0292, Japan, E-mail: kurakake@fubac.fukuyama_u.ac.jp

Received January 19, 1999; Revised June 3, 1999; Accepted June 9, 1999

Abstract

Bagasse, corn husk, and switchgrass were pretreated with ammonia water to enhance enzymatic hydrolysis. The sample (2 g) was mixed with 1–6 mL ammonia water (25–28% ammonia) and autoclaved at 120°C for 20 min. After treatment, the product was vacuum-dried to remove ammonia gas. The dried solid could be used immediately in the enzymatic hydrolysis without washing. The enzymatic hydrolysis was effectively improved with more than 0.5 and 1 mL ammonia water/g for corn husk and bagasse, respectively. In bagasse, glucose, xylose, and xylobiose were the main products. The adsorption of CMCase and xylanase was related to the initial rate of enzymatic hydrolysis. In corn husks, arabinoxylan extracted by pretreatment was substantially unhydrolyzed because of the high ratio of arabinose to xylose (0.6). The carbohydrate yields from cellulose and hemicellulose were 72.9% and 82.4% in bagasse, and 86.2% and 91.9% in corn husk, respectively. The ammonia/water pretreatment also benefited from switchgrass (*Miscanthus sinensis* and *Solidago altissima* L.) hydrolysis.

Index Entries: Corn husk; bagasse; switchgrass; pretreatment; ammonia water; cellulase.

Introduction

Corn husks and sugarcane bagasse are well known agricultural and industrial wastes. Carbohydrates in their biomass are a renewable resource that may be used to produce ethanol. The enzymatic hydrolysis of carbo-

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

hydrate polymers in lignocellulosic materials requires pretreatment to improve enzyme accessibility, decrease the lignin content, and decrease cellulose crystallinity. These two lignocelluloses have advantages compared to wood, because of their soft structure, low lignin content, and low cellulose crystallinity. Glucose and xylose are the main monosaccharides released from the lignocellulose and can be fermented to ethanol by yeast *(1,2)*. Alternatively, xylose can be converted to the sweetener xylitol *(3,4)*.

As a pretreatment, chemical methods using acid and alkaline reagents are considered industrially economical *(5–10)*. In this study, we proposed pretreatment with ammonia/water. Alkaline reagents like ammonia solubilize lignin and hemicellulose. Although many chemical pretreatments have the problem of chemical recovery and neutralization, the removal and recovery of ammonia is relatively easy. In our treatment, lignocellulose is heated as a wet solid containing ammonia water, and then ammonia was effectively removed by vacuum-drying. The pretreated sample was immediately enzymatically hydrolyzed without washing. In this study, the ammonia pretreatment was used for corn husks, bagasse, and two varieties of switchgrass (*Miscanthus sinensis* and *Solidago altissima* L.) that are widely distributed in Japan. The pretreatment effect was determined based on lignin removal, sugar composition released, and sugar recovery.

Materials and Methods

Materials

Corn husk (finer than 80 mesh) and bagasse (finer than 36 mesh) were obtained from Sanwa Cornstarches LTD and Ajinomoto LTD, respectively. The starch in the corn husks was completely removed by enzymatic digestion with 400 U α-amylase and 170 U glycoamylase per gram of husk (wet material). The starch hydrolysis was done at 60°C for 24 h in 0.1 *M* acetic acid buffer (pH 6.0) including with 1.1 m*M* calcium acetate and 3.4 m*M* sodium chloride. Alcohol precipitate from commercial cellulase (Meicelase; *Trichoderma viride* origin, Meijiseika LTD.) was used to hydrolyze the pretreated lignocellulose. The specific activity was 6000 U CMCase and 4700 U xylanase per gram of the enzyme preparation. Xylobiose and xylotriose for high-pressure liquid-chromatography (HPLC) analysis were purchased from Wako Pure Chemical Industries LTD. All other chemicals were reagent grade.

Determination of Carbohydrates and Klason Lignin Contents in Lignocellulose

Analytical methods of carbohydrates and Klason lignin contents were based on NREL standard procedures 002 and 003, respectively. In carbohydrates, lignocellulose sample was incubated at 30°C with 72% sulfuric acid for 2 h and then autoclaved at 120°C for 1 h after diluting the acid to 4% with deionized water. The mixture was neutralized with calcium car-

bonate and filtered. The released sugars were analyzed by HPLC. In Klason lignin, lignocellulose sample was incubated at 20°C with 72% sulfuric acid for 2 h and then boiled for 4 h after diluting the acid to 3% with deionized water. The residue was filtered, dried at 100°C for 2 h, and weighed.

Pretreatment with Ammonia Water

The sample (2 g) and 0.25–0.28 g NH_3/g solution (1–6 mL) were mixed in 25-mL vial and capped. The vessel was autoclaved at 120°C for 20 min. The temperature was raised to 120°C from 100°C at 2.5°C/min. After the incubation at 120°C for 20 min, it was cooled down to 100°C at the rate of 1.4°C/min. The heated mixture was vacuum-dried after air-drying in a hood to remove ammonia. The dried solid was used in the enzymatic hydrolysis.

Enzymatic Hydrolysis

Fifty milligrams of pretreated sample and 1.1 mL of 1.8 mg Meicelase/mL (10.8 U CMCase/mL and 8.5 U xylanase/mL) were incubated at pH 5.0 and 40°C for 24 h. After centrifugation at 1500*g* for 10 min, the supernatant was analyzed for sugar by HPLC.

Treatment with Trifluoroacetic Acid (TFA)

TFA treatment was carried out to determine the amount of soluble high-arabinose arabinoxylan of corn husk, which enzymes cannot hydrolyze. Enzymatically unhydrolyzed arabinoxylan was hydrolyzed completely to arabinose and xylose by TFA and the sugars were analyzed by HPLC. After enzymatically hydrolyzing the pretreated corn husk, 0.2 mL of supernatant from the reaction mixture was mixed with 0.02 mL TFA in a 10-mL test tube. The capped tube was incubated at 100°C for 2 h. Then the mixture was freeze-dried and dissolved with 1 mL water for HPLC analysis.

Adsorption of CMCase and Xylanase on the Pretreated Lignocellulose

Sample (50 mg sample/mL) was incubated with 2 mg Meicelase/mL (12 U CMCase/mL and 9.4 U xylanase/mL) at pH 5.0 and 40°C for 5–20 min after premixing at 0°C for 10 min. The centrifuge supernatant was analyzed for enzyme activity (E). The activity of the adsorbed enzyme (E_{ads}) was obtained by subtraction from total activity (E_t). CMCase and xylanase activities were determined using substrates of 1% CMC (carboxymethyl cellulose) and oat spelts xylan in 0.1 *M* acetic acid (pH 5.0), respectively, where the reaction was done at 40°C for 10 min. The enzyme action was stopped by adding 1 *M* sodium carbonate (0.5 mL) to the mixture (1 mL) and the concentration of reducing sugar produced was determined by 3,5-dinitrosalicylic acid *(11)*. One unit for CMCase or xylanase was defined as the amount of enzyme that produced 1 µmol of reducing sugar in 1 min.

Table 1
Composition of Lignocellulosic Materials

Lignocellulose	Cellulose (%)	Arabinoxylan (%)	Ara/Xyl (g/g)	Klason lignin (%)
Bagasse	45.0	26.0	0.073	19.6
Corn husk	21.8	43.2	0.590	10.9
Miscanthus sinensis	44.0	27.7	0.132	18.8
Solidago altissima L.	41.3	20.9	0.028	17.3

Analysis of Sugars

Enzymatic hydrolyzates were analyzed by HPLC using the following conditions: column, GLC610 (Hitachikasei LTD); mobile phase, water; flow rate, 1.0 mL/min; and detector, Hitachi model L-3300 differential refractive index monitor.

Results and Discussion

Table 1 shows the composition of the four lignocellulosic materials. Carbohydrate polymers were expressed with the anhydrous forms. HPLC analysis showed that the hemicellulose in all lignocellulosics were mostly arabinoxylan. Corn husk has much more arabinoxylan and its ratio of arabinose to xylose (Ara/Xyl) is very high. In contrast, the arabinoxylan of *Solidago altissima* L. has little arabinose.

Figure 1 shows the pretreatment effect for bagasse and corn husk as a function of ammonia water loading. The enzymatic hydrolysis was substantially improved by adding ammonia water, but the sugar composition produced during the enzymatic hydrolysis was different between bagasse and corn husk. In bagasse, the effect was almost unchanged at more than 1 mL ammonia water/g, where glucose from cellulose and xylose and xylobiose from xylan were mainly produced. On the other hand, in corn husks, glucose formation required only 0.5 mL ammonia water/g, but sugars released from arabinoxylan were not essentially improved by pretreatment. As shown in the HPLC charts in Fig. 2, the high-molecular-weight peak was detected at a 5-min retention time on gel chromatography for pretreated husk (A). In the enzymatic hydrolysis, the peak was shifted toward retention of smaller molecules; monosaccharide peaks were detected (B). When the reaction mixture was treated by TFA, the high-molecular-weight peak disappeared and the xylose and arabinose peaks appeared (C). This finding suggests that the high-molecular-weight peak is arabinoxylan and it dissolved by pretreatment with ammonia water. Because the arabinose composition of arabinoxylan extracted from corn husk is very high (the ratio of arabinose to xylose was 0.6), the xylanase attack of the xylan bone is largely depressed by the steric hindrance of arabinose side chains. However, this soluble arabinoxylan can be used

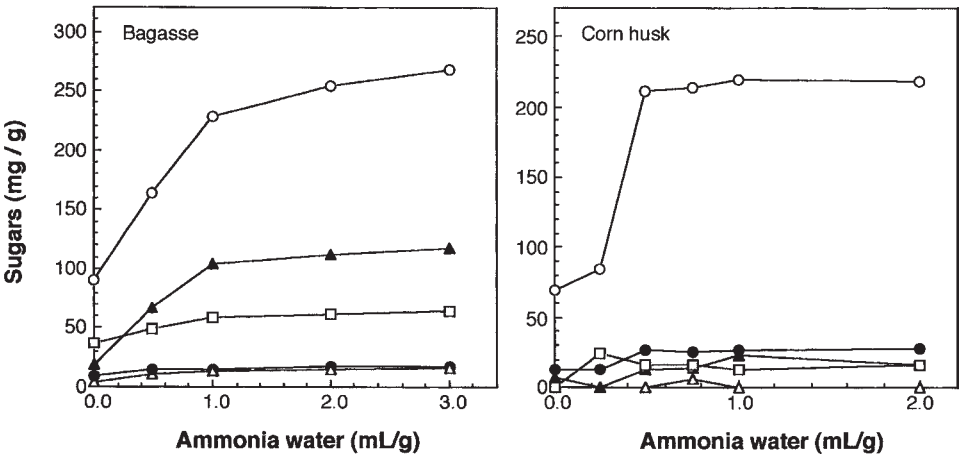

Fig. 1. Effect of pretreatment with ammonia water on enzymatic hydrolysis of bagasse and corn husk. Pretreatment: 0.5–3.0 mL/g ammonia water, 120°C, 20 min. Enzymatic hydrolysis: 45 mg substrate/mL, 1.8 mg Meicelase/mL, pH 5.0, 40°C, 24 h. Symbols: (○) glucose, (△) xylotriose, (▲) xylobiose, (□) xylose, (●) arabinose.

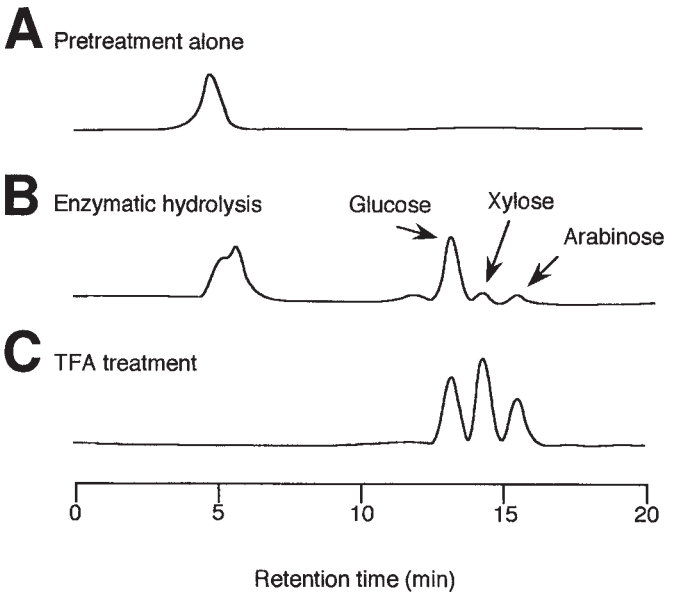

Fig. 2. HPLC charts of the reaction mixture from the pretreated corn husk. HPLC: column, GL C610; carrier, water; flow rate, 1.0 mL/min; temperature, 60°C.

to add dietary fiber in food. Table 2 presents the recovery sugars from corn husk and bagasse in pretreatment of 2 mL ammonia water/g biomass. Glucose yields of 255 mg or 218 mg were obtained from 1 g of raw bagasse or corn husk, respectively. Xylobiose and xylotriose from bagasse was 127 mg/g and soluble arabinoxylan from husk was 360 mg/g. The yields from cellulose and hemicellulose were 49.7% and 70.9% in bagasse, and

Table 2
Effect of Pretreatment with Ammonia Solution on Enzymatic Hydrolysis[a] (mg/g)

Sample	Glc	X3	X2	X1	Ara	sol. AX	Ara/Xyl (g/g)	Total
Bagasse	255	14.8	112.0	61.9	17.5	0.0	—	461
Corn husk	218	0.0	16.4	16.2	27.6	360.0	0.60	638

[a]Pretreatment: 2 mL/g ammonia water (25–28%), 120°C, 20 min.
Enzymatic hydrolysis: 45 mg substrate/mL, 1.8 mg Meicelase/mL, pH 5.0, 40°C, 24 h. Glc, glucose; X3, xylotriose; X2, xylobiose; X1, xylose; Ara, arabinose; sol. AX, soluble arabinoxylan.

Table 3
Limit of Enzymatic Hydrolysis for Pretreated Bagasse[a] (mg/g)

Run	Glc2	Glc	X3	X2	X1	Ara	Total
1 (24 h)	27.8	228.0	15.5	115.0	57.1	16.4	460.0
2 (48 h)	0.0	82.0	0.0	22.4	0.0	1.8	106.0
3 (72 h)	0.0	35.6	0.0	10.0	0.0	1.1	46.8
Total	27.8	346.0	15.5	147.0	57.1	19.3	613.0

[a]Pretreatment: 2 mL/g ammonia water (25–28%), 120°C, 20 min
Enzymatic hydrolysis: 50 mg substrate/mL, 2.0 mg Meicelase/mL, pH 5, 40°C, 24 h Glc2, cellobiose; Glc, glucose; X3, xylotriose; X2, xylobiose; X1, xylose; Ara, arabinose.

86.2% and 91.9% in corn husk, respectively. Their yields were based with the original raw materials.

Limits of the enzymatic hydrolysis for corn husk and bagasse were investigated using 2 mL ammonia water/g. As shown in Table 3, Run 1 is the first reaction for 24 h and the residual solid was hydrolyzed for 24 h again after washing by water (Run 2). The operation was repeated again (Run 3). The time in each run represents the accumulated time. The hydrolysis of corn husk reached the limit in the first reaction (Run 1; the data not shown), but that of bagasse was about 75% in the total of three Runs. Sugar yields from bagasse improved to 72.9% from cellulose and 82.4% from hemicellulose in the three runs. The solid residues in the final hydrolysis of bagasse and corn husk were 18.9% and 9.9%, respectively, in which lignin content was subtracted. This finding suggests that high sugar recovery from the pretreated bagasse could be achieved by optimizing of enzymatic hydrolysis conditions such as enzyme concentration, reaction time, and reaction temperature.

Table 4 presents the adsorption of CMCase and xylanase on pretreated corn husk and bagasse. The adsorption of each enzyme on bagasse (E_{ads}/E_t) was increased by pretreatment with ammonia water. The residue after the enzymatic hydrolysis in Table 3 did not have much affinity to enzymes. This finding means that CMCase and xylanase does not adsorb on lignin, which the dominant component in the residue. In corn husk, the xylanase adsorption was not improved by pretreatment, which is different than

Table 4
Adsorption of CMCase and Xylanase on Pretreated Bagasse and Corn Husk[a]

	Sample	E_{ads}/E_t (%)		Initial hydrolysis rate (m*M*/min)
		CMCase	Xylanase	
Bagasse	Untreated	0.00	15.5	1.30
	Pretreated	33.6	34.2	3.30
	Residue	0.00	7.77	0.00
Corn husk	Untreated	0.00	0.00	1.14
	Pretreated	40.5	0.00	4.82

[a]Pretreatment: 2 mL/g ammonia water, 120°C, 20 min.
Adsorption: 5% sample, 2 mg Meicelase/mL (12 U CMCase/mL, 9.4 U xylanase/mL), vol 1 mL, 40°C—5 min after 0°C—10 min.

Table 5
Adsorption on Bagasse Pretreated with Ammonia Water[a]

Ammonia water (mL/g)	Initial formation rate (m*M*/min)		E_{ads}/E_t (%)	
	Glucose	X3, X2, and X1	CMCase	Xylanase
Untreated	0.555	0.444	0.00	0.00
0.5	0.845	0.714	13.9	3.70
1.0	0.924	0.836	28.7	13.9
2.0	0.941	0.924	41.5	19.9
3.0	0.925	0.990	42.1	22.2

[a]Pretreatment: 2 mL/g ammonia water, 120°C, 20 min.
Adsorption: 5% sample, 2 mg Meicelase/mL (12 U CMCase/mL, 9.4 U xylanase /mL), vol 1 mL, 40°C—20 min after 0°C—10 min.

CMCase. Arabinoxylan of husk contains more arabinose in side chains, which inhibits xylanase adsorption on the xylan bone. The initial hydrolysis rate of these lignocellulosics was related to enzyme adsorption.

Table 5 presents the relationship between enzyme adsorption on bagasse and the amount of ammonia water in pretreatment. The adsorption of CMCase and xylanase in 20 min increased with increasing ammonia water and is related to the pretreatment effect. This means that the surface areas of cellulose and arabinoxylan are increased by pretreatment *(12,13)*. The effect of ammonia water is attributed to the degradation of lignin and solubilization of hemicellulose by alkaline reagent. The cellulose surface area was exposed by releasing lignin and hemicellulose.

In addition to corn husks and bagasse, two varieties of switchgrass (*Miscanthus sinensis* and *Solidago altissima* L.; Susuki and Seitakaawadachisou in Japanese, respectively) were pretreated with ammonia water and the sugar recoveries were investigated. These were selected as useful biomass, because of their large size (2–3 m) and their wide growing areas. The switchgrass was cut to small size by a blender and then ground to less

Table 6
Pretreatment of Switchgrasses with Ammonia Water[a]

Pretreatment	Glc2	Glc	X3	X2	X1	Ara	Total
Miscanthus sinensis (Susuki; mg/g)							
Untreated	0.0	60.6	0.0	0.0	34.4	2.5	97.5
Treated	27.2	245.3	15.3	119.6	63.9	22.4	493.6
Solidago altissima L. (Seitakaawadachisou; mg/g)							
Untreated	0.0	48.1	0.0	8.4	35.8	0.7	93.0
Treated	13.8	186.0	6.7	72.4	51.1	1.1	331.2

[a]Pretreatment: 2 mL/g ammonia water, 120°C, 20 min
Enzymatic hydrolysis: 50 mg substrate/mL, 2.0 mg Meicelase/mL, pH 5.0, 40°C, 24 h
Glc2, cellobiose; Glc, glucose; X3, xylotriose; X2, xylobiose; X1, xylose; Ara, arabinose.

than 0.4 mm in diameter (36 mesh) by a Cyclotec 1093 sample mill (Tecator LTD). As shown in Table 1, which indicates the arabinose ratio in arabinoxylan, *Miscanthus* has almost the same value as oat spelts xylan and bagasse, but arabinoxylan of *Solidago* has a low arabinose residue. Table 6 presents the sugar recovery from these switchgrasses pretreated with 2 mL ammonia water/g at 120°C. The primary sugars produced were glucose (from cellulose) and xylobiose (from arabinoxylan), which were similar to bagasse. The yield of total sugars from *Miscanthus* (494 mg/g) was larger than that from *Solidago* (331 mg/g). The yields from cellulose and arabinoxylan were 55.2% and 66.8 % in *Miscanthus*, and 43.7% and 54.3% in *Solidago*, respectively. To enhance the sugar yield, the enzymatic hydrolysis conditions must be investigated.

Conclusion

As a result, ammonia water pretreatment is appropriate for the enzymatic hydrolysis of lignocellulosic materials because of its easy operation, low cost, simple apparatus, high sugar recovery, and so on. Corn husk and bagasse are useful resources because of their large production rate and soft structure. Furthermore, it was found that switchgrasses could be also used as a biomass feedstock.

References

1. Puls, J., Poutanen, K., Korner, H. U., and Viikari, L. (1985), *Appl. Microbiol. Biotechnol.* **22,** 416–423.
2. Saha, B. C., Dien, B. S., and Bothast, R. J. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 115–125.
3. Alive, L. A., Filipe, M. G. A., Silva, J. B. A. E., Silva, S. S., and Prata, A. M. R. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 89–98.
4. Dominguez, J. M., Gong, C. S., and Tsao, G. T. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 49–56.

5. Lyer, P. V., Wu, Z. W., Kim, S. B., and Lee, Y. Y. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 121–132.
6. Torget, R., Hatzis, C., Hayward, T. K., Hsu, T. A., and Philippidis, G. P. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 85–101.
7. Branka, B., Costas, G. B., Donald, M., and Alexander, W. M. (1991), *J. Sci. Food Agric.* **56,** 195–214.
8. Carrasco, J. E., Navarro, M. C. S. A., Saez, P. S. F., and Martinez, J. M. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 23–34.
9. Chang, V. S., Burr, B., and Holtzapple, M. T. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 3–19.
10. Wang, L., Dale, B. E., Yurttas, L., and Goldwasser, I. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 51–66.
11. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
12. Ooshima, H., Kurakake, M., Kato, J., and Harano, Y. (1992), *Appl. Biochem. Biotechol.* **37,** 165–176.
13. Kurakake, M., Ooshima, H., Kato, J., and Harano, Y. (1994), *Bioresource Technology* **49,** 247–251.