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Comparison of Microbial Inhibition and Enzymatic Hydrolysis Rates of Liquid and Solid Fractions Produced from Pretreatment of Biomass with Carbonic Acid and Liquid Hot Water

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Abstract

This research quantified the enzymatic digestibility of the solid component and the microbial inhibition of the liquid component of pretreated aspen wood and corn stover hydrolysates. Products of liquid hot water and carbonic acid pretreatment were compared. Pretreatment temperatures tested ranged from 180 to 220°C, and reaction times were varied between 4 and 64 min. Both microbial inhibition rates and enzymatic hydrolysis rates showed no difference between pretreatments containing carbonic acid and those not containing no carbonic acid. Microbial inhibition increased as the reaction severity increased, but only above a midpoint severity parameter of 200^0C for 16 min. Both the rates and yields of enzymatic hydrolysis displayed an increase from the lowest tested reaction severity to the highest tested reaction severity.

Index Entries: Carbonic acid; pretreatment; hydrolysis; corn stover; aspen wood.

Introduction

Conversion of lignocellulosic material to ethanol requires hydrolysis of carbohydrate polymers to their constituent sugars. Enzymatic hydrolysis is a common approach to hydrolysis and offers the benefits of mild

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reaction conditions and selective hydrolysis. To achieve useful rates of enzymatic hydrolysis, the lignocellulose must first be pretreated to reduce the recalcitrance of the substrate to hydrolysis. Pretreatment accomplishes many alterations of the biomass. Depending on the technology chosen, these effects typically include, to varying degrees; hydrolysis of the hemicellulose, solubilization of lignin and carbohydrate oligomers, and increased accessibility of the cellulose to cellulase enzymes *(1)*. Several pretreatment methods have been explored to varying degrees. The most commonly reported technologies include dilute-acid pretreatment, in which H_2SO_4 used in low concentrations (on the order of 1%) and at temperatures usually less than 200°C *(2–6)*, and steam explosion, which exposes the substrate to steam at elevated temperature and then explosive decompression to physically break apart the plant fibers *(7–9)*. Often, steam explosion is coupled with acid catalysis by impregnating the substrate with sulfur dioxide prior to steam treatment *(10–12)*. Other techniques include ammonia fiber explosion, which breaks down the lignin using ammonia and explosive decompression *(13–15)*, treatment with organic solvents *(16,17)*; and treatment with liquid hot water *(18–21)*. Some methods that have been examined less thoroughly include treatment with supercritical fluids *(22)* and carbonic acid *(23–25)*.

Steam explosion and dilute-acid pretreatment have undergone research and development for many years. Much of this research has been devoted to fuel production from biomass. Dilute-acid pretreatment offers good performance in terms of recovering hemicellulose sugars but suffers from its use of H_2SO_4. H_2SO_4 is highly corrosive and its neutralization results in copious production of solid wastes *(25)*. In situations in which landfill costs are high, disposing of the gypsum precipitate can be prohibitively expensive. The calcium sulfate resulting from neutralization also has problematic solubility characteristics in that it becomes less soluble at higher temperatures, such as those encountered in a reboiler *(25)*. Compared to dilute-acid pretreatment, steam explosion makes no use of H_2SO_4 and is less corrosive both to equipment and as a recovery product. However, steam explosion yields lower amounts of hemicellulose sugars. Studies suggest an 80% recovery of five-carbon sugars with dilute-acid pretreatment compared with 65% with steam explosion *(3,26)*. SO_2-catalyzed steam explosion achieves higher recovery of hemicellulose sugars, but this again introduces a reliance on sulfur acids and the requisite neutralization.

One process that may offer benefits of acid catalysis without the drawbacks of H_2SO_4 is the use of carbonic acid. The pH of carbonic acid is determined by the partial pressure of CO_2 in contact with water, and thus it can be neutralized by releasing the reactor pressure. Carbonic acid is relatively mild and hence does not offer the same hydrolytic capability of H_2SO_4. However, van Walsum *(25)* has demonstrated that at temperatures on the order of 200°C, carbonic acid does exhibit a catalytic effect on the hydrolysis of xylan. Van Walsum *(25)* observed an enhanced release of xylose and low-degree-of-polymerization xylan oligomers compared to pretreatment

using hot water alone. Puri and Mamers *(24)* compared steam explosion of biomass with and without CO_2 pressurization and reported enhanced enzymatic degradation with the carbonic acid-enhanced steam explosion. Contrary to these results, McWilliams and van Walsum *(23)* reported that compared to liquid hot water, carbonic acid offered no improvement in xylose yields for pretreatment of aspen wood. They proposed that this may have been owing to the high level of endogenous acid produced by the highly acetylated substrate and suggested that a less acidic substrate may show benefits from carbonic acid. This idea was confirmed by Shi and van Walsum *(27)*, who observed enhanced hydrolysis activity for carbonic acid pretreatment of corn stover, noting both increased xylose and furan yields when liquid hot water pretreatment was pressurized with CO_2.

The present study investigated the inhibition of *Saccharomyces cerevisiae* by the liquid hydrolysate and the kinetics of enzymatic hydrolysis of the solid components produced by the pretreatment of aspen wood and corn stover by liquid hot water and hot carbonic acid. Inhibition of yeast was determined by measuring the rate of glucose consumption by yeast growing in hydrolysates produced at various reaction severities. The enzymatic hydrolysis rates of pretreated solids was determined by measuring rates of sugar accumulation of enzyme-digested pretreated solids.

Materials and Methods

Experimental Design

A series of laboratory experiments compared products from reactions of biomass with water with those of biomass with water and carbonic acid. The three parameters that determined reaction severity were the reaction temperature, reaction duration, and carbonic acid concentration (psi of CO_2) *(25)*. The parameters tested were 180, 190, 200, and 220°C; 0 (at room temperature) and 800 psi (at room temperature); and 4, 8, 16, 32, and 64 min. Using the severity function as defined by Overend and Chornet *(28)*,

$$t \times \exp\left(\frac{\mathrm{T} - 100}{14.75}\right) \tag{1}$$

in which t is the time, min and T is the temperature in °C, and the severity conditions for these parameters varied from $R_o = 1813.8$ to $R_o = 109{,}242.9$. Broad categories of low, medium, and high severities were determined using the Log of the severity function (Log R_o). These broad categories were defined as having the following Log R_o: low (3.3–4.1), medium (4.2–4.5), and high (4.6–5.0). Table 1 shows some results representing the basic layout of the experimental design and inhibition/hydrolysis results in these severity ranges.

Preparation of Feedstock

Aspen wood chips were kindly supplied by the USDA Forest Products Laboratory (Madison, WI) and corn stover by the National Renewable

Table 1
Selected High-, Medium-, and Low-Severity Yeast Inhibition/
Enzymatic Hydrolysis Experiments on Aspen Wood and Corn Stover

	Severity parameters[a]					Inhibition[b]			Enzyme hydrolysis[c]			Final pH range
	T	*t*	*P*	R_o	Log R_o	No. of rep	No. of exp	%inh	No. of rep	No. of exp	T+24 (g/L yld)	
Aspen Wood												
	180	8	0	1813.78	3.3	2	1	4	6	4	0.58	5.69–4.97
	180	8	800	1813.78	3.3	2	1	0	2	1	0.55	5.69–4.97
	200	16	0	14,076.33	4.2	6	1	26	6	4	1.31	5.69–4.97
	200	16	800	14,076.33	4.2	6	1	22	6	4	1.71	5.69–4.97
	220	32	0	109,242.90	5.0	4	1	92	1	1	1.97	5.69–4.97
	220	32	800	109,242.90	5.0	4	1	84	1	1	2.09	5.69–4.97
Corn stover												
	180	8	0	1813.78	3.3	2	1	0	2	1	0.65	5.87–4.97
	180	8	800	1813.78	3.3	2	1	0	2	1	0.09	5.87–4.97
	200	16	0	14,076.33	4.2	4	2	4	4	2	1.06	5.87–4.97
	200	16	800	14,076.33	4.2	4	2	6	4	2	1.06	5.87–4.97
	220	32	0	109,242.90	5.0	2	1	18	2	1	1.5	5.87–4.97
	220	32	800	109,242.90	5.0	2	1	24	2	1	1.56	5.87–4.97

[a] R_o, severity = $t \times \exp[(T - 100)/14.75]$.
[b] No. of rep, number of replicates of the sample analysis; no. of exp, number of experiments in which the samples were generated; % inh, percent of microbial inhibition.
[c] T + 24 g/L yld, g/L of glucose produced at T + 24 of the experiment.

Energy Laboratory (Golden, CO). CO_2 was standard laboratory grade, and water was of standard laboratory deionized quality. Feedstock was first ground in a manual grinder to reduce particle size and then further ground in a domestic coffee grinder. Particles were sifted to give a uniform particle size of 0.6–1 mm. Moisture content of the ground biomass was determined by oven drying at 100°C for several days.

Pretreatment

Two 1.25-g samples of uniform particle-size feedstock were each weighed out and placed in separate 150-mL, 316 stainless steel reactors, along with 100 mL of deionized water each. The reactors were filled and emptied by removing a swage connection on one end. One reactor was pressurized with 800 psi of CO_2 at room temperature, and the other was left with air in the headspace at atmospheric pressure. For reactions using CO_2, a stainless steel tubing connection and valve with pressure gage were fitted to the reactor. Two sand baths (model SBL 2D in (Techne, Oxford UK,), with temperature controller (model TC-8D; Techne) were used for temperature control, one heated to the desired reaction temperature, and the second to 40°C above the desired reaction temperature. The higher-temperature sand bath was used for preheating the reaction vessels for 3 min to quickly attain the desired reaction temperature *(25)*. After preheating, the reactors were transferred to the reaction-temperature sand bath for the desired reaction duration. The reaction was quenched in an ice bath immediately after the reaction was complete. Previous research by McWilliams and van Walsum, *(23)* had determined that a temperature range of 180 to 220°C was optimal for xylose production from aspen wood. Reaction times ranged from 4 to 64 min.

Preparation of Hydrolysates

Solids were filtered out from the hydrolysate samples using a vacuum filter and microfilter paper. The filtrate was further clarified by centrifuging with a Fisher Marathon 21000R centrifuge and 04-976-006 rotor at 4000*g*, generating approx 3000 RCF, for 15 min at 15°C. The solids remaining on the filter paper were washed with deionized water three times (12 mL od deionized water total), with the final rinse dewatered under vacuum for 3 min prior to shutting off the vacuum. The filtered solids were placed in weighing tins and stored in a 100% humidity equilibrium chamber for 72 h. Half of each sample was analyzed for dry weight by drying in an oven at 101°C for 72 h. Dried portions of the samples were then available for quantitative saccharification, while the remaining moist sample was used for the enzymatic hydrolysis tests.

Microbial Culturing

A new batch of yeast malt agar (Y-3127; Sigma, St. Louis, MO), yeast malt broth (Y-3752; Sigma) and yeast (Fleishman's) were prepared for each

experiment according to the manufacturers directions. With each experiment, a new batch of baker's yeast was prepared according to the manufacturer's directions: two and a quarter teaspoons of yeast was added to 1/4 cup of water at 37°C with 1 teaspoon of glucose. The mixture was left to stand for 10 min. Once growth of yeast was confirmed, the mixture was plated for isolation under a laminar flow hood using fresh yeast agar plates. An isolated colony from the plate was aseptically transferred into the 10 mL of broth in the culture tube using a loop. The culture tubes were placed into the incubator at 30°C for 48 h.

Preparation of Test Vials

Twenty milliliters of the previously generated liquid hydrolysate was placed in serum vials containing from 0 to 100% hydrolysate, with the balance being deoinzed water. Dry yeast broth (21 g/L) and dextrose (10 g/L) were weighed and placed in each serum vial to serve as nutrient and energy sources. Vials were purged with N_2 for 30 s, sealed, and placed in an autoclave for 20 min at 121°C. The pH for each of these samples was also tested and was within the preferred tolerance range of yeast, varying from 4.97 for the most severe pretreatment to 5.87 for the least severe pretreatment *(29)*.

Inhibition

Batch culture experiments were performed to measure the rate of glucose consumption. Once the seed yeast had been incubated for exactly 48 h, the serum vials were each injected with 0.2 mL of freshly vortexed yeast. The moment of inoculation became time zero ($t = 0$) for the experiment. Viable cell counts taken at time zero showed the initial cell concentration to be on the order of 100,000 colony-forming units/mL. Samples were taken every 2 h over the next 12 h, except for the first 4 hours, and analyzed for glucose.

Glucose Assay

The glucose assay used was the Infinity™ Glucose Reagent Kit from Sigma. The procedure used was Procedure 17-UV provided with the kit. For each sample, 0.2 mL of liquid was removed from the serum vials and placed in a 1-mL microcentrifuge tube. The glucose assay was found to give a linear response up to 3 g/L of glucose, so samples were sufficiently diluted to put their glucose content into this range, depending on the anticipated concentration of the sample. Next, 1500 µL of glucose reagent was placed into a 2-mL cuvet for each sample. Prior to adding the sample, UV measurements of the reagent were made at 340 nm. Then, 15 µL of each sample was added to its dedicated cuvet. The cuvets were covered and incubated for 14 min (at room temperature). The absorbance was again measured at 340 nm. The difference in absorption units was then used to determine glucose content. A glucose standard of 2 g/L was run at each sampling time. A control was also used for each yeast inhibition experiment by running a sample that contained 0% hydrolysate but all the other components of the nutrient broth.

For the enzymatic hydrolysis samples, the control used contained citrate buffer, preservative, and cellulase enzymes.

Quantitative Saccharification

Quantitative saccharification was done in accordance with NREL LAP-002 *(30)*. Duplicate samples of approx 0.4 g were dispensed into separate test tubes. A standard of pure cellulose (Avicel) was analyzed in triplicate each experiment. Tubes and vials were placed on ice during manipulations to minimize reaction during untimed periods. H_2SO_4 (72%) was added to each of the tubes to create an acid-to-sample ratio of 0.01 mL/mg, after which they were placed in a reciprocal water bath set at 30°C. The tubes were immersed in the shaker bath for 2 h and stirred every 15 min with dedicated glass stirring rods. At the end of the 2 h period, each sample was diluted with deionized water into a serum vial to give a final concentration of 0.27 mL/mg of sample. Each serum vial was then capped, sealed, and autoclaved for 1 h. Once the autoclaving was complete, the serum vials were cooled and analyzed for glucose using the enzymatic glucose assay.

Enzymatic Hydrolysis Tests

A pH 5.0 citrate buffer solution was prepared with 0.5% sodium benzoate as a preservative. Taking into account the moisture and cellulose content of each pretreated sample, vials were prepared to achieve a glucose level no greater than 2 g/L (assuming 100% conversion of cellulose) in 20 mL of buffer solution. Each sample was done in duplicate. Cellulase (Iogen IG) was added at a loading of 20 cellulase units/g of solid residue. Quantitative saccharification of the residues found that most samples, prepared across various severities, had a cellulose content of approx 64%; thus, the cellulase loading translates to about 31 U/g of cellulose. β-Glucosidase (Novo 188) was added at a ratio of 5 U/U of cellulase. The addition of the enzymes defined time zero. Immediately after taking the samples at time zero, the testing vials were placed in a shaking water bath at 40°C. The serum vials were removed for testing every 2 h for up to 12 h. They were further sampled at 24 h, 48, and 120 h.

Results

Inhibition Testing

Two metrics were used to quantify inhibition: total consumed glucose and consumption rate. Figures 1–3 show average values derived from replicate samples within single experiments.

Figure 1 shows that with aspen wood as the substrate, glucose consumption by yeast was inhibited by the hydrolysate and that the inhibition increased as the severity of the pretreatment increased. However, there was no difference in the amount or rate of glucose consumed between samples that were pretreated with CO_2 and samples pretreated without CO_2.

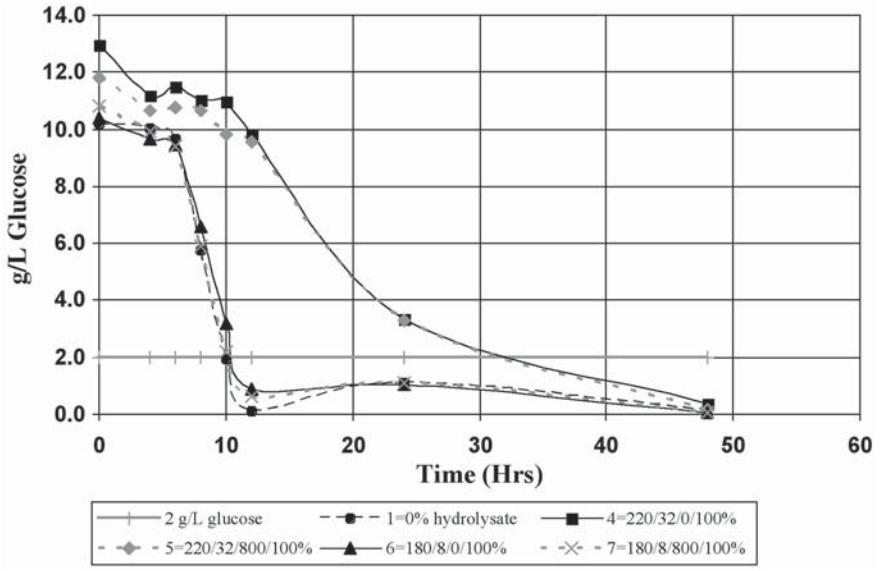

Fig. 1. Glucose consumed for samples with and without CO_2 for aspen wood.

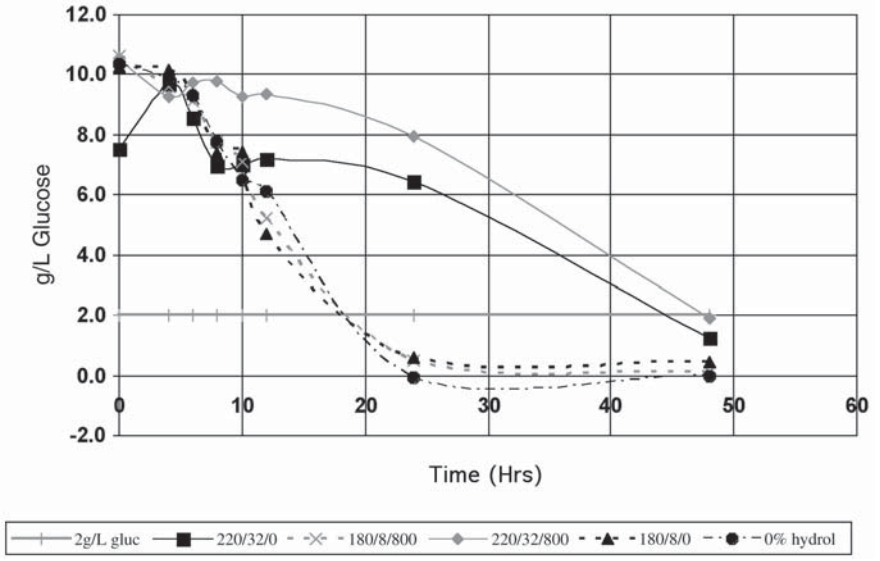

Fig. 2. Glucose consumed for samples with and without CO_2 for corn stover.

Figure 2 shows that for corn stover at the highest tested severity parameter of 220°C for 32 min, inhibition of the yeast regarding to the control was as extreme as with the aspen wood samples shown in Fig. 1. It took more than double the amount of time for the higher-severity sample yeast to consume 50% of the glucose than in all the other samples, just as with the aspen wood sample at 220°C for 32 min.

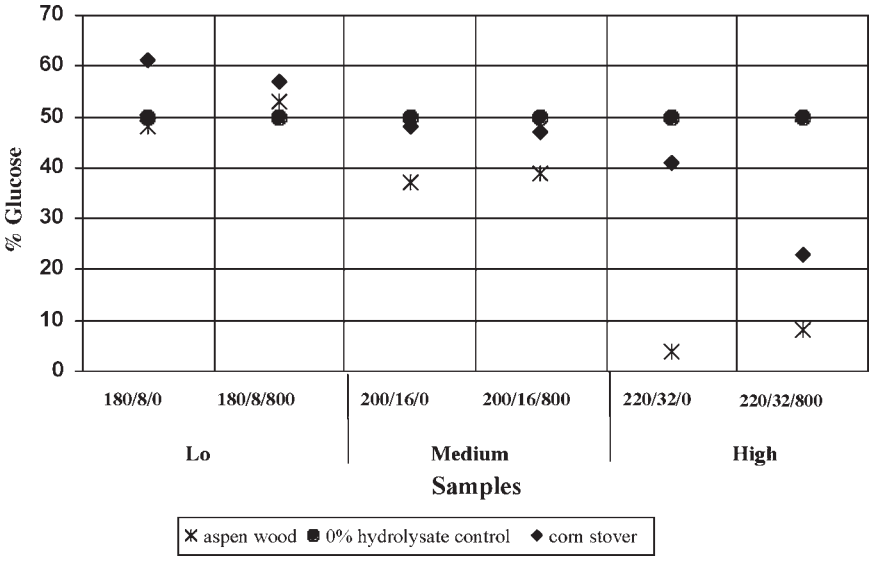

Fig. 3. Glucose consumption comparing aspen wood and corn stover for low-, medium-, and high-severity pretreatment.

At the lowest tested severity parameter of 180°C for 8 min, there was almost no inhibition when compared to the control that was run at each experiment. However, at the highest tested severity parameter of 220°C for 32 min, there was an almost 50% reduction in the rate of glucose consumption by the yeast. This division of inhibition as it correlates to severity is well defined for both the aspen wood and corn stover samples. However, between the aspen wood and corn stover samples there appeared to be a difference in inhibition rates, as a comparison of Figs. 1 and 2 shows. However, for this distinction to be conclusive, a side-by-side experiment would need to be conducted, giving a more standardized inoculum between the two experiments. Both Figs. 1 and 2 show the lowest and highest tested severity parameters. The response of mid-level severity was similar to that observed for the low level severities (data not shown).

Figure 3 shows a comparison of the glucose consumption among a low-severity (Log R_o = 3.3), medium-severity (Log R_o = 4.2), and high-severity (Log R_o = 5.0) pretreatment with and without carbonic acid. Glucose consumption for different severity hydrolysates was measured at the time when the 0% control hydrolysate had achieved 50% utilization. A trend is shown that the yeast was more inhibited as the severity of the pretreatment increased. Figure 3 also shows a difference between corn stover samples and aspen wood samples for the same pretreatments. Aspen wood was more inhibitory than corn stover for all three pretreatment conditions, and the difference between the two continued to increase as the severity of the pretreatment increased.

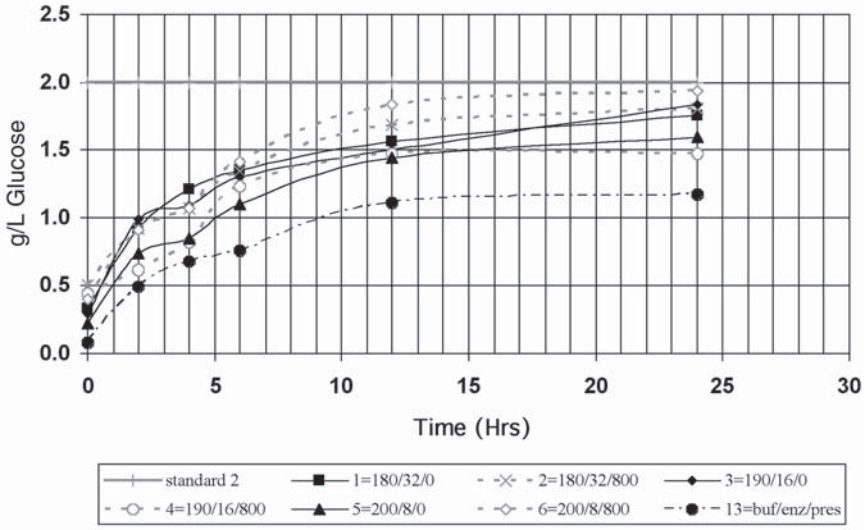

Fig. 4. Rate and yield of enzymatic hydrolysis vs reaction severity of medium-severity pretreatments only for aspen wood samples.

Enzymatic Hydrolysis Rates

Two metrics of hydrolysis efficacy were used: total recovered glucose and reaction rate.

Different combinations of time and temperature parameters that result in a low to mid range severity (Log R_o ~ 3.84) are shown in Fig. 4. The relative consistency between the experiments indicates that the severity of the different conditions is comparable and also that there appears to be no significant difference between samples pretreated with or without carbonic acid. After 24 h there was little difference in the yield, and this remained so even when the testing continued for 120 h (data not shown), the time it is thought to take for enzymes to achieve about 90% hydrolysis. Figure 5 shows that reaction severity played a major role in the rate and yield of enzymatic hydrolysis and that as the reaction severity increased, so too did the rate and final yield of the enzymatic hydrolysis throughout the range of severities. Increased severity also appeared to enhance initial glucose concentrations, which indicates that some glucose was released during pretreatment. In Fig. 6, the grams per liter of glucose produced from enzymatic hydrolysis was determined for the low, medium and high severities at t + 24. Aspen wood consistently produced more glucose than did the corn stover, and more glucose was produced with increased severity for both substrates.

Discussion

Inhibition

The midpoint pretreatment condition of 200°C for16 min could be an optimal condition for reduced microbial inhibition. For severities around

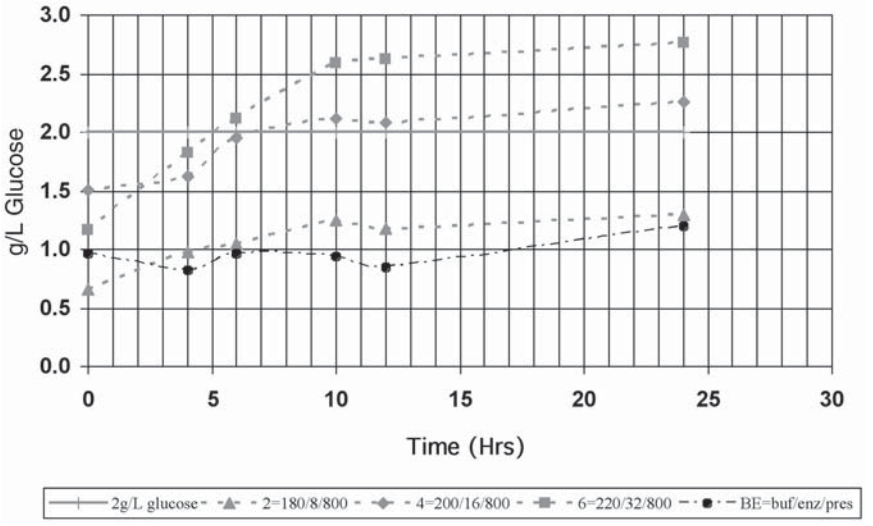

Fig. 5. Rate and yield of enzymatic hydrolysis vs reaction severity of low-, medium-, and high-severity pretreatment for corn stover samples.

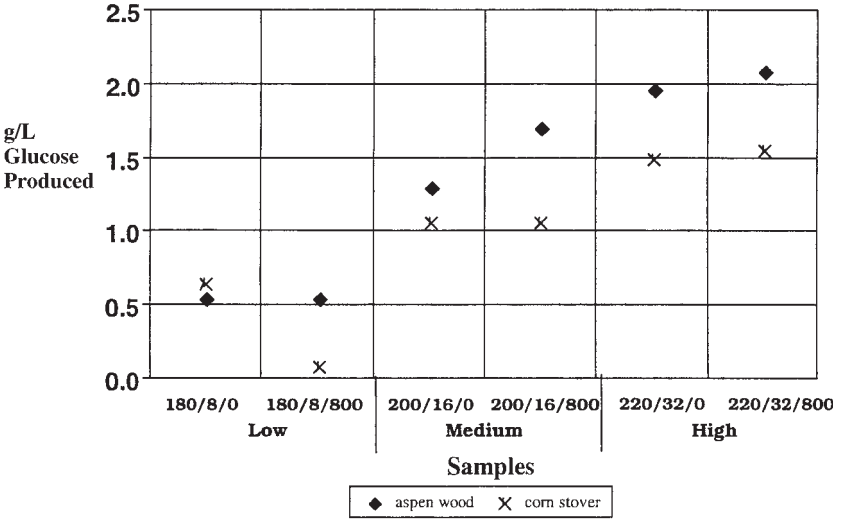

Fig. 6. Glucose produced from enzymatic hydrolysis at t = 24 h comparing aspen wood and corn stover for low-, medium-, and high-severity pretreatment.

the midpoint and below, there was no improvement in the reduction of microbial inhibition. This is promising because McWilliams and van Walsum *(23,31)* found that the midpoint severity of 200°C for 32 min to produce the optimal yield of xylose monomers during pretreatment: lower severities produced less; higher severities led to degradation.

It is possible that a toxicity threshold is not achieved at lower severities, reducing the inhibition from these conditions. The corn stover samples were consistently less inhibitory than the aspen wood samples. This is likely owing to the fact that fewer acids are inherent in the corn stover biomass than in the aspen wood biomass. For example, it was found that the pH of the corn stover hydrolysates was higher than the pH of aspen hydrolysates. Further testing is warranted to investigate the difference in the inhibition of the substrates and also to determine whether scatter is to blame for the appearance of a difference between pretreatments with and without carbonic acid when assessing the percent inhibition from Fig. 3. Pretreatment severity showed a marked effect on the degree of inhibition suffered by yeast cultures, but the presence or absence of carbonic acid appears to have had no influence on inhibition. Between the aspen wood and corn stover samples, there appeared to be a difference in inhibition rates.

Hydrolysis Rates

It is clear that the midpoint severity of 200°C for 32 min that produces the maximum xylose recovery as found by McWilliams and van Walsum *(23,31)* from the pretreatment step is not the optimal severity for enzymatic hydrolysis. The rates and yields of enzymatic hydrolysis continued to increase as the reaction severity of the pretreatment increased. It is possible that the enzymatic hydrolysis rates and yields would continue to increase past the maximum severity that was tested, 220°C for 32 min. A possible explanation for this result is that the more severe reaction conditions are continuing to break down the hemicellulose and solubilize the lignin, allowing the enzymes more and easier access to the cellulose without destroying the cellulose. Thus, optimization of this pretreatment appears to involve a compromise among maximized xylose yield, minimized inhibition, and maximal rates of enzymatic hydrolysis. The differences between the substrates and their glucose yield at t +24 is very interesting. A possible cause of this phenomenon is that the lower pH of the aspen hydrolysate enhances the pretreatment effectiveness as time continues. These acids may assist in the degradation of the hemicellulose and lignin, allowing more exposure of the cellulose for the enzymes to hydrolyze. Further investigation of the differences shown between the substrates is warranted.

Conclusion

Both microbial inhibition rates and enzymatic hydrolysis rates showed no differences between pretreatments containing carbonic acid and those not containing carbonic acid. Additionally, when the microbial inhibition and enzymatic hydrolysis rates were tested at varying reaction severities and between different substrates, this remained true.

When microbial inhibition rates were examined with increasing reaction severity, the inhibition increased as the reaction severity increased, but

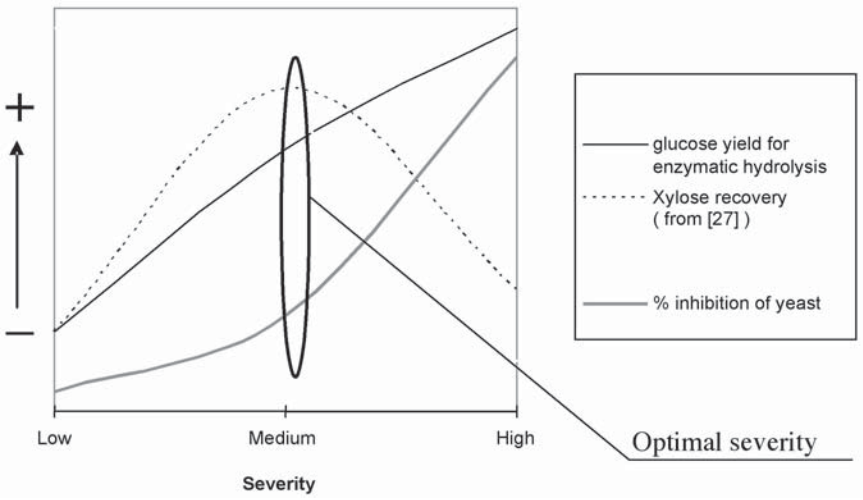

Fig. 7. Optimization of severity parameters.

only above the midpoint severity parameter. Below the midpoint severity parameter, there was little to no inhibition to the yeast. When enzymatic hydrolysis rates and yields were examined with increasing reaction severity, both the rates and yields displayed an increase from the lowest tested reaction severity to the highest tested reaction.

It is possible to theorize that between the two tests performed, there is an optimal severity that will maximize the glucose yield and rate from enzymatic hydrolysis while minimizing the inhibition to the yeast. This can further be optimized when using the severity parameters that produce the greatest amount of xylose monomer as detailed in previous research by McWilliams and van Walsum *(23)*. Figure 7 provides a possible view of the optimization of the severity parameters. While the inhibition of yeast is still minimal in the midseverity range, the amount of xylose is at its highest point. Finally, the glucose produced from enzymatic hydrolysis is at a median point.

Acknowledgments

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References

1. McMillan, J. D., (1994), in *ACS Symposium Series No. 566*, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., American Chemical Society, Washington, DC, pp. 292–324.

2 Bouchard, J., Overend, R. P., and Chornet, E., (1992), *J. Wood Chem. Technol*. **12(3),** 335–354.
3 Quang, A. N., Tucker, M. P., Keller, F. A., and Eddy, F. P., (2000), *Appl. Biochem. Biotechnol*. **84–86,** 561–576.
4 Torget, R., Hatzis, C., Hayward, T. K., Hsu, T., and Phillipidis, G.P., (1996), *Appl. Biochem. Biootechnol*. **57/58,** 85–101.
5 Torget, R. W., Kim, J. S., and Lee, Y. Y., (2000), *Ind. Eng. Chem. Res*. **39,** 2817–2825.
6 Wooley, R., Ruth, M., Glassner, D., and Sheehan, J., (1999), *Biotechnol. Prog*. **15,** 794–803.
7 Boussaid, A. L., Esteghlalian, A. R., Gregg, D. J., Lee, K. H., and Saddler, J. N., (2000), *Appl. Biochem. Biotechnol*. **84–86,** 693–705.
8 Brownell, H. H. and Saddler, J. N., (1987), *Biotechnol. Bioeng*. **29,** 228–235.
9 Montane, D., Farriol, X., Salvado, J., Jollez, P., and Chornet, E., (1998), *J. Wood Chem. Technol*. **18(2),** 171–191.
10 Clark, T. A., Mackie, K. L., Dare, P. H., and McDonald, A. G., (1989), *J. Wood Chem. Technol*. **9(2),** 135–166.
11 Mackie, K. L., Brownell, H.H., West, K. L., and Saddler, J. N., (1985), *J. Wood Chem. Technol*. **5(3),** 405–425.
12 Schell, D., Nguyen, Q., Tucker, M., and Boynton, B., (1998), *Appl. Biochem. Biotechnol*. **70–72,** 17–24.
13 Dale, B. E., Weaver, J., and Byers, F. M., (1999), *Appl. Biochem. Biotechnol*. **77–79,** 35–45.
14 Holtzapple, M. T., Lundeen, J. E., Sturgis, R., Lewis, J. E., and Dale, B. E., (1992), *Appl. Biochem. Biotechnol*. **34/35,** 5–21.
15 Wang, L., Dale, B. E., Yurttas, L., and Goldwasser, I., (1998), *Appl. Biochem. Biotechnol*. **70–72,** 51–66.
16 Avgerinos, G. C. and Wang, D. I. C.,(1983), *Biotechnol. Bioeng*. **15,** 67–83.
17 Bouchard, J., Lacelle, S., Chornet, E., Vidal, P. F., and Overend, R. P., (1993), *Holzforschung* **47(4),** 291–296.
18 Allen, S. G., Kam L. C., Zemann, A. J., and Antal Jr., M. J. (1996), *Ind. Eng. Chem. Res*. **35(8),** 2709–2715.
19 Allen, S. G., Schulman, D., Lichwa, J., Antal, M. J., Laser, M., and Lynd, L.R., (2001), *Ind. Eng. Chem. Res*. **40,** 2934–2941.
20 Mok, W. S. L. and Antal Jr., M. J. (1992), *Ind. Eng. Chem. Res*. **31(4),** 1157–1161.
21 van Walsum, G. P., Allen, S. G., Spencer, M. J., Laser, M. S., Antal, M. J., and Lynd, L.R., (1996), *Appl. Biochem. Biotechnol*. **54/55,** 157–170.
22 Sasaki, M., Fang, Z., Fukushima, Y., Adschiri, T., and Arai, K., (2000), *Ind. Eng. Chem. Res*. **39,** 2882–2890.
23 McWilliams, R. C. and van Walsum, G. P., (2002), *Appl. Biochem. Biotechnol*. **98–100,** 109–134.
24 Puri, V. P. and Mamers, H., (1983), *Biotechnol. Bioeng*. *25,* 3149–3161.
25 van Walsum, G. P. (2001), *Appl. Biochem. Bioetechnol*. *91–93,* 317–329.
26 Heitz, M., E. Capek-Menard, P. G. Koeberle, J. Gagne, E. Chornet, E., Overend, R. P., and Taylor, E. Y. (1991), *Bioresour. Technol*. **35,** 23–32.
27 Shi, H. and van Walsum, G. P. (2004) *Bioresourc. Technol.*, in press.
28 Overend, R. P. and Chornet, E., (1987), *Philos. Trans. R. Soc. Lond*. *A321:* 523–536.
29 Palmqvist, E. and Hähn-Hagerdal, B. (2000), *Bioresour. Technol*. **74,** 17–24.
30. Ruiz, R. and Ehrman, T. (1996), *Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography*, Laboratory Analytical Procedure No. LAP-002, National Renewable Energy Laboratory, Golden, CO.
31. McWilliams, R. (2002), MS thesis, Department of Environmental Studies, Baylor University, Waco, TX.