Chapter 6

Unconventional Relationships for Hemicellulose Hydrolysis and Subsequent Cellulose Digestion

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Cellulosic biomass can be pretreated with dilute sulfuric acid to recover high yields of sugars directly from hemicellulose and subsequently by enzymatic hydrolysis of the residual cellulose, and these sugars can be used to produce fuels and chemicals with unique and powerful economic, environmental, and strategic benefits. Pretreatment is costly for such biological routes, and we seek to better understand hydrolysis kinetics to support emerging applications and needed technology advances. Hemicellulose removal is affected by solids concentration and flow through the solids, contrary to customary first-order reaction models. We also measure a wide range of oligomer species, particularly at lower acid levels, that most kinetic models ignore or oversimplify, and a model is proposed to more accurately predict their behavior. The solubility of oligomers is also being measured by a new technique to better understand its effect on kinetics. New data suggests that lignin alterations during hemicellulose hydrolysis are important in improving enzymatic hydrolysis of cellulose.

Introduction

Biological production of fuels and commodity chemicals from inexpensive and abundant sources of cellulosic biomass can provide unparalleled environmental, economic, and strategic benefits *(1,2)*. For example, enzymatic hydrolysis of the cellulose in these materials to glucose followed by fermentation to ethanol is an economically attractive route to production of alternative liquid transportation fuels *(3,4,5,6,7)*. However, biomass must be pretreated to open up its structure prior to such biological operations so that high yields vital to economic success can be realized. Pretreatment is currently one of the most expensive steps in bioconversion routes, and advanced pretreatment technologies are needed to significantly reduce costs, improve cellulose digestibility, simplify upstream and downstream operations, and provide the potential for additional revenues from co-products *(8,9,10,11)*. Better knowledge of pretreatment systems would facilitate such advances and accelerate commercial applications by giving practitioners and financial organizations greater confidence in scale-up *(9)*.

Dilute Acid Hydrolysis

A number of organizations favor hemicellulose hydrolysis by dilute sulfuric acid for pretreatment because high sugar yields can be realized from hemicellulose during pretreatment and from cellulose in subsequent enzymatic hydrolysis of the solid residue *(11,12,13,14,15,16,17)*. Compared to water-only processes, sulfuric acid pretreatments increase the ratio of monomeric to oligomeric sugars and can produce monomeric sugar yields of up to 90% of the theoretical maximum *(19,20,21,22,23)*. Novel technologies based on flowing liquid through solid biomass have been shown to produce higher hemicellulose sugar recoveries, greater lignin removal, less inhibitors in the hydrolyzate liquid, and highly digestible cellulose when compared to conventional systems *(25,26,27,28,29)*. However, the high water consumption required for such flow systems results in excessive energy consumption for pretreatment and product recovery, and development of satisfactory equipment would be challenging. Nonetheless, understanding the cause of the enhanced performance of flow systems could lead to novel advanced pretreatments that reduce costs.

Data and kinetic models of dilute acid pretreatment are vital to provide a foundation for understanding hemicellulose hydrolysis and the cause of enhanced performance by flow system systems. Initial hemicellulose hydrolysis models were adapted from Saeman's first-order homogeneous kinetic model of cellulose hydrolysis in a dilute acid batch system *(30)* and later modified to include two different fractions of hemicellulose, one of which is more easily hydrolyzed than the other *(31)*. Oligomers were eventually included as reaction intermediates in a few studies *(32,33)*, but these models either ignored oligomers or treated them as only one or two discrete compounds. Although other models have been devised over the years *(34,35,36)*, they all evolve from the same first-order kinetic representation, and none reported in the literature is robust enough to adequately describe the changes in observed performance for different reactor configurations.

Jacobsen and Wyman studied the effect of solids loading on the total yield of xylose as both monomers and oligomers for hydrolysis of sugarcane bagasse in water at 200°C and found a statistically significant increase in yield by reducing solids loading from 1% to 0.5% *(37)*. Others have shown that sugar recovery increases with water content in batch systems *(38,39)*. Such results suggest that flowthrough operations benefit from the large amounts of water applied, but a complete explanation has not yet been established. In addition, the effect of solids concentration is not consistent with the Saeman model or any of its derivatives *(30,32)*.

To reconcile these differences, we have proposed that pretreatment models should combine reaction with mass transfer and solubility limitations so that the concentration gradient between the solid and the liquid phases is taken into account. Furthermore, we have applied depolymerization kinetics to account for the production of sugar monomers and oligomers observed during the hydrolysis of cellulosic biomass, particularly at low acid levels. Some aspects of these studies will be summarized in this chapter.

Enzymatic Hydrolysis of Pretreated Cellulose

Several structural and compositional attributes of biomass are thought to influence the enzymatic hydrolysis of cellulose to glucose including cellulose crystallinity, lignin levels, hemicellulose removal, accessible surface area of cellulose, and the presence of acetyl groups *(40,41)*. However, the complex structure of biomass makes it difficult to discern the relative importance of these features and their roles, and reducing one barrier to digestion can alter the importance of others. For example, removing hemicellulose also removes acetyl groups and usually changes the lignin left in the material making it difficult to ascertain which factor was most influential in improving performance. Various studies have reported that cellulose hydrolysis improves with increased lignin removal although differences were reported in the degree of lignin removal needed *(42,43,44)*. The ratio of syringyl to guaiacyl lignin groups was also shown to have important effects on enzymatic digestibility *(45)*. It is probable that one of the more significant factors is the impact of lignin on fiber swelling which in turn influences cellulose accessibility *(46,47)*. Lignin has also been claimed to depolymerize and then repolymerize in a different morphology during hemicellulose hydrolysis *(48,49)*.

The removal of lignin not only increases cellulose accessibility but also enhances cellulase availability. Lignin and its complexes physically and chemically resist enzymatic attack, and condensed lignin also has the ability to adsorb protein from aqueous solutions *(50)*. Thus, it appears that lignin removal not only opens up more space for enzymes but also reduces non-specific enzyme adsorption, improving the efficacy of hydrolysis *(41, 51,52,53,54)*.

On the other hand, several studies showed a direct relationship between cellulose digestion and hemicellulose removal. Knappert et al showed that removal of hemicellulose in poplar by dilute acid hydrolysis increased the susceptibility of cellulose to enzymes *(12)*. Grohmann et al also showed a direct relationship between hemicellulose removal and cellulose digestion and concluded that although lignin removal could enhance digestibility, it was not necessary to achieve good cellulose conversion *(55,56)*. However, some substrates required higher temperatures for effective cellulose hydrolysis at the same degree of hemicellulose removal, suggesting that hemicellulose is not the only factor impacting digestibility, while other studies do not support a role for hemicellulose in changing cellulose digestibility *(16,57,58)*. It is important to note that lignin could be altered at the high temperatures typically employed for hemicellulose hydrolysis, thereby impacting cellulose digestion rates *(16)*.

As part of our pretreatment research, we have evaluated the digestibility of corn stover cellulose following pretreatment and found that it changes significantly with pretreatment reactor configuration. Furthermore, we have observed that these differences in performance can be related to modification or removal of lignin as well as removal of hemicellulose, and this chapter will include a summary of some of these findings along with our results for hemicellulose hydrolysis.

Materials and Methods

Sample Preparation

The National Renewable Energy Laboratory (NREL) in Golden, Colorado graciously provided corn stover from a large lot they obtained from Harlan, Iowa and maintained at controlled conditions. This material was milled to pass through a 2 mm opening and then screened to obtain a –420 +250 µm fraction which was stored in plastic Ziploc bags and kept in a freezer (-20°C) for all tests.

The composition of the corn stover was determined through application of NREL LAP procedures 001, 002, and 012 *(59,60,61)*, and this substrate was 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 by weight.

Reactors

For water-only tests, batch reactors were constructed by cutting stainless steel or Hastelloy C276 tubing (0.5" OD x 0.035" wall thickness) into 4" sections that were fitted with Swagelok couplings and removable threaded end caps, giving a total reactor volume of 9.5 mL. For dilute acid tests, Hastelloy C276 tubing was used, and Teflon plugs were inserted in the ends to protect the stainless steel caps as utilized by researchers at Auburn University *(35)*.

Work was also performed with a mixed batch reactor to gain insight into the effects of agitation on batch systems and the possible influence of fluid velocity on hemicellulose removal. For these tests, a 1-L Parr bomb constructed of Carpenter-20 (Parr Instruments, Moline, IL) was fitted with a flat-blade impeller on a one-piece shaft and operated at varying speeds using a Parr DC motor drive (A1750HC, Parr Instruments, Moline, IL) *(62)*.

Two flowthrough systems were employed in this work: a smaller reactor (1/2-in ID × 1.84-in length with an internal volume of 3.6 mL) and a larger reactor (1/2-in ID × 6-in length with an internal volume of 14.3 mL). All reactor parts were obtained from Maine Valve and Fitting Co., Bangor, ME. A 1/8-in stainless steel thermocouple (Omega Engineering Co., Stamford, CT) was installed at the outlet of the reactor to monitor temperature, and 316 stainless steel tubing was used as a preheating coil (1/4-in OD × 0.35 inch wall), to connect the reactor with other components of the system, and as a cooling coil (1/8-in OD × 0.028 inch wall). The preheating coil was long enough to allow the incoming water to reach the desired temperature before it entered the reactor. To operate the flowthrough unit, about 2 grams of corn stover was loaded into the reactor, and the reactor was then connected to the system. Distilled water at room temperature was pumped through the reactor to purge air, completely wet the biomass in the reactor, and pressurize the system to the set pressure. Then, the reactor and preheating coil were submerged for 2 minutes in a 4-kW fluidized sand bath (model SBL-2D, Techne Co., Princeton, NJ) set at a temperature of 100°C and then moved to a second sand bath set at the target reaction temperature. Flow was maintained until the desired reaction time, at which point the reactor and preheating coil were transferred to an ice water bath to stop the reaction *(63,64)*.

Solubility Measurements

The solubility of monomeric and oligomeric sugars was measured by an inline refractive index detector (AFAB Enterprises, Eustis, FL) attached to a 1" stainless steel tee with a plug at the bottom and a rubber stopper to cover the top. During operation, this device was loaded with a known mass of water and sugar, placed on a programmable hotplate/stirrer, and covered with styrofoam insulation to minimize heat losses. The RI signal rapidly increased with temperature as the sugars dissolved and more slowly once they were all in solution. Thus, the solubility could be determined from the total amount of sugar and water added and the temperature at which the slope of the RI signal versus temperature changed. Solubilities were also measured by a standard method in which a vial containing a sugar/water mixture was submerged in a constant temperature water bath and samples taken at equilibrium were analyzed by HPLC and gravimetric methods *(65)*.

Enzymes

Cellulase (Spezyme CP, Lot No. 301-00348-257, Genencor, Palo Alto, CA) at 28 FPU/mL supplemented with β-glucosidase (Novozyme 188 at 250 β-glucosidase IU/mL, Sigma, St. Louis, MO) at a ratio of 1:1.75FPUase:CBUase was used for all hydrolysis experiments. Enzymatic treatments were performed at 60 FPU/g cellulose with the activity as calculated by adding the activities of both the Spezyme CP and Novozyme 188 *(66)*.

Enzymatic Hydrolysis

Enzymatic hydrolysis of pretreated cellulose from both the batch and flowthrough systems was conducted at a 2% solids concentration (g dry weight/100 mL) in 50 mM acetate buffer (pH 4.8) containing 40 µg/mL tetracycline and 30 µg/mL cycloheximide. Flasks were pre-incubated at 50°C in water using an orbital shaker bath (3540, Barnstead International, Dubuque, IA) at 150 rpm for 10 minutes, and the enzymes were added to start the hydrolysis after acclimation. Aliquots of 0.5 mL were taken at different times (0, 4, 24, 48, 72 h), immediately chilled on ice, and centrifuged at 5000 G for 10 min. Total sugar analyses were carried out on the resultant supernatants.

Analytical Procedures

Sugar and acid insoluble lignin content of solids were determined using the Klason lignin procedure published as NREL LAP 003 and 014 *(67,68)*. The sugar concentrations were measured with a high performance liquid chromatography system (Waters 2695, Milford, MA) equipped with a pulsed refractive index detector (Waters 2410, Milford, MA). The column was equilibrated with deionized water at a flow rate of 0.6 mL/min. An Aminex HPX-87P (Bio-Rad, Sunnyvale, CA) column was used for sugar separations.

Results and Discussion

Effects of Flow Rate on Corn Stover

Total solids removal

As presented in Table 1 and Figure 1, our results show that the rate of total solids removal increased with both temperature and flow rate for both water-only and dilute acid pretreatment of corn stover. However, for water-only pretreatment, about 94-97% of the total material was accounted for in all runs at 180°C after 16 minutes, suggesting little of this loss in mass was due to decomposition at this temperature. However, the overall mass recovery decreased with increasing temperature, especially for batch and low flow rate runs, indicating that more mass was lost at these operating conditions. These results show that increasing flow rate increases removal of solids, supporting the idea that at longer residence times and higher temperatures, dissolved sugars decomposed to furfural and other volatile degradation products. It is expected that most of the overall loss in mass was due to dissolved xylan, as much less glucan was dissolved over this range of temperatures and times (Table 1).

Table 1. Total mass dissolution (TMD), total mass remaining (TMR), xylan remaining, and glucan remaining in the solid residue for water-only pretreatment of corn stover.

Temp. (°C)	*Flow rate (mL/min)*	*Time (min)*	*TMD (%)*	*TMR (%)*	*OMB[1] (%)*	*Xylan remaining (%)*	*Glucan remaining (%)*
180	0	12	13.3	82.7	96.0	91.3	102.3
		16	18.1	78.9	97.0	80.0	101.5
180	10	12	27.0	66.5	93.5	60.8	
		16	32.4	62.3	94.7	42.5	99.4
200	0	12	22.5	63.5	86.0	40.1	101.0
		16	23.4	62.2	85.6	29.9	99.9
200	1	12	28.4	56.6	85.0	36.4	-
		16	32.7	53.0	85.7	29.4	99.5
200	10	12	51.2	48.5	99.7	14.1	99.3
		16	52.4	43.3	95.7	9.5	98.5
220	0	12	28.7	55.2	83.9	13.5	-
		16	30.2	53.1	83.3	8.7	98.8

OMB[1]: overall mass balance = TMD + TMR

Xylan removal

Xylan removal increased with flow rate and temperature for both water-only and very dilute acid pretreatment, as illustrated by Figure 1. As can be seen from this figure, flowthrough pretreatment of corn stover with hot water at a flow rate of 10 mL/min removes more xylan at the same temperature and time than batch pretreatment of stover with 0.05 wt% sulfuric acid. Although it has been postulated that autohydrolysis of hemicellulose is catalyzed by acetic and other organic acids released during the breakdown of hemicellulose *(69)*, this mechanism cannot fully explain flowthrough reactor behavior in which hemicellulose solubilization is enhanced by flow without acid addition, especially at high flow rates. At such high flow rates, the acids will have little time to act before they are swept from the reactor, and the large volumes of water used also decrease the concentration of organic acids. In addition, other studies demonstrated that adding supplemental acetic acid did not accelerate the solubilization of hemicellulose during pretreatment of biomass with hot water, suggesting that the organic acids released are not the primary hydrolytic agent *(70,71)*.

Almost all of the dissolved xylan was in oligomeric form for water-only pretreatment of corn stover over a temperature range of 160-220°C. Addition of very small amounts of acid accelerated hydrolysis of xylose oligomers to monomers and increased the monomeric fraction, but the largest portion of the overall xylan dissolved remained in oligomeric form. These results demonstrate that oligomers are important intermediates during hemicellulose hydrolysis, especially at no acid or very low acid conditions. The yield of oligomers also increased tremendously with flow rate, suggesting that flow rate accelerates solubilization of hemicellulose. Together, these studies demonstrate that factors other than acid concentration and temperature impact hemicellulose hydrolysis, and although a detailed mechanism responsible for this behavior is still under investigation, we postulate that mass transfer plays an important role in hemicellulose hydrolysis, with this effect being especially important at no acid or very low acid conditions *(37,63)*.

Lignin removal

Our results show the amount of lignin removed increased with flow rate and a positive correlation was observed between xylan and lignin removal for flowthrough pretreatment, as shown in Figure 2. Consistent with observations by others that lignin and hemicellulose are covalently linked in native materials, we expect that hemicellulose oligomer-lignin compounds are released initially. In addition, these hemicellulose oligomer-lignin materials are expected to be soluble at high temperatures and can therefore be swept from a flowthrough system, especially with the large volume of water at high flow rates. However, when held at reaction conditions, the hemicellulose oligomer-lignin compounds are expected to break down to separate sugar oligomer and lignin fragments that can in turn form sugar monomers and monomeric lignin, respectively, depending on temperature, acid concentration, and residence time. The lignin species formed have a limited solubility and can form other low solubility products through condensation reactions. Thus, for batch runs with longer residence times, the overall result is dramatically lower lignin removal. For intermediate flow rates, some of the hemicellulose oligomer-lignin compounds are removed while the remaining portions have enough time to react to insoluble lignin-based products. Such a mechanism would explain the relationship between xylan and lignin removal shown in Figure 2 and why lignin removal is impacted by flow rate and temperature *(63)*.

Effect of Solids Concentration

The effect of solids loading on hemicellulose hydrolysis was studied by Jacobsen and Wyman *(37)* and Stuhler *(71)*. Water-only batch tube experiments with corn stover were performed at 200°C for 15 minutes at solids loadings of 5 and 21%, and a number of performance criteria including total solids remaining and total soluble xylan oligomer yields were evaluated *(71)*. Statistically significant differences in several results were observed at a 95% confidence level, as summarized in Figure 3.

It is interesting to note that xylan remaining in the solids decreases with increasing solids loading, which is opposite of what would be expected if solubility were limiting. A more likely reason for this is that the increased hydronium ion concentration at higher solids loadings accelerates sugar release. On the other hand, solubilized oligomers and total mass removal decrease while monomers and furfural increase with increased solids loading, consistent with this hypothesis.

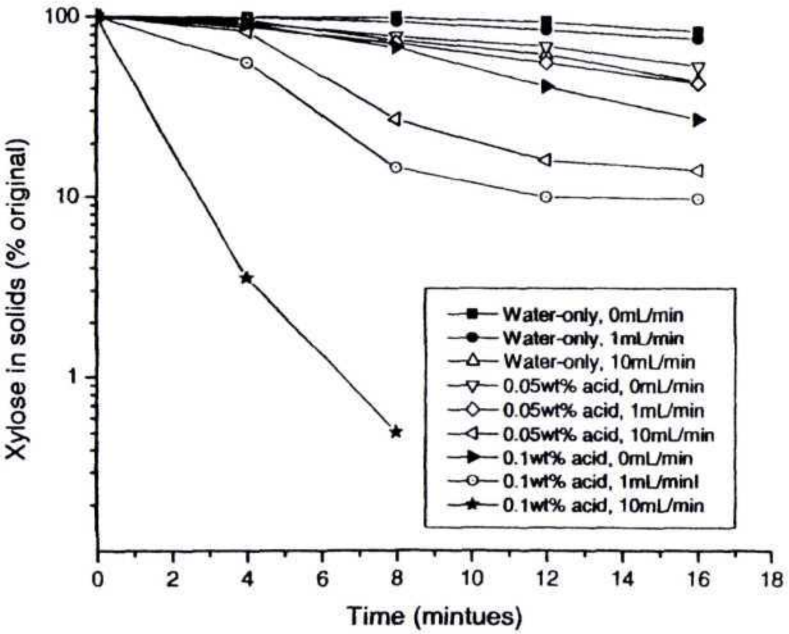

Figure 1. Effect of flow rate and acid concentration on xylose remaining in the solids at 180°C

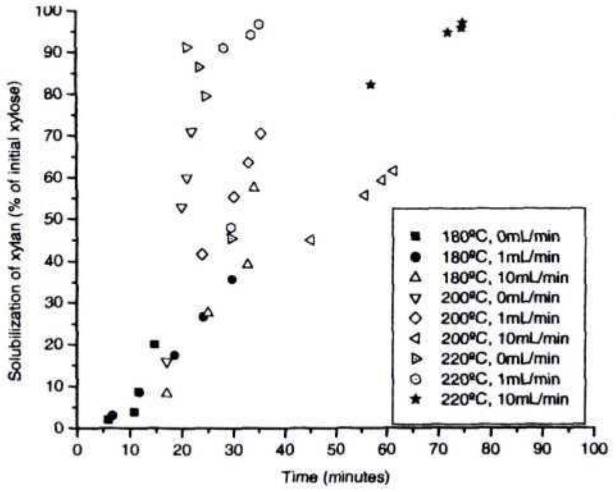

Figure 2. The effect of flow rate and temperature on the relationship between solubilization of xylan and lignin for water-only hydrolysis.

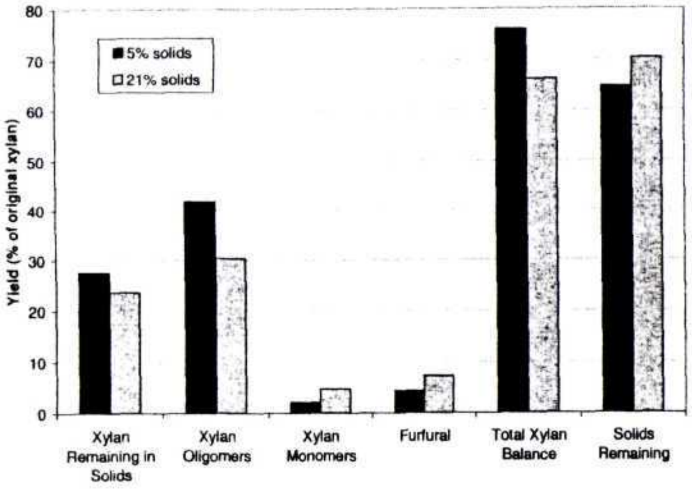

Figure 3. Effect of solids concentration on xylan hydrolysis yields after 15 minutes at 200°C.

The significant decrease in the xylan balance with increasing solids has two possible explanations: the degradation reactions are more complete (as evidenced by the higher furfural content) or higher concentrations of other solubilized components such as lignin remove more sugars from solution through condensation reactions. It is most likely that the observed effect results from a combination of these two mechanisms.

Traditional hemicellulose hydrolysis kinetic models cannot account for a change in hemicellulose sugar yields with solids concentration and suffer from inconsistencies that bring into question their mechanistic accuracy. Thus, although current models can be useful for a given flow regime, their ability to describe different systems such as flowthrough reactors on a consistent basis is unproven *(29)*.

Modeling Mass Transfer Effects

A model which includes the effects of mass transfer on hemicellulose hydrolysis has been developed by Brennan and Wyman with the following equations applied to describe a batch system *(62)*.

$$\frac{dH}{dt} = -k_1 H \tag{1}$$

$$\frac{dX_n}{dt} = k_1 H - k_d A\left(\frac{X_n}{V_p} - \frac{X_s}{V_t}\right) \tag{2}$$

$$\frac{dX_s}{dt} = k_d A\left(\frac{X_n}{V_p} - \frac{X_s}{V_t}\right) - k_4 X_s \tag{3}$$

$$\frac{dD}{dt} = k_4 X_s \tag{4}$$

Equation [1] expresses the reaction of hemicellulose, *H*, in the solid phase over time *t* to form oligomers X_n, where k_1 is a rate constant with units of time^{-1}.

Equation [2] is the differential equation describing the amount of soluble but still undissolved oligomers inside the particle, X_n, by accounting for their accumulation by chemical reaction and removal by mass transfer across a diffusive boundary layer. k_d is a mass transfer coefficient (length^2/time), X_s is the amount of xylan oligomers dissolved in the bulk solution (moles or mass), A is the surface area of a solid particle (length^2), V_p is the volume of the solid phase (length^3), and V_t is the total volume of the bulk solution (length^3). Equation [3] is the differential balance describing mass transfer of solubilized xylan into the bulk solution and its degradation to furfural, tars, etc. with k_4 being the degradation rate constant. Equation [4] is the differential balance for the chemical degradation of solubilized xylan to degradation products, D. Together, Equations 1-4 account for the effects of both mass transfer and chemical reaction during hemicellulose hydrolysis, a process that has been perceived to be completely reaction-controlled in the past.

Table 2 summarizes the mass transfer coefficients determined for this model for batch tubes, stirred batch, and flowthrough reactor configurations and shows that the diffusive mass transfer coefficient k_d increases in this order of reactor type. However, one would expect the mass transfer coefficient to follow such a pattern as flow is increased. On the other hand, although the rate constants for conventional models based on only chemical reaction can also be fit to data from these three reactor types, rate constants for these models should only depend on temperature, and these variations would not be expected. Thus, coupling mass transfer to reaction appears to provide a more meaningful explanation for the effects of flow on performance, but further work is needed to fully develop and evaluate this approach.

Oligomer Solubility

One of the factors that we need to integrate into our mass transfer model is the solubility limits of the sugars and oligomers involved and how they are influenced by the presence of other species. However, there is surprisingly little information available in the literature on the solubility of pure sugars in a two component (i.e., single sugar/water) mixture and almost none for sugar oligomers. For this reason, the in-situ solubility device described earlier was developed for measuring oligomer solubilities and was tested using β-cyclodextrin, an inexpensive starch oligomer of limited solubility *(72,73)*. Data was collected at various solids levels using both in-situ and water bath methods, with the results shown in Figure 4. The 95% confidence intervals on the data from this device intersect those of the literature data at every temperature except for at 55°C, where it is slightly higher. The in-situ method had a tighter confidence range than the water bath method, possibly because the latter method requires filtering a solution and removing it from a hot environment before analysis while the former method measures solubility instantly. We are now investigating using a much smaller apparatus to collect data with significantly less of the expensive oligomers needed for this research.

Table 2. Mass transfer coefficient k_d (cm^2/sec) determined for various reactor configurations for corn stover treated at 180 °C.

Reactor Type	*Water-Only*	*0.05wt% H_2SO_4*	*0.1wt% H_2SO_4*
Batch Tube	0.0025	-	0.0036
Stirred Batch	0.0058	-	-
Flowthrough – 1 ml/minute	0.004	0.0075	0.02
Flowthrough – 10 ml/minute	0.011	0.025	0.15

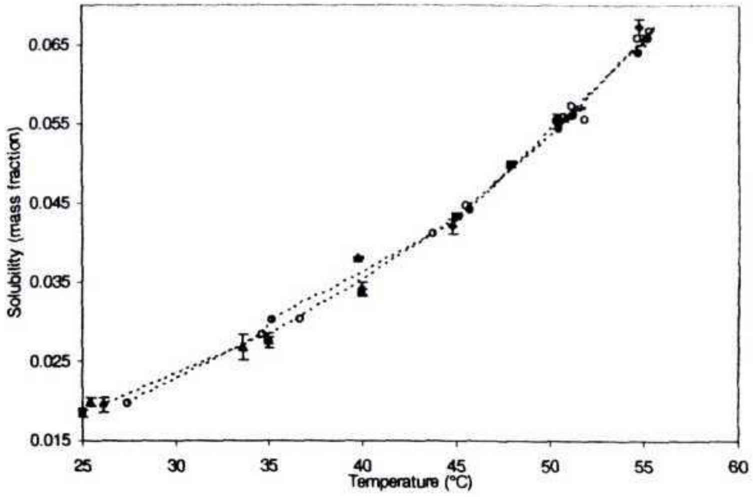

Figure 4. Solubilities of β-cyclodextrin obtained with the in-situ device (□), compared to isothermal water bath data (□)and literature values (◆ (72) and ■(73)). The dotted lines are 95% confidence intervals for the in-situ data.

An important goal of this element of our research is to develop tools to predict the solubility of oligomers at pretreatment temperatures at which it is difficult to collect solubility data. In this regard, we were able to estimate the experimental solubility data of low molecular weight xylo-oligomers using the ideal solubility law. However, many of the physical parameters needed in this model are unrecorded and had to be estimated. Subject to this limitation, the solubilities for xylobiose through xylohexaose at room temperature were predicted to be over 1% by mass and increased to greater than 55% at 120°C. This projection implies that solubility is not expected to be a limiting factor in water-only hydrolysis for oligomers with a degree of polymerization less than 6 *(65)*. However, additional data and modeling are needed to substantiate this finding and extend the method to higher molecular weight oligomers.

Oligomer Production, and Hydrolysis

We also studied the autohydrolysis of xylan at 200°C in hot water to help clarify the behavior of hemicellulose hydrolysis. After 5 minutes, around 37% of the potential xylose was present as soluble oligomers, as determined by post-hydrolysis of the resulting liquid fraction. These oligomers appeared to be high molecular weight compounds with a degree of polymerization (DP) greater than 10 because they could not be analyzed directly on our ion-moderated partition (IMP) chromatography column. However, after reaction for 10 minutes, most of the oligomers had a DP of less than ten and could be measured by the IMP column *(74)*.

To understand how the soluble oligomers react in solution, the kinetics of water-only hydrolysis of pure oligomers of DP 1 to 5 was followed at 200°C. Disappearance of each of these oligomer species could be described well by first-order homogeneous kinetics. It was further found that the decomposition rate constants of xylopentaose and xylotetraose were found to be similar. The rate constants of xylotriose and xylobiose were about two-thirds of the rate of xylotetraose and xylopentaose. Xylose decomposed at about half the rate of xylobiose. The formation of shorter oligomers from each of these pure oligomers was also tracked, and it was found that the release of lower DP species from the higher DP oligomers could not be accurately described unless degradation (products other than xylose or its DP 2 to 5 oligomers) of a significant fraction of the oligomers was integrated into the kinetic pathway. This result could help account for why yields are low for hemicellulose hydrolysis without acid addition.

Depolymerization Model

A primary limitation of traditional kinetic models is their inability to describe the time course distribution of oligomeric species of varying chain lengths that were observed in the experiments described in the previous sections. Because of the polymeric nature of hemicellulose, we would expect a distribution of hemicellulose fragments with varying chain lengths, and by adapting an approach developed by Simha *(75)*, the hydrolysis of corn stover hemicellulose was modeled as a depolymerization reaction *(35)*. Assuming that all bonds in the xylan chain are cleaved at the same rate, the instantaneous distribution of depolymerization fragments can be expressed by the following differential equation:

$$\frac{dN_j}{dt} = 2k_h \sum_{i=j+1}^{n} N_i - k_h (j-1) N_j \qquad (5)$$

or

$$N_j = N_n^0 (1-\alpha)^{(j-1)} \alpha [2 + (n-j-1)\alpha] \quad (6)$$

in which k_h is the hydrolysis rate constant, N_n^0 is the initial concentration of polymer of initial length n, N_j = molar concentration of a fragment of length j, and $\alpha = 1 - e^{-k_h t}$.

Figure 5a reveals that this depolymerization model shows trends consistent with data but is not particularly accurate. However, a modified model was developed in which the right-hand side of Equation [5] was multiplied by a first-order reactivity term, $a = e^{-kt}$, with a being the reactivity, k_a a proportionality constant, and t the time, and application of this model did a much better job of describing the time course of xylan hydrolysis, as shown in Figure 5b. The fact that declining bond reactivity describes the data well suggests that the original assumption that all xylan bonds are broken equally at random is not valid. An obvious next step is the determination of individual bond energies within the hemicellulose molecule and incorporation of this information into a modified model.

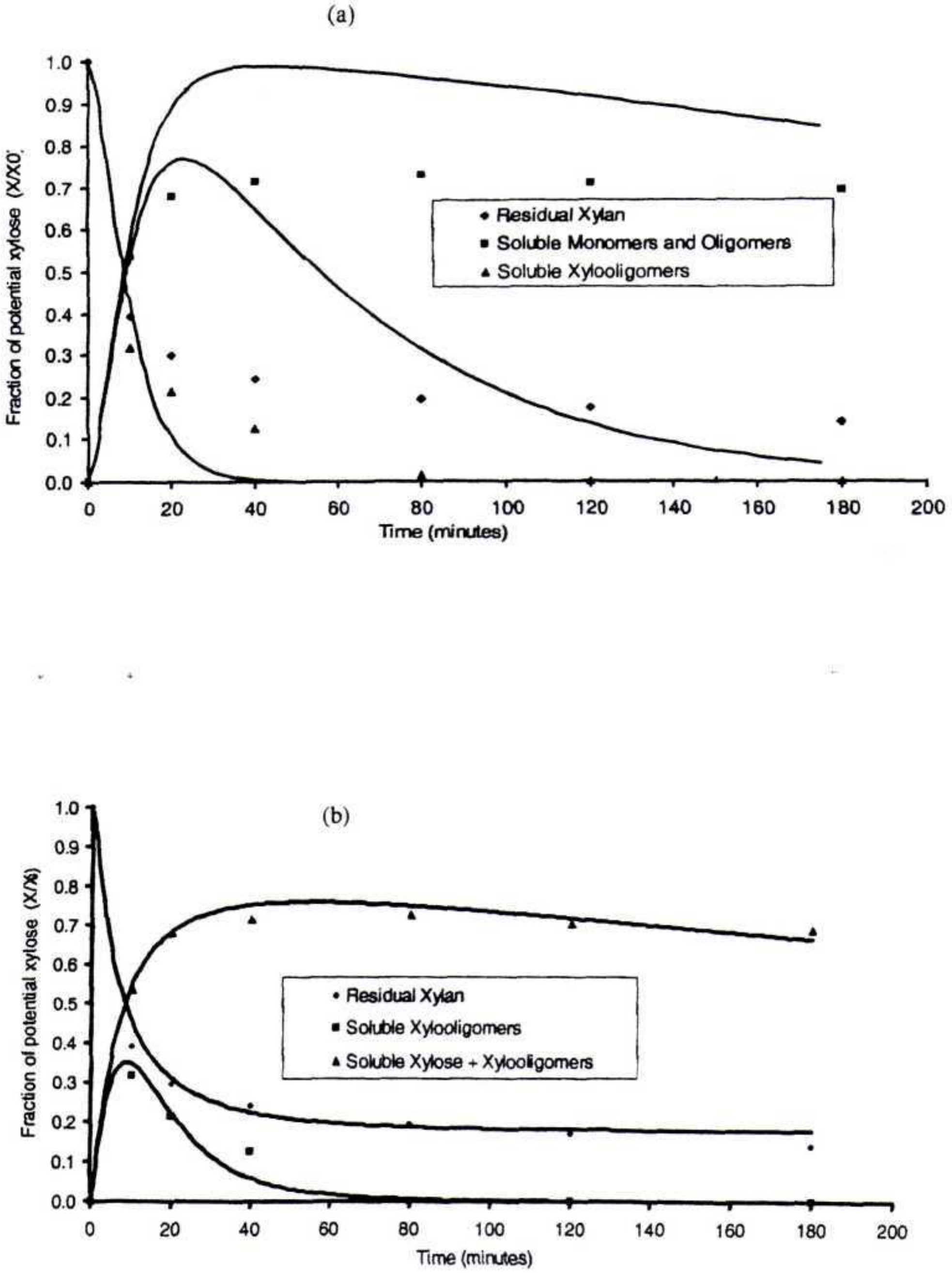

Figure 5. Comparison of an unmodified and modified depolymerization model applied to batch hydrolysis of corn stover at 140 °C with 0.5% sulfuric acid added.

The Relationship between Digestibility and Hemicellulose and Lignin Removal

Figure 6a shows the relationship of enzymatic digestibility of cellulose to xylan removal for both water-only and very dilute acid hydrolysis pretreatment in flowthrough and batch reactors. When no acid was used, less xylan removal was required to achieve the same enzymatic digestibility of cellulose in the solid residue from a flowthrough reactor as from a batch system. For example, removal of only about 20% of the xylan in a flowthrough reactor without addition of acid achieved a cellulose digestibility of approximately 55% while 78% of the xylan had to be removed in a batch system to achieve about the same yield. Alternatively, the digestibility of cellulose for corn stover pretreated in a flowthrough system was about 20% higher than that from the batch system at the same level of xylan removal. Because digestibilities peak at high xylan removal, this difference decreased when xylan removal was greater than about 85%.

Figure 6a also indicates that less xylan removal is needed for the flowthrough reactor to achieve the same level of enzymatic digestibility as a batch system when very dilute sulfuric acid is added. However, the relative advantage of flowthrough operation decreased with addition of sulfuric acid, and flowthrough reaction gave about a 10% greater enzymatic digestibility compared to the batch approach, with the differences again declining at high xylan removal levels. Nonetheless, acid addition improved the extent of enzymatic digestion of cellulose for both flowthrough and batch operations.

While the enzymatic digestibility of cellulose could be directly related to xylan removal for both batch and flowthrough operations, its relationship to lignin removal was not as consistent, as shown in Figure 6b. In particular, lignin removal tended to peak at about 20% in batch reactors with acid added and at about 30% when corn stover was treated without acid. Nonetheless, cellulose digestibility was as high as 90% for batch operations. On the other hand, cellulose digestibility was found to increase almost linearly with lignin removal for corn stover pretreated by the flowthrough reactor with or without added sulfuric acid, and the digestibility reached nearly 100% at the maximum of about 75% lignin removal observed for flowthrough pretreatment. In addition, while adding acid to the batch system decreased lignin removal but increased digestibility, the relationships between lignin removal and cellulose digestibility were quite similar for flowthrough operation whether acid was added or not.

Coupling the relationship between removal of xylan and lignin in Figure 2 with the digestibility results in Figure 6 leads us to conclude that adding dilute acid to biomass may enhance lignin solubilization, but for batch operations that lignin may precipitate as different species that interfere less with enzyme action than native lignin. The result would be that acid addition improves cellulose digestion relative to an uncatalyzed batch reactor while not being as effective as flowthrough systems that remove much more solubilized lignin before it can condense. Furthermore, it could be that lignin modification is more important than xylan dissolution and that the latter provides a convenient marker of lignin alterations that improve cellulose digestibility. This leads us to believe that lignin modification is important to enhance the digestibility of cellulose and that lignin removal provides even greater benefits. Of course, removing lignin also reduces non-productive binding of cellulase, increasing its efficiency during hydrolysis, and we believe it likely that enhancing accessibility of cellulose and reduction of nonproductive binding of enzyme both improve performance. Unfortunately, it is difficult to prove this mechanism, but further research is planned to clarify these possibilities *(64)*.

Conclusions

Our results show that increasing flow rate in a flowthrough reactor significantly enhanced xylan removal for pretreatment of corn stover with just hot water or when acid was added. In addition, yields of xylose monomer and oligomers increased with decreasing solids concentration in batch systems. This variation in xylose removal with flow rate and solids concentration is not consistent with predictions from first-order homogeneous kinetic models that incorporate only the effects of temperature, acid concentration, and time. Furthermore, these variations cannot be attributed to acetic acid released from biomass during hydrolysis as this effect should decrease, not increase, with flow rate. Our preliminary models show that coupling chemical reaction of xylan to soluble oligomers in the solids with mass transfer of these oligomers into solution followed by further reaction once in solution could more appropriately account for the change in reaction rate with flow rate and solids concentration. A novel apparatus is being applied to gather new data on oligomer solubility to better understand whether solubility limitations could also play an important role in this sequence. We have measured a range of oligomer chain lengths during xylan hydrolysis, and soluble oligomers were projected to directly degrade to nonsugars such as furfural as well as form lower DP species. Although existing models do not consider oligomer reactions in any detail, we have found that a depolymerization model can describe their basic features provided consideration is given to a change in reactivity as the reaction progresses. Further research targets enhancing our data and bringing these observations about oligomer behavior into a single analytical framework that can explain the effects of solids concentration and flow rate on hemicellulose hydrolysis on a consistent basis.

Lignin removal was limited to about 30% for a batch reactor without acid addition and dropped to about 20% when dilute sulfuric acid was used, and the enzymatic digestibility of cellulose in the residual solids increased with xylan removal for both, consistent with observations by others. On the other hand, introducing flow of liquid through the solids enhanced removal of lignin

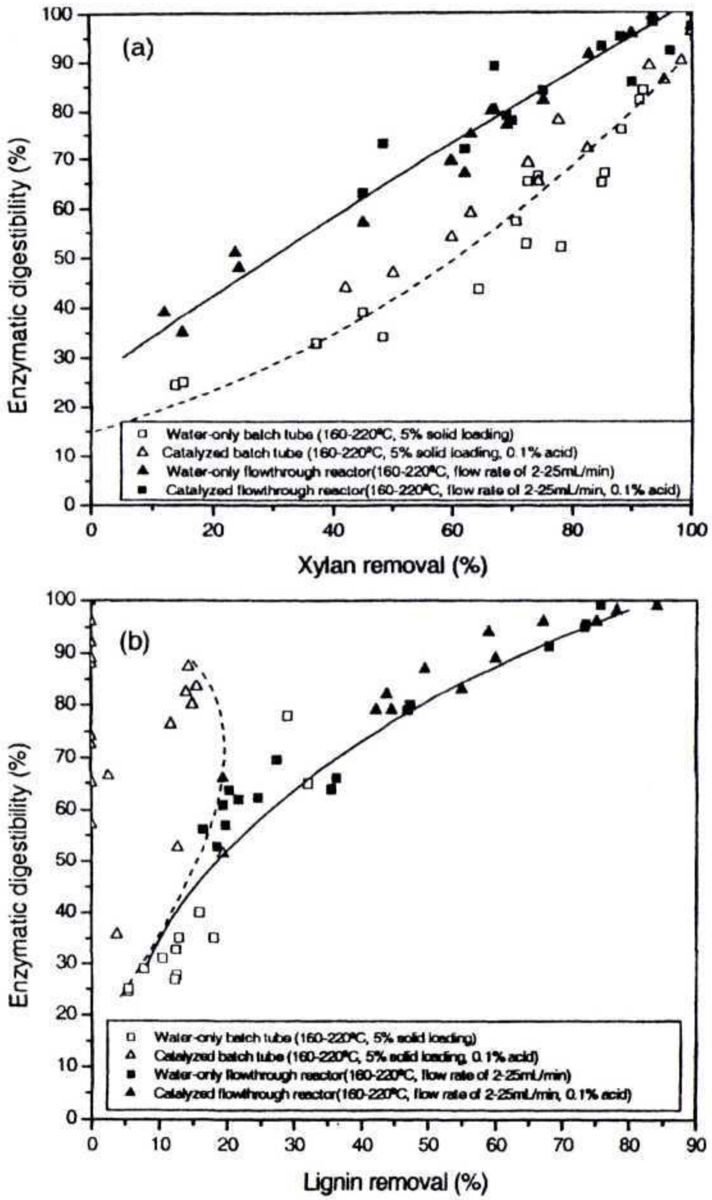

Figure 6. Effect of xylan removal (a) and lignin removal (b) on enzymatic digestibility of cellulose produced by hemicellulose hydrolysis of corn stover at the conditions noted.

considerably compared to batch operations, with as much as 70% of the total lignin being removed at high flow rates. In addition, the enzymatic digestibility of the cellulose in the solids from a flowthrough reactor was always greater than that from a batch system at the same degree of xylan removal. These flowthrough findings imply that lignin removal augments digestibility. Yet, adding acid to a batch system also increases cellulose digestibility even though lignin removal drops. The direct relationship observed between removal of lignin and xylan at high flow rates for flowthrough operations leads us to believe that lignin is solubilized during hemicellulose hydrolysis in both batch and flowthrough systems but that much of the lignin will react and precipitate back on the solid surface unless it is removed during pretreatment. Nonetheless, we believe that modification of lignin in this way improves the accessibility of enzymes to cellulose, increasing digestion yields. However, removal of lignin altogether before it can reform on the surface prevents it from adsorbing enzyme and from interfering with enzyme action, further enhancing performance. Although difficult to prove, lignin disruption or removal may actually be more important than xylan dissolution, and the latter may provide a simple marker of lignin alteration in batch systems. Our research continues to explore these relationships with the goal of improving the effectiveness of pretreatment in recovering hemicellulose sugars and enhancing cellulose digestibility.

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References

1. Lynd, L.R.; Cushman, J.H.; Nichols, R.J.; Wyman, C.E. *Science (Washington, DC).* **1991**, 251(4999), 1318-23.

2. Wyman, C.E.; Goodman, B.J. *Appl. Biochem. Biotechnol.* **1993**, 39, 41-59.
3. Lynd, L.R. *Ann. Rev. Energy Environ.* **1996**, 21, 403-465.
4. Wright, J.D. *Chem. Eng. Prog.* **1988,** 84(8), 62-74.
5. Wyman, C.E. In *Handbook on Bioethanol: Production and Utilization;* Wyman, C.E. Ed.; Taylor & Francis, Washington, DC, 1996: pp 1-18.
6. Lynd, L.R.; Wyman, C.E.; Gerngross, T. U. *Biotechnol. Prog.* **1999,** 15, 777-793.
7. Hinman, N.D.; Schell, D.J.; Riley, C.J.; Bergeron, P.W.; Walter, P.J. *Appl. Biochem. Biotechnol.* **1992**, 34/35, 639-649.
8. Wright, J.D. In *Energy from Biomass and Wastes XII*; Klass, D.L.; Eds., : Institute of Gas Technology: Chicago, IL. 1989, pp1247-1296.
9. Wyman, C.E. *Ann. Rev. Energy Environ.* **1999,** 24, 189-226.
10. Wyman, C.E. *Biotechnol. Prog.* **2003,**19(2), 254-262.
11. Grethlein, H.E. US patent 4237226, 1980.
12. Knappert, D.; Grethlein, H.; Converse, A.O. *Biotechnol. Bioeng.* **1980**, 22(7), 1449-63.
13. Heitz, M.; Capekmenard, E.; Koeberle, P.G.; Gagne, J.; Chornet, E.; Overend, R.P.; Taylor, J.D.; Yu, E. *Bioresour. Technol.* **1991,** 35(1), 23-32.
14. Ramos, L.P.; Breuil, C.; Saddler, J.N. *Appl. Biochem. Biotechnol.* **1992,** 34-35, 37-48.
15. Wooley, R.; Ruth, M.; Glassner, D.; Sheehan, J. *Biotechnol. Prog.* **1999,** 15, 794-803.
16. Torget, R.; Werdene, P.; Himmel, M.; Grohmann, K. *Appl. Biochem. Biotechnol.* **1990,** 24-25, 115-26
17. Lee, Y.Y.; Wu, Z.; Torget; R.W. *Bioresour. Technol.* **1999**, 71(1), 29-39.
18. Torget, R.W.; Kim, J.S.; Lee, Y.Y. *Ind. Eng. Chem. Res.* **2000**, 39(8), 2817-2825.
19. Torget, R.; Werdene, P.; Himmel, M.; Grohmann, K. *Appl. Biochem. Biotechnol.* **1990,** 24-25, 115-26.
20. Grohmann. K.; Torget, R.; Himmel, M. *Biotechnol. Bioeng.* **1985,** 15, 59-80.
21. Hsu T-A. In *Handbook on Bioethanol: Production and Utilization;* Wyman, C.E. Ed.; Taylor & Francis, Washington, DC, 1996: pp 179-212.
22. McMillan, J.D. In *Enzymatic Conversion of Biomass for Fuels Production.* Himmel, M.E.; Baker, J.O.; Overend, R.P. Eds, ACS Symposium Series 566, American Chemical Society, Washington, DC, 1994, 566, 292-324.
23. von Sivers, M.; Zacchi, G. *Bioresour. Technol.***1995,** 51, 43-52.
24. Bobleter, O. *Prog. Polym. Sci.* **1994**, 19(5), 797-841.
25. Bonn, G.; Oefner, P.J.; Bobleter, O. *Fresenius' Z. Anal. Chem.* **1988**, 331(1), 46-50.
26. Torget, R.; Hatzis, C.; Hayward, T.K.; Hsu, T-A.; Philippidis, G.P. *Appl. Biochem. Biotechnol.* **1996**, 57/58, 85-101.
27. Hoermeyer, H.F.; Schwald, W.; Bonn, G.; Bobleter, O. *Holzforschung*. **1988**, 42(2), 95-98.
28. Laser, M.; Schulman, D.; Allen, S.G.; Lichwa, J.; Antal, M.J.; Lynd, L.R. *Bioresour. Technol.* **2001**, 81(1), 33-44.
29. van Walsum, G.P.; Allen; S.G.; Spencer, M.J.; Laser, M.S.; Antal, M.J. Jr.;, Lynd, L.R. *Appl. Biochem. Biotechnol.* **1996**, 57/58, 157-170.
30. Saeman, J.F.; Bubl, J.L.; Harris, E.E. *Ind. Eng. Chem. Res.* **1945**,17, 35-7.
31. Kobayashi, T.; Sakai, Y. *Mokuzai Toka Shingikai Hokoku*. **1956**, 5, 1-9.
32. Conner, A.H.; Lorenz, L.F. *Wood and Fiber Sci.* **1986**, 18(2), 248-263.
33. Garrote, G.; Dominguez, H.; Parajo, J.C. *Proc. Biochem.* **2001,** 36, 571-578.
34. Jacobsen, S.E.; Wyman, C.E. *Appl. Biochem. Biotechnol.* **2000**, 84-86, 81-96.
35. Lloyd, T.; Wyman, C.E. *Appl. Biochem. Biotechnol.* **2003**, 105-108, 53-67.
36. Stuhler, S.L.; Wyman, C. E. *Appl. Biochem. Biotechnol.* **2003**, 105-108, 101-114.
37. Jacobsen, S.E.; Wyman, C.E. *Ind. Eng. Chem. Res.* **2002**, 41(6), 1454-1461.
38. Allen, S.G.; Schulman, D.; Lichwa, J.; Antal, M.J. Jr.; Jennings, E.; Elander, R. *Ind. Eng. Chem. Res.* **2001**, 40(10), 2352-2361.
39. Allen, S.G.; Schulman, D.; Lichwa, J.; Antal, M.J. Jr.; Laser, M.; Lynd, L.R. *Ind. Eng. Chem. Res.* **2001**, 40(13), 2934-2941.
40. Chang, V.S.; Holtzapple, M. T. *Appl. Biochem. Biotechnol.* **2000**, 84-86, 5-37.
41. Converse, A.O. In *Bioconversion of Forest and Agricultural Plant Residues.* Saddler, J.N.; Eds., C.A.B. International: Wallingford, UK, **1993,** pp 93-106.
42. Gharpuray, M.M.; Lee,Y-H; Fan, L.T. *Biotechnol. Bioeng.* **1983**, 25, 157-172.
43. Gould, J.M. *Biotechnol. Bioeng.* **1984.** 26(1), 46-52.
44. Yang, B.; Boussaid, A.; Mansfield, S.D.; Gregg, D.J.; Saddler, J.N. *Biotechnol. Bioeng.* **2002,** 77(6), 678-684.
45. Yamaguchi, A.; Shimizu, K.; Fujii, T. *Mokuzai Gakkaishi.* **1994,** 40(11), 1208-13.
46. Nelson, R. *J. Polym. Sci.* **1971**, 36, 305-320.
47. Mooney, C.A.; Mansfield, S.D.; Touhy, M.G.; Saddler, J.N. *Biores. Technol.* **1998**. 64, 113-119.

48. Donaldson, L.A.; Wong, K.K.Y.; Mackie, K.L. *Wood Sci. Technol.* **1988**, 22, 103-114.
49. Tanahashi, M. *Wood Res.* **1990**, 77, 49-117.
50. Kawamoto, H.; Nakatsubo, F.; Murakami, K. *Mokuzai Gakkaishi.* **1992,** 38(1), 81-4.
51. Girard, D.J.; Converse, A.O. *Appl. Biochem. Biotechnol.* **1993**, 39/40, 521-533.
52. Grethlein, H.E.; Allen, D.C.; Converse, A.O. *Biotechnol. Bioeng.* **1984,** 26(12), 1498-505.
53. Lu, Y.P.; Yang, B.; Gregg, D.; Saddler, J.N.; Mansfield, S.D. *Appl. Biochem. Biotechnol.* **2002**, 98, 641-654.
54. Ooshima, H.; Burns, D.S.; Converse, A.O. *Biotechnol. Bioeng.* **1990**, 36, 446-452.
55. Grohmann, K.; Himmel, M.; Rivard, C.; Tucker, M.; Baker, J. Torget, R.; Graboski, M. *Biotechnology and Bioengineering Symposium 14(Symp. Biotechnol. Fuels Chem.)* **1984**, 6, 137-57.
56. Grohmann, K.; Torget, R.; Himmel, M. *Biotechnol. Bioeng.* **1985**,15, 59-80.
57. Fan, L.T.; Lee, Y.H.; Gharpuray, M.M. *Adv. Biochem. Eng.* **1982**, 23, 157-87.
58. Millett, M.A.; Baker, A.J.; Satter, L.D. *Biotechnology and Bioengineering Symposium 6(Enzym. Convers. Cellul. Mater.: Technol. Appl., Symp. Proc., 1975).* **1976**,125-53.
59. Ehrman, T. *Laboratory Analytical Procedure No.001*, National Renewable Energy Laboratory: Golgen, CO. 1994.
60. Ehrman, T. *Laboratory Analytical Procedure No.012*, National Renewable Energy Laboratory: Golden, CO. 1994.
61. Ruiz, R.; Ehrman, T. *Laboratory Analytical Procedure No.002*, National Renewable Energy Laboratory: Gloden, Co. 1996.
62. Brennan, M. A. M.S. thesis, Thayer School of Engineering of Dartmouth College, Hanover, NH, 2003.
63. Liu, C.G.; Wyman, C.E. *Ind. Eng. Chem. Res.* **2003**, 42(21), 5409-5416.
64. Yang, B.; Wyman, C. E. *Biotechnol. Bioeng*. (accepted Nov. 2003).
65. Gray, M.C.; Converse, A.O.; Wyman, C.E. *Appl. Biochem. Biotechnol.* **2003**, 105-108,179-193.
66. Adney, B.; Baker, J. *Laboratory Analytical Procedure No.006*, National Renewable Energy Laboratory: Gloden, CO.1996.
67. Ruiz, R.; Ehrman, T. *Laboratory Analytical Procedure No.014*, National Renewable Energy Energy Laboratory: Gloden, CO.1996.
68. Templeton, D.; Ehrman, T. *Laboratory Analytical Procedure No.003,* National Renewable Energy Laboratory: Gloden, CO. 1995.
69. Lora, J.H.; Wayman, M. *Tappi J.* **1978**. 61, 47-50.
70. Bobleter, O.; Grif, M.; Huber, C. De patent 4401992, 1994.
71. Stuhler, S.L. M.S. thesis, Thayer School of Engineering of Dartmouth College, Hanover, NH, 2002.
72. Jozwiakowski, M.J.; Connors, K. A. *Carb. Res.* **1985**, 143, 51-59.
73. Jennings, D.W.; Rousseau, R.W. *Carb. Res.* **1995**, 273(2), 243-248.
74. Li, X.; Converse, A.O.; Wyman, C.E. *Appl. Biochem. Biotechnol.* **2003,** 105-108, 515-522.
75. Simha, R. *J. Appl. Phys.* **1941,** 12, 569-78.

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