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Microbial Pretreatment of Biomass

Potential for Reducing Severity of Thermochemical Biomass Pretreatment

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Abstract

Typical pretreatment requires high-energy (steam and electricity) and corrosion-resistant, high-pressure reactors. A review of the literature suggests that fungal pretreatment could potentially lower the severity requirements of acid, temperature and time. These reductions in severity are also expected to result in less biomass degradation and consequently lower inhibitor concentrations compared to conventional thermochemical pretreatment. Furthermore, potential advantages of fungal pretreatment of agricultural residues, such as corn stover, are suggested by its effectiveness in improving the cellulose digestibility of many types of forage fiber and agricultural wastes. Our preliminary tests show a three- to five-fold improvement in enzymatic cellulose digestibility of corn stover after pretreatment with *Cyathus stercoreus*; and a ten- to 100-fold reduction in shear force needed to obtain the same shear rate of 3.2 to 7 rev/s, respectively, after pretreatment with *Phanerochaete chrysosporium*.

Index Entries: Microbial pretreatment; fungal pretreatment; corn stover; enzymatic hydrolysis.

Introduction

In many enzyme-based biomass conversion processes to ethanol or other chemicals, a thermochemical pretreatment step is required to disrupt the lignocellulosic structure of biomass, and partially solubilize polysaccharides *(1,2)*. Pretreatment improves the susceptibility of holocellulosic polysaccharides to enzymatic hydrolysis. Thermochemical pretreatment has been identified as a unit operation that is the second highest (after feedstock) cost component in enzyme-based conversion of corn stover to ethanol *(3)*. The cost centers are steam, chemicals, and expensive corrosion-resistant

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pretreatment reactors. A major reduction in cost of pretreatment would have a significant impact on the overall cost of ethanol production.

Literature Highlights on Fungal Pretreatment of Hardwood and Softwood Feedstock

Eriksson and Vallander *(4)* and Messner and Srebotnik *(5)* reported as much as a 23% reduction in pulp-refining energy required by incubating spruce and pine chips for 2-wk with the white-rot fungus *Phanerochaete chrysosporium* at 35–40°C. It was suggested that fungal pretreatment disrupts the wood structure apparently by partially breaking down the lignin/carbohydrate complex.

Akhtar et al. *(6,7)* found an average 47% reduction in refining energy required in mechanical pulping after a 4-wk treatment with *Ceriporiopsis subvermispora* sp. on aspen wood chips and a 30% reduction in extractable resin compared to controls. Energy savings of 37% on loblolly pine were obtained. The weight losses on each were about 6% on aspen and 5% for loblolly pine after 4 wk of fungal treatment.

Kashino et al. *(8)* reported the effects of treating coarse mechanical pulp with an as-yet unidentified white-rot fungus, *IZU-154*. Seven-d treatment with whole culture reduced refining energy of beech pulp by 50–66% in 7 d for bleached and unbleached pulp, respectively, and by about 33% for softwood pulp (spruce and pine), which was treated for 10–14 d. The hardwood lignin loss was 17.5%, with a weight loss of 4.3%, and the softwood lost 5.3% lignin and 1.5% weight. The lignin loss for both *Coriolus versicolor* and *P. chrysosporium* was about half of that for *IZU-154*: about 6–7% for *Coriolus*, and about 9 % for *Phanerochaete*. The refining energy was measured by the number of revolutions in a PFI mill (Process Facilities Inc., Philadelphia, PA) required to develop a measured amount of "freeness", as well as by electrical refining energy consumed. The inoculum for the coarse pulp was the mycelium of potato dextrose broth–grown fungi fragmented in water for 30 s in a sterile Waring blender, or the whole broth culture. It was found that whole broth, or adding additional glucose to the blended mycelium inoculum, provided no added benefit. The moisture content recommended for each pulp is 75% beech, 89% spruce, and 86% pine.

Sawada et al. *(9–11)* investigated the effects of fungal pretreatment and steam explosion pretreatment on enzymatic saccharification of beech wood meal. Their work indicates that fungal pretreatment followed by less severe steaming maximizes enzymatic saccharification. The investigators suggested that the lignin network covering the holocellulose (cellulose and hemicellulose) is broken down by successive fungal pretreatment and steam explosion pretreatments, which together maximize subsequent enzymatic saccharification of beech wood meal. *P. chrysosporium* (ATCC 34541) was incubated without agitation at 37°C with 5.0 g of meal (unspecified dryness), 15 mL of Kirk's broth (2.0 g of glucose, 0.02 g of ammonium tartrate, 0.02 g of yeast extract, 5.0 mL of 0.4 *M* phthalate buffer pH 4.5,

1.0 mL of Kirk's salt solution, and 100 mL water) in 300-mL Erlenmeyer flasks. The type of closure on the flasks was unspecified. One gram of fungal pretreated biomass was then subjected to steam explosion at 170–230°C for 0–30 min.

The maximum saccharification of beech wood meal (9.5%) occurred at 28 d of fungal pretreatment and became less thereafter, possibly, Sawada et al. *(9–11)* explained, because degraded lignin combined with or coated the remaining holocellulose. Fungal pretreatment combined with steam explosion improved saccharification, compared to steam explosion alone by 20–100 % of the polysaccharide in the wood. However, 17 % of the holocellulose degraded during fungal pretreatment, and there was an unspecified holocellulose loss during steam explosion at optimum 215°C for 6.5 min.

Literature Highlights on Fungal Pretreatment of Agricultural Wastes, Grasses and Forage Crops

Karunanandaa et al. *(12)* studied the biodegradability of crop residues colonized by white-rot fungi. Colonization of maize (*Zea maize L.*) and rice straw for 15 and 30 d by three fungi and a cellulaseless mutant of *P. chrysosporium* were compared for dry matter (DM) digestibility. *Cyathus stercoreus* improved the in vitro cellulose digestibility of maize and rice straw by 37 and 45%, respectively, and DM loss was only 3.3%. The wild and mutant strains of *Phanerochaete* indiscriminately degraded both hemicellulose and cellulose. No direct correlation was found between lignin degradation and improved digestibility.

Akin et al. *(13)* found that microbial delignification with white-rot fungi improves forage digestibility relative to nonfungal treated controls. *C. subvermispora fp*-90031 improved in vitro ruminal digestion of Bermuda grass by 80% in 72 h. Of four other fungi—*Phellinus pini RAB-83-19*; *Phanerochaete chrysosporium K-3*; and two cellulaseless mutants, 3113, and 85118, derived from K-3—only K-3 improved the digestibility of Bermuda grass and produced losses of lignin. The other three organisms did not improve the digestibility of the forage. *C. subvermispora* extensively removed ester-linked *p*-coumaric and ferulic acids as well as non-ester-linked aromatics from growing (parenchyma) and more recalcitrant, nongrowing (sclerenchyma) plant cell walls. Further, it appears that the fungi improve mass transport into the biomass.

Hadar et al. *(14)* reported that biodelignification of cotton straw by the edible "oyster mushroom", *Pleurotus ostreatus*, followed by 36 h of in vivo ruminal digestion removed 2.2 times more organic material than nonfungal pretreated controls.

The concept that fungal pretreatment lowers the energy requirements of thermomechanical pulping of lignocellulosic biomass could potentially be applied to enzyme-based biomass conversion processes to lower the thermochemical pretreatment severity. Lower pretreatment severity is

expected to translate directly into lower chemical consumption, and because of lower temperature requirements, possibly lower steam costs. These reductions in severity could also reduce the capital costs and result in lower biomass degradation and consequently lower inhibitor levels. The reason for the success of fungal pretreatment, or "seasoning" as it is called in the pulp industry, is that such pretreatment of wood chips reduces electrical power consumption of mechanical pretreatment, as well as the chemical requirements. This potential for fungal pretreatment can be explained by the ability of certain fungi to disrupt the plant cell wall chemistry, resulting in partial breakdown of the lignin/carbohydrate complex. Furthermore, fungal pretreatment can readily be incorporated into the feedstock handling area of biomass-to-ethanol processes.

From this summary of relevant literature, we present preliminary experimental results of the effect of fungal pretreatment on the enzymatic cellulose digestibility of corn stover, and a concept of applying fungal pretreatment to bioconversion of lignocellulosic biomass to ethanol.

Materials and Methods

Fungal Collection

Based on our interest in the articles that we have reviewed, we collected two fungi for evaluation: (1)*C. stercoreus* NRRL 6473 (delignification of maize), and (2)*P. chrysosporium* BKM-F-1767; FPL (reference wild strain, delignification)

Handling of Fungal Strains

Two possible approaches for handling fungal strains are to apply the key fungi directly to the biomass, and to produce the enzymes and then apply enzymes to the biomass. We applied the former approach since it has been most successful in the literature.

Culturing and Preservation

Preliminary procedures were developed for propagation and preservation of the fungal cultures. As soon as the fungal strains arrived from either the Forest Products Lab or the Agricultural Research Service, they were grown on potato dextrose agar (PDA, Difco, Detroit, MI). Once the cultures covered most of the PDA plate, they were transferred vegetatively by cutting pieces out of the agar with a sterile scalpel, or using a sterile, sharp cork borer.

Preservation of Fungus in Glycerol

The PDA pieces were placed into a sterile test tube and resuspended using 1 part culture and adding at least 2 parts 50% (w/v) sterile glycerol, making at least 25% (w/v) glycerol. The mixture was well blended with a glass rod so glycerol covered the fungus. A small amount of glycerol was added to rinse the rod. A drop of Tween-80 was added if the glycerol

Table 1
Media Preparation for Fungal Pretreatment[a]

Chemical	Quantity (g)
$NaNO_3$	3.0
KCl	0.5
$MgSO_4 \cdot 7H_2O$	0.5
$FeSO_4 \cdot 7\ H_2O$	0.5
KH_2PO_4	1.0
Glucose	20.0
Distilled and deionized water	Make up to 1 L (resulting pH 6.0)

[a]Adapted from Demain and Solomon *(15)*.

did not wet the fungus and was reblended. The mixture was vortexed to mix well, and 1.5 mL was serologically pipeted into cryovials, and frozen at –70°C. Freeze preservation was evaluated; all cultures survived freezing and thawing, and grew well when plated on PDA. The cork borers were kept from corroding by washing and draining them quickly and rapidly drying in a 100°C oven. Sterilize fast at 121°C and dry.

Preparation of Stover

Coarsely milled corn stover was washed to remove dirt, drained, and air-dried to about 30% moisture. It was then well mixed (coned and quartered), portioned, and frozen. To obtain homogeneous material, approx 2 kg of frozen, coarsely milled corn stover was mixed with crushed dry ice (using 1 part crushed dry ice to 1 part biomass); the mixture was then milled in a laboratory Wiley mill through a 2-mm mesh. The milled stover was stored unsealed in plastic bags in a freezer. When all the dry ice had sublimed (when it was observed that the bag was not expanding), the bags were sealed and kept frozen until needed.

Preparation and Inoculation of Corn Stover Biomass

Mycelial cultures were grown on PDA agar. Approximately 1 in.^{2} of a fresh agar culture was minced and suspended in 5 mL media at pH 6 (*see* Table 1, ref. *15*) and then homogenized using a sterile glass rod until uniformly dispersed in the buffer. Large quantities were prepared in a small, sterilized Waring blender. Mycelial culture homogenates were used promptly. Spore suspensions could be kept for up to 1 wk in a refrigerator (4°C).

Approximately 100 g (dry basis) of Wiley-milled corn stover was autoclaved for 20 min at 121°C. The two fungi, *P. chrysosporium* (wild-type) and *C. stercoreus* were compared for their ability to pretreat corn stover vs. a control (i.e., corn stover incubated without fungi). The fungi pretreatment was carried out in triplicate using 250-mL DeLong Erlenmeyer flasks capped with stainless steel Morton closures (Bellco, Vineland, NJ). Three control flasks (without fungal inoculum) were also run simultaneously.

Ten grams of washed and frozen-milled corn stover was added to each flask. The total solids (TS) of stover added was 70.0%, so each flask contain approx 7.0 g of bone-dry stover. Water was added to bring the stover up to about 88% moisture. The flasks were autoclaved at 121°C for 1 h, and water was added to replace evaporated water. To each flask was added 20 µL of 12 mg/mL tetracycline-HCl (prepared in 70% ethanol) to prevent bacterial contamination. Five milliliters of a 1:1 PD agar:buffer homogenate was used as inoculum. No extra nitrogen was added. Initial flask weights were obtained, and the flasks were incubated at 27°C at 150 rpm. These cultures still lost moisture, even though the rest of the incubator was filled with flasks containing only water to increase the ambient relative humidity. The weight of the flasks was periodically monitored and distilled deionized water was added every 2 to 3 d to maintain constant weight.

After 29 d, the flasks were weighed and distilled and deionized water was added to give 90% moisture. The flasks were incubated for 1 h. Then the cultures were harvested (the contents of three flasks of each culture were combined) and centrifuged. Free liquid was pipeted off, filtered through a 0.45-µm filter, and analyzed for soluble protein and glucose. The solids were washed twice, and samples were obtained for dry weight and cellulose digestibility assay.

Roller Bottle Tests

A roller bottle test was done to evaluate the performance of roller bottles as the next scale-up from shake flask. One hundred grams of frozen-milled corn stover (30% TS) was weighed out into each of two 1-L sterile plastic roller bottles (Bellco). One was inoculated with 10 mL of *Phanerochaete* wild-type homogenate, and the other with 10 mL of distilled deionized water. The bottles had Kraft-wrapped foam caps and were rotated at about 5 rpm. After 35 d, weight loss was replaced by distilled deionized water. Each bottle was emptied into a 1-L beaker, using hooked spatulas to retrieve as much of the contents as possible. Free water was used when possible to rinse the bottles. No additional water was added. The consistencies and viscosities were measured for each batch.

Assays

Enzymatic cellulose digestibility and viscosity assays were conducted to estimate the effectiveness of biopretreatment and potential reduction in the severity required for thermochemical pretreatment.

Enzymatic Cellulose Digestibility

Two types of control samples were included in the enzymatic cellulose digestibility evaluation. The fungal pretreatment control sample was incubated at the same temperature for the same residence time but without fungal inoculation. Another type of control sample, the enzyme digestibility control, is the frozen-milled corn stover without incubation. The first two control samples and the fungal-pretreated corn stover

samples were washed with water to remove soluble components by blending 1 part, by weight, of corn stover with 4 parts of warm distilled deionized water, and incubated at 45°C for 1 h. The slurry was vacuum filtered using 0 .45 µm-pore filter.

The cake was then stored in sealed containers and frozen. A corn stover sample containing 0.10 g of cellulose (dry wt basis) was added to a 20-mL labeled glass scintillation vial, with plastic-lined caps. In addition, a milled corn stover sample as is (i.e., not incubated) containing 0.10 g of cellulose was added to another vial for a digestibility control. To each vial, 5.0 mL of 0.1 *M*, pH 4.8, sodium citrate buffer and 40 µL (400 µg) of tetracycline were added to control the pH and minimize the potential for a bacterial infection. Distilled and deionized water was added to make up a total of 9.93 mL. The vials were capped and kept at 50°C until cellulase enzyme was added. Commercial cellulase enzyme (CPN) was added to the vials at dose equivalent to approx 15, 25, and 60 filter paper units (FPU)/g of cellulose.

The vials were capped and incubated with gentle shaking (100 rpm) at 50°C until the release of soluble sugars leveled off, usually between 72 and 168 h. A 0.5-mL sample was taken daily using large-mouth serological pipet, filtered through 0.45-µm filters. The glucose concentration was measured by YSI (Yellow Springs, OH).

Spectrophotometric Estimate of Soluble Protein

The second portion of free liquid pipeted off, and filtered was read on a Milton Roy Spectronic Genesys 5 UV spectrophotometer, at 260 and 280 nm, using a quartz cell, to get an estimate of enzyme protein concentration (less nucleic acid), based on the original method of Warburg and Christian *(16)* (*see* ref. *17*). The following equation was used to estimate the enzyme protein concentration, by measuring absorbance at 280 and 260 nm:

$$\text{Protein (mg / mL)} = 1.55A_{280} - 0.76A_{260}$$

Viscosity of Corn Stover Slurry

A number of articles have reported that less mechanical energy is required for pulp refining after fungal pretreatment. Because the particle size of corn stover was visually reduced after fungal pretreatment, viscosity measurement was thought to be a quick, but less rigorous, test than digestibility to determine the extent of the pretreatment.

Thomas-Stormer Viscometer

The TS range for the Thomas-Stormer viscometer (Thomas Scientific, Swedesboro, NJ) is from a few percent to as high as 30%, depending on the suspended solids and substance tested. If the material exhibits true viscosity, then the time in s is measured for 100 revolutions of the rotor, and the viscosity is read from graphs of standard viscous substances, determined in the same apparatus. Whether the material exhibits true viscosity can be

Table 2
Enzymatic Cellulose Digestibility of Fungal-Pretreated Corn Stover

	Flask no.	Enzyme loading (FPU/g cellulose)	Cellulose digested (%) 16 h	22 h	112 h	136 h	Dry Insoluble solids loss (%)[b]
Digestibility control:	1	15	0.3	1.2	10.2	9.5	N/A
no fungal culturing or no incubation	2	25	0.7	1.4	7.2	9.4	
	3	60	8.4	9.6	18.1	16.1	
Incubation control:	4	15	1.2	1.6	3.8	6.9	5.6 ± 0.7
no fungal culture present during	5	25	2.2	2.6	5.0	6.1	5.6 ± 0.7
29-d incubation at 27°C	5	60	6.3	6.7	11.0	12.1	5.6 ± 0.7
Cyathus: pretreatment,	6	15	8.3	10.2	24.6	24.6	8.3 ± 1.1
29-d incubation at 27°C	8	60	20.5	22.1	34.0	35.7	8.3 ± 1.1
Phanerochaete: pretreatment,	9	15	0.3	0.5	4.9	3.4	20.0 ± 1.8
29-d incubation at 27°C	10	25	2.0	2.3	4.5	5.8	20.0 ± 1.8
	11	60	5.6	6.1	9.8	12.0	20.0 ± 1.8

[a]Not available.
[b]Insoluble solids loss as a result of incubation control or fungal pretreatment before enzymatic hydrolysis.

determined by plotting a graph of laminar flow rates of the fluid vs force on the fluid. This can be done for the Stormer viscometer by plotting revolutions per second vs weight applied, for at least four weights. For a true viscous substance, the plot is a straight line through the origin. Other graphic forms represent various types of "plastics." If a straight line is not produced, viscosities need to be determined at more than one force (weight). Known amounts of distilled deionized water may be added to low-moisture biomass, in both samples and controls, to reduce the TS to the range for measurement of viscosity with the Stormer.

Bostwick Consistometer

The Consistometer (CSC Scientific, Fairfax, VA) is used to determine the consistency of viscous materials such as jellies and sauces by measuring the distance that the material flows under its own weight in a given time interval. This may not work for a slurry if the solids dewater. It can be calibrated against known standards.

Results and Discussion

Shake Flask Tests

At least three major conditions, and their ranges, need to be satisfied to support fungal pretreatment of biomass: aeration, carbon-to-nitrogen (C:N) ratio, and moisture level. These conditions usually vary widely, depending on the biomass and organisms. It is well known that fungi require a certain amount of air to disrupt lignocellulosic bonds. A range of C:N ratios is important to favor disruption of lignocellulosic bonds over fungal growth, where fungal growth is the utilization of the carbon and nitrogen to support the increase in fungal cell mass. Third, the correct ranges of moisture level, or water activities are required not only to support fungal pretreatment, but also to disfavor bacterial growth. Certain bacteria may be desirable for optimal mixed cultural communal activity, but this topic is beyond the scope of this first phase of study.

Table 2 compares the cellulose digestibility of fungal-pretreated corn stover with control samples at varying enzyme loadings. The cellulose digestibility of the incubation control stover (using cellulase loadings of 15, 25, and 60 FPU/g of cellulose) ranged from 1.2 to 12.1% of theoretical. The digestibility of *Cyathus*-pretreated corn stover ranged from 8.3 to 35.7%, or between 6.9 and 3.0 times that of the controls, respectively. *Phanerochaete* pretreatment did not increase the cellulose digestibility. The digestibilities of washed raw stover were similar to those of the controls. The average insolable dry solids losses (±2 SDs) after 29 d of incubation without culture, with *Cyathus* and with *Phanero-chaete*, respectively, are shown in the last column of Table 2. The liquid that was pipetted off was divided into two parts and analyzed for glucose and enzyme protein. The glucose concentration was not detected. The enzyme protein concentratiosn (less nucleic acid) are given in Table 3.

Table 3
Estimated Protein Concentration in Liquid Obtained from Fungal Pretreated Corn Stover

Sample source	Protein concentration (mg/mL)
Corn stover control (incubated without fungi)	40
Cyathus-pretreated corn stover	34
Phanerochaete-pretreated corn stover	166

These protein values are expected from the literature since *Phanerochaete* is known to be a prolific enzyme producer. *Cyathus* produced no net protein relative to the control. The lowest moisture level they dropped to before water was added back during the 29 d was about 77%. The fungi appeared to be present when viewed under the microscope. No bacterial contamination was observed in any of the flasks.

The flask weight loss discussed earlier contains CO_2 loss from fungal activity on corn stover. As shown in Table 2, the 29-d incubation with *Cyathus* caused an 8.3% dry-wt loss, and *Phanerochaete* caused a weight loss of almost 20%. However, the stover controls lost an average of 5.6%. This is an expected weight loss owing to water-soluble extractives dissolved out of the stover during the 29-d incubation. Compared to the controls, the net weight loss owing to the *Cyathus* was only 2.7%, and about 14.5% for *Phanerochaete*. Shorter incubation time and selection of the appropriate fungal species should reduce the dry weight loss. Furthermore, one would also want to know what component(s) make up the weight loss since loss of carbohydrates results in lower ethanol yield and lignin loss could mean less boiler fuel.

However, the problem here, which will need to be solved before accurate work can be done in the future, is that we are not able to detect moisture loss simply by weighing the flasks, unless we know the DM loss, and fungal cell mass gain. Studies in the literature solved this problem by controlling the humidity so that there is no significant moisture loss. We could estimate the DM loss from CO_2 emission. That is possible, but difficult to do in small shake flasks while they are being aerated. One could use a mass spec if one obtained a uniform and high enough air flow rate. Or, one could attempt to trap the CO_2. The DM loss could at best only be estimated unless one knows the solubilization rate of each: hemicellulose, cellulose and lignin, and cell mass gain.

At small scale, studies in the literature use an environmentally controlled incubator, or they make them by enclosing the flasks in a closed chamber, or even plastic bags, while sparging the flasks by pumping water-saturated air through tubing to each flask. At large scale, the biomass is stacked in piles over tarpaulin-lined depressions. Drainage is pumped out and sprayed over the piles, at frequent intervals, occasionally adding makeup water, or the piles are covered with tarpaulins.

Table 4
Force vs Time Measurements of Fungal-Pretreated and Control Corn Stover Slurries

	Force(*g*)	Time (s)
Phanerochaete-treated corn stover	5	29.7
	10	28.0
	30	23.7
	60	18.8
	80	14.3
Corn stover control	250	No flow
(incubated without fungi)	300	21.3
	400	16.6
	500	13.0
	600	11.0

Roller Bottle Tests

A roller bottle test was done at the same time as the second shake flask test to evaluate the performance of roller bottles for fungal pretreatment. The consistencies and viscosities were measured for each batch.

Consistencies

The stover control took 20 s to travel 2.5 cm, and 40 s to travel 3.5 cm at 12°C on the Bostwick Consistometer, before it began to noticeably dewater. The *Phanerochaete* pretreated material ran the full course (24 cm) in 0.5 s. The slurry of fungal-pretreated corn stover became noticeably more fluid before adding any water back, as compared to the control. The approx 80-fold difference in flow velocities between the two consistencies is quite dramatic to the unaided eye.

Viscosity

Viscosity of the stover slurries (diluted with distilled deionized water to 10% [w/w]) was measured at 15°C using the Thomas-Stormer viscometer. Experimental data of force vs time per 100 revolutions for *Phanerochaete*-pretreated corn stover and corn stover with no fungal pretreatment are presented in Table 4 and Fig. 1. Figure 2 shows the data transformed into the apparent viscosity, or fluid flow characteristic, which shows the type of "plastic" that the slurry flow describes. The unpretreated stover behaves like an "inverted plastic," while the *Phanerochaete*-pretreated stover flows like a pseudoplastic. More important, Fig. 2 illustrates the great reduction in force required to agitate the *Phanerochaete*-pretreated stover as compared with the unpretreated stover. The reduction in force ranges from almost a 10-fold reduction at the higher shear rates to a greater than 100-fold reduction at the low shear rates.

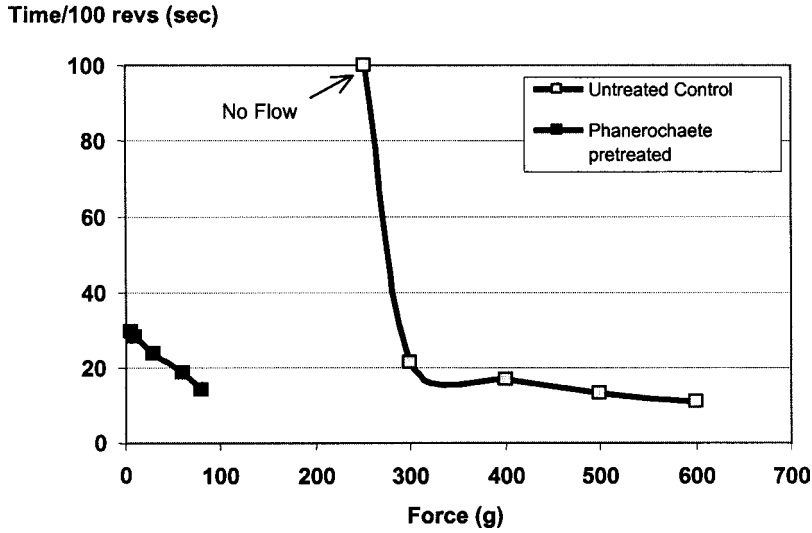

Fig. 1. Viscosity measurements of 10% (w/w) corn stover slurry using Thomas-Stormer viscometer.

Proposed Performance Criteria for Fungal Pretreatment

We expect that application of fungal pretreatment to achieve the objective of reduced steam pretreatment severity requires the following general criteria:

1. Low mass loss of feedstock, especially carbohydrates.
2. Low costs—particularly nutrients, air and a reasonably short residence (storage) time.
3. Robustness; that is, the fungi must compete with the natural flora.
4. Lower thermochemical pretreatment severity (i.e., significant reduction in one or more of the following parameters: temperature, acid or alkali concentration, and residence time).
5. Improved enzymatic cellulose digestibility.
6. Noninhibitory to fermenting organisms.

Figure 3 shows a conceptual configuration for incorporating fungal pretreatment in bioethanol production. Simulation of process and equipment options should be carried out to evaluate the potential process improvements with application of fungal pretreatment. Possible process improvements include the following:

1. Lower pretreatment severity requirements result in lower operating costs (e.g., size reduction, acid, steam) and pretreatment reactor cost (e.g., lower temperature, pressure, and acid concentration, result in less expensive material of construction).
2. Higher enzymatic cellulose digestibility and higher ethanol fermentation yield because of lower level of inhibitors as a result of lower-severity pretreatment.

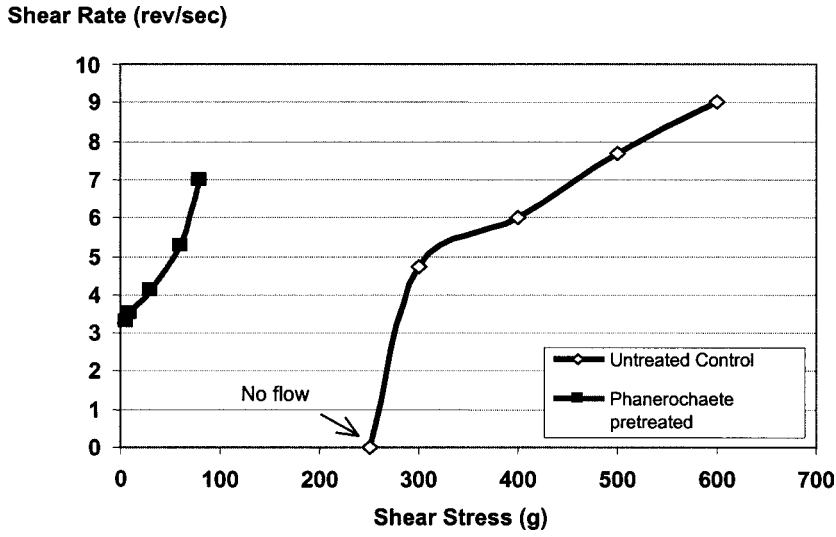

Fig. 2. Flow characteristics of 10% (w/w) corn stover slurries.

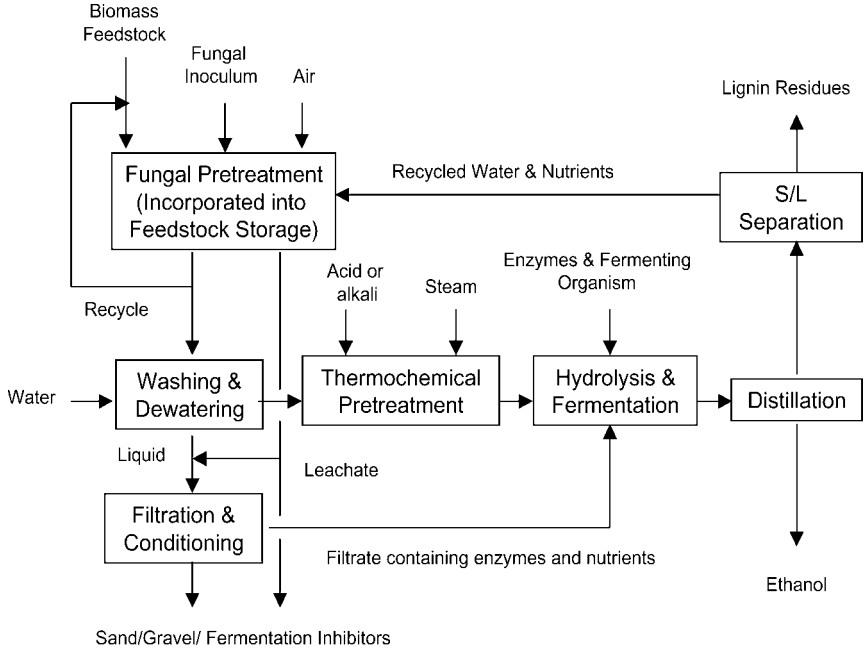

Fig. 3. Conceptual block flow diagram of bioethanol production using fungal pretreatment.

3. Lower enzyme requirements owing to the combined fungal and thermochemical pretreatments and supplemental enzyme produced in the fungal pretreatment step.

As practiced in the pulp industry, onetime high-dose inoculation of the biomass feedstock is required to establish a colony of desirable fungus (or fungi). The fungal population could be maintained by blending a small portion of the fungal-containing biomass with incoming feed. The process simulation of various process options would set the targets and priority for research activities to confirm the technical and economic feasibility.

Conclusion

Based on the preliminary results, there is not a clear correlation between digestibility improvement and viscosity reduction. However, we believe that these impressive fungal pretreatment improvements should translate into significant reduction in size-reduction (e.g., milling) energy requirements or the severity of steam pretreatment needed to solubilize corn stover polysaccharides. Recommendations for further work include fine tuning the fungal pretreatment conditions and time needed to result in significant reduction in viscosity and improvement in cellulase digestibility at small scale, with minimal carbohydrate loss; scaling up of the fungal pretreatment to 5-gal pails or 55-gal drum to generate sufficient material for evaluation of the impact on thermochemical pretreatment severities required to achieve high hemicellulose solubilization and cellulose digestibility; optimizing a symbiotic fungal pretreatment using two or more fungi; and since the key requirement for the small-scale work is a controlled, constant-humidity incubator, maintaining constant humidity while providing mixing, controlled temperature, and airflow for the aerobic fungi.

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