0273-2289/98/70-72—0077$10.75

Dilute Acid Pretreatment of Softwoods

Scientific Note

Q. A. NGUYEN,* M. P. TUCKER, B. L. BOYNTON, F. A. KELLER, AND D. J. SCHELL

Biotechnology Center for Fuels and Chemicals, National Renewable Energy Laboratory, Golden, CO 80401

ABSTRACT

Selective thinning of forests in the western United States will generate a large, sustainable quantity of softwood residues that can be an attractive feedstock for fuel ethanol production. The major species available from thinning of forests in northern California and the eastern Rocky Mountains include white fir *(Abies concolor)*, Douglas fir *(Pseudotsuga menziesii)*, and Ponderosa pine *(Pinus ponderosa)*. Douglas fir chips were soaked in 0.4% sulfuric acid solution, then pretreated with steam at 200–230°C for 1–5 min. After pretreatment, 90–95% of the hemicellulose and as much as 20% of the cellulose was solubilized in water, and 90% of the remaining cellulose can be hydrolyzed to glucose by cellulase enzyme. The prehydrolysates, at as high as 10% total solid concentration, can be readily fermented by the unadapted yeast *Saccharomyces cerevisiae* D_5A.

Index Entries: Biomass; ethanol; pretreatment; softwood; bioconversion.

INTRODUCTION

After decades of human intervention in suppressing forest fires, large quantities of small-diameter trees and underbrush have overcrowded forests in the western United States, and created a severe fuel-loading problem. The resulting forest fires are often catastrophic. They not only destroy important natural resources, but endanger property, pollute the air, and can lead to soil erosion and flooding. Recognizing the need to reduce this severe fuel loading, the United States and various state forest service agencies have started selective thinning operations in national and state forests. Thinning millions of acres of overgrown forest areas would be expensive,

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

and the small-diameter trees to be removed are of low economic value to the current forest-products industry. Furthermore, the volume of wood generated from forest-thinning operations would be too large to be absorbed by traditional uses such as producing wood composite products, firewood, compost, and mulch.

A potential use of forest thinnings is to convert it into fuel ethanol and cogenerated electricity. This option has merit in that it can convert the enormous quantity of available biomass to an excellent fuel oxygenate such as ethanol. Additionally, there are synergistic benefits to locating biomass ethanol plants next to biomass power generation facilities. Such combinations would strengthen the process economics of both technologies and provide an ecologically sound solution to alleviate the forest fuel-loading problem.

In contrast to hardwoods and herbaceous materials, published data on softwood conversion to ethanol are limited. Little attention has been paid to softwoods, perhaps because of softwoods have high lignin contents that cannot be fermented to ethanol, and pretreatment processes successfully developed for herbaceous materials do not necessarily work well for softwoods. Sulfur dioxide-catalyzed steam pretreatment of spruce *(1)* and *Radiata* pine *(2)* was reported to greatly enhance the separation of major wood components. Most of the hemicellulose is rendered water soluble, and the cellulose can be enzymatically hydrolyzed to glucose.

The predominant softwood species available from forest thinning operations in the western United States are white fir *(Abies concolor)*, Douglas fir *(Pseudotsuga menziesii)*, and Ponderosa pine *(Pinus ponderosa)*. The objective of this study was to determine the potential ethanol yield from softwood via dilute sulfuric acid pretreatment and enzymatic hydrolysis. We determined sugar recoveries after pretreatment, glucose yields from enzymatic hydrolysis of water-washed substrates, and fermentability of the prehydrolysate. Douglas fir was selected as the model feedstock in our preliminary work.

MATERIALS AND METHODS

Feedstock Preparation

Softwood forest thinnings of Douglas fir and Ponderosa pine of 7–20-cm diameter were selected and harvested by Colorado State Forest Service agents (Golden district) from a north-facing slope in Golden Gate Canyon near Golden, Colorado. After being harvested and transported, the segregated logs were debranched, manually debarked, and chipped using a 65-hp Brush Bandit mobile knife chipper (Foremost, Remus, MI). The wood chips were then milled in a rotary knife mill (model 10 × 12, Mitts and Merrill, Saginaw, MI) equipped with a 3/8-in (9.5-mm) rejection screen, and cone/quartered blended. The fines were removed by screening through

a 2-mm screen. The final chips were packed in polyethylene-lined drums and stored at −20°C.

Milled and screened Douglas fir chips were soaked in dilute sulfuric acid (0.35–0.4%) solution at 60°C for 4 h, and drained overnight to approx 40% solids before being pretreated.

Pretreatment

All pretreatment experiments were performed using a 4-L steam-explosion reactor equipped with a steam jacket, a 4-in (10-cm) ball valve at the top for loading biomass, a 2-in (5-cm) ball valve at the bottom for discharging the contents of the reactor, two steam-injection ports near the top and bottom, and K-type thermocouples inserted near the top and bottom for reactor-temperature measurements. The reactor was made of Hastelloy C-22™ to resist corrosion. The reactor temperature was controlled at or near the desired value by using a pressure-control valve to control the steam-supply pressure. At the beginning of each experiment, the reactor was preheated to near the desired operating temperature by admitting steam into the jacket and cycling steam repeatedly through the reactor. A batch of preweighed acid-soaked chips (approx 1 kg wet weight) was then loaded into the reactor and saturated steam admitted (defined as time zero). After a predetermined cooking time, the steam was shut off, then the contents of the reactor (cooked wood chips, condensate, and steam) were discharged into a cooled flash tank with the flash vapor condensed in a separate condenser. The contents of the flash tank were emptied and blended, then the flash-tank surfaces were rinsed with water and collected for analysis. Portions of the pretreated solids were stored at −20°C for digestibility and fermentation assays. The pretreated materials were processed into liquor samples (obtained by pressing the liquid from the wet samples) and water-insoluble solid samples (obtained by extensively washing the samples) for chemical analyses and enzyme digestibility assays.

Analysis of Wood and Water-Insoluble Solids

Dry weights were determined by oven drying at 105°C to constant weight *(3)* and Klason acid-insoluble lignin and acid-soluble lignin were determined by standard methods *(4,5)*. Anhydrosugars in the whole wood and pretreated solids were determined by a procedure slightly modified from that developed at the U.S. Forest Products Laboratory *(4,6)*. Ash in the wood and pretreated solid residues was analyzed by standard gravimetric methods *(7)*.

Analysis of Liquor

Organic acids, glycerol, hydroxymethyl furfural (HMF), and furfural in the liquor and rinsate fractions were determined by ion-moderated par-

tition chromatography using Bio-Rad Aminex HPX-87H columns (Bio-Rad, Hercules, CA) *(4,8)*. Monomeric sugars were determined by HPLC (high-pressure liquor chromatography) using Bio-Rad Aminex HPX-87P columns. Oligomeric sugars in the liquor and rinsate fractions were converted to monomers using 4% H_2SO_4 hydrolysis at 121°C for 1 h, followed by determining monomeric sugars with the Bio-Rad HPX-87P column and correcting for sugar losses *(4,8)*.

Enzymatic Hydrolysis

Extensively washed pretreated wood samples were tested for enzymatic digestibility with Iogen cellulase (Iogen Super Clean cellulase, lot no. BRC 191095, Iogen, Ottawa, Ontario, Canada). The filter paper activity of the enzyme was assayed at 91 FPU/mL and the β-1 glucosidase activity at 198 IU/mL. The digestibility assays were perform at pH 4.8 with an enzyme loading of 60 FPU/g of cellulose in prewarmed (50°C) 10-mL reaction cocktails that contained solids equivalent to 1% cellulose, 50 m*M* citrate buffer, 40 mg/mL tetracycline, and 30 mg/mL cycloheximide. The antibiotics were to minimize contamination *(9,10)*. Duplicate reaction vials were incubated at 50°C with mixing at 120-rpm rotation at a 45° angle and compared to controls that contained 1% Solka-Floc, grade NF-FCC (Fiber Sales and Development, Urbana, OH) and enzyme blanks. One-half milliliter samples were removed at time zero, 2, 4, 6, 18, 24, 48, 72, 96, and 168 h. Glucose levels were determined with a YSI Model 2700 Select Biochemistry Analyzer equipped with immobilized glucose oxidase membranes (Yellow Springs Instruments, Yellow Springs, OH). Samples were centrifuged at 12,000*g* for 5 min and diluted to keep the glucose readings below the 2.50 g/L level used to calibrate the instrument.

Fermentability and Simultaneous Saccharification and Fermentation Performance

A fermentability test was carried out on whole slurry of pretreated wood samples. Thawed samples of pretreated wood were adjusted to pH 5.5 with $Ca(OH)_2$ then autoclaved at 121°C for 30 min in 250-mL DeLong (Fisher Scientific, Pittsburgh, PA) culture flasks. Sterile 10X solutions of yeast extract, peptone, and glucose (50 g/L, 100 g/L, and 500 g/L, respectively) were added to each flask to bring the total solids to 10 or 15% in a total of 150 g. The flasks were inoculated with a 15-h culture of *Saccharomyces cerevisiae* D_5A at a 10% level (w/w). Inoculated flasks were placed on a rotary shaker that operated at 30°C and 150 rpm. Sugar levels in samples were analyzed by HPLC using an HPX-87P column with deionized water as the eluant and refractive-index monitoring. Ethanol and fermentation byproducts were analyzed using an HPX-87H column with 0.01 *N* H_2SO_4 as the mobile phase and refractive-index monitoring *(8)*.

Standard simultaneous saccharification and fermentation (SSF) assays were carried out on exhaustively washed pretreated solids to determine the potential ethanol yield from the cellulose fraction. Cellulase enzyme (Iogen) at 25 IU/g of cellulose was added to each flask, which also contained 10 g/L yeast extract and 5 g/L peptone (Difco Laboratories, Detroit, MI) and enough solids to give 30 g/L cellulose in the 100-mL cocktail. The D_5A pre-inoculum was started in yeast extract peptone dextrose media (YPD), pH 5.0, from a frozen culture and incubated at 37°C for 6 h. A 10% volume of the preinoculum was used to start an overnight inoculum (12–16 h) in YPD, pH 5.0, mixed at 150 rpm and 37°C. The SSF was initiated by adding a 10% volume of the overnight inoculum and enzyme to flasks sealed with bubble traps. Samples were taken at time 0 and at 24-h intervals. SSF assays of whole slurry samples at 10% solid concentrations were also carried out to determine the effect of inhibitors present in the hydrolysate on ethanol yield.

RESULTS

Pretreatment Results

Five pretreatment conditions tested during the first run ranged from temperatures of 200 to 230°C and residence times of 125 to 305 s. The chips were impregnated with 0.4% sulfuric acid at 60°C for 4 h and then drained overnight to approx 40% solid before pretreatment. Depending on the pretreatment temperature and time, the solid contents of pretreated materials ranged from 25 to 30% based on wet weight. Compositional data were collected only on the liquid fraction of the pretreated material. Water used to rinse the flash tank (rinsate) was not collected during these experiments. A summary of the results is shown in Table 1. Solids recovery is the fraction of the original wood weight recovered in the pretreated material and should be less than 100% because of loss of volatile components. Recovery will also be low because rinsate was not collected. Solids solubilized is the fraction of the original wood lost as volatile or as water-soluble components, such as sugars, soluble lignin, and nonvolatile decomposition products. The sugar and byproduct yields and solids solubilized are also low because rinsate was not collected, however, the error will be small (6–9%, based on mass balance experiments, data not shown) and will be assumed to not affect a relative comparison of the results.

The results show that as pretreatment severity increases, the solids recovery decreases, and the solid solubilized increases. Both results are expected. Using xylose yield as an indication of hemicellulose hydrolysis, the maximum value occurred at 201°C and 305 s. The higher temperature conditions produced lower xylose yields and higher furfural yields, which indicated that the material was overcooked. However, it is not possible from this data to identify the maximum yield conditions. The glucose yields are difficult to interpret because glucose is produced from both

Table 1
Sugar Yields from Liquid Fractions of Pretreated Douglas Fir[a]

Exp. #	Temp. (°C)	Time (s)	Solids Recovery (%)	Solids solubilized (%)	Glucose[2] Yield (%)	Xylose[2] Yield (%)	HMF Yield (%)	Furfural Yield (%)
1	201	125	93.4	30.9	6.8	35.0	0.5	2.7
2	201	305	88.0	32.4	20.9	61.7	1.6	7.1
3	230	305	60.2	60.3	29.0	12.8	4.9	10.2
4	231	125	70.2	59.0	40.8	30.2	4.7	11.2
5	216	215	83.0	44.2	33.2	43.0	3.1	9.4

[a] Wood chips impregnated with 0.4% H_2SO_4.
[b] % of theoretical amount in wood, based on total sugars (monomeric + oligomers).

the cellulose and hemicellulose. Assuming a mannan to glucan ratio of 3:1 in the hemicellulose, the maximum glucose yield from the hemicellulose glucan is approx 8% based on wood weight. Glucose yields higher than this are from cellulose.

The experiment was set up as a two-factor (temperature and time), two-level factorial experiment with one center point, so it can be analyzed using the techniques of experimental design. The effects of temperature and time on solids solubilized (similar for hydrolysis of hemicellulose) and furfural yield (similar for hydroxymethyl furfural [HMF] yield) are shown in Figs. 1 and 2, respectively. The results indicate that temperature has a significant effect on all responses and that time has little or no effect on solids solubilized and hemicellulose yields. Time does become a more significant factor for HMF and furfural yields, but temperature still dominates. These results are true only within the tested range of variables.

An additional experiment was performed to obtain complete mass balance information on one set of pretreatment conditions. Ten batches of Douglas fir chips that had been impregnated with 0.35% sulfuric acid were pretreated at 212°C for 105 s. Rinsate was collected and analyzed in this experiment to complete the material balance. Table 2 presents the feedstock composition and component yield calculations based on the measured component concentrations in the pretreated solids, pretreated liquor, and flash vapor. The only decomposition products measured were HMF and furfural. The unaccounted-for fraction is material not accounted for by the indicated components. Additionally, it is assumed that HMF was produced only from glucan-derived glucose and furfural only from xylose. The large unaccounted-for fraction for most of the sugars is partially from: not accounting for byproduct production that is HMF or furfural; from production of byproducts not measured; and from experimental error.

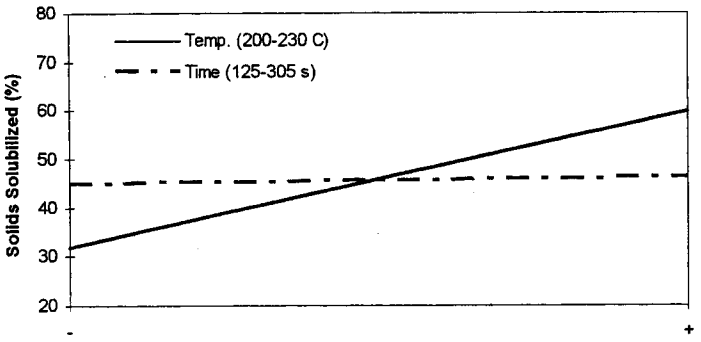

Fig. 1. Effects of pretreatment temperature and time on solids solubilized (%).

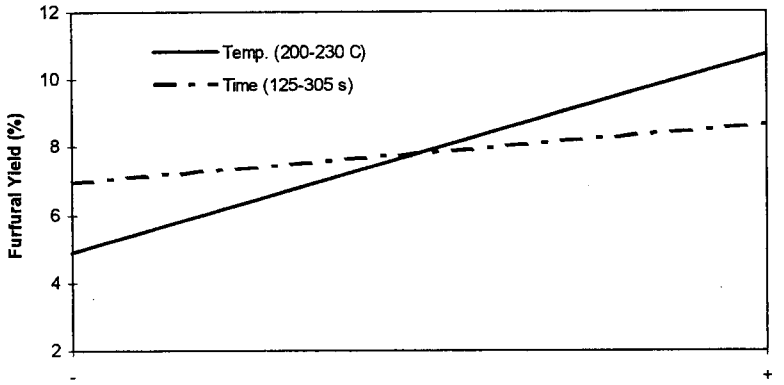

Fig. 2. Effects of pretreatment temperature and time on HMF and furfural yields (%).

The results show that more than 90% of the hemicellulose was solubilized during the pretreatment. The hemicellulosic sugar yields ranged from 56 to 63%, except for arabinose at 36%. A large fraction of the remaining hemicellulose is unaccounted for reasons discussed above.

Seventy percent of the glucan, which consisted of hemicellulosic glucan and cellulose, was not converted during the pretreatment. Twenty-three percent was converted to soluble sugars, and the rest to HMF or was unaccounted for. The mass recovery (mass accounted for by known products divided by mass input) based on individual component measurements for glucan was good at 96.8% and was 86.5% for all measured components (carbohydrates, lignin, and ash). The value is low because of the low recoveries for the hemicellulosic sugars (approx 60%). However, because they are only a small fraction of the feedstock, errors in these mass balances contribute less to the overall mass balance.

Enzymatic Hydrolysis Results

The effect of pretreatment time on the enzymatic hydrolysis glucose yield from Douglas fir is presented in Fig. 3. For a pretreatment tempera-

Table 2
Feedstock Composition and Component Yields of Pretreated Douglas Fir (Pretreatment at 212°C, 105 s, 0.35% H_2SO_4)

	Feedstock Composition (%)	Yields after Pretreatment (%)
Glucan	44.1	
Unconverted		70.6
To Monomeric Glucose		22.0
To Oligomeric Glucose		1.1
To HMF		3.1
Unaccounted For		3.2
Mannan	13.2	
Unconverted		3.4
To Monomeric Mannose		55.8
To Oligomeric Mannose		7.2
Unaccounted For		33.6
Galactan	3.7	
Unconverted		1.6
To Monomeric Galactose		63.4
To Oligomeric Galactose		4.3
Unaccounted For		30.6
Xylose	6.0	
Unconverted		0.4
To Monomeric Xylose		59.4
To Oligomeric Xylose		0
To Furfural		13.5
Unaccounted-For		30.0
Arabinan	3.0	
Unconverted		0.8
To Monomeric Arabinose		36.3
To Oligomeric Arabinose		0
Unaccounted For		64.4

ture of 212°C and sulfuric acid concentration of 0.4%, the 65-s pretreatment time resulted in 32% solubilization of wood and 91% solubilization of hemicellulose in water. Longer pretreatment times (105–185 s) resulted in 37 to 40% solubilization of wood and more than 95% of hemicellulose. When 91% solubilization of hemicellulose was achieved, the enzymatic digestibility of cellulose in pretreated Douglas fir was 65% of theoretical. With 95% solubilization of hemicellulose, the cellulose digestibility increased to 85%.

Fermentability and SSF Results

The results of fermentability tests of pretreated Douglas fir samples from the first pretreatment run are shown in Table 3. Ethanol yields were calculated by dividing the net ethanol produced by the theoretical amount based on total hexose sugars (glucose, mannose, and galactose). Fermentation performance was best for the less severe pretreatments (samples 1, 2,

Table 3
Effects of Solid Concentration on Ethanol Yield from Pretreated Douglas Fir[a]

Sample	Pretreatment Temperature (C)	Pretreatment Duration (s)	Solids Conc. (wt. %)	Hexose[2] Conc. (g/L)			Ethanol Produced (g/L)		Ethanol Yield % of theo.	
				0h	9h	46h	9h	46h	9h	46h
1	201	125	10	74.2	10.8	0.9	25.6	23.1	68	61
			15	82.5	45.4	1.7	25.2	33.3	60	79
			20	97.5	84.8	6.4	2.5	35.3	5	71
2	201	305	10	66.9	15.2	1.0	25.8	27.3	76	80
			15	87.8	40.2	4.7	20.2	32.4	45	72
			20	103.2	69.5	7.0	13.7	38.5	26	73
3	230	305	10	62.0	68.9	69.4	0.2	0.0	1	0
			15	80.0	84.3	84.7	0.0	0.0	0	0
			20	87.7	87.0	93.1	0.3	0.0	1	0
4	231	125	10	76.2	75.4	72.6	0.8	0.4	2	1
			15	95.0	94.4	94.8	0.0	0.0	0	0
			20	109.8	102.0	111.9	0.0	0.0	0	0
5	216	215	10	74.3	38.6	2.5	15.4	28.2	40	74
			15	87.2	79.2	3.9	3.4	36.4	8	82
			20	106.3	105.0	140.3	1.0	5.1	2	9
Glucose Control	N/A	N/A	N/A	44.4	0.0	0.0	14.3	12.4	63	55

[a] Wood chips impregnated with 0.4% H_2SO_4.
[b] Hexose includes glucose added and hexose (glucose, mannose, and galactose) present in the wood hydrolysate.

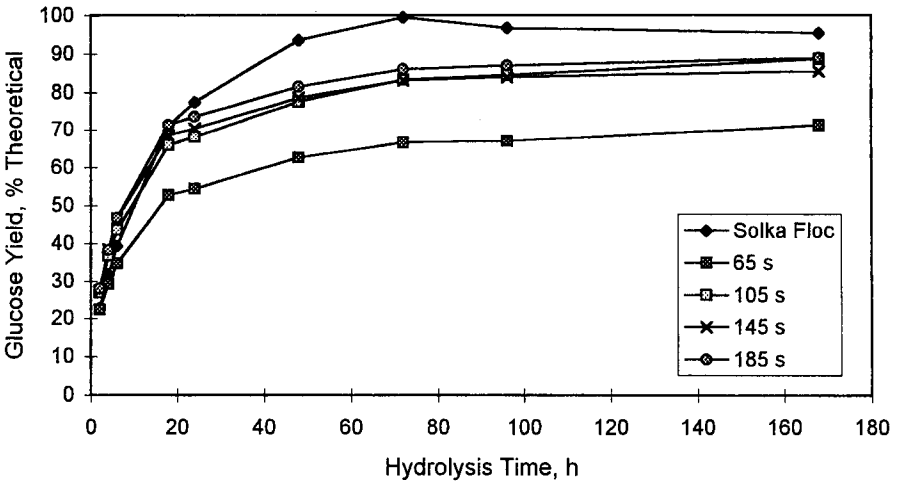

Fig. 3. Enzymatic hydrolysis glucose yield of pretreated Douglas fir (pretreatment at 212°C, 65–185 s, 0.4% H_2SO_4; enzymatic hydrolysis at 1% cellulose, 60 FPU/g cellulose, and 50°C).

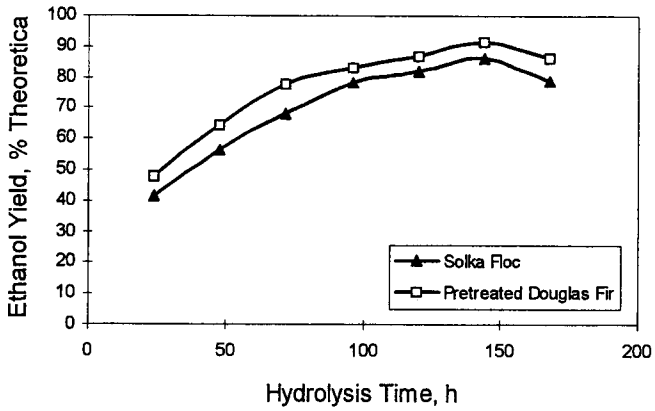

Fig. 4 Ethanol yield during SSF of pretreated Douglas fir pretreatment at 212°C, 105 s, 0.35% H_2SO_4; extensively washed with water; SSF at 3% cellulose, 25 FPU/g cellulose, *S. cerevisiae* D_5A yeast, and 37°C.

and 5) and at solids concentrations lower than 15%. For the more severe pretreatments (i.e., high temperatures and longer residence times), samples 3 and 4, the yeast was severely inhibited even at 10 wt% total solids loading.

Figure 4 shows the ethanol yield from SSF of the Douglas fir pretreated at 212°C for 105 s. With more than 95% of the hemicellulose removed after pretreatment, the ethanol yield from the cellulose portion of the pretreated wood sample was comparable to that of Solka-Floc (Fiber Sales and Development, Urbana, OH) (i.e., 80–85% of theoretical). Enzymatic hydrolysis and SSF of whole-slurry-pretreated Douglas fir at 10% solid concentrations (data not shown) indicated inhibition of both enzyme activity and fermentation, which resulted in lower ethanol yields (i.e., 60–70% of theoretical). We have since successfully adapted the D_5A yeast to a 21% solids hydrolysate from Douglas fir pretreated at 212°C, 0.35% sulfuric acid, and 105 s *(11)*.

DISCUSSION

The results of our preliminary work show that dilute sulfuric acid pretreatment effectively renders most of the hemicellulose fraction of Douglas fir soluble in water and the cellulose fraction highly digestible by cellulase enzyme. However, the pretreatment conditions that favor high enzyme digestibility substantially degrade the hemicellulosic sugar. To maximize hemicellulose recovery and minimize formation of inhibitors such as furfural and HMF, the pretreatment temperatures need to be lower than those used in these experiments. This suggests a two-stage pretreatment method, with the first stage operating at a lower temperature (e.g., 170–190°C), and a washing step between stages to remove the hemicellulosic sugars before carrying out the second-stage pretreatment at a higher

temperature (e.g., 210–230°C). However, this method, would increase the complexity and cost of the pretreatment process.

Another key issue is the presence of bark and needles. In forest-thinning operations, the normal practice is to chip the whole tree, which results in high bark and needle content in the chips. Because bark and needles contain high levels of extractives that may inhibit enzymes and the fermenting organism, whole tree chips may not be readily converted. We are currently investigating the effect of bark and needle content on the performance of pretreatment and fermentation. The trade-off between the additional cost of debarking and reduced ethanol yield caused by bark and needles will be evaluated.

ACKNOWLEDGMENT

Chemical analyses of hydrolysis and fermentation samples by F. Posey-Eddy, J. Hora, L. W. Brown, R. Ruiz, and C. I. Ehrman are greatly appreciated. Solid compositional analyses were performed by Hauser Laboratories (Boulder, CO). This work was funded by the Biochemical Conversion Element of the Office of Fuels Development of the US Department of Energy.

REFERENCES

1. Schwald, W., Smaridge, T., Chan, M., Bruil, C., and Saddler, J. N. (1989), in *Enzyme Systems for Lignocellulose Degradation*, Coughlan, M. P., ed., Elsevier, New York, pp. 231–242.
2. Clark, T. A. and Mackie, K. L. (1987), *J. Wood Chem. Technol.* **7(3),** 373–403.
3. TAPPI Test Methods (1991), T 210 cm-86, *Weighing, Sampling, and Testing Pulp for Moisture*, TAPPI, Atlanta, GA.
4. Moore, W. E. and Johnson, D. B. (1967), *Procedures for the Chemical Analysis of Wood and Wood Products*, Forest Products Laboratory, US Department of Agriculture, Madison, WI.
5. TAPPI Test Methods (1994–1995), T 222 om-88, *Acid-Insuluble Lignin in Wood and Pulp*, TAPPI, Atlanta, GA.
6. Grohmann, K., Himmel, M., Rivard, C., Tucker, M., Baker, J., Torget, R., and Graboski, M. (1984), *Biotechnol. Bioeng. Symp.* **14,** 137.
7. TAPPI Test Methods (1991), T 211 om-85, *Ash in Wood and Pulp*, TAPPI, Atlanta, GA.
8. Ehrman, C. I. and Himmel, M. E. (1994), *Biotechnol. Techniques* **87(2),** 99–104.
9. Ghose, T. K. (1987), *Pure Appl. Chem.* **59,** 257–268.
10. Grohmann, K., Torget, R., and Himmel, M. (1986), *Biotechnolo. Bioeng. Symp.* **17,** 135–151.
11. Keller, F. A., Bates, D., Ruiz R., and Nguyen, Q., *Nineteenth Symposium on Biotechnology for Fuels and Chemicals*, Colorado Springs, Colorado, May 4–8, 1997.