Effect of Xylan and Lignin Removal by Batch and Flowthrough Pretreatment on the Enzymatic Digestibility of Corn Stover Cellulose

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Abstract: Compared with batch systems, flowthrough and countercurrent reactors have important potential advantages for pretreating cellulosic biomass, including higher hemicellulose sugar yields, enhanced cellulose digestibility, and reduced chemical additions. Unfortunately, they suffer from high water and energy use. To better understand these trade-offs, comparative data are reported on xylan and lignin removal and enzymatic digestibility of cellulose for corn stover pretreated in batch and flowthrough reactors over a range of flow rates between 160° and 220°C, with water only and also with 0.1 wt% sulfuric acid. Increasing flow with just water enhanced the xylan dissolution rate, more than doubled total lignin removal, and increased cellulose digestibility. Furthermore, adding dilute sulfuric acid increased the rate of xylan removal for both batch and flowthrough systems. Interestingly, adding acid also increased the lignin removal rate with flow, but less lignin was left in solution when acid was added in batch. Although the enzymatic hydrolysis of pretreated cellulose was related to xylan removal, as others have shown, the digestibility was much better for flowthrough compared with batch systems, for the same degree of xylan removal. Cellulose digestibility for flowthrough reactors was related to lignin removal as well. These results suggest that altering lignin also affects the enzymatic digestibility of corn stover. © 2004 Wiley Periodicals, Inc.

Keywords: xylan removal; lignin removal; flowthrough reactor; batch hydrolysis; enzymatic digestibility; pretreatment

INTRODUCTION

Bioconversion of cellulosic biomass to fuels and commodity products has many potential benefits (Lynd et al., 1999), with enzymatic hydrolysis of cellulose to glucose, followed by fermentation to ethanol, a particularly attractive route to low-cost sustainable liquid transportation fuels (Wyman, 1999). However, enzymatic hydrolysis may be the most complex step in the bioconversion process because of the combination of substrate-related and enzyme-related effects. Although the hydrolysis mechanism and the relationship between the structure and function of various cellulases have been studied extensively (Chang and Holtzapple, 2000; Converse, 1993; Klyosov, 1990; Lynd et al., 2002; Reese et al., 1950), the complex biomass structure confounds understanding of the relative importance of these features and their roles, and reducing one barrier to digestion can enhance or disguise the importance of others.

Various studies have reported that cellulose hydrolysis improves with increasing lignin removal, although differences have been reported in the degree of lignin removal needed (Converse, 1993; Gharpuray et al., 1983; Gould, 1984; Grohmann et al., 1986; Yang et al., 2002). Lignin has also been claimed to depolymerize and then repolymerize during hemicellulose hydrolysis, although undoubtedly in a different morphology, which could change its impact on cellulose digestion (Donaldson et al., 1988; Tanahashi, 1990). Because condensed lignin can adsorb protein from aqueous solutions (Kawamoto et al., 1992), lignin removal should also improve performance by reducing nonspecific adsorption of cellulase enzymes (Converse, 1993; Lu et al., 2002; Ooshima et al., 1990; Sutcliffe and Saddler, 1986).

Nevertheless, several investigations have shown a direct relationship between cellulose digestion and xylan or hemicellulose removal, with some concluding that lignin removal is not necessary for good cellulose conversion (Grohmann et al., 1984, 1989; Knappert et al., 1980). However, some substrates have required higher temperatures for effective hemicellulose removal, suggesting that hemicellulose is not the only factor impacting digestibility, and some evidence does not support a role for hemicellulose in changing cellulose digestibility (Fan et al., 1982; Millett et al., 1976; Tsao et al., 1978).

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A few research teams have shown that reactors that employ countercurrent flow of liquid and solids, or flow of water through the solids, can recover virtually theoretical yields of hemicellulose (Allen et al., 2001b; Bobleter et al., 1976; Hoermeyer et al., 1988; Mok and Antal, 1992, 1994) and remove about 60% of the lignin (Allen et al., 2001a). Pretreatment of cellulosic biomass in a flowthrough reactor also provides more digestible cellulose than from a batch system, and the time required to digest the pretreated cellulose drops significantly (van Walsum et al., 1996). Adding very dilute acid to a flowthrough reactor further reduces the digestion time by about 67% compared with α-cellulose or batch acid-treated biomass (Torget et al., 1996). Because flowthrough reactors remove more lignin than batch systems, this research was directed at developing data on xylan removal, lignin removal, and the enzymatic digestibility of cellulose for corn stover pretreated in batch and flowthrough systems with the goal of gaining better insight into how solids composition influences cellulose digestion.

MATERIALS AND METHODS

Sample Preparation

Corn stover was graciously provided by the National Renewable Energy Laboratory (Golden, Colorado) from a large standard supply they had developed from a source in Harlan, Iowa. This material was milled to pass through a 2-mm opening and then screened using a RoTap (RX-29) separator (Soiltest, Inc., Chicago, IL). The −420/+250-μm fraction was retained in a plastic Ziploc bag and kept in a freezer (−20°C) for use in all tests. The composition of the corn stover was determined through application of NREL LAP 001, 002, and 012 procedures (Ehrman, 1994a, 1994b; Ruiz and Ehrman, 1996a) and found to contain 37.8% glucan, 21.3% xylan, 1.6% arabinan, 3.8% mannan, 1.4% galactan, 17.8% lignin, 7.8% ash, and 5.8% moisture content.

Batch Tube Reactors

To withstand the dilute acid concentrations used in this study, the batch reactors were made of Hastelloy (C276) tubing. However, because Hastelloy caps are very expensive, Teflon plugs were inserted in both ends, which were then capped with stainless-steel Swagelok fittings (Maine Valve and Fitting Co., Bangor, ME) in a procedure recommended by Professor Y. Y. Lee from Auburn University. The working volume of these tubular reactors was 13.4 mL. Thermocouple probes were inserted along the centerline of the tubes and pushed 3/16 in. and 3 in. into the reactor. Reaction temperatures of 160°, 180°, 200°, and 220°C were applied at 0.0 wt% and 0.1 wt% H_2SO_4 with the solids concentration held at 5 wt%. Because temperature transients can impact biomass hydrolysis in reactor tubes, a three-bath procedure was developed to minimize these effects based on a thermal modeling approach (Stuhler, 2002). The sequence began by preheating each tube in boiling water for 2 min, followed by immediate transfer to a sand bath set at a temperature, T_1, selected through our modeling approach to minimize heat-up times. After the tube was held for a specified time in this sand bath, it was transferred to a second sand bath set at the target reaction temperature T_2. The time the tube was put into the second sand bath was arbitrarily set as zero reaction time. After being subjected to the target temperature for a given time, the reactors were quickly transferred to an ice-water bath to quench the reaction. Next, the tubes were removed from the water and dried, and the end caps and Teflon plugs were removed. The contents were then pushed out and separated into liquid and solid fractions by filtration for analysis.

Flowthrough Reactors

The flowthrough systems employed in this work were 1/2-in. i.d. × 6-in. length with an internal volume of 14.3 mL. These units were constructed of 316 stainless-steel parts using VCR (Swagelock Corp.) fittings, including one VCR male union (1/2 in), two gasket filters (316 stainless-steel, average pore size 5 μm), two VCR glands (1/2 in. × 1/2 in.), two VCR nuts, and two VCR reducing fittings (1/2 in. × 1/8 in.). All reactor parts were obtained from the Maine Valve and Fitting Co. A 1/8-in. stainless-steel thermocouple (Omega Engineering Co., Stamford, CT) was installed at the outlet of the reactor to monitor temperature. Stainless-steel tubing (316) was used as a preheating coil (1/4-in. o.d. × 0.035-in. wall) and to connect the reactor with other system components as well the cooling coil (1/8-in. o.d. × 0.028-in. wall). The preheating coil was long enough to allow the incoming water to reach the desired temperature before it entered the reactor, as measured experimentally. A high-pressure pump (Acuflow Series III Pumps, Fisher) with a flow rate range of 0 to 40 mL/min, a pressure gauge (pressure range 0 to 1500 psi; Cole-Parmer Instrument Co., Vernon Hills, IL), and a back-pressure regulator (Maine Valve and Fitting Co.) were used to control flow through the system.

To operate the flowthrough unit, about 2 g of corn stover was loaded into the reactor, which was then connected to the system. Distilled water at room temperature was pumped through the reactor to purge air and then used to pressurize the reactor to a set pressure of 350 to 400 psig. The loaded biomass was completely wetted by this procedure. The preheating procedure was the same as for the batch tube.

Enzymes

Cellulase (Spezyme CP, Lot No. 301-00348-257 at 28 FPU/mL, Genencor, Palo Alto, CA) supplemented with β-glucosidase (Novozym 188 at 480 β-glucosidase IU/mL and 5 FPU/mL, Sigma Co., St. Louis, MO) at a ratio of 1:1.75 (FPUase:CBUase) was used for all hydrolysis experiments. Enzymatic treatments were performed at

60 FPU/g cellulose with the activity as calculated by adding the cellulase activities of both the Spezyme CP and Novozym 188 (Adney and Baker, 1996).

Enzymatic Hydrolysis

Enzymatic hydrolysis of pretreated cellulose from both the batch and flowthrough systems was conducted at 2% solids concentration (grams dry weight per 100 mL) in 50 m*M* acetate buffer (pH 4.8) containing 40 μg/mL tetracycline and 30 μg/mL cycloheximide. Flasks were preincubated at 50°C on the rotary shaker at 150 rpm for 10 min, and the enzymes were added to start the hydrolysis after acclimation. Aliquots of 0.5 mL were taken at different timepoints (0, 4, 24, 48, 72 h), immediately chilled on ice, and centrifuged at 5000*g* for 10 min. Total sugar analyses were performed on the resultant supernatants.

Analytical Procedures

Sugar content and acid-insoluble lignin were determined for the solids using the Klason lignin procedure published as NREL LAP 003 and 014 (Ruiz and Ehrman, 1996b; Templeton and Ehrman, 1995). The sugar concentrations were measured with a high-performance liquid chromatography system (Model 2695, Waters, Milford, MA) equipped with a pulsed refractive index detector (Model 2410 Differential Refractometer, Waters). The column was equilibrated with de-ionized water at a flow rate of 0.6 mL/min. An Aminex (Model HPX-87P, Bio-Rad, Sunnyvale, CA) column was used for sugar separations.

Severity Parameters

A severity parameter and modified severity parameter were used to unify our data obtained at different combinations of temperature, time, and acid concentration. The severity parameter for water-only hydrolysis is defined as (Overend and Chornet, 1987):

$$R_0 = t \cdot \exp[(T - 100)/14.75] \quad (1)$$

where t is reaction time (minutes), and T is the hydrolysis temperature (degrees Celsius). The modified severity parameter is (Chum et al., 1988):

$$M_0 = t \cdot A^n \cdot \exp[(T - 100)/14.75] \quad (2)$$

where A is the acid concentration (weight percent), and n is an arbitrary constant.

RESULTS AND DISCUSSION

This study examined how changes in xylan and lignin removal for batch and flowthrough pretreatment at temperatures of 160° to 220°C, solid retention times of 0 to 300 min, acid concentrations of 0.0 wt% and 0.1 wt%, and flow rates of 0 to 25 mL/min affected the enzymatic digestibility of corn stover cellulose. Particular emphasis focused on determining how digestibility changed between the two systems for similar amounts of xylan or lignin removal.

Xylan Removal

As expected, xylan removal increased with severity. Figure 1 reveals that >90% of the total xylan was removed when $\log R_0$ was >4.3 for batch operation or >4.1 for flowthrough. Furthermore, the flowthrough system removed more xylan than the batch system at the same severity, especially in the range of $3.5 < \log R_0 < 4.5$ with hot water alone. Figure 2 shows that adding dilute sulfuric acid increased total xylan removal, with >90% removed when $\log M_0$ was >2.2 for batch and >2.0 for flowthrough pretreatment. Thus, the relative benefits of introducing flow were less with than without acid. However, sulfuric acid addition, even at these low concentrations, had a major effect on performance for both batch and flowthrough systems. Overall, total xylan removal increased with flow rate, acid concentration, temperature, and time, and flow rate appeared to affect xylan solubilization in a manner similar to the other three variables.

Lignin Removal

As shown in Figure 3 for pretreatment in the flowthrough system with water only, lignin removal correlated well with $\log R_0$, reaching about 85% lignin removed at $\log R_0 = 4.8$. On the other hand, lignin removal for the batch operation rose to about 20% at $\log R_0 = \sim 3.4$ and then changed little with further increases in severity, with a maximum value of about 30% lignin removal at $\log R_0 = 4.5$.

An unexpected observation for batch pretreatments, as shown by comparing Figures 3 and 4, was that Klason

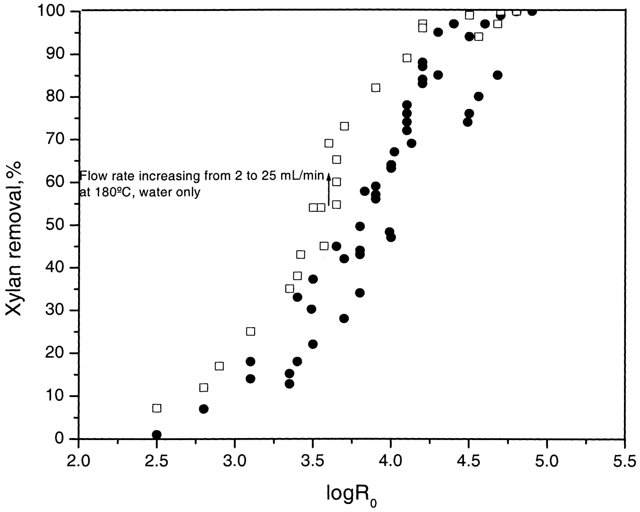

Figure 1. Effect of severity parameter on xylan removal for batch tube and flowthrough pretreatment of corn stover at 160° to 220°C with water only: (●) batch tube at a 5% solids concentration; (□) flowthrough reactor at flow rates of 2 to 25 mL/min.

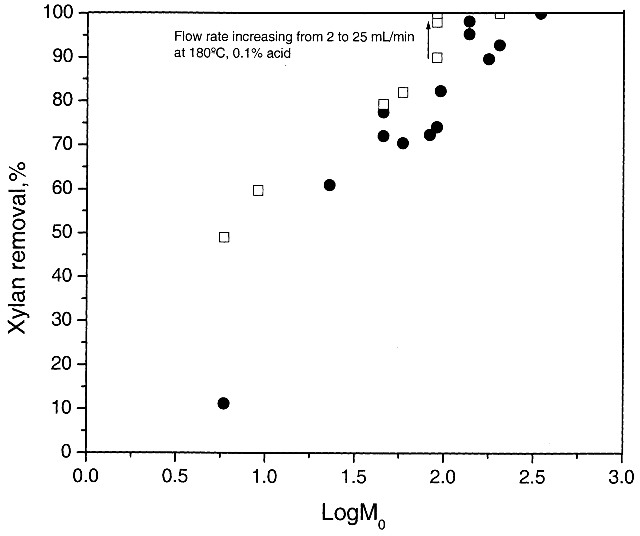

Figure 2. Effect of modified severity parameter on xylan removal for batch tube and flowthrough pretreatment of corn stover at 160° to 220°C with 0.1 wt% sulfuric acid: (●) batch tube at a 5% solids concentration; and (□) flowthrough reactor at flow rates of 2 to 25 mL/min.

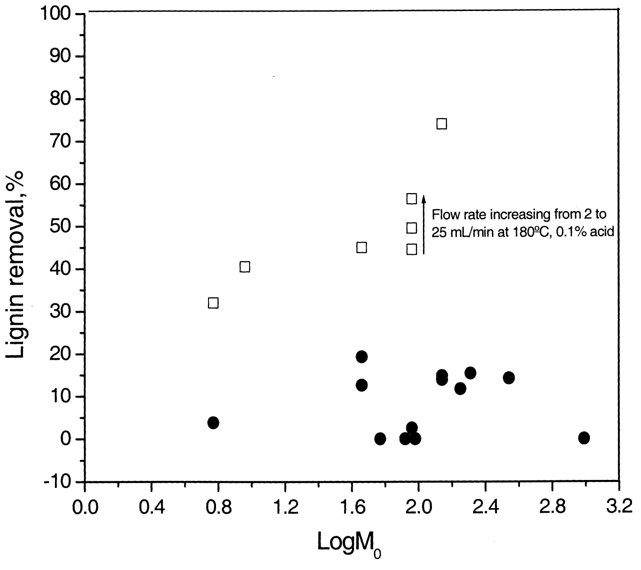

Figure 4. Effect of modified severity parameter on lignin removal for batch tube and flowthrough pretreatment of corn stover at 160° to 220°C with 0.1 wt% sulfuric acid: (●) batch tube at a 5% solids concentration; and (□) flowthrough reactor at flow rates of 2 to 25 mL/min.

lignin removal for dilute-acid operation was less than that with only hot water at the same temperature. Moreover, for dilute-acid batch pretreatment, we found that Klason lignin removal decreased as reaction time increased at higher temperatures (>200°C), and the maximum lignin removal peaked at about 18% at $\log M_0 = 1.65$. By comparison, at each severity, flowthrough pretreatment removed a much larger fraction of the original Klason lignin. A possible explanation for these differences is that lignin is dissolved as hemicellulose is hydrolyzed, and acid addition accelerates these reactions. However, acid could accelerate lignin reactions to insoluble compounds even more, resulting in less lignin removal for batch operations (Shevchenko et al., 1999; Wu et al., 1998). On the other hand, for flowthrough systems, dissolved lignin could be swept from the reactor before it can react and precipitate, resulting in substantially more lignin removal (Liu and Wyman, 2003).

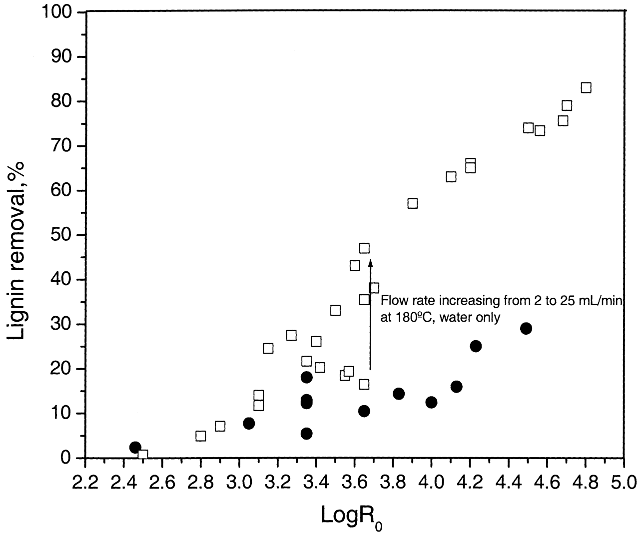

Figure 3. Effect of severity parameter on lignin removal for batch tube and flowthrough pretreatment of corn stover at 160° to 220°C with water only: (●) batch tube at a 5% solids concentration; and (□) flowthrough reactor at flow rates of 2 to 25 mL/min.

Enzymatic Digestibility

As shown in Figures 5 and 6, enzymatic digestibility could be related to the severity parameter or the modified severity for both batch and flowthrough reactors. As $\log R_0$ increased from 2.50 to 4.68, the enzymatic digestibility increased from 21% to 83% for corn stover pretreated with water only in a batch system, and from 42% to 97% over the same severity range for flowthrough operation. Adding acid, the digestibility increased from 36% to 87% over a $\log M_0$ range of between 0.65 and 2.45 for batch, and from 55% to 96% for $\log M_0$ from 0.65 to 2.20 with flowthrough. For flowthrough pretreatment, digestibility increased significantly for materials treated at higher flow rates in addition to higher temperatures or acid concentrations.

Overall, the descending order of enzymatic digestibility for different pretreatment approaches at similar conditions was as follows: catalyzed flowthrough > water-only flowthrough ≥ catalyzed batch > water-only batch. Furthermore, increasing flow rate enhanced digestibility more for > water-only flowthrough pretreatment than when acid was added. For example (Table I), with flow rates of 0, 2, 7.5, and 25 mL/min at 180°C for water-only hydrolysis, the digestibility was about 53.2%, 57.8%, 66.4%, and 79.7%, respectively, at 72 h. At the same conditions, but with 0.1 wt% H_2SO_4, the digestibility increased to 67.3%, 86.9%, 92.3%, and 95.8%, respectively. To provide different lignin and hemicellulose removal levels, the reactor was run for 20 min

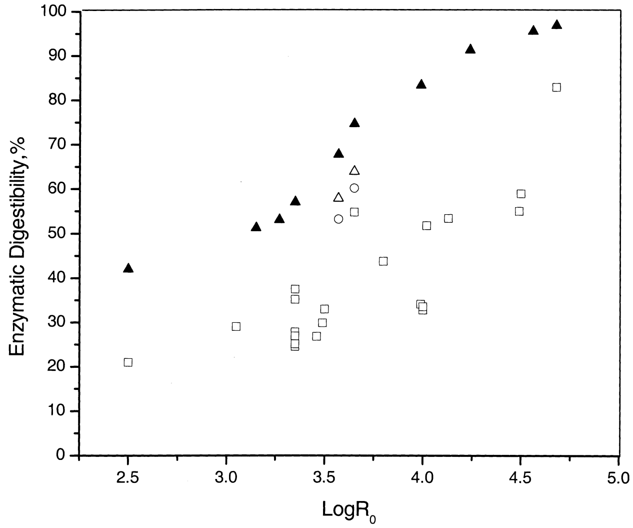

Figure 5. Enzymatic digestibility vs. logR_0 for batch and flowthrough pretreatment of corn stover at 160° to 220°C with only water: (□) batch tube at a 5% solids concentration; (○) flowthrough reactor at a flow rate of 2 mL/min; (△) flowthrough reactor at a flow rate of 7.5 mL/min; and (▲) flowthrough reactor at a flow rate of 25 mL/min.

at 180°, 200°, and 215°C with a flow rate of 25 mL/min. The pretreated corn stover cellulose digestibility was found to be 79.7%, 91.4%, and 98.4%, respectively, after 72 h with an enzyme loading of 60 FPU/g cellulose.

Relating Digestibility to Xylan and Lignin Removal

For batch and flowthrough pretreatment of corn stover, Figure 7 shows that enzymatic digestibility can be related to xylan removal. However, when only water is used, much less xylan must be removed to achieve the same cellulose digestibility for the flowthrough than for the batch system. For example, 55% digestibility was obtained when only 45% xylan was removed for flowthrough, whereas batch required removal of about 72% of the xylan to achieve the same digestibility. Alternatively, cellulose digestibility was about 20% greater for the flowthrough system than for batch at the same level of xylan removal over the range of 20% to 75% xylan removal. When sulfuric acid was added, the advantage for flowthrough pretreatment narrowed somewhat, resulting in about a 10% greater enzymatic digestibility than for the batch approach at the same xylan removal level. Furthermore, the advantage of flowthrough

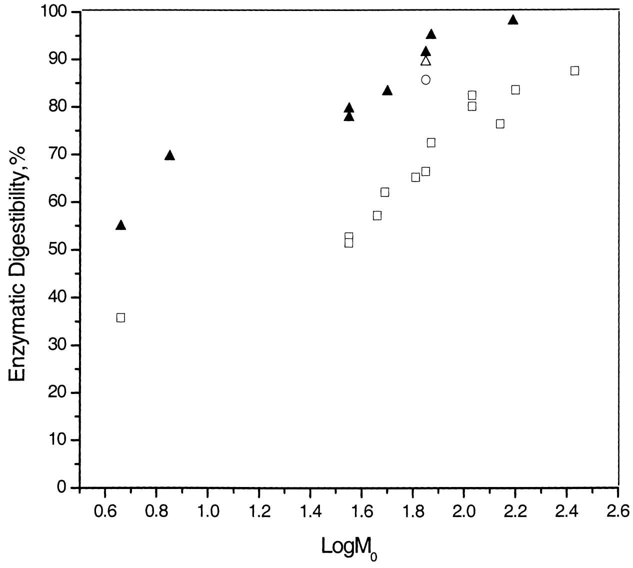

Figure 6. Enzymatic digestibility vs. logM_0 for batch and flowthrough pretreatment of corn stover at 160° to 220°C with 0.1 wt% sulfuric acid: (○) batch tube at a 5% solids concentration; (△) flowthrough reactor at a flow rate of 2 mL/min; (□) flowthrough reactor at a flow rate of 7.5 mL/min; and (▲) flowthrough reactor at a flow rate of 25 mL/min.

Table I. Effect of flow rate on lignin removal, xylan removal, and enzymatic digestibility after 20-min pretreatment.

Acid loading (wt%)	Flow rate (mL/min)	Temperature (°C)	Xylan removal (%)	Lignin removal (%)	Enzymatic digestibility (%)
0	0	180	25.4	12.1	53.2
0	2	180	49.2	19.7	57.8
0	7.5	180	62.7	36.2	66.4
0	25	180	66.5	47.3	79.7
0	25	200	100	56.3	91.4
0	25	215	100	73.2	98.7
0.1	0	180	71.7	1.5	67.3
0.1	2	180	94.8	41.2	86.9
0.1	7.5	180	96.2	61.5	92.3
0.1	25	180	100	68.4	95.8

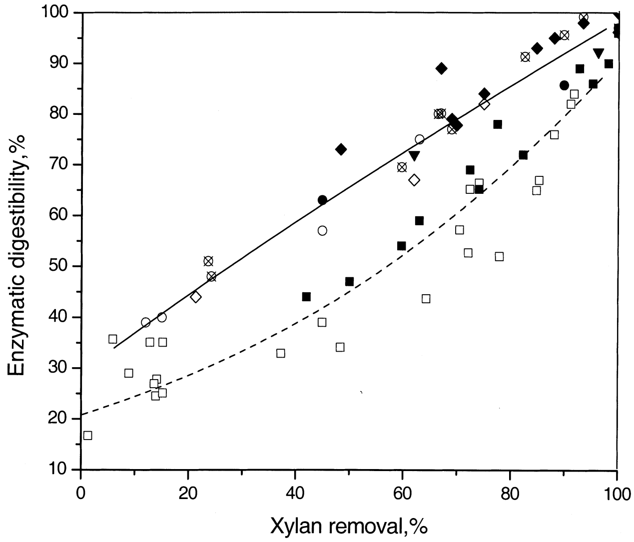

Figure 7. Effect of xylan removal on enzymatic digestibility for batch and flowthrough pretreatment of corn stover at 160° to 220°C with only water and 0.1 wt% sulfuric acid: (□) water-only batch tube at a 5% solids concentration; (■) dilute acid batch tube at a 5% solids concentration; (○) water-only flowthrough reactor at a flow rate of 2 mL/min; (◇) water-only flowthrough reactor at a flow rate of 7.5 mL/min; (⊠) water-only flowthrough reactor at a flow rate of 25 mL/min; (●) dilute acid flowthrough reactor at a flow rate of 2 mL/min; (▼) dilute acid flowthrough reactor at a flow rate of 7.5 mL/min; and (◆) dilute acid flowthrough reactor at a flow rate of 25 mL/min.

declined as xylan removal increased. As expected, when pretreated at same time and temperature, acid addition improved the enzymatic hydrolysis of pretreated cellulose for both flowthrough and batch operations. Overall, the digestibility tended to follow the upper solid diagonal line for flowthrough and the lower dashed line for batch operations, showing that, for a given xylan removal, flowthrough improved digestibility considerably compared with batch operation.

We also considered whether cellulose digestibility could be related to lignin removal, as shown in Figure 8. Although digestibility reached as high as about 90% for acid addition in the batch operation, lignin removal was no more than about 20%, and little correlation was obvious between digestibility and lignin removal. For batch pretreatment with water only, digestibility was related to increased lignin removal, but <30% of the lignin was removed at the maximum digestion of about 83%. In contrast, cellulose digestibility was strongly related to lignin removal for flowthrough operation, with or without acid addition. Furthermore, whereas the digestibility for batch pretreatment did not exceed 90%, it reached virtually 100% when about 75% of the lignin was removed for flowthrough operation, implying that lignin removal augmented digestibility.

Together, the results in Figures 7 and 8 could be interpreted to mean that cellulose digestibility is affected by xylan removal only for batch operations, whereas either or both xylan and lignin removal enhance cellulose digestibility for flowthrough operations. However, this would imply that lignin reacts to soluble products for flowthrough, but not batch operation. On the other hand, data from our laboratory reported elsewhere show that lignin and hemicellulose removal are directly related at high flow rates for flowthrough operations (Liu and Wyman, 2003). This observation supports the idea that, during hemicellulose hydrolysis, lignin continually reacts into soluble products that react further to form insoluble materials, which precipitate onto biomass unless removed from the reactor first (Liu and Wyman, 2003). Adding sulfuric acid could accelerate the release of soluble lignin compounds into solution but could also speed their conversion to insoluble species even more, thus limiting the amounts dissolved. Furthermore, substrates from flowthrough pretreatment attained 90% digestibility once xylan and lignin removals were about 80% and 70%, respectively, whereas batch systems, with <30% lignin removal, required >90% xylan removal to attain similar cellulose conversion.

These two observations suggest that lignin is disrupted during batch pretreatment into forms that precipitate onto the solids but interfere less with enzyme action than native lignin. This concept is consistent with microscopic observations of pretreated biomass surfaces (Robert Torget, personal communication). On the other hand, lignin removal in the flowthrough system provides greater accessibility of cellulose to cellulase and also reduces nonproductive binding of cellulase to lignin.

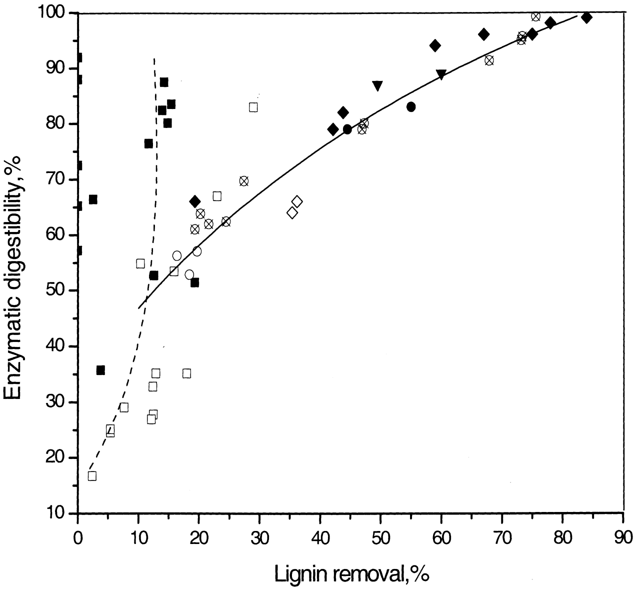

Figure 8. Effect of lignin removal on enzymatic digestibility for batch and flowthrough pretreatment of corn stover at 160° to 220°C with water only and 0.1 wt% sulfuric acid: (□) water-only batch tube at a 5% solids concentration; (■) dilute acid batch tube at a 5% solids concentration; (○) water-only flowthrough reactor at a flow rate of 2 mL/min; (◇) water-only flowthrough reactor at a flow rate of 7.5 mL/min; (⊠) water-only flowthrough reactor at a flow rate of 25 mL/min; (●) dilute acid flowthrough reactor at a flow rate of 2 mL/min; (▼) dilute acid flowthrough reactor at a flow rate of 7.5 mL/min; and (◆) dilute acid flowthrough reactor at a flow rate of 25 mL/min.

CONCLUSIONS

Increasing severity (time, temperature, and acid concentration) and flow of liquid through biomass enhanced xylan removal and cellulose digestibility for corn stover pretreatment, supporting previous observations that enzymatic hydrolysis can be related to xylan removal. Flow also substantially increased lignin removal, and cellulose digestibility was always greater at the same xylan removal level for the flowthrough reactor than that for the batch system, implying that lignin removal augments digestibility. However, for batch pretreatment, adding acid reduced lignin removal but increased cellulose digestibility. In contrast, for the flowthrough system, adding acid increased both lignin removal and digestibility compared with water-only flowthrough operation.

These results suggest that a large fraction of lignin reacts to soluble products that react further to form insoluble species if left in the reactor. Adding acid to the batch system appears to promote both lignin dissolution and precipitation. In either event, the net result is to disrupt the lignin and improve access of cellulose to enzymes. Flowthrough operation removes lignin before it can condense onto the biomass surface, enhancing the accessibility of cellulose and its digestibility even more.

Overall, it appears that lignin modification is important to enhance cellulose digestibility and that lignin removal provides even greater benefits. Unfortunately, based on these data, it is still difficult to rate the relative importance of hemicellulose versus lignin removal. At one extreme, hemicellulose removal may be vital with additional benefit from reducing lignin levels. At the other extreme, lignin alteration could be essential to high digestibility, with hemicellulose disappearance simply a marker of lignin disruption during batch pretreatment. Of course, another possibility is that removing lignin reduces nonproductive binding of cellulase, increasing its efficiency during hydrolysis. It is likely that all of these together enhance performance; thus, further research is planned to clarify these possibilities.

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