Enzymatic Hydrolysis of Corn Stover Pretreated by Combined Dilute Alkaline Treatment and Homogenization

Y. Li, R. Ruan, P. L. Chen, Z. Liu, X. Pan, X. Lin, Y. Liu, C. K. Mok, T. Yang

Abstract. *Corn stover, the most abundant agricultural residue in the U.S., is a potential feedstock for production of bioethanol because of its high content of carbohydrates, but an efficient pretreatment is required prior to enzymatic hydrolysis. In this study, a combination of NaOH treatment and homogenization was used as a pretreatment to enhance the enzymatic hydrolysis of corn stover. The combined pretreatment increased the enzymatic hydrolysis of corn stover five times compared to the control. The effectiveness of such pretreatment was found to be a function of NaOH concentration and particle size. Within the NaOH concentration range of 0.1 to 1.0 N, best performance of this combined pretreatment was achieved at 0.3 N NaOH. There is a significant cross effect of homogenization and NaOH treatment. Among the three particle sizes tested (the particle size was not directly measured; it passed through screens with openings of 2 mm, 0.707 mm, and 0.25 mm respectively), 2 mm was found to maximize the economic benefit of the pretreatment.*

Keywords. *Biomass, Corn stover, Homogenization, Hydrolysis, Pretreatment.*

Corn stover, which includes the leaves, stalks, and cobs of corn plant, is the most abundant agricultural residue in the U.S. and therefore has the potential to become an industrial feedstock for ethanol. A critical step in production of ethanol from lignocellulosic biomass such as corn stover is the conversion of cellulose and hemicellulose to simple sugars usable by fermentation yeasts. Effective conversion of cellulose to simple sugars relies on a number of factors including the composition and structure of the feedstock, pretreatment used, type and loading of cellulase used, cellulose crystallinity, etc.

Cellulose and hemicellulose are largely protected from attack by enzymes. This inaccessibility to attack is mainly due to the association of these polysaccharides with lignin and with each other, all of which act as a barrier shielding the polysaccharides (Torget et al., 1991; Moniruzzaman, 1996). The highly organized crystalline structure of cellulose itself poses another obstacle to hydrolysis. The limitation of available sites of enzymatic attack may arise from the fact that the average size of the capillaries in biomass is too small to allow the entry of large enzyme molecules, and the enzymatic attack is confined, therefore, to the external surface (Abraham and Kurup, 1997).

Due to the resistance to enzymatic attack mentioned above, the lignocellulosic biomass must be pretreated before it can be enzymatically hydrolyzed. An ideal pretreatment should result in reduction of crystallinity and lignin content and an increase in surface area, should limit the formation of inhibition byproducts, and should be cost effective. Different processes, such as mechanical, chemical, thermochemical, and biological pretreatment, have been used for the pretreatment of lignocellulosic biomass (Sun and Cheng, 2002). Different pretreatment processes require different extent of size reduction. Generally, grinding raw material down to a particle size on the order of 1 mm is required before the chemical pretreatment. Costs and energy requirements for particle size reduction increase geometrically with decreasing particle size. The grinding process can account for one–third of the energy requirements of the entire process (Walsum et al., 1996).

Various pretreatment processes have been applied to the pretreatment of corn stover. Effective pretreatment processes that have been reported include dilute sulfuric acid (Schell et al., 2003), ammonia (Kim et al., 2003; Wang et al., 1998), lime (William and Holtzapple, 2000), and hot water (Liu and Wyman, 2003; Weil et al., 1998) treatment. It is difficult to compare the performance and economics of these various approaches due to differences in feedstocks tested, chemical analysis methods, and data reporting methodologies. Recently, a group of pretreatment researchers across North America have begun to collaborate to investigate different pretreatment approaches on a common basis to allow meaningful comparison (Dale et al., 2003).

NaOH treatment causes lignocellulosic biomass to swell, leading to an increase in the internal surface area, a decrease in the degree of crystallinity, and disruption of the lignin structure (Fan et al., 1987). Varga at al. (2002) reported that

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1%, 5%, and 10% NaOH treatments reduced lignin content in the solid fraction by 91.4%, 93.7%, and 95.9%, respectively; reduced hemicellulose in the solid fraction by 65.9%, 79.2%, and 88.2%, respectively; and reduced cellulose by 30.8%, 41.9%, and 53.3%, respectively. The stronger alkaline pretreatment caused more solubilization of cellulose and hemicellulose, and a slight increase in lignin reduction. Considering economic and environmental aspects, dilute NaOH treatment would be much more suitable than a concentrated NaOH treatment. Combination of dilute NaOH treatment and other treatments seems more efficient. For example, corn stover pretreatment of dilute NaOH (2%) combined with irradiation (500 kGy) caused the glucose yield to increase from 20% for NaOH treatment only to 43% (Chosdu et al., 1993). Combination of thermochemical pretreatment and enzymatic hydrolysis of corn stover was regarded as the most efficient means to produce fermentable sugars (William and Holtzapple, 2000).

The extent of accessible surface area and the average size of the capillaries are two of the major factors limiting efficient hydrolysis of biomass. Physical pretreatment, such as ball milling, significantly increased yield and selectivity of saccharification. Degree of crystallinity was found to decrease linearly with respect to time of ball milling (Sidiras and Koukios, 1989). A simultaneous ball milling and enzymatic hydrolysis could improve the rate of saccharification and/or reduce the amount of enzyme required to attain total hydrolysis of the carbohydrate moieties (Mais et al., 2002).

Homogenization can change substrates from large, obvious particles down to a homogeneous watery mash. The structural and functional properties analysis of the homogenization of alkaline–pretreated corn stover showed increase in surface area and pore diameter. The degree of crystallinity was also found to decrease after homogenization (Gu et al., 2001).

The present work was an attempt to study the enzymatic hydrolysis of corn stover pretreated by combined alkaline treatment and homogenization. The effects of homogenization, NaOH concentration, and corn stover particle size on enzymatic conversion were studied.

Methods

Pretreatment

The ground corn stover samples with initial moisture content of 11% (d.b.) and varying particle sizes (particle size was not directly measured, but particles passed through screens with openings of 2 mm, 0.707 mm, and 0.25 mm, respectively) provided by AURI (Agriculture Utilization Research Institute, Waseca, Minn.) were used in all experiments. The samples were stored in a cool room at 1°C to 4°C before use. The raw material contained about 38% cellulose, 21% hemicellulose, and 18% lignin.

Approximately 200 g (dry matter) of ground corn stover was suspended in NaOH solution. The concentration of NaOH solution used was ranged from 0.1 N to 1.0 N. The concentration of the suspension was 100 g corn stover/L NaOH solution. The suspension was kept at room temperature for 4 h and stirred every 0.5 h. The solid fraction was collected and repeatedly washed with water until the pH of the solid reached around 7.0. A centrifugal extractor with a filter bag of 30 µm openings (model 1, Bock Engineered Products, Inc., Toledo, Ohio) was used to collect the solids during washing. The pretreated material was stored at 0°C to 4°C for later use. Three samples of the pretreated material were dried at 105°C for 24 h and then weighed for moisture content determination.

Corn stover pretreated with NaOH solution was dispersed with an Ultra–Turrax homogenizer (IKA–Werke, Staufen Breisgau, Germany) in the combined pretreatment of NaOH treatment and homogenization. Homogenization was operated at a concentration of 15 g dry solid/L for 5 min at 8000 rpm. The homogeneous watery mash obtained was sealed and stored at 0°C to 4°C in a cool room for later enzymatic hydrolysis. Triplicate samples of the obtained slurry were dried at 105°C for 24 h and weighed for solid concentration measurement.

Enzymatic Hydrolysis

Enzymatic hydrolysis methods similar to those used in evaluating steam explosion pretreatment of softwood (Ballesteros et al., 2000) were used here. Both the solid material obtained from NaOH treatment and the slurry obtained from the combined NaOH treatment and homogenization were enzymatically hydrolyzed to evaluate the effectiveness of the pretreatments. Treated corn stover was mixed with ultrafiltered water in a 2 L fermenting vessel (New Brunswick Co., Inc., Edison, N.J.) to produce 1 L substrate with a concentration of 13 g dry matter of pretreated solid/L. The substrate was autoclaved for 15 min at 120°C and cooled down to 50°C for enzymatic hydrolysis. Initial pH was adjusted to 4.8 using buffer solutions. The suspension was kept at 50°C and agitated at 150 rpm during hydrolysis.

Commercial enzyme solution with cellulase activity of 90 to 115 GCU/mL (Spezyme CP, Genencor International, Inc., Rochester, N.Y.) was used in the enzymatic hydrolysis experiments. The enzyme loadings used in the experiments were 20, 40, 60, 80, and 100 GCU/g substrate. In all cases, pretreatment and hydrolysis experiments were performed in two replicates, and results are presented as a mean value. Total hydrolysis was 60 h. Triplicate replicates were taken for analysis at 0, 12, 24, 36, 48, and 60 h. Samples were centrifuged at 10,000 rpm for 15 min. The supernatants were used for sugar measurement.

Sugar Measurement

Sugar concentration was measured using an YSI–27 sugar analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). Glucose standard solutions (200 and 500 mg/dL) were used for instrument calibration. The mean results of triplicate analysis were presented as the glucose concentration.

Results and Discussion

Enzyme Loading

To determine a suitable enzyme loading for enzymatic hydrolysis of corn stover pretreated by combined NaOH treatment and homogenization, experiments with different loading of cellulase (20 to 100 GCU cellulase/g substrate) were performed. For the 2 mm corn stover pretreated by combined 1.0 N NaOH treatment and homogenization, the maximum glucose yield was achieved with cellulase loading of 100 GCU/g substrate (fig. 1). Increases of glucose yield

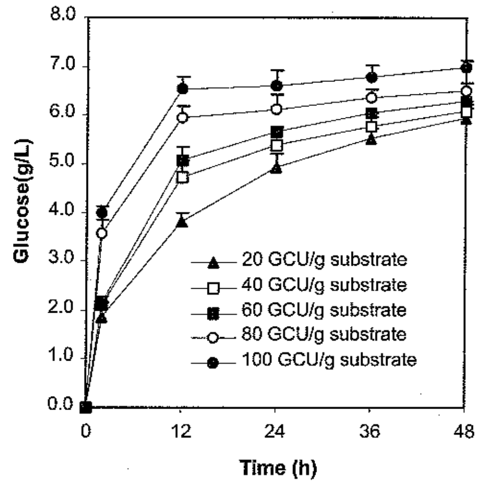

Figure 1. Effect of enzyme loading on enzymatic hydrolysis (raw material = 2 mm corn stover, pretreatment conditions = 1.0 N NaOH + homogenization, hydrolysis conditions = 50°C, pH 4.8).

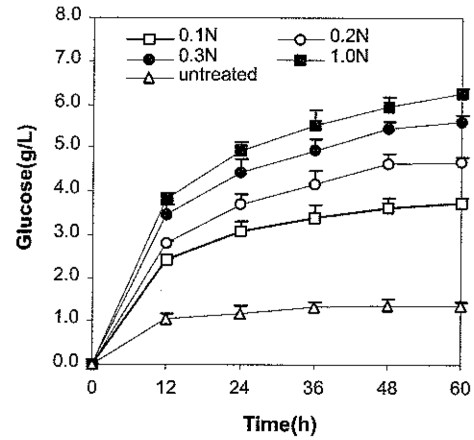

Figure 2. Effect of NaOH concentration on enzymatic hydrolysis of corn stover pretreated by combined NaOH treatment and homogenization (raw material = 2 mm corn stover, pretreatment conditions = NaOH treatment + homogenization, hydrolysis conditions = 20 GCU cellulase/g substrate at 50°C, pH 4.8).

and saccharification rate were observed with the increase of enzyme loading. Increasing the enzyme loading from 20 to 100 GCU/g substrate increased glucose yield by 20% after 48 h of saccharification. However, because high enzyme loading will significantly increase cost, use of high enzyme concentrations may not be economically justified (Vega et al., 1991). An optimum cellulase loading needs to be selected according to the pretreatments used.

Alkaline Concentration

Glucose yield of enzymatic conversions of 2 mm corn stover pretreated by combined alkaline treatment and homogenization is shown in figure 2. Increase in hydrolysis conversion of corn stover was observed with increasing NaOH concentration. After 60 h of enzymatic hydrolysis, the glucose concentrations were 1.35, 3.71, 4.66, 5.60, and 6.25 g/L for untreated material, 0.1 N, 0.2 N, 0.3 N, and 1.0 N NaOH treated material, respectively. The combined alkaline treatment and homogenization pretreatment increased the enzymatic hydrolysis of corn stover 3 to 5 times more than the control. The highest glucose yield (6.25 g/L) was obtained when the corn stover was pretreated by a combination of 1.0 N NaOH treatment and homogenization. The glucose yield only increased by 12% when the NaOH concentration increased from 0.3 N to 1.0 N, while the glucose yield increased by 20% when the NaOH concentration increased from 0.2 N to 0.3 N, and by 25% when the NaOH concentration increased from 0.1 N to 0.2 N. The increment of glucose yield decreased with the increase of NaOH concentration. High NaOH concentration will increase the chemical cost and the cost for waste liquor treatment. Thus, NaOH concentrations in the range of 0.1 N to 0.3 N are recommended for the combination pretreatment of corn stover with NaOH treatment and homogenization.

Homogenization

The effects of homogenization on the enzymatic hydrolysis of 2 mm corn stover pretreated by varying concentration of NaOH solution are shown in figure 3. It can be seen from figure 3 that the homogenization increased the glucose yield of corn stover pretreated by dilute NaOH treatment. However, the homogenization had little effect on the glucose yield of corn stover pretreated by 0.5 N and 1.0 N NaOH (fig. 3d and 3e). After 60 h of enzymatic hydrolysis, the increases of glucose concentration due to homogenization were 1.06, 1.3, 1.6, 0.47, and 0.5 g/L for the 0.1, 0.2, 0.3, 0.5, and 1.0 N NaOH treated corn stover, respectively (fig. 3f). Homogenization increased glucose concentration by 40%, 39%, 40%, 8.1%, and 8.6% for the 0.1, 0.2, 0.3, 0.5, and 1.0 NaOH treated corn stover, respectively. The effect of homogenization on the glucose yield of NaOH pretreated corn stover was smaller as NaOH concentration increased. The most significant effect of homogenization was obtained in the case of 0.3 N NaOH pretreated corn stover. The glucose yield of corn stover pretreated by combined 0.3 N NaOH treatment and homogenization was almost the same as that of 1.0 N NaOH treatment only. The increase in glucose yield resulting from homogenization when corn stover was pretreated with 0.1 N and 0.2 N NaOH was little lower than that with 0.3 N NaOH treatment. Adequate chemical pretreatment is required to obtain the best performance of homogenization.

The main effects of NaOH treatment and homogenization and interaction effect of NaOH treatment and homogenization on glucose yield were studied through F statistics. The F values of the NaOH treatment, homogenization, and NaOH treatment–homogenization interaction were 57.0, 45.5, and 138.7, respectively. All three effects were statistically significant. The NaOH treatment by homogenization interaction had the largest F, followed by the main effects of NaOH treatment and homogenization, clearly indicating a cross effect of homogenization and NaOH treatment. Hydrolysis of corn stover at lower NaOH concentration has the potential to reduce the cost of chemicals and waste sludge treatment. Considering both economical and environmental aspects, the combination of dilute NaOH treatment and homogenization is recommended for pretreatment of corn stover.

Particle Size

Effects of particle size and homogenization on the glucose yield of varying particle sizes of corn stover pretreated by 0.3 N NaOH are shown in figure 4. For the corn stover samples treated only with 0.3 N NaOH , the glucose yield increased

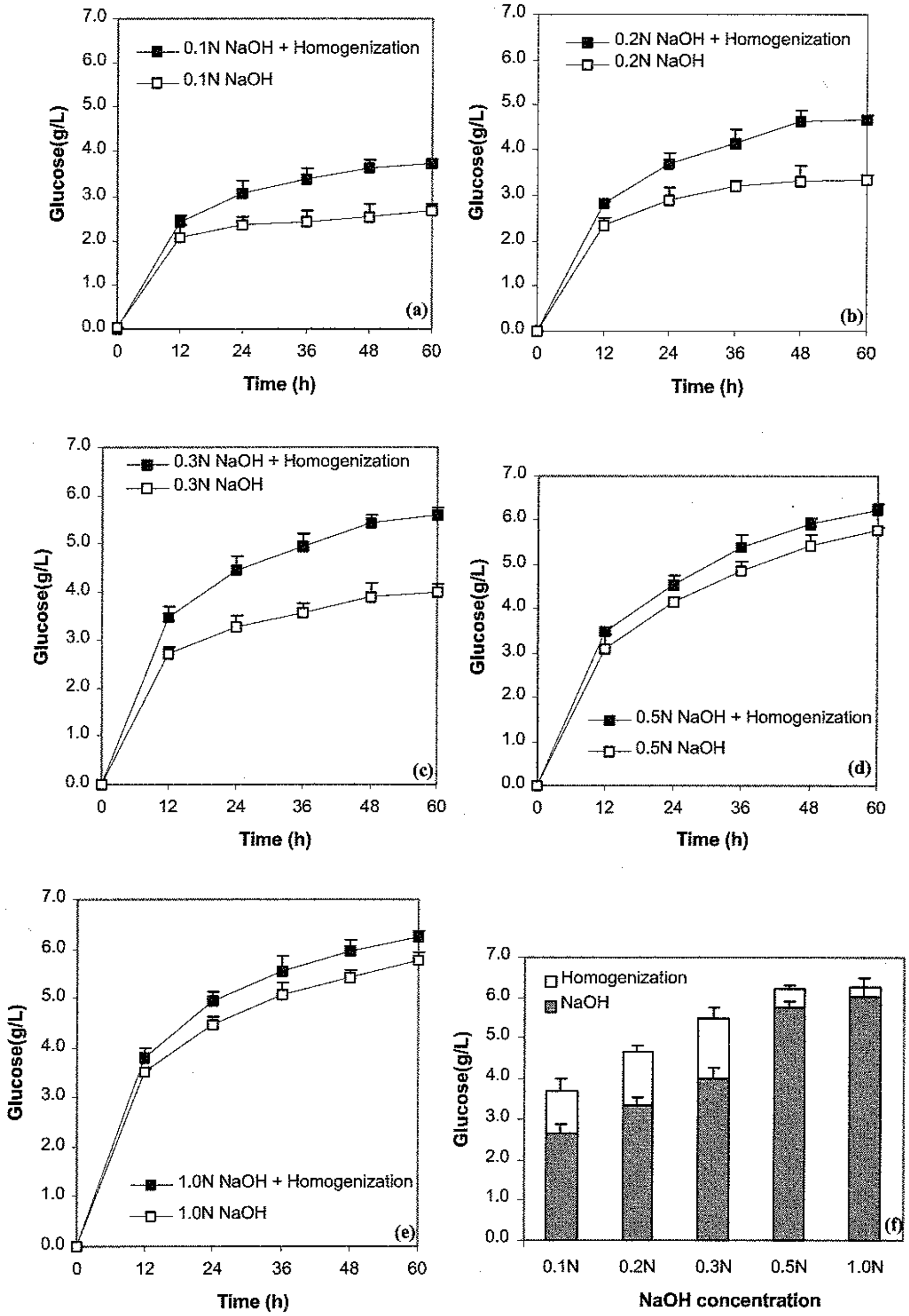

Figure 3. Effect of homogenization process on enzymatic hydrolysis as a function of alkaline treatment at different NaOH concentrations: (a) 0.1 N, (b) 0.2 N, (c) 0.3 N, (d) 0.5 N, (e) 1.0 N, and (f) glucose concentration after 60 h enzymatic hydrolysis at different NaOH concentrations. Enzymatic hydrolyses were conducted with 20 GCU cellulase/g substrate at temperature of 50°C and pH of 4.8.

with decreasing particle size. After 60 h of enzymatic hydrolysis, the glucose yield for the 0.3 N NaOH pretreated 2 mm corn stover was 4.0 g/L. When the particle size was reduced to 0.707 mm, a glucose yield increase of 1.2 g/L (30%) was obtained. However, with the further reduction in particle size to 0.25 mm, an additional increase of only 0.5 g/L (9.6%) in glucose yield was obtained. Therefore, in the case of pretreatment with dilute NaOH only, grinding corn stover to 0.707 mm is sufficient. Further decreasing particle size may not be economically justified. The increases in glucose yield due to homogenization were 1.5, 1.0, and 0.2 g/L for the 0.3 N NaOH pretreated corn stover with particle sizes of 2, 0.707, and 0.25 mm, respectively. The most significant increase in glucose yield due to homogenization was obtained with 2 mm corn stover. The improvement in the glucose yield by homogenization

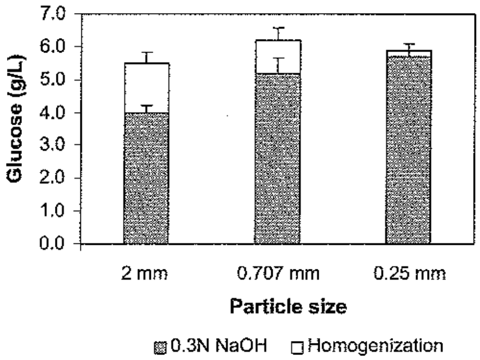

Figure 4. Effects of homogenization and particle size on enzymatic hydrolysis of corn stover (hydrolysis conditions = 20 GCU cellulase/g substrate at 50°C, pH 4.8).

decreased with decreasing particle size. Since size reduction increases the production costs, a particle size of 2 mm or larger is recommended when combined pretreatment of dilute NaOH treatment and homogenization.

In the case of corn stover pretreated with combined 0.3 N NaOH treatment and homogenization, the effect of particle sizes reduction from 2 mm to 0.25 mm on the glucose yield was not significant (fig. 4b). After 60 h of enzymatic hydrolysis, the glucose yield for the 2 mm corn stover was 5.5 g/L. When the particle size was reduced to 0.707 mm, glucose yield increased by 0.7g/L. No further increase in glucose yield was observed when particle size was further reduced to 0.25 mm.

Conclusions

The effect of combined NaOH treatment and homogenization on the enzymatic hydrolysis of corn stover was studied. When the homogenization was applied to dilute NaOH treated samples, the saccharification increased as much as 40%. The lower the NaOH concentration used, the higher the effect of homogenization on the enzymatic hydrolysis of the corn stover. The most significant effect of homogenization was obtained when 0.3 N NaOH was used. The effect of NaOH treatment by homogenization interaction had the largest F value, followed by the main effects of NaOH treatment and homogenization. All these three effects were statistically significant.

For corn stover with the particle sizes used in this study, the larger the particle size, the higher the effect of homogenization on the enzymatic hydrolysis. The most significant increase in glucose yield due to homogenization was obtained with the 2 mm corn stover. It can be concluded that the homogenization not only decreased the particle size of the corn stover but also increased the surface area and porosity, and possibly reduced the degree of crystallinity.

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References

Abraham, M., and G. M. Kurup. 1997. Pretreatment studies of cellulose wastes from optimization of cellulase enzyme activity. *Applied Biochem. and Biotech.* 62(2–3): 201–211.

Ballesteros, I., J. M. Oliva, A. A. Navarro, A. Gonzalez, J. Carrasco, and A. Ballesteros. 2000. Effect of chip size on steam explosion pretreatment of softwood. *Applied Biochem. and Biotech.* 84–86(6): 97–110.

Chosdu, R., N. Hilmy, T. B. Erlinda, and B. Abbas. 1993. Radiation and chemical pretreatment of cellulosic waste. *Radiat. Phys. Chem.* 42(4–6): 695–698.

Dale, B. E., R. T. Elander, M. Holtzapple, M. R. Ladisch, Y. Y. Lee, T. Eggeman, and C. E. Wyman. 2003. Comparative data from application of leading pretreatment technologies to corn stover. In *25th Symposium on Biotechnology for Fuels and Chemicals*, Biomass Pretreatment and Hydrolysis Session, Oral Presentation 6A–06. Breckenridge, Colo.: National Renewable Energy Laboratory.

Fan, L. T., M. M. Gharpuray, and Y. H. Lee. 1987. *Cellulose Hydrolysis Biotechnology Monographs*, 57. Berlin, Germany: Springer.

Gu, L., R. Ruan, P. Chen, W. Wilcke, and P. Addis. 2001. Structure–function relationships of highly refined cellulose. *Trans. ASAE* 44(6): 1707–1712.

Kim, T. H., J. S. Kim, C. Sunwoo, and Y. Y. Lee. 2003. Pretreatment of corn stover by aqueous ammonia. *Bioresource Tech.* 90(1): 39–47.

Liu, C. G., and C. E. Wyman. 2003. The effect of flow rate of compressed hot water on xylan, lignin, and total mass removal from corn stover. *Ind. and Eng. Chem. Res.* 42(21): 5409–5416.

Mais, U., A. Esteghlalian, J. N. Saddler, and S. D. Mansfield. 2002. Enhancing the enzymatic hydrolysis of cellulosic materials using simultaneous ball milling. *Applied Biochem. and Biotech.* 99(1–3): 815–832.

Moniruzzaman, M. 1996. Effect of steam explosion on the physicochemical properties and enzymatic saccharification of rice straw. *Applied Biochem. and Biotech.* 59(3): 283–297.

Schell, D. J., J. Farmer, N. Newman, and J. D. McMillan. 2003. Dilute–sulfuric acid pretreatment of corn stover in pilot–scale reactor: Investigation of yields, kinetics, and solids enzymatic digestibilities. *Applied Biochem. and Biotech.* 105(1–3): 69–85.

Sidiras, D. K., and E. G. Koukios. 1989. Acid saccharification of ball–milled straw. *Biomass* 19(4): 289–306.

Sun, Y., and J. Cheng. 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresource Tech.* 83(1): 1–11.

Torget, R., P. Walter, M. Himmel, and K. Grohmann. 1991. Dilute–acid pretreatment of corn residues and short rotation woody crops. *Applied Biochem. and Biotech.* 28/29: 75–85.

Varga, E., Z. Szengyel, and K. Reczey. 2002. Chemical pretreatments of corn stover for enhancing enzymatic digestibility. *Applied Biochem. and Biotech.* 98(1–3): 73–87.

Vega, J. L., K. T. Klasson, E. C. Clausen, J. L. Gaddy. 1991. The saccharification of corn stover by cellulase from *Penicillium funiculosum*. *Bioresource Tech.* 35(1): 73–80.

Walsum, G. P., S. G. Allen, M. J. Spencer, M. S. Laser, M. J. Antal, and L. R. Lynd. 1996. Conversion of lignocellulosics pretreated with liquid hot water to ethanol. *Applied Biochem. and Biotech.* 57/58: 157–169.

Wang, L., B. E. Dale. L. Yurttas, and I. Goldwasser. 1998. Cost estimates and sensitivity analysis for the ammonia fiber explosion process. *Applied Biochem. and Biotech.* 70–72: 51–66.

Weil, J. R., A. Sarikaya, S.–L. Rau, J. Goetz, C. M. Ladisch, M. Brewer, R. Hendrickson, and M. R. Ladisch, 1998. Pretreatment of corn fiber by pressure cooking in water. *Applied Biochem. and Biotech.* 73(1): 1–17.

William, E. K., and M. T. Holtzapple. 2000. Using lime pretreatment to facilitate the enzymatic hydrolysis of corn stover. *Biomass and Bioenergy* 18(3): 189–199.