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Effect of Sulfuric and Phosphoric Acid Pretreatments on Enzymatic Hydrolysis of Corn Stover

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Abstract

The pretreatment of corn stover with H_2SO_4 and H_3PO_4 was investigated. Pretreatments were carried out from 30 to 120 min in a batch reactor at 121°C, with acid concentrations ranging from 0 to 2% (w/v) at a solid concentration of 5% (w/v). Pretreated corn stover was washed with distilled water until the filtrate was adjusted to pH 7.0, followed by surfactant swelling of the cellulosic fraction in a 0–10% (w/v) solution of Tween-80 at room temperature for 12 h. The dilute acid treatment proved to be a very effective method in terms of hemicellulose recovery and cellulose digestibility. Hemicellulose recovery was 62–90%, and enzymatic digestibility of the cellulose that remained in the solid was >80% with 2% (w/v) acid. In all cases studied, the performance of H_2SO_4 pretreatment (hemicellulose recovery and cellulose digestibility) was significantly better than obtained with H_3PO_4. Enzymatic hydrolysis was more effective using surfactant than without it, producing 10–20% more sugar. Furthermore, digestibility was investigated as a function of hemicellulose removal. It was found that digestibility was more directly related to hemicellulose removal than to delignification.

Index Entries: Corn stover; enzymatic hydrolysis; H_3PO_4; pretreatment; H_2SO_4.

Introduction

The United States and other industrialized countries of the world are dependent on imported oil. Oil imports continue to increase, threatening the strategic security of these countries *(1)*. For instance, according to a June 23, 2001 article in *Time* magazine,the United States imports almost 60% of

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its current oil supply. Furthermore, the transportation sector in the United States is particularly dependent on oil, with approx 97% of transportation energy being derived from petroleum. Few substitutes exist for petroleum for transportation usage *(2)*.

One of the more promising alternative fuels to replace gasoline is ethanol. To have a significant impact on our current oil consumption, ethanol must be both inexpensive and plentiful *(3)*. Lignocellulosic biomass such as agricultural residues, wood, and crops are abundant renewable materials for the production of sugars for subsequent fermentation. Of the agricultural residues, corn stover is the most abundant, with annual US production rates of 150,000,000 t/yr *(4)*.

The polysaccharide fraction of agricultural residues can be hydrolyzed in reaction media using acids or enzymes as catalysts *(5)*. Complete hydrolysis of cellulose yields the easily fermentable glucose, allowing biomass to be a potential renewable energy source *(4)*. The enzymatic approach to hydrolyzing cellulose to glucose is receiving attention because enzymes can achieve high yields, since they do not catalyze glucose degradation reactions common in the acid process. However, the cellulose must be accessible to the enzyme. The accessibility depends on the reagent and the severity of the pretreatment process. Therefore, effective pretreatment is an essential prerequisite to improve the rate and yields of saccharification.

One potential method of converting corn stover to ethanol involves removing the hemicellulose sugars by an acid process. Then, after washing the sugars from the solids, the solids are subjected to enzymatic hydrolysis. Previous results *(6)* have shown that acid is an effective pretreatment reagent for subsequent enzymatic hydrolysis of hardwood. However, high enzyme loadings are required for high conversion. Hence, the use of enzyme should be as low as possible. Toward this aim, it has been reported that the addition of nonionic surfactants, especially Tween-80, improved cellulase activity and preserved enzyme for recycling *(7, 8)*.

Knappert et al. *(9)* pretreated corn stover with acid concentrations from 0 to 1.2% for 0.22 min at 180–220°C using a cellulase loading of 40 filter paper units (FPU)/g of substrate at a solids concentration of 2.5%. Good cellulose conversions (70–100%) were obtained after 2 d of enzymatic hydrolysis. Schell et al. *(4)* pretreated corn stover with a 1.0 wt% solution of H_2SO_4 and 140–180°C steam for 5–20 min using a cellulase loading of 40 FPU/g of substrate at a 2% solids concentration. After 5 d of enzymatic hydrolysis, the cellulose digestibility was 78%.

Thus, the primary objectives of the present study were to compare the compositions of hydrolysate liquids and solids after mild pretreatment with H_2SO_4 and H_3PO_4s, and to compare the enzymatic digestibility of the H_2SO_4 and H_3PO_4 pretreated solids. In these experiments, the temperature was held constant, and residence time and acid concentrations were varied. The sugar yields after pretreatment and after enzymatic hydrolysis were investigated.

Materials and Methods

Materials

Chips of corn stover (the residue remaining in the field after corn kernels are harvested from the cob), supplied by Agricultural Research Development and Education Center (Colorado State University, Fort Collins, CO), were ground to an average size of 20–60 mesh (0.25–0.84 mm) using a laboratory knife mill. The milled corn stover was used directly in chemical pretreatment studies. Untreated, milled corn stover contained 40.8% glucan and 25.8% xylan. Solid corn stover was analyzed for moisture, sugars, Klason lignin, and ash by National Renewable Energy Laboratory (NREL) standard procedures (Analytical Procedures #001–005) *(10)*.

Pretreatment

The corn stover was prepared for acid pretreatment by presoaking the particles at a 5% (w/v) solids concentration at room temperature in a 0–2% (w/v) solution of sulfuric or H_3PO_4s, overnight. The presoaked slurry was then pretreated at 121°C in an autoclave reactor with residence times ranging from 30 to 120 min. At the end of the pretreatment, the contents were filtered and the solids washed with distilled water until the filtrate pH reached 7.0. This was followed by surfactant swelling of the cellulosic fraction in a 0–10% (w/v) solution of Tween-80 (polyoxyethylene sorbitan monooleate, CAS# 9005-64-5) at room temperature for 12 h. The swollen cellulosic fraction was again filtered and washed to neutral pH with distilled water. Wet samples were stored in plastic Petri dishes at –20°C prior to enzymatic hydrolysis. Effects on digestibility owing to freezing were not studied.

Enzyme and Enzymatic Hydrolysis

Enzymatic digestibility measurements were performed on several acid-treated and control samples (untreated) using NREL standard procedure (Standard Procedure #009) *(10)*. Commercially produced cellulase and β-glucosidase (Novo Nordisk, Bagvard, Denmark) were used. Celluclast (80 FPU/mL, 80 mg of protein/mL) and Novozym 188 (792 cellobiase units (CBU)/mL, 73 mg of protein/mL) were used for cellulose hydrolysis with a volume ratio of 5 FPU of celluclast/CBU of Novozym to alleviate end-product inhibition by cellobiose. The amount of washed solids required to give 0.2 g of cellulose in 20 mL was added to a 150-mL flask. The buffer for the digestion was 0.05 *M* citrate (pH 4.8), containing 800 μg of tetracycline. The cellulase enzyme loading was adjusted to 40 FPU/g biomass of cellulose in the flask. The contents of the flask were preheated to 50°C before the enzyme was added. Then, the flask was placed in a shaker incubator operating at 50°C and 150 rpm. Using the same method, untreated substrate control was placed in the incubator. Samples were taken every 24 h of the hydrolysis and subjected to heat denaturation (boiling water for 15 min) to inactivate and precipitate the enzyme. These samples were analyzed for

Table 1
Composition of Untreated and Pretreated Corn Stover[a]

Acid Conc. (w/v)	Solid remaining (%)	Glucan (%)[b]	Xmg (%)[b]	Klason lignin and ash (%)
Untreated	100.0	40.4	25.8	18.7
No acid (water)	76.3	46.8	26.6	23.1
0.5% H_2SO_4	57.0	61.1	8.4	29.5
1.0% H_2SO_4	54.9	60.3	6.6	29.7
2.0% H_2SO_4	51.8	61.9	ND	31.1
0.5% H_3PO_4	68.7	51.8	23.3	24.9
1.0% H_3PO_4	63.9	53.4	17.5	26.0
2.0% H_3PO_4	60.8	55.4	14.3	26.9

[a]Pretreatment conditions were as follows: presoaking, 12 h; temperature, 121°C; sulfuric and phosphoric acid concentrations (w/v), 0–2%; reaction time, 120 min; solid content (w/v), 5%.

[b]All sugar contents are based on the original oven-dried untreated biomass and expressed as glucan, xylan, mannan, and galactan equivalents.

[c]ND=not determined.

glucose content and digestibility using a high-performance liquid chromatography (HPLC) and YSI analyzer.

Analytical Methods

All experimental analyses were done in triplicate following NREL standard procedures *(10)*. Carbohydrate products were measured by HPLC using a Bio-Rad Aminex HPX-87H column (conditions: 0.6 mL/min, 65°C, and 0.005 *M* H_2SO_4) and the YSI model 2300 glucose analyzer (YSI, Yellow Springs, OH). Since the HPX-87H column does not resolve xylose, mannose, and galactose, the three components were presented as xylose + mannose + galactose (Xmg). The quantity of Xmg was calculated on a xylose basis, because xylose consists of more than 90% of these three components *(11)*.

Results and Discussion

Pretreated Corn Stover Composition

The results of analysis of the pretreated corn stover are shown in Tables 1–3. Table 1 provides compositions of treated and untreated corn stover. The percentages of glucan (approx 61%), and lignin and ash (approx 30%), did not change with the increase in H_2SO_4 concentration. However, the percentage of Xmg decreased with increasing H_2SO_4 concentration. With H_3PO_4 pretreatment, the percentage of glucan did not remain constant as the concentration of the acid was increased; it increased with the acid concentration. However, there was still a significant amount of Xmg (14.3%) left with 2% (w/v) H_3PO_4. With both pretreatment methods, the mass fraction of corn stover was significantly reduced.

Table 2
Sugar Recoveries in Pretreated Corn Stover Liquors[a]

Acid conc. (w/v)	Gluca (%)[b]n	Xmg (%)[b]
Untreated	—	—
No Acid (water)	3.2	12.4
0.5% H_2SO_4	6.0	71.3
1.0% H_2SO_4	6.4	74.8
2.0% H_2SO_4	6.2	89.5
0.5% H_3PO_4	4.5	30.2
1.0% H_3PO_4	5.0	48.8
2.0% H_3PO_4	5.7	58.1

[a]Enzymatic hydrolysis conditions were as follows: 1% (w/v) solid concentration; 40 FPU of cellulase/g of cellulose for 96 h at 50°C.

[b]All sugar contents are based on the original oven-dried untreated biomass and expressed as glucan, xylan, mannan, and galactan equivalents.

Table 3
Cellulose Digestibility of Pretreated Corn Stover Solids

Acid conc. (w/v)	Digestibility at 72 h (%)
Untreated	10.8
No Acid (water)	24.8
0.5% H_2SO_4	55.4
1.0% H_2SO_4	58.7
2.0% H_2SO_4	75.6
0.5% H_3PO_4	42.1
1.0% H_3PO_4	45.8
2.0% H_3PO_4	56.0

Table 2 provides sugar recoveries from the pretreated liquors. The percentages of glucan in the liquor using both the pretreatment methods remained approx the same (approx 5%). However, Xmg recovery was significantly higher (approx 90% using 2% H_2SO_4) for H_2SO_4 pretreatment than H_3PO_4 pretreatment (approx 58% using 2% H_3PO_4). This indicates that at mild concentrations, sugar yield is higher for H_2SO_4 pretreatment.

Table 3 represents the cellulose digestibility (measured at 72 h) of the pretreated corn stover solids. Again, the digestibility increased with higher acid concentrations, and the H_2SO_4 pretreatment method showed higher digestibility compared with the H_3PO_4 pretreatment method.

Increasing the severity of the pretreatment conditions, apparently because a greater fraction of the hemicellulose sheath surrounding cellulose is dissolved, enhances the enzymatic digestibility of the pretreated substrates. This allows the enzyme to have greater access to the cellulose.

In general, the more severe pretreatment conditions lead to more dissolution of the xylan, and more production of the toxic material for fermentation such as acetic acid *(12)*. At a temperature of 121°C, there was a decrease in xylan content in solids with increasing acid concentration. In 2% (w/v) H_2SO_4 for 120 min, little xylan was left after the pretreatment. H_2SO_4 was more effective at dissolving the hemicellulose fraction than H_3PO_4. On the other hand, the more severe conditions may result in the conversion of more of the cellulose to glucose and subsequent conversion to 5-hydroxymethylfurfural, which is an inhibitory component for subsequent fermentation *(13)*. The desired dilute-acid pretreatment conditions are those that produce the highest ethanol yields in a subsequent fermentation process that is influenced by the enzymatic hydrolysis of the pretreated substrate, xylose yield, and presence of toxic byproducts.

H_2SO_4 Pretreatment

Moderate temperature was investigated at low acid concentrations, which would result in savings in catalyst and acid neutralization costs, as well as material costs of constructing the reactor. This method would also reduce the problems and costs associated with handling the gypsum formed during the neutralization *(14)*.

H_2SO_4 effectively solubilized the hemicellulosic portion of the corn stover and increased the digestibility of the cellulose that remained in the solid residues. As shown in Table 1, the relative percentage of glucan was near 62%, and the xylose remaining was approx 1%. Thus, about 90% of the xylose was recovered with 2 % (w/v) H_2SO_4 treated for 120 min (Table 2). When the H_2SO_4 concentration and residence time were increased from 0.5 to 2% (w/v) and from 30 to 120 min respectively, hemicellulose recovery (=[hemicellulose amount recovered in liquid phase]/ [initial hemicellulose content]) was increased by 30%. However, delignification was only 22% with 2% (w/v) acid concentration and a residence time of 120 min.

H_3PO_4 Pretreatment

Compared with H_2SO_4 treatment, H_3PO_4 treatment had considerably lower hemicellulose degradation. Compositions of hydrolysate liquors from various pretreatments are given in Table 2. In addition, both acid pretreatments had approx the same effect on the lignin content of the corn stover. At 121°C and 120 min, the hemicellulose recovery increased from 30 to 48%, and finally to 58% at 0.5, 1, and 2% acid concentrations, respectively.

The difference of both acid-treated corn stovers seems to be as much a function of reaction time as acid concentration. At moderate temperatures, H_3PO_4 pretreatment resulted in a lower digestibility and hemicellulose conversion (20 and 30%, respectively), which suggests that H_3PO_4 as a reagent for pretreatment is not very effective. Under relatively low temperatures (121°C) and under similar conditions, the results showed that H_2SO_4 treatment is a better method compared with H_3PO_4 treatment.

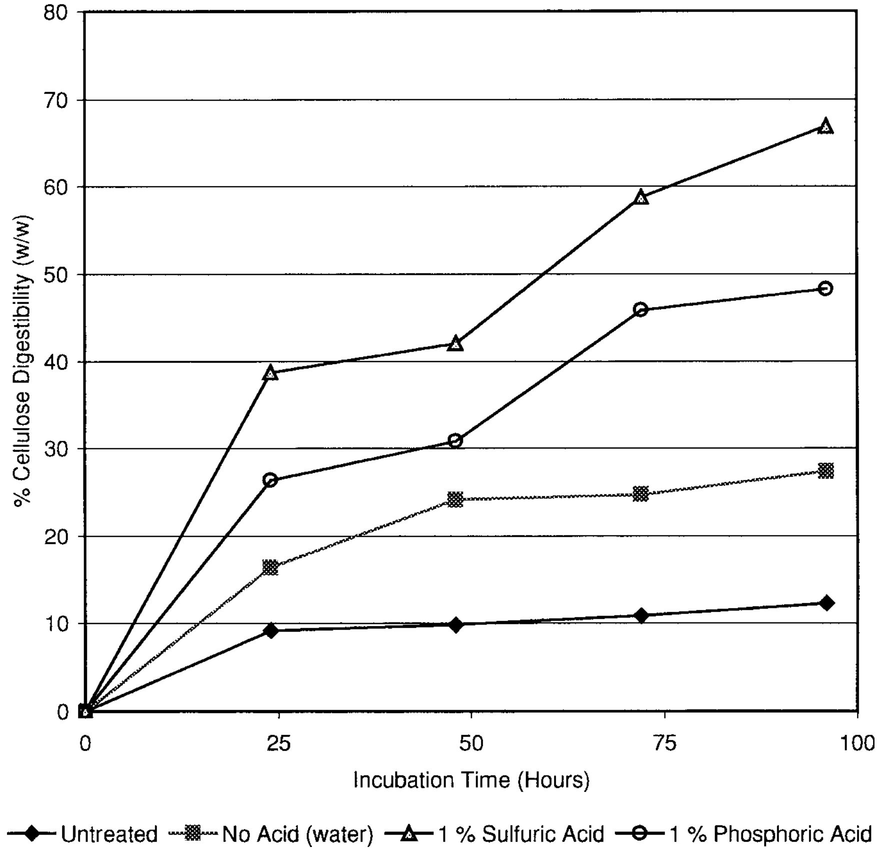

Fig. 1. Cellulose digestibility from enzymatic hydrolysis of corn stover pretreated for 120 min at 1% (w/v) acid concentration.

Digestibility as Function of Time

The digestibility shown in Table 3 is the percentage of released glucose during hydrolysis at 72 h. However, to investigate the overall trend in enzymatic digestibility, samples were taken every 24 h for 96 h. Figures 1 and 2, respectively, show the cellulose digestibility of 1 and 2% (w/v) acid-treated corn stover at the pretreatment reaction time of 120 min. As the pretreatment reaction time was increased from 30 to 120 min, the cellulose digestibility increased up to 62 and 18% in 2% (w/v) acid concentration for H_2SO_4 and H_3PO_4, respectively (data not shown).

Increasing acid concentration from 0.5 to 2% (w/v) increased the degree of digestibility from 56 to 80%, and from 42 to 60% for H_2SO_4 and H_3PO_4, respectively, after pretreatment at 121°C for 120 min. Figures 1 and 2 show the effects of acid concentration on digestibility. The digestibility increased as the acid concentration and residence time were increased. The digestibilities from various H_2SO_4 treatments were higher than those of the H_3PO_4 treated samples. When the 24-h digestibility of H_2SO_4 treatments

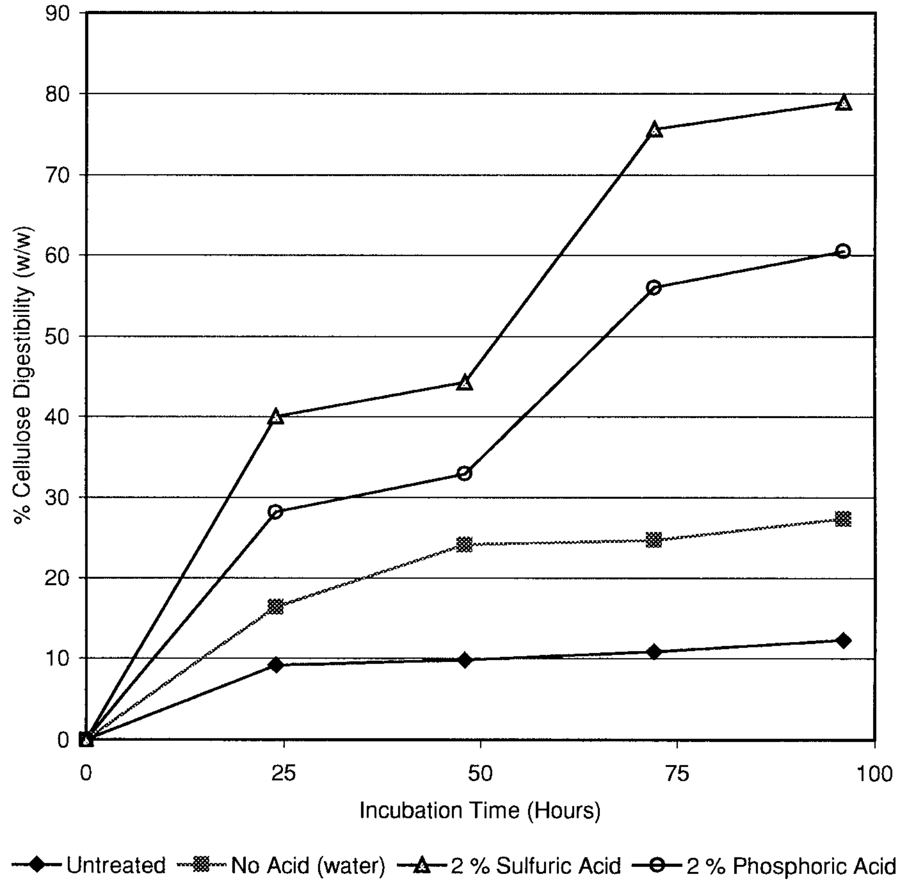

Fig. 2. Cellulose digestibility from enzymatic hydrolysis of corn stover pretreated for 120 min at 2% (w/v) acid concentration.

are compared with those of the H_3PO_4 treatment, it is found that initial digestibility was affected somewhat by the extent of hemicellulose removal, resulting in increased initial rate. From Fig. 2 it is also apparent that the percentage of digestibility (at 96 h, and 2% [w/v] concentration) is approx 25% higher for the H_2SO_4 than for the H_3PO_4 pretreatment process.

Effect of Surfactant on Enzymatic Hydrolysis

Figure 3 shows the effect of different concentrations of Tween-80 on the enzymatic hydrolysis yield of 2% (w/v) acid–pretreated corn stover. Tween-80 clearly aided the enzymatic hydrolysis of the pretreated corn stover; the average rate and extent of conversion with Tween-80-treated samples were higher than for Tween-free samples. At the loading level of 0–10% (w/v) solution of Tween-80, the total sugar yield increased up to a maximum of 15% when compared to results obtained in the absence of the additive in the 96-h period. It was observed that the addition of Tween-

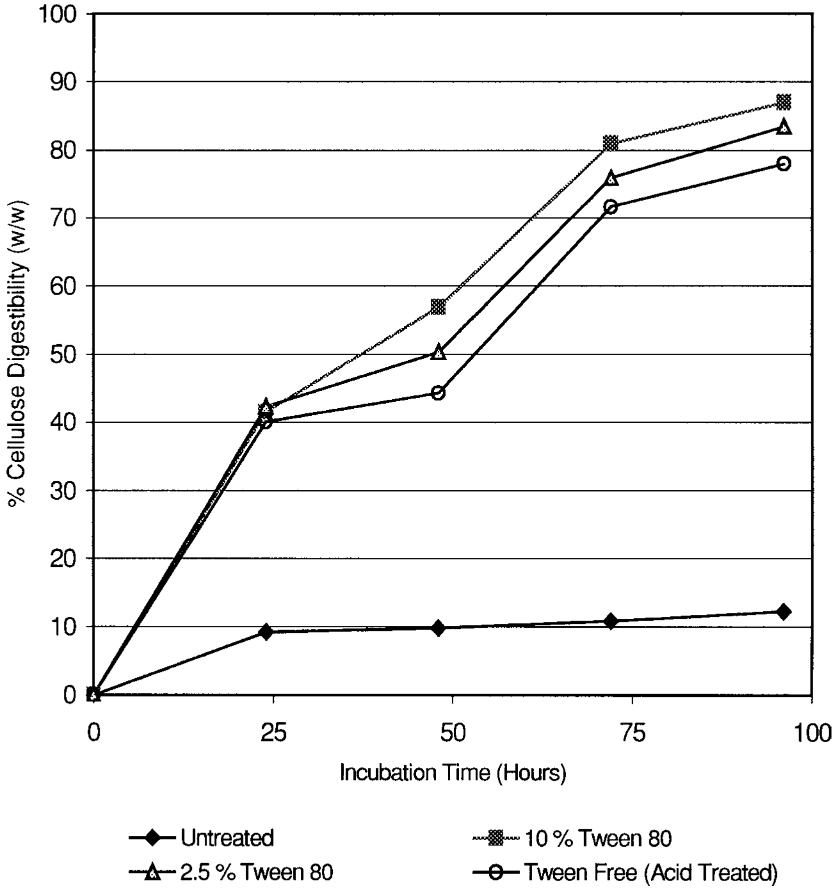

Fig. 3. Cellulose digestibility from enzymatic hydrolysis of corn stover pretreated for 120 min at 2% (w/v) H_2SO_4 followed by surfactant swelling at room temperature.

80 increased the sugar yield proportional to the concentration of the surfactant, and it was found that Tween-80 promoted the availability of the reaction that increased the hydrolysis rate, as was reported by Kaar and Holtzapple *(7)* and Castanon and Wilke *(8)*.

Digestibility as Function of Hemicellulose Removal

Figure 4 shows 96-h enzymatic digestibility as the function of the amount of hemicellulose removed by dilute-acid pretreatment of the corn stover. The data were obtained after treating corn stover with H_2SO_4 or H_3PO_4 without the addition of Tween-80. Here, the points are experimental raw data and the solid lines represent nonlinear regression model prediction using Excel.

The digestibility increased approximately linearly with increasing removal of xylan. In the case of H_2SO_4, hemicellulose removal was about 1.5 times higher than that in the H_3PO_4 treatment.

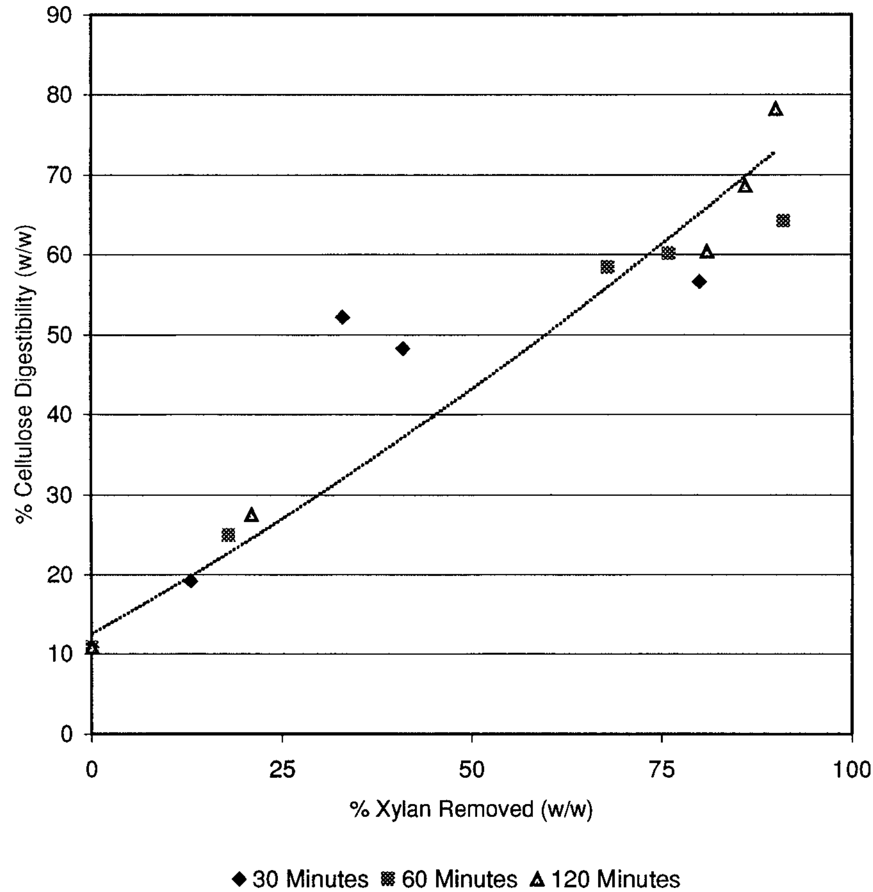

Fig. 4. Cellulose digestibility as a function of percentage xylan removed. Pretreatment conditions were 0.5–2% H_2SO_4 for 30–120 min at 121°C. Enzyme hydrolysis conditions were 1% (w/v), cellulose loading and 40 FPU/g of cellulose for 96 h at 50°C.

Several studies have reported that hemicellulose *(7)* or lignin *(15,16)* hinders enzyme adsorption on cellulose. The results of our study indicate that the digestibility may be directly related to hemicellulose removal.

Conclusion

Recovery of xylose using H_2SO_4 pretreatment of corn stover was 10–30% greater in comparison with that obtained with H_3PO_4 pretreatment under similar comditions. There was a linear relationship between cellulose digestibility and percentage of xylan removed from the solid. An increase in H_2SO_4 concentration (1–2% [w/v]) in the pretreatment step increased cellulose digestibility by approx 25%. This research also confirms the previous results in the literature that Tween-80 is an effective surfactant aid, which increases cellulose digestibility. The addition of

10% Tween-80 increased digestibility of H_2SO_4 (2% [w/v])-pretreated corn stover by 15% compared with that of the Tween-free process.

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