0273-2289/97/6801-02—0021$13.00

Pretreatment of Yellow Poplar Sawdust by Pressure Cooking in Water

JOE WEIL,[1,2] AYDA SARIKAYA,[1] SHIANG-LAN RAU,[1,3] JOAN GOETZ,[3] CHRISTINE M. LADISCH,[3] MARK BREWER,[1] RICK HENDRICKSON,[1] AND MICHAEL R. LADISCH*,[1,2]

[1]*Laboratory of Renewable Resources Engineering;* [2]*Department of Agricultural and Biological Engineering;* [3]*Textile Science–CSR, Purdue University, West Lafayette, IN 47907*

Received August 9, 1996; Accepted January 13, 1997

ABSTRACT

The pretreatment of yellow poplar wood sawdust using liquid water at temperatures above 220°C enhances enzyme hydrolysis. This paper reviews our prior research and describes the laboratory reactor system currently in use for cooking wood sawdust at temperatures ranging from 220 to 260°C. The wood sawdust at a 6–6.6% solid/liquid slurry was treated in a 2 L, 304 SS, Parr reactor with three turbine propeller agitators and a proportional integral derivative (PID) controller, which controlled temperature within ±1°C. Heat-up times to the final temperatures of 220, 240, or 260°C were achieved in 60–70 min. Hold time at the final temperature was less than 1 min. A serpentine cooling coil, through which tap water was circulated at the completion of the run, cooled the reactor's contents within 3 min after the maximum temperature was attained. A bottoms port, as well as ports in the reactor's head plate, facilitated sampling of the slurry and measuring the pH, which changes from an initial value of 5 before cooking to a value of approx 3 after cooking. Enzyme hydrolysis gave 80–90% conversion of cellulose in the pretreated wood to glucose. Simultaneous saccharification and fermentation of washed, pretreated lignocellulose gave an ethanol yield that was 55% of theoretical. Untreated wood sawdust gave less than 5% hydrolysis under the same conditions.

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

Index Entries: Wood; water pretreatment; enzyme; hydrolysis; fermentation; pretreatment; cellulose; glucose; ethanol.

INTRODUCTION

Bobleter et al. *(1)* first used water for pretreatment to enhance susceptibility of lignocellulosic material to enzymatic hydrolysis. High-temperature steam, sometimes combined with pressurized inert gas, and added H_2SO_4, is another well-documented hydrothermal pretreatment *(2)*. Hydrothermolysis studies, such as those by Haw et al. *(3)*, Hormeyer et al. *(4)*, and Walch et al. *(5)*, have shown that hot water removes and solubilizes hemicellulose, particularly when its hydrolysis is catalyzed by small quantities of acid. Solubilization of some of the lignin also occurs at temperatures above 180°C. Brownell and Saddler *(6)* reported that steam pretreatment of lignocellulosic material was at least as effective for pretreatment of aspen chips as steam explosion, and that neither the explosion or temperatures above 190°C were necessary. Mok and Antal *(7)* found that hemicellulose will dissolve at 200–230°C. Amorphous cellulose was also removed from the lignocellulose at this temperature range. van Walsum et al. *(8)* pretreated fresh sugarcane bagasse, aspen chips, and mixed hardwood flour using liquid hot water at 220°C; hemicellulose solubilization was nearly complete, but solubilization of cellulose was less than 10%.

Microcrystalline Cellulose Pretreatment

The development of conditions that give reproducible swelling of microcrystalline cellulose at temperatures above 200°C was initially carried out in our laboratory in 5.5-mL, high-pressure, stainless steel reaction tubes filled with a 16.8% slurry of microcrystalline cellulose (Avicel®, FMC, Philadelphia, PA) in water. The Avicel consisted of 98–99% cellulose (including 5–10% hydroxy methyl cellulose), with the balance being xylan. The tubes were preheated in a sand bath at 190°C for 4 h. A swollen form of the cellulose resulted, having an apparent volume of up to 4 times of that of freshly suspended material. At these conditions, hydrolysis during pretreatment was minimal. This led to a procedure in which preheating of the tubes at 190°C in a first sand bath was followed by heating to 220–230°C in a second sand bath. Larger samples were needed for analytical purposes, so the pretreatment was scaled up to a 150-mL vol in a microprocessor-controlled, high-pressure, stirred-tank batch reactor *(9)*.

The runs with the microcrystalline cellulose were repeated, with the larger volume allowing more detailed sample analysis, and the reactor system facilitating better control of the temperature and pH. During cooking, the pH of the aqueous slurry of Avicel was found to quickly drop to about 3.0, and subsequent enzymatic hydrolysis gave only 25–30% conversion,

which is the same as for untreated Avicel. The remaining solid material turned dark brown when held for 10 min at 220°C. Apparently, the Avicel was rapidly degraded during pretreatment by the hot water. Control of the pH in the range from 5.5–7.0 by addition of KOH gave 50% conversion of the pretreated cellulose to glucose upon enzymatic hydrolysis *(10)*. The results with microcrystalline cellulose indicated a need for a better understanding of pH effects during cellulose pretreatment. A model was developed that suggested that a pH close to 7.0 could minimize degradation of cellulose in water *(11)*.

Lignocellulose Pretreatment

Cowpea and rapeseed plant (lignocellulosic) materials were cooked in water at 180°C, and the hemicellulose was significantly solubilized. Liquid chromatographic analysis of the supernatant fraction indicated that breakdown of the hemicellulose to mono- and disaccharides was minimal. Hot-water pretreatment of rapeseed stems and soybean hulls at 180°C enhanced the extent of enzymatic hydrolysis, and resulted in 90% conversion to glucose.

Recrystallization, which is promoted by drying of pretreated cellulose, can cause the cellulose to lose its enhanced susceptibility to hydrolysis. Rapeseed stems and soybean hulls retained their substantial enhancement in susceptibility to enzymatic hydrolysis, even when the pretreated samples were air-dried. We hypothesized that the noncellulosic constituents in these biomass materials interfere with recrystallization by serving as intercalating agents. Intercalating agents would insert themselves between hydrated cellulose chains, thereby interfering with the realignment of cellulose polymer chains (i.e., recrystallization) *(9,10)*. The practical impact of this discovery is that the susceptibility of the cellulose to enzyme hydrolysis can be maintained even after pretreatment conditions are removed.

Steam Explosion vs Water Pretreatment

In steam explosion, steam penetrates the lignin, hemicellulose, and cellulose. The mixture is explosively decompressed and the resulting expansion increases cellulose accessibility *(12)*. The operating conditions promote acid formation and result in degradation of cellulose by autohydrolysis. Hydrolysis is considered to be an important and necessary aspect of the steam pretreatment *(12,13)*, and acid addition is recommended to promote this effect *(2)*. Our approach to water pretreatment, by comparison, has the goal of maintaining a liquid phase (under pressure)

during pretreatment, while keeping the pH between 5.0 and 7.0, in order to minimize and, preferably, to avoid cellulose hydrolysis and sugar degradation reactions *(9,10,14–16)*.

Current Research

This paper presents our experience in developing a 2-L bench scale reactor system for pressure cooking of wood sawdust at temperatures of up to 260°C, and gives baseline data for cellulose cooked in water in the absence of pH control. Water is shown to be an effective pretreating agent that enhances enzymatic hydrolysis and simultaneous saccharification and fermentation of cellulose to ethanol, compared to untreated materials.

MATERIALS AND METHODS

Standard chemical and analysis procedures were used to analyze the compositions of the various cellulose and fermentation streams *(17)*. The yellow poplar wood sawdust was provided by National Renewable Energy Laboratory (NREL), in 1 × 5 mm particle size, and was stored in a freezer until use. The procedures used in this work are summarized below.

Analytical Methods

The determination of total solids, carbohydrates, acid insoluble/soluble lignin, and ash in the pretreated and untreated samples of biomass, and the quantity of ethanol, glycerol, acetate, and lactate in the fermentation broth, were determined using the standard analysis and testing procedures 001, 003, 004, 008, and 009 provided by the NREL (17). Minor modifications to the procedures are indicated as appropriate.

Total Solids/Moisture

NREL standard procedure 001 was followed. The large particles (1 × 5 mm) of biomass sample were milled in a Thomas-Wiley mill using a 35-mesh screen. A larger mesh size than the recommended 40 mesh was used to match the particle size in the NREL standard hardwood CES sample (18 mesh = 1.0 mm). A 1–5 g sample of the biomass was weighed in an aluminum foil weighing dish, placed in a convection oven at 105 ± 3°C, and dried to constant weight (±0.1%). The oven-dried sample was cooled in a desiccator and weighed to obtain the difference in weight because of moisture.

Carbohydrates

NREL standard procedure 002, which utilized HPX-87C liquid chromatography columns, was followed. Cellobiose was detected, in addition

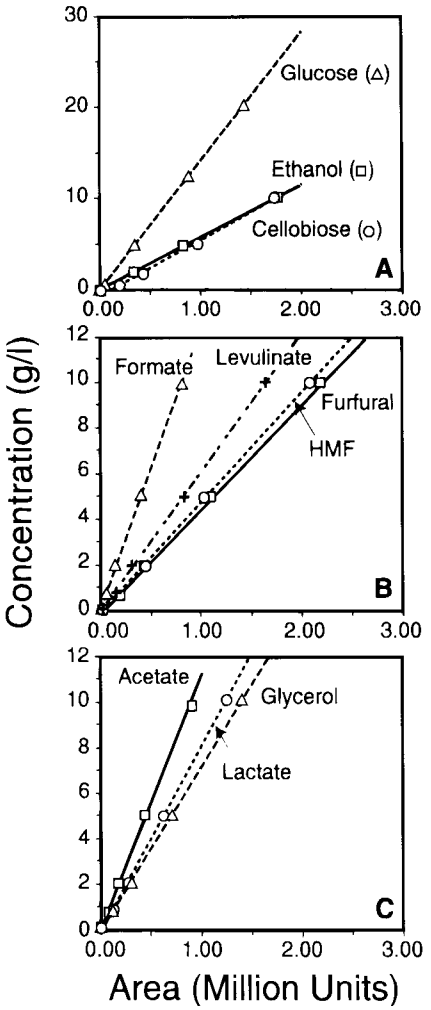


Fig. 1. Liquid chromatography calibration curves for **(A)** sugars/ethanol; **(B)** sugar degradation products; **(C)** organic acids and glycerol. Column: HPX-87C; Operating conditions: 10 µL; sample volume, eluent of 5 m*M* H_2SO_4 in deionized water at flow rate of 0.6 mL/min, column temperature at 60°C.

to glucose, xylose, and arabinose. The biomass sample was treated at 30°C with 72% sulfuric acid for 2 h and then autoclaved at 121°C, after diluting the acid to 4% with deionized water. The hydrolysate was neutralized with calcium carbonate, vacuum filtered, and run in the HPLC column. A blank was run in the HPLC column (operated at 60°C) to determine the retention time of the eluting acid peak. Standardization curves were developed for the major components of the pretreatment supernatant and the fermentation broth (Fig. 1A–C). High-purity sugar standards were used as controls to

quantitate the degradation. The equation to calculate the percent sugar recovered was modified from that given in procedure 002 to reflect a w/w ratio. The modified equation is:

$$\% \text{ sugar recovered} = (\text{conc. detected by HPLC, mg/mL})(\text{volume of filtrate, mL/ known amount of sugar before hydrolysis, mg}) \quad (1)$$

Carbohydrate, Acid, and Ethanol Analysis

Liquid chromatography was used to measure the concentrations of glucose, cellobiose, ethanol, glycerol, acetate, and lactate. Analysis was based on a 1090 Hewlett-Packard HPLC equipped with a Bio-Rad HPX-87C column (operated at 60°C). An isocratic flow rate of 0.6 mL/min of 5 mM sulfuric acid solution was used for all analyses. Linear standard curves were obtained for this system (Fig. 1A–C).

Klason Lignin

NREL standard procedure 003 was followed, in which the biomass sample was thoroughly mixed in 72% sulfuric acid and then allowed to stand for 2 h at 20°C. The hydrolysate was diluted to 3% acid and boiled for 4 h in a distilling column in a hood. The flask/distilling column was used in place of a microreflux condenser in a manifold. The Kimax brand distilling column (Fisher Scientific, Pittsburgh, PA) had straight 24/40 T-joints and a 300-mm jacket. The solution was vacuum-filtered through a filtering crucible. The residue was dried at 105 ± 3°C for 2 h. The resulting weight was corrected for the acid-insoluble ash (determined by igniting the contents at 575 ± 25°C) in order to obtain the ash-free dry wt.

Acid Soluble Lignin

NREL standard procedure 004 was used. The filtrate saved in procedure 003 was directly analyzed using a spectrophotometer. The absorbance was measured at 205 nm, using the 10-mm light path cuvet. When the absorbance was not in the range 0.2–0.7, the filtrate was diluted accordingly with 3% sulfuric acid.

Ash

Aluminum weighing pans of 30 mL were used, instead of the 50-mL pans outlined by the NREL Analytical Procedure 005. This allowed more pans to be accommodated in the oven. A 0.5–1.0 g sample of biomass was placed in a crucible, ignited in a muffle furnace at 575 ± 25°C, cooled in a desiccator, and weighed. The crucibles were labeled by etching identification marks into them, since a permanent marker was found to wear off at the high temperature used.

Unfermented Insoluble Solids

NREL standard procedure 008 was followed. A 10-mL initial sample volume was used to calculate solids, rather than the volume after washing.

Enzyme

The cellulase enzyme (Spezyme CP), used in this study, was provided by NREL. The cellulase enzyme (Spezyme, Lot #41-95034-004) was sterilized by filtering it through a Gelman Vacu Cap 90 filter (0.20 μm) into sterilized bottles, in which the enzyme was stored at 4°C until use. The most recent NREL analysis placed the enzyme's activity at 59 (IFPU)/mL. Our assay showed an activity of 48 IFPU/mL and was based on NREL procedure #006. The higher value of 59 was used in our calculations.

Enzymatic Hydrolysis

The enzymatic hydrolysis was carried out using NREL procedure 009, with the modification that 100 mg of dry material, instead of 100 mg of cellulose, was used. The hydrolysis was carried out by suspending 100 mg of wood or cellulose (dry wt basis) in 10.0 mL of water, citrate buffer, and enzyme, and incubating for 48 h at pH 4.8 and 50°C. Sodium azide at 200 mg/L was added to the buffer to prevent microbial growth.

Pretreatment Reactor

The goal of this research is to pretreat lignocellulosic material using water at selected temperatures and pH, while minimizing cellulose degradation. The research reported here establishes a baseline case for pretreating wood sawdust in the absence of pH control. A Parr, 304 SS reactor (Model 4843, Moline, IL) was used in this research to treat wood sawdust slurries at 6–6.6% (solid/liquids). The reactor had a volume of 2 L, with three turbine propeller agitators and a PID temperature controller (±1.0°C). Water was circulated through a serpentine cooling coil to cool the reactor contents at the end of each run. A bottoms port, and two inlet ports, allow sampling of the pretreated material or addition of reagents to the reactor.

Procedure

A weight of 100 g of wood sawdust (at 15–20% moisture) was soaked in 1500 mL of deionized water in the reactor overnight (about 16 h) prior to pretreating. The resulting solids content (based on dry w/vol) was between 6.0 and 6.6%. A heat-up time of 70–75 min was needed to attain pretreatment temperatures of 220, 240, and 260°C (Fig. 2). The set-point temperature was set 2°C higher than the target temperature, since this resulted in attaining the target temperature within 70 min.

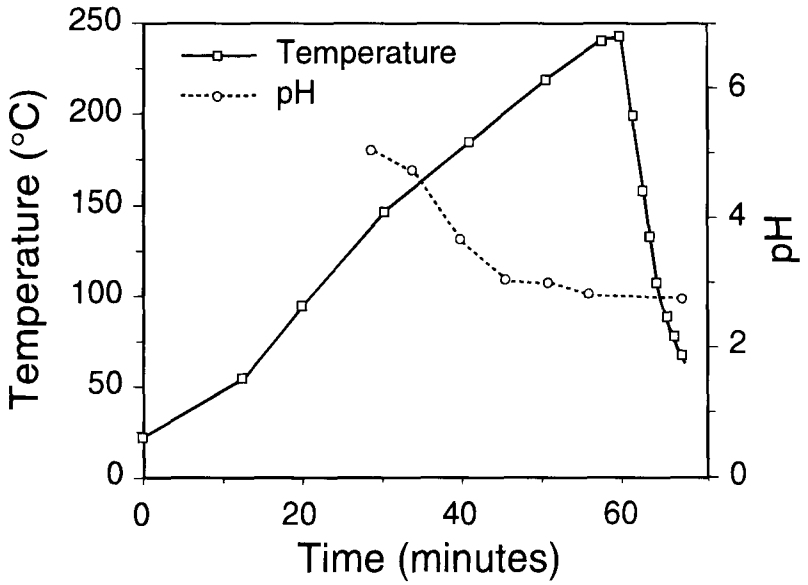


Fig. 2. Typical temperature and pH profile as a function of time during cooking in wood sawdust in water.

The run started when the power to the heating jacket that surrounded the reaction chamber was turned to "high." Temperature recordings were taken every 10 min, and samples were taken from the bottoms port at temperature increments of 20°C starting at 140°C until the target temperature was attained. These samples were allowed to cool to room temperature and the pH was then measured using a Markson pH meter with a glass electrode probe, which had previously been calibrated at pH 4.0 and 7.0 using standard solutions at 22°C.

Once the reactor reached the target temperature, the power switch was turned off and the water valve was turned on within 60 s. Tap water, flowing through the serpentine coil, cooled the reactor contents to below 150°C in less than 3 min. Previous research had determined that cellulose hydrolysis is minimal at 150°C. Although the reactor contents were quickly cooled, the head plate of the reactor vessel itself was too hot to handle. Hence, the reactor required an additional 2 h before it was cool enough to be moved and opened. At this point, the electricity was turned off, agitation was discontinued, and the reaction vessel removed from the heating jacket. The vessel was then disassembled and the treated lignocellulosic slurry was removed, placed into plastic Nalgene containers with screw-on lids, and stored in the refrigerator until hydrolysis or analysis was carried out. The time between the completion of the pretreatment and initiation of hydrolysis was 12 h or longer.

Table 1
Mixtures for Simultaneous Saccharification and Fermentation of α-Cellulose and Pretreated Wood Sawdust

Component	Volume in mL	
	α-Cellulose	Pretreated wood sawdust
Water already present in the biomass	0.17	31.76
10X YP, pH 5.0	10.00	10.00
S. cerevisiae D_5A inoculum	10.00	10.00
Spezyme (59 IFPU[a]/mL)	1.22	1.27
Volume of deionized water added (mL)	78.86	46.97
Total volume (mL)	99.95	100.00

[a]IFPU denotes international filter paper units as given by procedure 6 in ref. 17.

Simultaneous Saccharification and Fermentation of Pretreated Wood Sawdust and α-Cellulose

The NREL Chemical Analysis & Testing Standard Procedure No. 008 was followed for lignocellulosic biomass hydrolysis and fermentation. The fermentation substrates were α-cellulose (100% cellulose, dry wt basis), with a moisture content of 3.17%, and pretreated wood sawdust, 50% cellulose (dry wt basis), with a moisture content of 37.8%. Solutions were prepared according to the protocol for simultaneous saccharification and fermentation of 3 g cellulose (dry wt basis) at an enzyme level of 25 IFPU/g cellulose. IFPU denotes international filter paper units, or international units based on hydrolysis of filter paper at the specific conditions associated with this procedure (6 in ref. *17*).

Procedure

The appropriate amount of biomass was placed into a 250-mL shake flask. Deionized water was added and the submerged biomass was gently pressed to remove the trapped air. The pH of the slurry was then adjusted to 5.0 ± 0.2 with either sodium hydroxide or phosphoric acid. A CO_2 trap (Triple Ripple, G. W. Kent, Ann Arbor, MI) was sterilized separately with the fermentation flask and assembled in a laminar flow hood. These items were autoclaved at 121°C for 20 min. The flask was then cooled to room temperature, and sterile 10X YP, pH 5.0 (10% v/v), yeast inoculum (10% v/v), and sterile-filtered cellulase enzyme were added aseptically and mixed to give the volumes shown in Table 1. Water was added to the CO_2 trap, and the whole fermentation system was placed in an incubator-shaker at

150 rpm and 38°C. As a control, the same composition of the fermentation system without the yeast inoculum was prepared and run under the same fermentation conditions.

The fermentations were run in triplicate for each biomass sample. At 24, 48, 72, 96, 120, 144, 168, and 192 h, 4 mL of slurry samples were taken aseptically from each fermentation flask. These samples were chilled in ice, microcentrifuged in a Fisher Scientific Marathon Micro A for 10 min at 4000 rpm, and the resulting supernatant was used for sample analysis.

Inoculum Preparation

Standard procedure 008 was followed to prepare the seed culture. A colony of *Saccharomyces cerevisiae* D_5A (from a YPD plate provided by NREL) was inoculated into 50 mL of sterile YPD-medium in a 250-mL baffled shake flask, and incubated at 38°C and 150 rpm for approx 10 h before measuring the optical density (OD) at 600 nm, pH, and residual glucose concentration.

To prepare the seed culture, 10 mL of inoculum from this first flask (YPD-medium) was added to 10 mL of sterile 10X YP-medium, 10 mL of sterile glucose solution (500 g/L), and 70 mL of sterile deionized water in a 250-mL baffled shake flask. The seed culture was incubated for 10 h at 38°C and 150 rpm. OD, pH, and glucose concentration were measured at the end of this period. This culture was then used to inoculate the flasks used in simultaneous saccharification and fermentation runs.

Washing of Pretreated Wood Sawdust

The pretreated wood sawdust, which was washed with 8 L of deionized water to remove fermentation inhibitors, is clearly indicated in the Results and Discussion section (an initial fermentation was performed on unwashed material). The procedure consisted of placing the wet, pretreated slurry on Whatman #1 Filter paper on a Bucher funnel and washing with 8 L of deionized water into a vacuum flask. Liquid chromatographic analysis of the supernatant, after pretreating and rinsing, showed no degradation products in the supernatant.

Dry Cell Weight Determination

Standard procedure 001, provided by NREL, was used to determine the dry wt of cells. Aluminum dishes were dried in the oven at 80°C overnight, cooled down to room temperature in a desiccator, and weighed (this weight was recorded as W_1). A 10 mL (V) sample from the fermentation flask was taken, centrifuged, and washed twice with 10 mL of DI water. It was then resuspended in 5 mL of DI water, transferred to the dish, dried overnight at 80°C, cooled down, and weighed. This weight (W_2) was also recorded. The dry wt of cells was calculated as $(W_2 - W_1)/V$ in g/mL.

Table 2
Composition of Yellow Poplar Wood Sawdust

Composition	per 100 g (dry wt)
As potential sugars (theoretical yield)	
Glucose	42.6
Xylose	19.5
Subtotal	62.1
As constituents of wood sawdust	
Cellulose	38.3
Xylan	17.2
Lignin	
Klason	22.7
Acid	4.0
Ash	0.5
Subtotal	82.7

RESULTS AND DISCUSSION

Pretreatment by Pressure Cooking in Water

Effect on pH and Cellulose Content

Wood sawdust (composition in Table 2) was pretreated in water at temperatures of 220, 240, or 260°C, respectively. Pretreatment increases the cellulose content of the composition of the solids. A pretreatment temperature of 260°C resulted in a lower cellulose content, compared to the maximum obtained at 240°C, but still higher than the starting untreated material. The hot water dissolves or otherwise removes 40.5 g of solids from the wood sawdust, thus increasing the cellulose content from 47.6% (untreated) to a maximum of 73.6% at 240°C (Fig. 3). The constituents extracted from the wood during pretreatment apparently buffer the pH at 3.0. This pH is less than the pH obtained for rapeseed stems pretreated at 200°C, and for rice, straw, and soybean hulls at 180°C, in which the final values were 4.3, 4.8, and 5.6, respectively *(16)*. Hence, addition of base is needed if the pH is to be kept between 5.5 and 7.0.

Material Balance at 240°C

An initial weight of 93.2 g of wood sawdust was charged to the reactor. Of this amount, 40% was solubilized and removed during pretreatment and subsequent rinsing with 8 L of deionized water. The corresponding cellulose content of the wood sawdust after pretreatment was 73.62%.

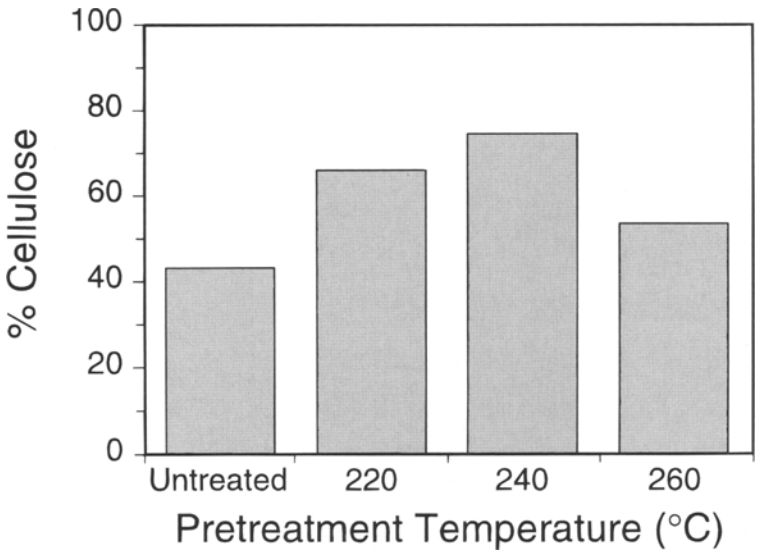


Fig. 3. Comparison of cellulose content after pretreatment, as a function of pretreatment temperature.

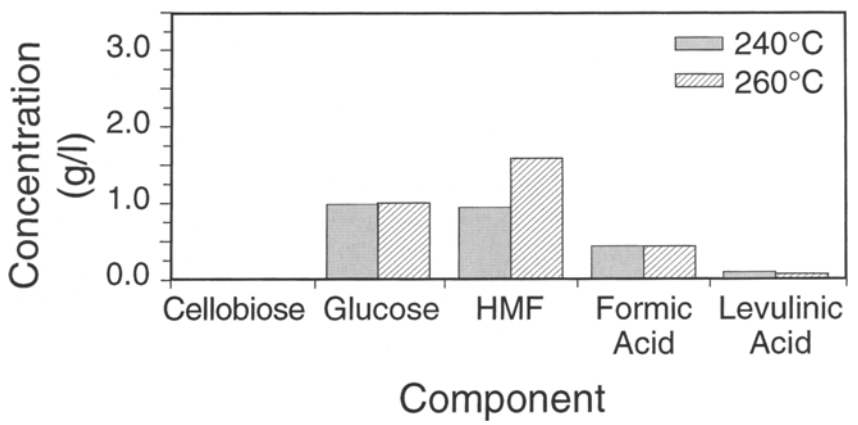
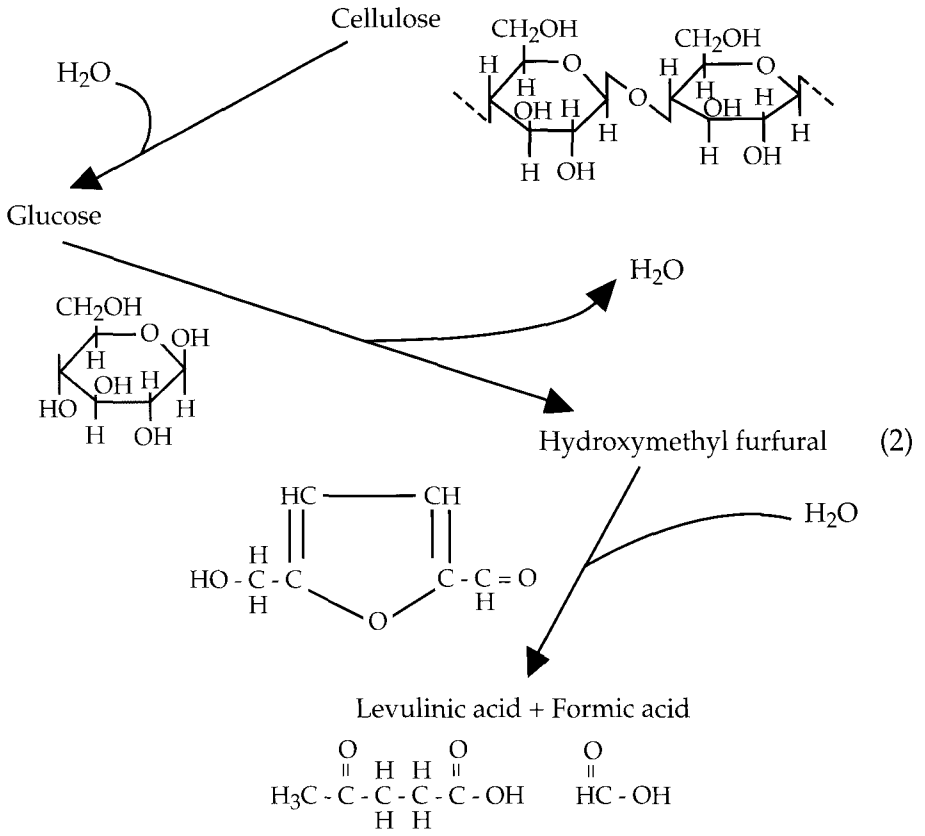


Fig. 4. Concentration of products of the glucose degradation pathway found in the supernatant from pretreated wood sawdust for pretreatment temperatures 240°C and 260°C.

After pretreatment, the hydroxymethyl furfural (HMF) concentration was 0.90 g/L (1.33 g; Fig. 4), which corresponds to a loss of 1.75 g, or about 4.3% of the cellulose. Liquid chromatographic analysis showed that 1.44 g of glucose was generated during pretreatment. This corresponds to 1.29 g of cellulose or 3.19% of the total cellulose. The accumulation of all the products along the cellulose degradation pathway accounts for a loss of 10.2% of the cellulose originally in the pretreatment reactor.



Material Balance at 260°C

For the pretreatment at 260°C, the total amounts of solids and cellulose in the pretreatment reactor were initially 65.6 and 28 grams, respectively. The cellulose weight after pretreatment and rinsing with 8 L water was 25.49 g, which corresponds to 8.5% loss (Table 3). Hydrolysis of cellulose occurred during cooking, as indicated by accumulation of glucose (0.97 g/L), as well as the glucose degradation products HMF (1.55 g/L), levulinic acid (0.04 g/L), and formic acid (0.4 g/L) (Fig. 4). The concentrations corresponding to pretreatment temperatures of 240 and 260°C are similar, with the exception of HMF, which was nearly two times greater at the higher temperature. The total loss of cellulose corresponding to concentrations of these degradation products would be about 20% with HMF, levulinic, and formic acids, corresponding to 10, 5, and 5%, respectively. The combination of hemicellulose dissolution, partial hydrolysis of cellulose, and redeposition of insoluble constituents translates into a net increase of the cellulose content to 54.9%.

Table 3
Material Balances for Wood Sawdust Subjected to Pressure Cooking in Water

Temperature (pressure) run	240°C (NPT011) Total	240°C (NPT011) Cellulose	240° (NPT016) Total	240° (NPT016) Cellulose	260° (NPT013) Total	260° (NPT013) Cellulose
Initial wt (dry, g)	88.61	37.78	93.22	39.75	65.62	27.98
Final wt (g)	49.17	36.20	59.73	43.39	46.46	25.49
% loss of solid material	44.5	4.20	35.9	NM[a]	29.0	8.9
g glucose/ g initial wt	0.34	–	0.42	–	0.38	–
g glucose/ g theoretical glucose	0.79	–	0.80	–	0.91	–

[a]Not meaningful (9.2% apparent weight gain).

Potential Role of pH Control

The first-order rate constants for the degradation of cellulose to glucose, glucose to HMF, and HMF to levulinic and formic acids are a function of temperature and pH *(18,19)*. Therefore, it would be optimal to keep the pretreatment temperature low and the pH close to neutral, in order to minimize degradation. During the course of the pretreatment run, the pH was measured at ambient temperature at 5–10 min intervals, as indicated in Fig. 2. When the temperature in the pretreatment vessel attained 180°C, the pH began to decrease until a value of pH 3.0 was attained. Even in the absence of pH control, the pH stayed at 3.0. Autohydrolysis is initiated at pH 2.7 *(18)*. Water can have the undesirable effect of acting as an acid catalyst and promoting the degradation of cellulose to glucose, and, eventually glucose to formic and levulinic acid. Evidence that water acts as an acid catalyst during pretreatment is consistent with the presence of acid hydrolysis/degradation products in the supernatant: glucose, HMF, formic acid and levulinic acid.

The pH at 240°C and the saturation pressure of water (492 psig) can be different from the pH of the same solution at room temperature, as recently illustrated by measurements of a 0.57 *M* NaCl solution. The pH was 5.8 at 400°C and 40 mPa and 4.2 at 25°C *(20)*. In our case, an analysis of dissolution constants for water showed that the pH at neutrality is calculated to decrease from 7.0 to 5.6 as temperature increases from 25 to 240°C. There is a difference, because there is a strong thermodynamic relationship between the dissociation constant and temperature, so that the dissociation product decreases, causing proton accumulation as temperature increases. This is illustrated by Kang and Seyfried et al. *(20)*. The pH values reported in this manuscript were all measured at 25°C.

The use of added acid to catalyze hydrolysis of hemicellulose and cellulose is well known and effective (*21*). However, undesirable degradation products form, as depicted in equation (*2*), with glucose disappearance accelerated by the presence of an added acid. Hemicellulose hydrolysis faces similar constraints, although formation of degradation products is less severe, because a lower hydrolysis temperature can be used. Minimal formation of furfural can be achieved by the addition of acid in a reverse-flow reactor configuration, and has been shown to exploit biphasic xylan hydrolysis kinetics at temperatures of 150–204°C, while removing all of the xylan (*22,23*).

More than 19 years have passed since we recognized that the disruption of the physical structure of cellulose, and generation of an amorphous form greatly enhances its hydrolysis to glucose by cellulosic enzymes (*24*). Many pretreatments have been shown to be successful, and enzymatic hydrolysis is enhanced by mild prehydrolysis conditions in which the acid hydrolysis selectively attacks the more amorphous regions and yields a more crystalline form of cellulose (*25*). In retrospect, the use of water for pretreatment fits this scheme, since it minimizes overt addition of large quantities of reagents that need to be removed at the end of the process. At the same time, control of the hydrolytic properties of water is important if the action of another technically successful, but environmentally challenging, cellulose solvent (cadoxen) is to be mimicked. This type of solvent minimizes covalent (chemical) changes in the structure of cellulose, while maximizing physical disruption and hydration of the physical structure, in order to obtain amphorous cellulose, which is susceptible to enzyme hydrolysis.

Intercalation Hypothesis

The appearance of furfural, acetic acid (Fig. 5), and xylose indicates the presence of hemicellulose. During pretreatment, the hemicellulose is solubilized, as well as being partially hydrolyzed by water and/or by acetate derived from hydrolysis of acetyl groups associated with the hemicellulose. The furfural accounts for only approx 27% of the potential xylose obtainable from the solubilized xylan fraction. The remaining xylan is assumed to be present as solubilized polymer and xylose.

This polymer may serve as an intercalating agent that interjects itself between hydrated (dissolved) cellulose chains. Hence, when pretreatment conditions are removed and the cellulose reprecipitates, the hemicellulose chains (or other soluble components) may become trapped in the structure of the cellulose, and inhibit its recrystallization after these conditions are removed. This is of significant practical importance, since inhibition of the recrystallization of cellulose allows the pretreated cellulose to be stored for hours or more, while still retaining its enhanced susceptibility to

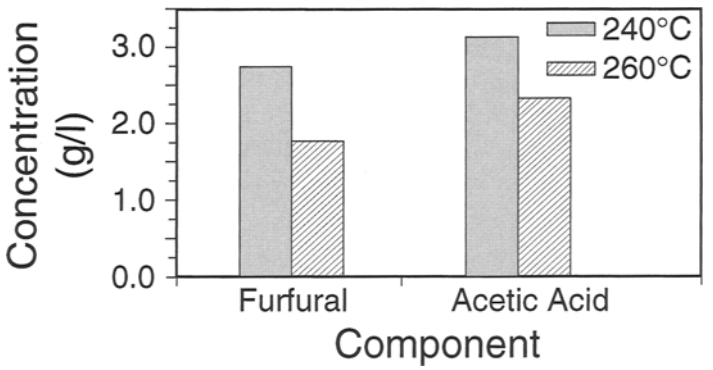


Fig. 5. Concentration of products of the hemicellulose degradation pathway found in the supernatant of pretreated wood sawdust for pretreatment temperatures at 240°C and 260°C.

hydrolysis. Xylan or other soluble components are referred to as intercalating agents. Xylan, if it penetrates into the structure of pretreated cellulose, can inhibit the formation of a tight crystalline conformation (after pretreatment), which is resistant to enzymatic hydrolysis.

Enhanced Enzymatic Hydrolysis

Enzymatic hydrolysis of wood sawdust cooked at 220°C gave 0.64 g glucose/g theoretical glucose, where the theoretical yield of glucose is based on the composition of the washed solids. The fractional glucose yield calculated from the cellulose initially present in the untreated lignocellulose can be lower, since some of the cellulose is lost during pretreatment and washing. At 240°C the yield was 0.77, and at 260°C the yield was 0.88 after 48 h (Fig. 6). The initial rate of glucose formation during enzyme hydrolysis appears to level off at 3.2 mg/h glucose for a pretreatment temperature at 240°C (Fig. 7). A pretreatment temperature of 260°C gives the highest conversion, but it also results in the highest amount of cellulose degradation (Figs. 3 and 4). Sawdust pretreated at 240°C gives the highest yield (0.77 g glucose/g theoretical glucose); degradation products are lower relative to 260°C. Therefore, wood sawdust, pretreated to 240°C, was fermented by simultaneous saccharification and fermentation.

Simultaneous Saccharification and Fermentation

Simultaneous saccharification and fermentation was performed using a genetically engineered yeast strain of *Saccharomyces cerevisiae*. Three g of cellulose were suspended in 100 mL of media (Table 1), and then fermented for 192 h. The control was α-cellulose, provided by NREL.

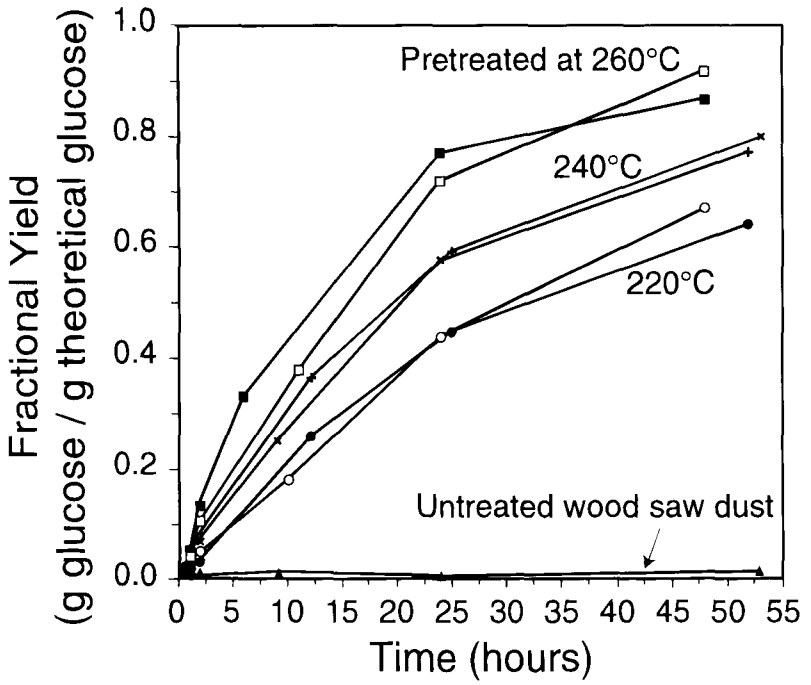


Fig. 6. Time-course for glucose yield during enzyme hydrolysis for yellow poplar wood sawdust pretreated to 220°C (○, ●), 240°C (×, +), and 260°C (□, ■). No KOH was added. Untreated wood sawdust was used as a control.

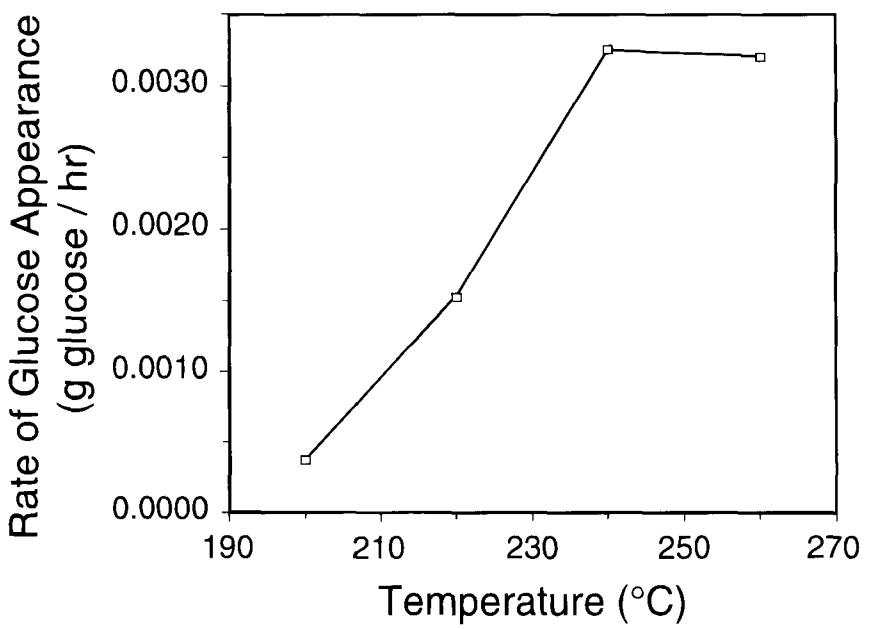


Fig. 7. Initial rate of glucose appearance between 0 and 2 h of enzyme hydrolysis. Temperature indicates maximum pretreatment temperature for pretreated wood sawdust. No KOH was added. Basis was 100 mg substrate (dry wt basis) in 10 mL of citrate buffer, enzyme, and water incubation mixture.

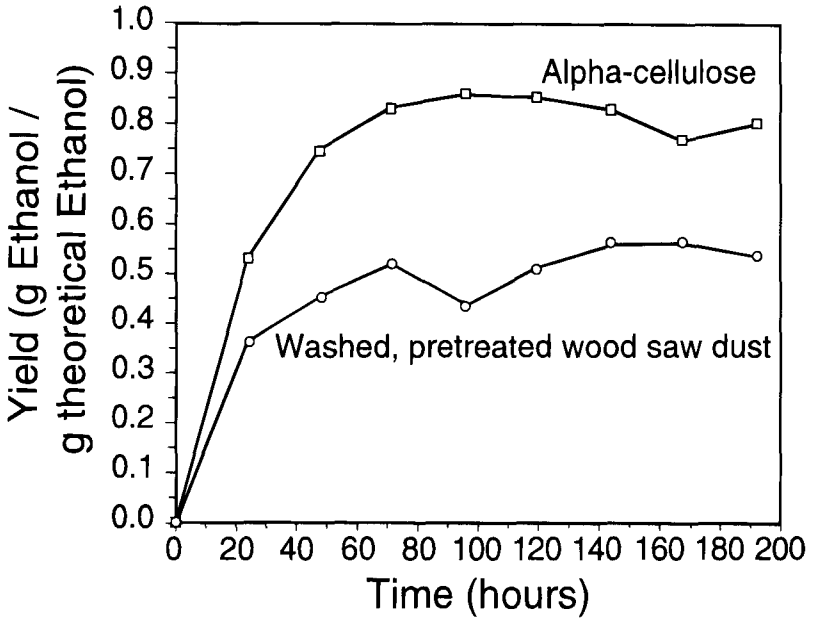


Fig. 8. Ethanol accumulation during simultaneous sacchirification and fermentation for α-cellulose and washed, pretreated wood sawdust. This fraction is based upon maximum theoretical yield of ethanol available from the substrate.

Wood sawdust that was pretreated at 240°C, but not washed prior to fermentation, gave 0.10 g ethanol/g theoretical ethanol after 192 h; the α-cellulose had an ethanol yield of 0.80. When washed and then fermented, pretreated wood sawdust gave a yield of 0.55 g ethanol/g theoretical ethanol. The α-cellulose again achieved around 80% theoretical conversion to ethanol (Fig. 8). The effective yields will probably be improved by use of yeast acclimated to the hydrolysate, since the presence of inhibitors, such as acetate, may be responsible for the lower yield (Fig. 9), as shown by Lawford and Rousseau for xylose fermentation *(26)*. The control run (without the yeast inoculum) with the wood sawdust, which was pretreated but not washed, gave an acetate value of 0.49 g/L at time zero, and 0.5 g/L at the end of 24 h of incubation. The acetate is formed during pretreatment and is probably the result of hydrolysis of acetyl groups from the hemicellulose. The acetic acid is not completely removed during washing of the pretreated wood sawdust, and is carried over into the fermentation. These results confirmed the presence of inhibitors in the fermentation medium. However, sugarcane bagasse, aspen, and mixed hardwood flour, pretreated with hot water, have recently been shown to give up to 90% conversion to ethanol by simultaneous saccharification and fermentation carried out for 2–5 d *(8)*. Hence, this pretreatment appears to be compatible with high fermentation yields.

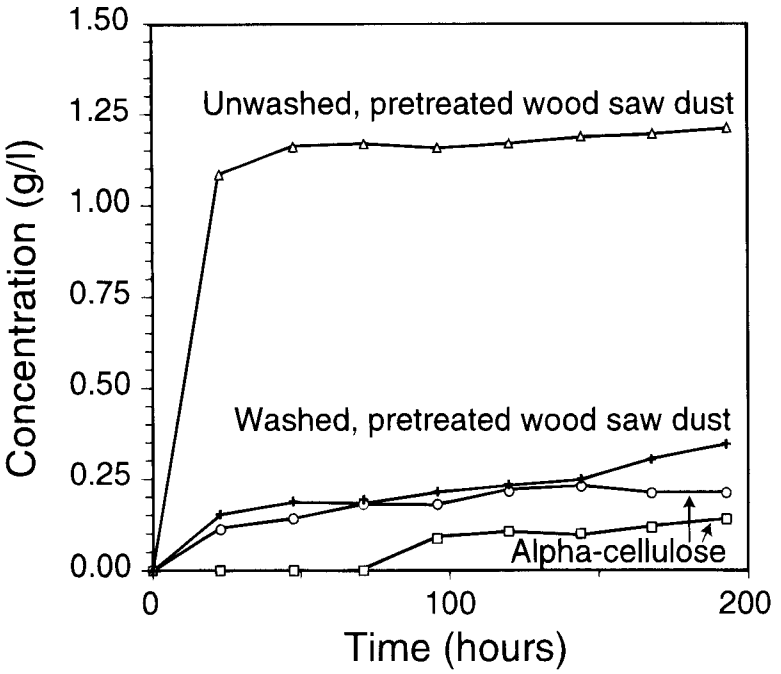


Fig. 9. Accumulation of acetate during fermentation of washed, pretreated wood sawdust (T = 240°C), unwashed, pretreated wood sawdust (T = 240°C) and α-cellulose.

CONCLUSIONS

The pretreatment of yellow poplar sawdust by pressure cooking in water showed that water is an effective pretreating agent that significantly enhances cellulose hydrolysis yields. Sawdust cooked at 240°C gave up to 90% conversion of cellulose to glucose; the conversion of cellulose to glucose in the untreated material was less than 5% at equivalent conditions. Simultaneous saccharification and fermentation of the pretreated wood using *S. cerevisiae* D_5A gave an ethanol yield that was 50% of theoretical. This lower yield, and control runs, reflects the presence of inhibitors from the pretreatment, and suggests that a microorganism that is acclimated to this particular hydrolysate may be needed to obtain a better yield. The pretreatment procedures, carried out in a 2 L Parr reactor, give useful baseline data, against which the effects of pH control during pretreatment, and temperature and hold time, can be compared.

ACKNOWLEDGMENTS

This work was carried out by Subcontract Number XAC-4-13511-01 of the National Renewable Energy Laboratory, Golden, CO. We thank Kyle

Beery and C. S. Gong for useful comments during preparation of this manuscript. The yellow poplar sawdust, and cellulase enzyme (Spezyme CP) were provided through the National Renewable Energy Laboratory.

REFERENCES

1. Bobleter, O., Niesner, R., and Rohr, M. (1976), *J. Appl. Polymer Sci.* **20,** 2083–2093.
2. Foody, P. (July 24, 1984), U.S. Patent 4,461,648.
3. Haw, J. F., Maciel, G. E., Linden, J. C., and Murphy, V. G. (1985), *Holzforschung* **39,** 99–107.
4. Hormeyer, H. F., Schwald, W., Bonn, G., and Bobleter, O. (1988), *Holzforschung* **42,** 95–98.
5. Walch, E., Zemann, A., Schinner, F., Bonn, G., and Bobleter, O. (1992), *Bioresource Technol.* **39,** 173–177.
6. Brownell, H. H. and Saddler, J. N. (1987), *Biotechnol. and Bioeng* **29,** 228–235.
7. Mok, W. S. L. and Antal, M. J. Jr. (1992), *Ind. Eng. Chem. Res.* **31,** 1157–1161.
8. van Walsum, G. P., Allen, S. G., Spencer, M. J., Laser, M. S., Antal, M. J., Jr., and Lynd, L. R. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 157–170.
9. Ladisch, M. R., Waugh, L., Westgate, P., Kohlmann, K., Hendrickson, R., Yang, Y., and Ladisch, C. (1992), Ladisch, M. R. and Bose, A., eds., American Chemical Society, Washington, DC, pp. 510–518.
10. Kohlmann, K. L., Westgate, P. J., Weil, J., and Ladisch, M. R. (1994), SAE 1993 Transactions. *Journal of Aerospace Section.* **102,** 1476–1483.
11. Weil, J., M.S.E. Thesis, Purdue University, May, 1993.
12. Beltrame, P. L., Carniti, P., Visciglio, A., Focher, B., and Marzett, A. (1992), *Biores. Technol.* **39,** 165–171.
13. Heitz, M., Capek-Menard, E., Koeberle, P. G., Gagne, J., Chornet, E., Overend, R. P., Taylor, J. D., and Yu, E. (1991), *Biores. Technol.* **35,** 23–32.
14. Weil, J., Westgate, P., Kohlmann, K., and Ladisch, M. R. (1994), *Enzyme Microb. Technol.* **16,** 1002–1004.
15. Kohlmann, K. L., Sarikaya, A., Westgate, P. J., Weil, J., Velayudhan, A., Hendrickson, R., and Ladisch, M. R. (1995), Penner, M. and Saddler, J., eds., American Chemical Society, Washington, DC, ACS Symp. Ser. No. 618, 237–255.
16. Kohlmann, K. L., Westgate, P., Velahudhan, A., Weil, J., Sarikaya, A., Brewer, M. A., Hendrickson, R. L., and Ladisch, M. R. (1996), *Adv. Space Res.* **18,** 251–265.
17. NREL, Chemical Analysis and Testing Standard Procedures, Golden, CO (1994).
18. Baugh, K. D. and McCarty, P. L. (1988), *Biotechnol. Bioeng.* **31,** 50–61.
19. Saeman, J. F. (1945), *Ind. Eng. Chem.* **37,** 43–54.
20. Kang, D. and Seyfried, W. E., Jr., (1996), *Science* **272,** 1634–1636.
21. Ladisch, M. R. (1989), in *Biomass Handbook,* Kitani O. and Hall C. W., eds., Gordon and Breach, New York, pp. 434–451.
22. Torget, R. and Hsu, T-A. (1994), *Appl. Biochem. and Biotechnol.* **45/46,** 115–123.
23. Torget, R., Hatzis, C., Hayward, T. K., Hsu, T-A., Philippidis, G. P. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 85–101.
24. Ladisch, M. R., Ladisch, C. M., and Tsao, G. T. (1978), *Science* **201,** 743–745.
25. Ladisch, M. R., Lin, K. W., Voloch, M., and Tsao, G. T. (1983), *Enzyme Microb. Technol.,* **5,** 82–102.
26. Lawford, H. G. and Rousseau, J. D. (1992), *Appl. Biochem. and Biotechnol.* **34/35,** 185–204.