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Ammonia Recycled Percolation as a Complementary Pretreatment to the Dilute-Acid Process

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ABSTRACT

A two-stage dilute-acid percolation (DA) was investigated as a pretreatment method for switchgrass. With use of extremely low acid (0.078 wt% sulfuric acid) under moderate temperature (145–170°C), hemicellulose in switchgrass was completely solubilized showing no sugar decomposition. The treated switchgrass contained about 70% glucan and 30% lignin. The high lignin content in the treated feedstock raises a concern that it may cause a high enzyme consumption because of irreversible adsorption of cellulase enzymes to lignin. This problem may be amplified in the SSF operation since it is usually run in fed-batch mode and the residual lignin is accumulated. The DA pretreatment was, therefore, combined with the ammonia recycled percolation (ARP) process that has been proven to be effective in delignification. The combined pretreatment essentially fractionated the switchgrass into three major components. The treated feedstock contained about 90% glucan and 10% lignin. The digestibility of these samples was consistently higher that that of DA treated samples. Further study on the interaction of cellulase with xylan and that with lignin has shown that the enzymatic hydrolysis of cellulose is inhibited by lignin as well as xylan. The external xylan was found to be a noncompetitive inhibitor to cellulose hydrolysis. The cellulase used in this study was proven to have the xylanase activity.

Index Entries: Pretreatment; delignification; dilute-acid; cellulase adsorption; xylan hydrolysis.

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INTRODUCTION

The primary purpose of pretreatment is to make the lignocellulosic substrate amenable to the action of cellulase. As such, the pretreatments are evaluated on the basis of the initial rate and the extent of cellulose hydrolysis. It is known that cellulase is adsorbed onto isolated lignin *(1)*, or the lignaceous residues even after complete hydrolysis of the cellulose component *(2–6)*. As a result, the cellulase enzyme becomes less efficient when it is applied to lignocellulosic substrates. The irreversible adsorption of cellulase to the lignin also makes it difficult to recover the enzyme. The cost of cellulase enzyme is one of the major cost items in the overall bioconversion process *(7)*. Obviously, removal of lignin and/or hemicellulose is an important factor that needs to be considered in the pretreatment. Although there has been some dispute over the relative influence of lignin and hemicellulose in enzymatic hydrolysis of lignocellulosic materials *(8,9)*, it is believed that lignin and hemicellulose are two main factors influencing the cellulose hydrolysis. They physically block the access of cellulase enzyme, adsorb it, and may even inhibit the reaction. Hence, removal of lignin and hemicellulose is a major occurrence in a number of known pretreatment methods. As one of such, a pretreatment method based on aqueous ammonia termed Ammonia Recycled Percolation (ARP) process was recently developed in the laboratory at Auburn University *(10)*. In this process, aqueous ammonia is used as a pretreatment in a flow-through packed-bed reactor (percolation reactor). Recent results on the ARP show that it is highly effective in removing lignin (80–85% of lignin removal for herbaceous biomass). It also meets other pretreatment criteria: 90–95% digestibility and a high retention of glucan *(11)*. Pretreatment by dilute sulfuric acid is also an established process. It is known for its effectiveness in removing hemicellulose *(12,13)*. The recent modeling work and process study suggest that the hemicellulose can be efficiently removed through a two-stage dilute acid percolation process *(14,15)*. These results further suggest that if the ARP and dilute-acid (DA) processes are combined, it can be improved to the point where the biomass is fractionated into the three main components. This investigation was undertaken to assess the effectiveness of the combined pretreatment of ARP and DA, and to elucidate the role of lignin and hemicellulose in the enzymatic hydrolysis of cellulose.

MATERIALS AND METHODS

Materials

Dry switchgrass milled and screened to 10–40 mesh was supplied by the National Renewable Energy Laboratory (NREL) and used as the lignocellulosic substrate. The composition of switchgrass as determined by

NREL Standard Procedures is as follows (% oven-dry biomass): glucan 35.23, xylan 17.26, galactan 1.41, arabinan 3.54, klason lignin 20.30, acid soluble lignin 3.30, ash 6.03, extractives 10.53, and other 1.9. The cellulase enzyme, Spezyme-CP, Lot No. 41-95034-004, was obtained from Environmental Biotechnologies, Menlo Park, CA. The specific activity of the enzyme as determined by the supplier is as follows: Filter paper activity = 64.5 FPU/mL, β-glucosidase activity = 57.6 p-NPGU/mL. Birch wood xylan (Sigma, St. Louis, MO) was used in hydrolysis experiment.

Experimental Setup and Operation

The details of the experimental apparatus for the pretreatment and the operation of ARP process have been previously reported *(11)*. A constant acid concentration (0.0784 wt%) and two different temperatures were applied in the two-stage dilute-acid process. A slight modification was made in that two additional feed reservoirs were added to the previous setup. One was for dilute acid and the other was for the deionized water. The latter was used to wash out the pretreatment agent remaining in the solid substrate.

Digestibility Test

Enzymatic hydrolysis of pretreated substrates was performed in 250 mL glass bottles at 50°C, pH 4.8, with a solid loading of 1% (w/v). It was agitated at 150 rpm on a Shaker Incubator. The enzyme loading of 60 IFPU/g glucan was applied. The enzymatic digestibility is defined as (total amount of glucose released) × 0.9/total glucan. A dehydration factor of 0.9 is used to convert the glucose to glucan.

Analytical Methods

The biomass samples were analyzed for sugar and lignin content following the procedure described in NREL-CAT Standard Procedures (No. 002–005 and LAP 010). Bio-Rad Aminex HPX-87H and HPX-87P HPLC columns were used for analysis of sugars and decomposition products. The sugar content in liquid sample was determined after the liquid sample was subjected to a secondary acid hydrolysis. The conditions in the secondary hydrolysis were: 4 wt% sulfuric acid, 121°C, and 1 h.

Enzyme Adsorption Test

The enzyme adsorption experiments were carried at 5°C to suppress the hydrolysis reaction. Sodium citrate (0.05*M*) was used as a buffer to keep the pH at 4.8. The protein in the solution was determined by the Bradford colorimetric method. Bovine serum albumin was used as the protein standard. The amount of adsorbed protein was calculated from the difference between the initial and final protein concentration in the supernatant.

RESULTS AND DISCUSSION

Effect of Hemicellulose on Cellulose Hydrolysis

Cellulose in lignocellulosic biomass is embedded in a sheath of hemicellulose and lignin. In order for the enzyme to attack the cellulose, it has to be first adsorbed on the surface of cellulose. In a previous study on the ARP it was found that the enzymatic digestibility of the lignocellulosic biomass is greatly enhanced by removal of the hemicellulose and lignin *(10,11)*. Since the hemicellulose and lignin were removed simultaneously in the ARP process, the isolated effect of them could not be identified. The role of the lignin in the enzymatic hydrolysis of lignocellulosic biomass has long been recognized. However, the role of the hemicellulose in hydrolysis of lignocellulosic biomass is still uncertain. Unlike cellulose, hemicellulose is a heteropolymer. It is distributed in each layer of the cell wall and functions as supporting material in the cell walls *(16)*. Grohmann et al. *(17)* reported that ester groups play an important role in the mechanism of plant cell wall resistance to enzyme hydrolysis. Kong et al. *(18)* examined the effects of cell-wall acetate, xylan backbone, and lignin on enzymatic hydrolysis of aspen wood. They found that acetyl groups and lignin were important barriers to enzyme hydrolysis, but the xylan backbone was not.

In this work, we studied the effect of the hemicellulose on the enzymatic digestibility of dilute-acid pretreated switchgrass. The pretreatment was carried out in a batch reactor with 0.0784 wt% sulfuric acid at 180°C applying five different levels of reaction time ranging from 5 to 45 min. The amount of hemicellulose solubilized increased with the reaction time whereas the amount of lignin solubilized stayed relatively constant. Removal of hemicellulose ranged from 30 to 88%. The plot of digestibility vs the percent hemicellulose removed (Fig. 1) indicates that the hemicellulose is an important factor influencing the hydrolysis of the lignocellulosic biomass. It is well known that the "cellulase" exhibits xylanase activity. However, it is unclear at this time whether the xylanase activity of cellulase comes from a separate enzyme(s) or from the cellulase itself. A recent study on cellulase protein indicates that a large number of the families of cellulases are polyfunctional, meaning that there are more than one specific catalytic domain in cellulase enzymes *(19)*. At least two different enzymes have been verified to possess activities on both CMC and xylan *(20,21)*. Johnston and Shoemaker *(22)* also reported that the endoglucanse has xylanase activity.

Shown in Fig. 2 is the effect of external xylan on cellulose hydrolysis at various levels of enzyme loading. The hydrolysis was conducted using the two substrates simultaneously with xylan to cellulose ratio of 0.4:1. It is clearly shown that with the enzyme loadings of 10 and 20 IFPU/g glucan, the cellulose hydrolysis is suppressed in the presence of the xylan. As the enzyme loading was raised to 40 and 60 IFPU/g glucan, the effect of xylan diminished.

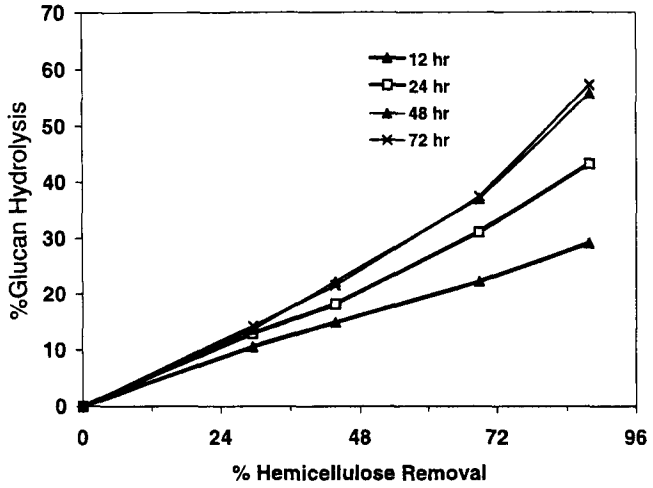

Fig. 1. Effect of hemicellulose content on enzymatic hydrolysis of switchgrass after dilute-acid batch pretreatment. Pretreatment condition: 0.05%(w/w) sulfuric acid, 180°C, reaction time 5–45 min.

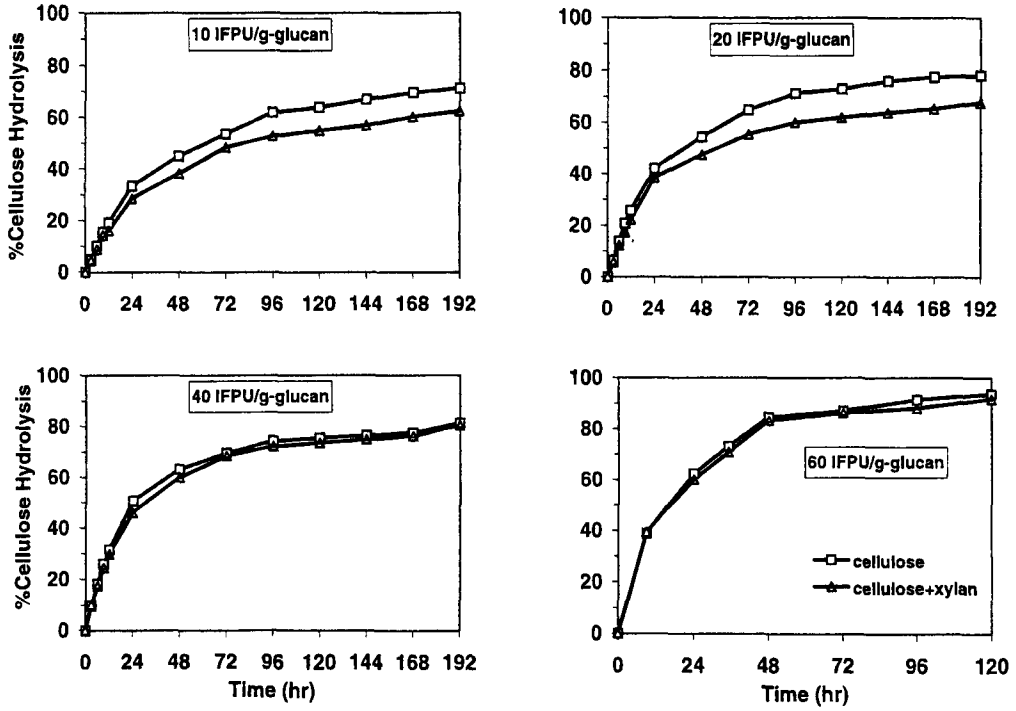

Fig. 2. Effect of xylan on enzymatic hydrolysis of cellulose. Weight ratio of xylan to cellulose = 0.4:1.

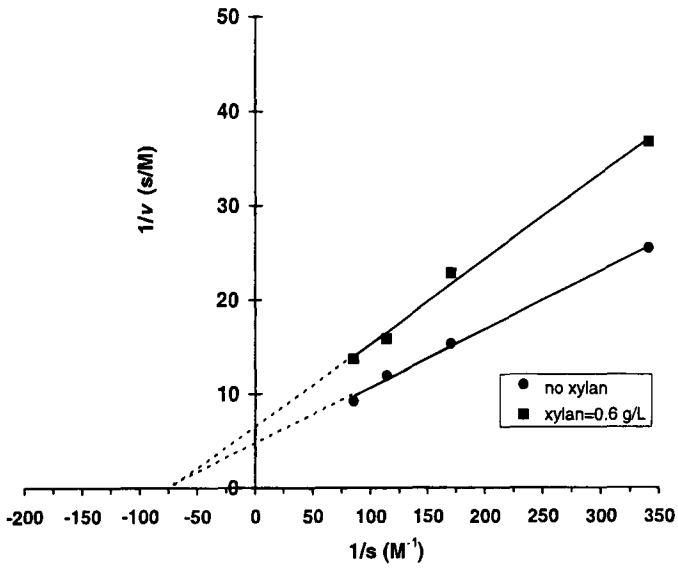

Fig. 3. Lineweaver–Burk plot of enzymatic hydrolysis of cellulose in presence of xylan. Hydrolysis conditions: 50°C, 40 IFPU/g glucan, pH 4.8, 1% (w/v) filter paper, time = 1.5 h.

The nature of the interaction between xylan and cellulase was further examined from a kinetic standpoint. An inhibition experiment was thus carried out using filter paper as a substrate. The enzymatic hydrolysis conditions were: 50°C, pH 4.8, 1% (w/v) filter paper, and 40 IFPU of cellulase/g glucan. The total amount of glucose and celloboise released from the filter paper at 90 min was used to calculate the initial rate of hydrolysis. The Lineweaver-Burk plot prepared from these results indicates that xylan inhibits cellulose hydrolysis in a noncompetitive inhibition mode (the vertical intercept and the slope increase in the presence of xylan) (Fig. 3). Under this kinetic pattern xylan and cellulose do not compete for the same active sites. The cellulase enzyme, most likely the endoglucanase, has active sites for xylan hydrolysis. It seems that adsorption of xylan onto the active sites somehow inhibits the cellulase activity.

Xylanase activity of cellulase was further tested using xylan as the only substrate (Xylan was from Sigma and its purity was not measured when this experiment was conducted). A peculiar reaction pattern was observed that the hydrolysis was very rapid for the first 12 h giving 40% conversion. The reaction, however, almost ceased after that period. It is unclear why there is a sudden stoppage of the reaction. One may speculate that xylose is a strong inhibitor to the enzyme or the substrate (commercial xylan) has a structure that limits the terminal digestibility. Addition of excessive amount of enzyme (total loading of 673 IFPU/g xylan) and a 140 h extension of reaction time increased the conversion

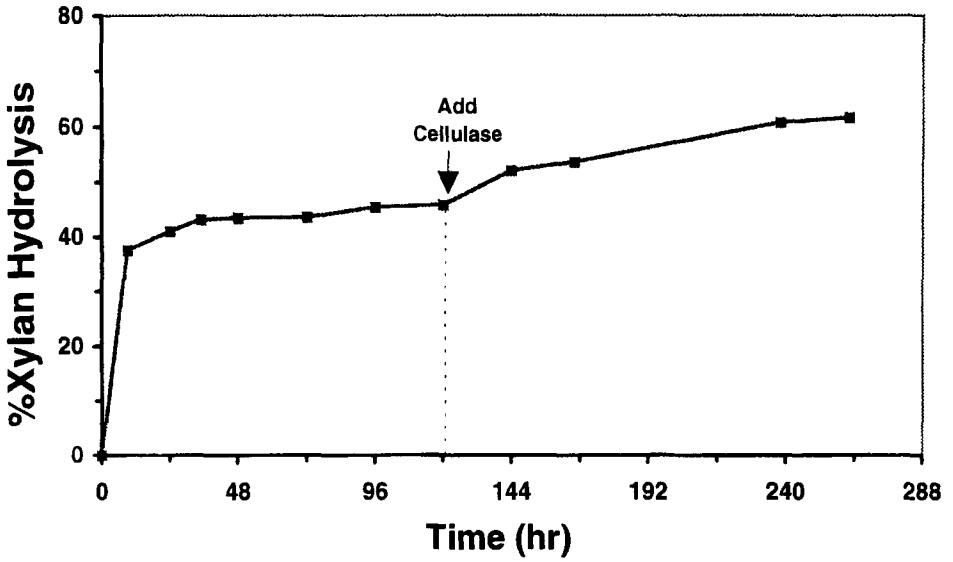

Fig. 4. Enzymatic hydrolysis of xylan by cellulase. Hydrolysis condition: 50°C, 20 IFPU/g-xylan, pH 4.8, 1% (w/v) xylan. The arrow indicates an additional 50 IFPU cellulase was added to the solution at 120 h. The total enzyme loading at this point is 673 IFPU/g xylan.

only by 15%. It appears that xylan plays a negative role in cellulose hydrolysis in two different ways. First, it adsorbs cellulase enzyme (formation of enzyme-substrate complex). This fraction of cellulase is, therefore, unavailable for cellulose hydrolysis. This effect applies for both internal xylan (Fig. 1) and external xylan (Fig. 2). Second, xylan or hemicellulose existing in a lignocellulosic substrate physically blocks the contact between cellulase and cellulose. It is our contention that hemicellulose has a profound effect on cellulose hydrolysis and its removal is a prerequisite for complete hydrolysis of cellulose in biomass.

Two-Stage Dilute-Acid Pretreatment

Dilute-acid treatment is known to be effective in removing hemicellulose from biomass. Recent studies along these lines including the ones from our laboratory suggest that two-stage treatment is highly effective in recovering hemicellulose sugars without decomposition *(14,15)*. The two-stage dilute-acid pretreatment of this work employed extremely low sulfuric acid (0.0784 wt%) with a reaction time of 10 min per stage. The results are summarized in Table 1. The solubilization of hemicellulose, cellulose, and lignin generally increase with temperature. When a low side temperature above 135°C and a high side temperature above 165°C were applied, the hemicellulose was completely removed. The treated solid thus contained lignin and glucan only. In addition to the solubilization of the hemicellulose, 40–48% of lignin and 16–35% glucan were also solubilized. The

Table 1
Two-Stage Dilute-Acid Pretreatment on Switchgrass[a]

Two-Stage Reaction Temperature	Solid Residue			Primary Hydrolyzate			
	% of Original Amount Removed			Hemicellulose Sugars			pH
	Lignin	Glucan	Hemicellulose	%Monomer	%Oligomer	%Furfural	
165°C + 195°C	48.23	35.00	100.00	43.51	55.28	trace	2.60
155°C + 185°C	47.38	18.83	100.00	39.63	54.47	trace	2.40
145°C + 175°C	43.21	17.47	100.00	37.10	61.90	none	2.37
135°C + 165°C	41.52	16.08	100.00	34.63	63.26	none	2.38
125°C + 155°C	43.90	14.57	65.11	36.40	63.61	none	2.23

[a]Data in the table based on the oven-dry untreated biomass.
Pretreatment condition: 0.0784 w/v% sulfuric acid, reaction time of 10 min for each stage, flow rate = 4.0 mL/min.

hemicellulose hydrolyzate contained both oligomers and monomers, the former being more than 50%. The oligomer content decreased as the reaction temperature was increased. Below 185°C, no furfural or hydroxymethylfurfural (HMF) was detected in the hydrolyzate. Two-stage process was applied because the hemicellulose in herbaceous biomass is biphasic *(14,15,23)*. In this experiment, we found that most of the hemicellulose (85% of original) and lignin (35 out of 45% total removal) were removed during the first stage.

Combined Pretreatment of ARP and DA

The ARP has been proven to be a highly effective pretreatment for switchgrass especially in deligninfication. In this work, we have shown that DA treatment is most effective in solubilizing hemicellulose. It is conceivable that combination of these two can fractionate biomass into the three major constituent polymers. The investigation of combined pretreatment of ARP and DA thus ensued. It was studied in both directions: DA followed by ARP (DA-ARP), and ARP followed by DA (ARP-DA). In the DA-ARP process, the two-stage DA conditions were: 0.0784 wt% sulfuric acid, low reaction temperature 140°C, high reaction temperature 170°C, reaction time of 10 min for each stage, flow rate of 4 mL/min. After the DA prehydrolysis, the reactor was washed on line with water, and then 10 wt% ammonia was pumped in and left for 4 h at room temperature for presoaking prior to the ARP operation. The ARP conditions were: 170°C, 10 wt% ammonia, 20 min. These reaction conditions were selected since they were the optimum conditions for the individual pretreatment. Similar reaction conditions and operation were applied to the ARP-DA process. The sugar and lignin balances in the combined pretreatment are shown in Tables 2 and 3. The hemicellulose is completely removed from the solid in both combined processes leaving the treated solid to contain glucan and lignin. In the DA-ARP process, all of hemicellulose is removed during the DA step whereas lignin is solubilized in both steps (37% in DA step and 45% in ARP step for a total of 82%). In the ARP-DA process, the two-thirds of the hemi-

Table 2
Sugar Balance in Switchgrass after the Combined Pretreatment of DA and ARP[a]

Pretreatment	Solid Residue					Acid Hydrolyzate or ARP Effluent				
	%wt	%glucan	%xylan	%galactan	%arabinan		%glucan	%xylan	%galactan	%arabinan
Untreated Switchgrass	100	35.23	17.76	1.41	3.54		0	0	0	0
DA-ARP	30.24	26.30	trace	0	0	DA	4.37	17.33	1.53	2.96
						ARP	0.24	0.24	0	0
ARP-DA	33.84	30.09	trace	0	0	ARP	2.44	11 05	1.19	2.78
						DA	2.43	6 59	0.17	0.91

[a]Data in the table based on the oven-dry untreated biomass. DA-ARP: dilute-acid pretreatment followed by the ammonia recycled percolation process. Pretreatment condition: DA: 140°C, 10 min + 170°C, 10 min, 0.0784 wt% sulfuric acid, 4 mL/min. ARP: 170°C, 10 wt% ammonia, 20 min, 4 mL/min. ARP-DA: ammonia recycle percolation process followed by the dilute-acid pretreatment. Pretreatment condition: ARP: 175°C, 10 wt% ammonia, 20 min, 4 mL/min. DA: 175°C, 0.0784 wt% sulfuric acid, 20 min 4 mL/min.

Table 3
Lignin Balance in Switchgrass after the Combined Pretreatment of DA and ARP[a]

Pretreatment		% Klason Lignin	% Acid Soluble Lignin	Total
Untreated Switchgrass		20.30	3.30	23.60
DA-ARP	Solid Residue	3.81	0.10	3.91
	DA Hydrolyzate	5.66*	3 08	8.74
	ARP Effluent	9.02*	1.54	10.56
ARP-DA	Solid Residue	3.78	0.07	3.85
	ARP Effluent	12.50*	4.67	17.17
	DA Hydrolyzate	0.00	0.36	0.36

[a]Data in the table based on the oven-dry untreated biomass. Pretreatment conditions same as Table 2.

*Klason lignin of DA hydrolyzate or ARP effluent is the lignin precipitated during the secondary acid hydrolysis.

cellulose is removed in ARP step and one-third hemicellulose is removed in the DA step. As for the lignin, a total of 83% of the initial lignin is removed, almost all of it is in the ARP step. In both combined pretreatment processes, the hemicellulose recovery was about 96%. Sugar decomposition was negligible in the ARP-DA process. However, about 4% of glucan is not accounted for in the DA-ARP process most likely by decomposition. One would thus prefer the pretreatment sequence of ARP followed by DA process to that of DA followed by ARP.

The enzymatic digestibility were determined for these samples. Results are shown in Fig. 5 and 6. A sample treated with DA only was included as a reference substrate. The composition of the DA treated sample was: 69.82% glucan and 30.28% lignin. At each level of enzyme loading, the digestibility of swichgrass after the combined pretreatment is seen to

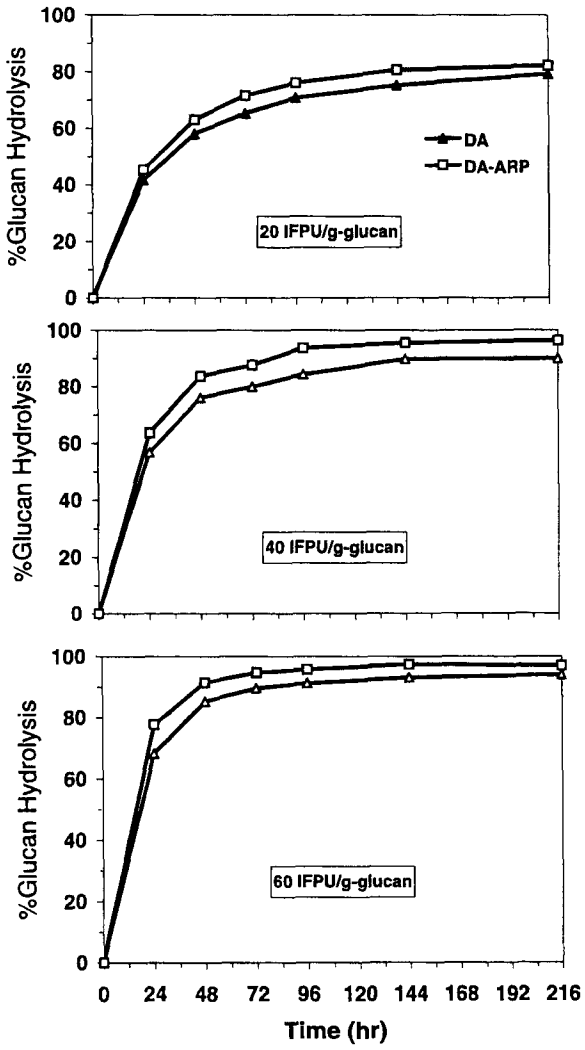

Fig. 5. Enzymatic hydrolysis of switchgrass after DA and DA-ARP pretreatment.

be higher than that of the sample treated with DA only. The digestibility results were quite similar in either sequence of the combined processes. We believe that the enhanced efficiency of cellulase enzymes observed here is directly linked to the low lignin content in the substrates that have been subjected to the combined pretreatment.

Enzyme Adsorption

Adsorption of enzyme onto lignocellulosic substrate is a required step in cellulose hydrolysis. Since we found some evidence that residual lignin affects the digestibility, it became in our interest to verify how the adsorption phenomena affects the enzymatic hydrolysis of cellulose. The following experiments address this issue.

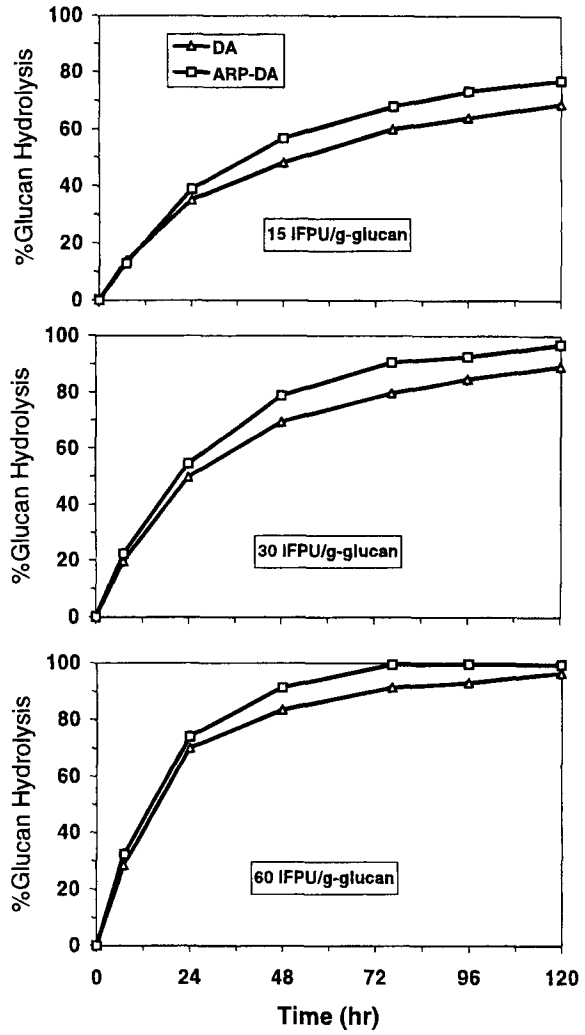

Fig. 6. Enzymatic hydrolysis of switchgrass after DA and ARP-DA pretreatment.

The enzyme adsorption capacity on cellulose was first studied covering the enzyme concentrations of 0.028–19.40 mg/mL. Figure 7 shows the enzyme (measured as protein) adsorption isotherm at 5°C. The enzyme adsorption on cellulose appears to follow the Langmuir multimolecular layer adsorption pattern. There are two stages of leveling off, one at the enzyme concentration of 0.056 to 0.084 mg/mL, the other at 5.89 to 12.10 mg/mL. It is followed by a steady increase not showing an upper limit of enzyme adsorption within this experiment range. Adsorption of cellulase enzyme on various substrates is presented in Table 4. The enzyme adsorption varies widely among the substrates. The amount of enzymes adsorbed is dependent on the nature and surface structure of substrates. Switchgrass upon pretreatment adsorbs more enzyme than the lignin residues and α-cellulose.

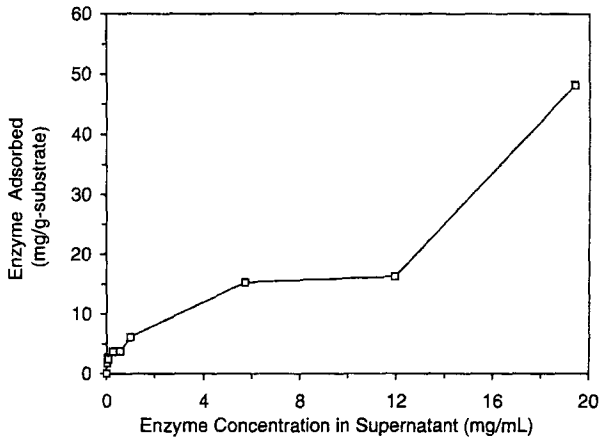

Fig. 7. Cellulase adsorption isotherm. Adsorption condition: 5°C, pH 4.8, 1% (w/v) cellulose, adsorption time = 1 h.

Table 4
Enzyme Adsorption on Various Substrates

ARP-DA treated switchgrass	Cellulase Adsorption
Substrates	(mg Protrein/g substrate)
DA treated switchgrass	14.51
ARP treated switchgrass	12.74
ARP-DA treated switchgrass	15.83
Cellulose*	3.52
Lignin from DA treatment	8.83
Lignin from ARP-DA Process	10.76
Indulin*	1.37

*All samples except those noted are kept wet before they are put into test. Adsorption condition: 5°C, 1 h 1% (w/v) substrate.

The effect of the isolated lignin on the cellulose hydrolysis is shown in Fig. 8. Commercial lignin (Indulin AT, Westvaco, North Charleston, SC), a dry lignin from the ARP process, and a wet lignin residue from the dilute-acid process were studied. The digestibility is seen to decrease when external lignin is added to the solution.

Adsorption of enzyme was further examined from an activity standpoint. The substrate used in the test was the switchgrass treated by dilute-acid in a batch mode. It contained 50% glucan, 7% xylan, and 38% lignin. After the substrate was suspended in the enzyme solution (1 IFPU/mL) for 2 h at 5°C, the solid substrate was separated from supernatant by vacuum filtration. Seventy five percent of total protein was found to be adsorbed to the solid substrate. The activity of cellulase in the supernatant and that of original enzyme solution were then measured by the enzymatic digestibility

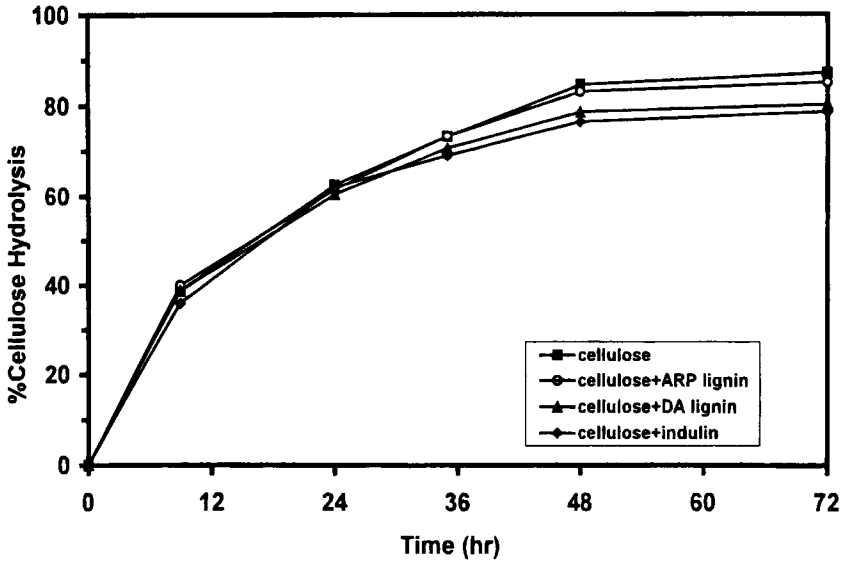

Fig. 8. Effect of external lignin on enzymatic hydrolysis of cellulose DA-lignin refers to the lignin residue of dilute-acid treated switchgrass after enzymatic hydrolysis. ARP-lignin refers to the lignin of switchgrass produced from the ARP process.

test. The data indicated that the supernatant had far less specific cellulase activity than that of original enzyme solution. Since 75% of the total protein is adsorbed onto the substrate, one expects to see 25% of enzyme activity retention in the supernatant, However, the supernatant showed only 15% of the initial activity. This result indicates that cellulase-substrate affinity promotes adsorption of cellulase enzymes much more so than the noncellulase proteins. This agrees with a recent finding by Lee et al. *(3)* that the activity of the recovered cellulase enzyme is much less than the value expected from the total recovered protein.

CONCLUSIONS

Hemicellulose in switchgrass can be completely solubilized and recovered through a two-stage dilute-acid percolation treatment conducted under an extremely low acid concentration (0.078 wt% sulfuric acid) and moderate reaction temperature (140–170°C). The combined pretreatment of ARP-DA on switchgrass has essentially fractionated the biomass into three major components. The treated feedstock contained glucan and a small amount of lignin. The combined sequence of ARP-DA showed better performance than DA-ARP in sugar decomposition. The cellulase enzyme used in this work (Spezyme-CP) was found to have activities on xylan as well as cellulose. However, it does not have sufficient xylanase activity to completely hydrolyze xylan. Both internal and external xylan have been proven to have a negative effect on cellulose hydrolysis. The xylan inhibits

the cellulose hydrolysis in a noncompetitive mode. Active cellulase is adsorbed more strongly onto the cellulosic substrates than inactive cellulase or noncellulase protein.

ACKNOWLEDGMENT

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