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Effect of Pretreatment Reagent and Hydrogen Peroxide on Enzymatic Hydrolysis of Oak in Percolation Process

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Abstract

The effect of pretreatment reagent and hydrogen peroxide on enzymatic digestibility of oak was investigated to compare pretreatment performance. Pretreatment reagents used were ammonia, sulfuric acid, and water. These solutions were used without or in combination with hydrogen peroxide in the percolation reactor. The reaction was carried out at 170°C for the predetermined reaction time. Ammonia treatment showed the highest delignification but the lowest digestibility and hemicellulose removal among the three treatments. Acid treatment proved to be a very effective method in terms of hemicellulose recovery and cellulose digestibility. Hemicellulose recovery was 65–90% and digestibilities were >90% in the range of 0.01–0.2% acid concentration. In both treatments, hydrogen peroxide had some effect on digestibility but decomposed soluble sugars produced during pretreatment. Unlike ammonia and acid treatments, hydrogen peroxide in water treatment had a certain effect on hemicellulose recovery as well as delignification. At 1.6% hydrogen peroxide concentration, both hemicellulose recovery and digestibility were about 90%, which were almost the same as those of 0.2% sulfuric acid treatment. Also, digestibility was investigated as a function of hemicellulose removal or delignification. It was found that digestibility was more directly related to hemicellulose removal rather than delignification.

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Index Entries: Pretreatment; ammonia; acid; water; hydrogen peroxide; enzymatic hydrolysis.

Introduction

Cellulose, a polymer of glucose, can be transformed into fermentable sugars by chemical or biochemical processes. Enzymatic hydrolysis, a method that is designed to produce glucose from cellulosic materials, has several advantages over acid hydrolysis. Enzyme reaction is very specific and does not produce undesirable byproducts. Also it is an energy-saving reaction since it takes place at mild temperatures. But enzymes are expensive and their reactions are slow. Even if the chemical and physical characteristics of native lignocellulosic material vary with the origin of raw material, its enzymatic hydrolysis is hindered by the following substrate-related factors *(1–3)*: cellulose in lignocellulosic biomass contains highly resistant crystalline structure, lignin and hemicellulose surrounding cellulose form a physical barrier, and sites available for enzymatic attacks are limited. Thus, effective pretreatment is an essential prerequisite to enhance the susceptibility of lignocellulosic residues to enzyme action.

The primary goal of any pretreatment process is to maximize digestibility. Various pretreatment methods have been investigated to improve enzymatic hydrolysis of woody biomass. Also, the economics of the bioalcohol process using various pretreatments have been evaluated *(4,5)*. These pretreatments are broadly classified into physical *(3)*, chemical *(6)*, and biologic *(7)* according to the principal mode of action on the substrates. Of the number of studies on the efficiency of enzymatic digestibility, there have been few comparing various pretreatment methods *(8)*. In the present study, ammonia, sulfuric acid, and water were employed as pretreatment reagents of woody biomass. It is generally known that an alkaline solution such as ammonia has a great capability in removing lignin but a relatively low capability in solubilizing hemicellulose, whereas acidic solution such as sulfuric acid has the opposite characteristics in removing the noncellulosic constitutes *(9,10)*. The results of water pretreatment are similar to those of acid. To overcome these problems, hydrogen peroxide was added to each pretreatment. The role of hydrogen peroxide is to promote removal of lignin and break bonds between lignin and carbohydrates *(11,12)*.

The purpose of the present study was to examine the effect of pretreatment reagent and hydrogen peroxide on enzymatic digestibility of oak. In addition, the effect of hemicellulose and lignin removal on enzymatic digestibility was investigated. The reactor used was a percolation reactor, which had a proven performance in our previous studies *(9,10)*. The chief advantage of this reactor is its ability to attain high sugar yield by minimizing sugar decomposition.

Materials and Methods

Materials

The oak chips supplied from Korea Institute of Energy Research (KIER) were ground to an average size of 20–60 mesh (0.25–0.84 mm) using a laboratory knife mill. Oak is one of the hardwood species indigenous to Korea. It is considered a renewable resource suitable for conversion to ethanol.

Pretreatment

The percolation system is composed of an aqueous ammonia reservoir, aqueous hydrogen peroxide reservoir, water reservoir, pump, programmable dry oven, reactor, and liquid holding tank. The details of experimental apparatus were described elsewhere *(13)*. A metering pump (TSP Minipump) supplied various solutions into the percolation reactor through a preheating coil. Ten grams of oak chips was presoaked with reaction solution overnight (over a 12-h period). To carry out the reaction, the oven temperature was initially set at 230°C. Also, nitrogen back-pressure was applied to the reaction system at selected pressure (2.758 MPa for ammonia and 1.379 MPa for sulfuric acid and water) to prevent the vaporization of each solution. After preheating for 20 min, the interior of the reactor reached the reaction temperature (170°C). Each reaction solution was pumped into the reactor while maintained at 170°C at a flow rate of 1 mL/min for 15–60 min. As soon as the reaction was completed, water was pumped into the reactor to remove residual sugars trapped in the biomass. After leaching for 20–50 min, the solid sample was discharged from the reactor and then dried at 105°C overnight for solid analysis. The liquid collected in the liquid-holding tank was measured for sugars after secondary hydrolysis.

Enzyme and Enzymatic Hydrolysis

Enzymatic digestibility was measured on the various extensively washed chemical-treated and control samples (untreated, α-cellulose) using National Renewable Energy Laboratory (NREL) standard Laboratory Analytical Procedure no. 009 *(14)*. Commercial cellulase and β-glucosidase (Novo Nordick, Bagvard, Denmark) supplied from KIER were used. Celluclast (80 IU or international filter paper units [IFPU]/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 4 IU of celluclast/CBU of Novozym to alleviate end-product inhibition by cellobiose. The amount of washed solids required to give 0.5 g of cellulose in 50 mL was added to a 250-mL flask. The buffer for the digestion was 0.05 *M* citrate, pH 4.8. The cellulase enzyme loading was adjusted to 60 IFPU/g of 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 on a water shaker bath operating at 50°C and 90 rpm. Using the same method, untreated substrate and α-cellulose were placed in the bath as control substrates. The hydrolysis was carried out for 96 h while removing 1 mL of sample every 24 h. The glucose content of this sample was analyzed using high-pressure liquid chromatography (HPLC), and then its digestibility was calculated.

Analytical Methods

Solid biomass samples were analyzed for moisture, sugars, klason lignin, and ash by NREL standard procedures (Laboratory Analytical Procedures no. 001-005) *(14)*. All experiments were done in duplicate. Sugars and decomposition products were measured by HPLC (Thermo Separation Products) using Bio-Rad Aminex HPX-87C (conditions: 0.6 mL/min, 85°C, and water) and 87H (conditions: 0.6 mL/min, 65°C, and 0.005 *M* sulfuric acid) columns. Since they do not resolve xylose, mannose, and galactose, the three components were presented as xylose + mannose + galactose (xmg). The quantity of xmg was calculated based on xylose analysis, because xylose consisted of >90% of these three components *(9)*.

Results and Discussion

Solutions of ammonia, sulfuric acid, and water were used singly or in combination with hydrogen peroxide for oak pretreatment. Each pretreatment was carried out at 170°C, using a percolation flow rate of 1 mL/min. The reaction time for each pretreatment was predetermined from our previous studies to maximize hemicellulose recovery and minimize sugar degradation *(9,10)*. Although enzymatic hydrolysis was performed for 96 h, the digestibility at 72 h was shown in Tables 1–3 to compare the pretreatment performance. Since xmg of oak comprises >90% of total hemicellulose, in this study we preferred the terminology of hemicellulose to xmg.

Ammonia Pretreatment

Ammonia has a number of favorable attributes regarding the processing of lignocellulosic biomass. It is very effective in removing lignin and can easily be recovered from aqueous mixtures owing to its high volatility. It also cleaves the lignin-hemicellulose bond and changes cellulose structure. Similar to most alkaline treatment, ammonia treatment does not cause significant loss of carbohydrate *(15)*.

Table 1 indicates the compositions of liquid hydrolysate and solid residue after ammonia treatment and percentage of digestibility at 72 h. When the ammonia concentration was increased from 0.5 to 20% (40 times of increment) as shown in Table 1, hemicellulose recovery (=hemicellulose recovered in liquid phase/initial hemicellulose) was increased from 19 to 30%. Also, it was found that ammonia effectively removed lignin, because delignification (=[initial lignin – residual lignin]/initial lignin) was increased from 34 to 66% as the ammonia concentration was increased from

Table 1
Composition of Ammonia-Treated Oak and Digestibility[a]

Ammonia (wt%)	H_2O_2 (wt%)	Solid remaining (%)	Glucan (%)		xmg (%)		Klason lignin (%)	Digestibility at 72 h (%)
			Liquid	Solid	Liquid	Solid		
Untreated biomass	—	100.0	—	48.3	—	18.5	22.9	7.2
0.5	0	76.6	0.5	48.0	3.6	13.5	15.0	51.5
5.0	0	72.0	0.6	46.9	4.4	12.7	11.8	55.0
10.0	0	70.9	0.6	46.0	4.8	12.4	10.2	65.4
20.0	0	66.8	0.9	45.4	5.5	12.1	7.9	66.1
10.0	1.7	69.4	0.9	43.5	5.6	11.3	9.7	66.2
10.0	3.3	69.1	1.2	40.1	5.8	10.8	7.5	78.4
10.0	6.6	54.4	1.6	38.6	6.8	8.4	5.4	87.6

[a]All sugar contents are based on the original oven-dry untreated biomass and expressed as glucan, xylan, mannan, and galactan equivalents (reaction conditions: 170°C, 60 min, 1.0 mL/min).

Table 2
Composition of Sulfuric Acid–Treated Oak and Digestibility[a]

H_2SO_4 (wt%)	H_2O_2 (wt%)	Solid remaining (%)	Glucan (%)		xmg (%)		Klason lignin (%)	Digestibility at 72 h (%)
			Liquid	Solid	Liquid	Solid		
Untreated biomass	—	100.0	—	48.3	—	18.5	22.9	7.2
0.01	0	68.5	1.7	45.4	12.1	4.5	17.5	90.2
0.1	0	65.9	1.9	45.7	14.9	1.9	17.9	92.2
0.2	0	64.2	2.8	44.4	16.6	0	16.9	93.8
0.1	0.4	64.9	2.3	44.4	15.4	1.6	17.4	90.6
0.1	0.8	63.9	2.9	45.2	14.6	1.7	17.2	92.5
0.1	1.6	61.3	4.1	43.5	13.4	0	15.9	100.0
0.1	3.2	58.2	4.1	41.7	13.6	0	14.8	100.0

[a]All sugar contents are based on the original oven-dry untreated biomass and expressed as glucan, xylan, mannan, and galactan equivalents (reaction conditions: 170°C, 15 min, 1.0 mL/min).

Table 3
Composition of Water-Treated Oak and Digestibility[a]

H_2O_2 (wt%)	Solid remaining (%)	Glucan (%)		xmg (%)		Klason lignin (%)	Digestibility at 72 h (%)
		Liquid	Solid	Liquid	Solid		
Untreated biomass	100.0	—	48.3	—	18.5	22.9	7.2
0	68.4	1.7	45.6	13.4	3.5	17.4	76.5
0.8	60.9	2.2	44.5	14.3	2.2	14.5	87.5
1.6	57.4	3.5	40.4	16.5	1.4	11.7	92.9
3.2	53.9	3.3	38.5	17.0	1.3	10.7	97.2

[a]All sugar contents are based on the original oven-dry untreated biomass and expressed as glucan, xylan, mannan, and galactan equivalents (reaction conditions: 170°C, 60 min, 1.0 mL/min).

0.5 to 20%. Meanwhile, residual cellulose content was not affected much by ammonia concentration, leaving it almost intact. The digestibility of the 20% ammonia–treated sample increased by nine times compared with that of the untreated sample. Thus, the ammonia solution used in the pretreatment could be considered an ideal pretreatment solution regarding delignification and very good sugar accountability (sugar in liquid + sugar in solid). However, ammonia pretreatment was not suitable for hemicellulose recovery because it left 70–80% of hemicellulose in the solid phase. Since hemicellulose and cellulose cannot be fermented simultaneously to alcohol, this low hemicellulose recovery is an undesirable attribute because another pretreatment to remove residual hemicellulose fraction is required for subsequent fermentation.

To alleviate this problem, hydrogen peroxide was added into the ammonia stream as an oxidant. As shown in Table 1, the effect of hydrogen peroxide was investigated at the fixed ammonia concentration, 10 wt%. As the concentration of hydrogen peroxide was increased, cellulose, hemicellulose, and lignin in solid phase were substantially decreased. But the amounts of hemicellulose and cellulose recovered in liquid phase were relatively small. This means that hydrogen peroxide decomposed hemicellulose as well as cellulose. Cellulose digestibility, however, greatly increased as hydrogen peroxide concentration increased.

Acid Pretreatment

Dilute sulfuric acid treatment is a very effective method in terms of hemicellulose recovery and cellulose digestibility. The main drawbacks of this method are sugar decomposition and corrosive action owing to the high temperature and low pH employed. Also, this process needs the neutralization step of residual sulfuric acid with lime, which must be disposed of. In spite of these problems, this pretreatment has been evaluated as the most competitive one for a commercial biomass-to-ethanol process *(8)*.

Table 2 shows the results obtained when oak was treated by sulfuric acid alone or 0.1% sulfuric acid with various hydrogen peroxide concentrations. The reaction time adopted in this treatment was 15 min, which is a quarter of that in the ammonia treatment. Compared to ammonia treatment, dilute-acid treatment has considerably higher hemicellulose removal and lower lignin removal. The hemicellulose recovery at 0.01 and 0.2% acid concentration was 65 and 90%, respectively. However, delignification was only 22% at a concentration of 0.1% acid, which was significantly lower than the values obtained in ammonia treatment. Even when hydrogen peroxide was added in the sulfuric acid stream, delignification was not increased much. In addition, it was found that hydrogen peroxide significantly decomposed the hemicellulose extracted into the liquid phase. By comparing the sugar balances of glucan and hemicellulose in ammonia treatment with those of acid treatment, it is apparent that hydrogen peroxide would degrade a certain sugar preferentially in each treatment; i.e., glucose in ammonia treatment and hemicellulose monomers in acid treatment.

In all cases studied in acid treatment, enzymatic digestibilities were >90%, which were significantly higher than the values obtained in ammonia treatment. This means that acid treatment is superior to ammonia treatment in terms of hemicellulose removal and enzymatic digestibility.

Water Pretreatment

In its natural state, hemicellulose exists in an amorphous form. Thus, it is easy to hydrolyze under mild reaction conditions or even in high-temperature water. The organic acids formed during water hydrolysis are acetic and formic acids derived from the cleavage of acetyl and methoxyl groups from hemicellulose *(16)*. These acids lower the pH to a range of 3.0 to 4.0, which permits the removal of hemicellulose from wood.

Table 3 shows the results of water or water–hydrogen peroxide treatment. The reaction time used in this treatment was 60 min because of low acidity found in hydrolysate. The hemicellulose recovery of water treatment was 72%, which was a little higher than that of 0.01% sulfuric acid. This could be owing to the four times longer reaction time. It is very difficult to achieve >70% of hemicellulose recovery in water treatment, because it is impossible to increase catalytic concentration to a certain degree using only water *(6)*.

Unlike ammonia and sulfuric acid treatments, hydrogen peroxide in water treatment has a certain effect on hemicellulose recovery as well as delignification. As the concentration of hydrogen peroxide increased from 0 to 0.8, 1.6, and 3.2%, hemicellulose recovery and delignification increased from 72 to 77, 89, and 92%, and from 24 to 37, 49, and 53%, respectively. In addition, enzymatic digestibility increased from 76.5 to 87.5, 92.9, and 97.2% in the same concentration change of hydrogen peroxide. This means that if hydrogen peroxide is added in the water stream, all three measured values (i.e., hemicellulose recovery, delignification, and enzymatic digestibility) can be increased significantly. When 1.6% hydrogen peroxide was added, both hemicellulose recovery and enzymatic digestibility were about 90%. These values were almost the same as those of 0.2% sulfuric acid treatment, and its delignification was 23% higher than that of 0.2% acid treatment.

As the concentration of hydrogen peroxide increased, it was observed that glucose degraded significantly, whereas hemicellulose was preserved well, as shown in Table 3. It was found that water–hydrogen peroxide treatment may bring a better result relative to sulfuric acid treatment if the amount of hydrogen peroxide is optimized. Also, this treatment can avoid the neutralization step that is required in dilute-acid treatment.

Digestibility as Function of Time

The enzymatic digestibilities shown in Tables 1–3 are percentage of digestibility at 72 h, which is suggested by NREL standard procedure. However, to investigate the overall trend of enzymatic digestibility,

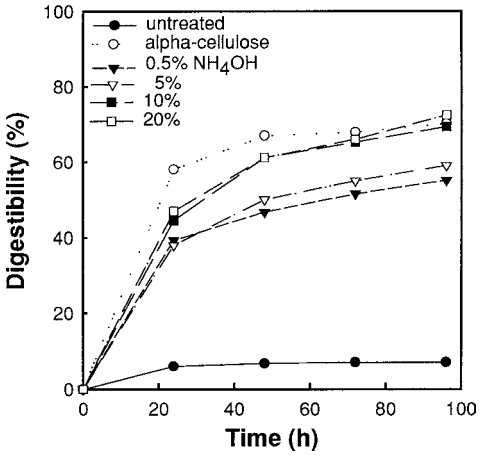

Fig. 1. Effect of ammonia concentration on enzymatic digestibility.

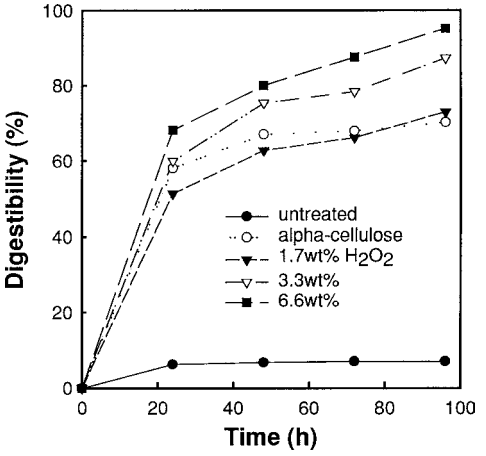

Fig. 2. Effect of H_2O_2 concentration in 10% ammonia solution on enzymatic digestibility.

samples were taken every 24 h for 96 h. The results are illustrated in Figs. 1–5. The untreated substrate and α-cellulose were used as the control substrates. Figure 1 shows the effect of ammonia concentration on digestibility. The digestibility increased as the ammonia concentration increased. The digestibilities from various ammonia treatments were much higher than those of the untreated sample, but they were generally lower than those of α-cellulose. Figure 2 presents the results of digestibilities obtained at various concentrations of hydrogen peroxide in 10% ammonia stream. The digestibility increased significantly as the concentration of hydrogen peroxide increased. Unlike ammonia treatment, ammonia–hydrogen peroxide treatments showed almost equal or higher digestibilities than those of α-cellulose. Note that measured digestibilities showed a gradually growing trend until 96 h in all cases.

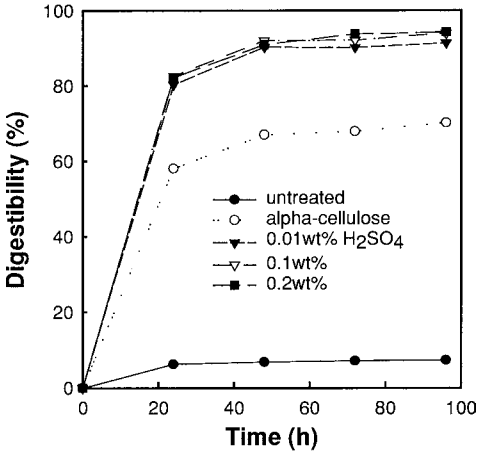

Fig. 3. Effect of acid concentration on enzymatic digestibility.

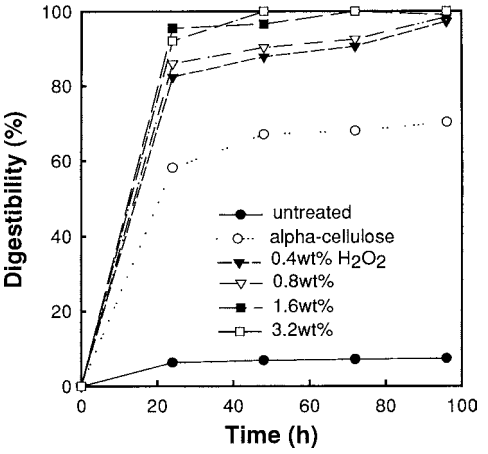

Fig. 4. Effect of H_2O_2 concentration in 0.1% sulfuric acid solution on enzymatic digestibility.

In the sulfuric acid treatment shown in Fig. 3, there was almost no difference in digestibilities as acid concentration was varied. In all cases digestibilities reached about 90% in 48 h and did not increase after that time. Figure 4 shows the effect of hydrogen peroxide on digestibility in 0.1% sulfuric acid stream. Digestibility increased as the concentration of hydrogen peroxide increased. When the 24-h digestibilities of acid–hydrogen peroxide treatments are compared with those of the acid treatment, it is found that initial digestibilities were affected somewhat by hydrogen peroxide, resulting in increasing initial rate. Figure 5 shows the effect of hydrogen peroxide on digestibility in water treatment. The digestibilities obtained from all cases are higher than those of α-cellulose. The digestibilities increased gradually at <0.8% hydrogen peroxide, as in the ammonia–

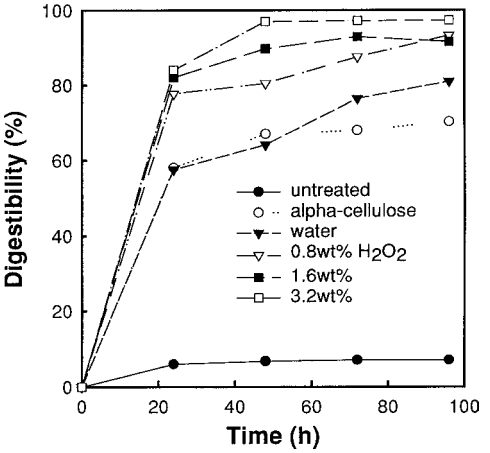

Fig. 5. Effect of H_2O_2 concentration in water on enzymatic digestibility.

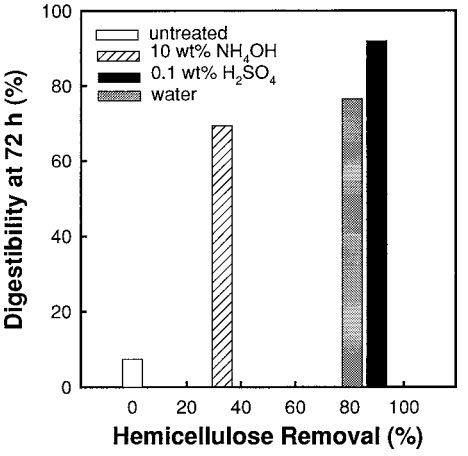

Fig. 6. Enzymatic digestibility vs percentage of hemicellulose removal.

hydrogen peroxide treatment but did not change after 48 h at >1.6% hydrogen peroxide.

Digestibility as Function of Hemicellulose Removal or Delignification

Figure 6 shows 72-h enzymatic digestibility as a function of hemicellulose removal (=[initial hemicellulose – residual hemicellulose]/initial hemicellulose). The data were obtained after treating with ammonia, acid, and water without the addition of hydrogen peroxide. Digestibility increased as hemicellulose removal increased. This can be explained in terms of the substrate-related aspect as follows: the chances of enzyme adsorption onto the cellulose surface are increased as hemicellulose

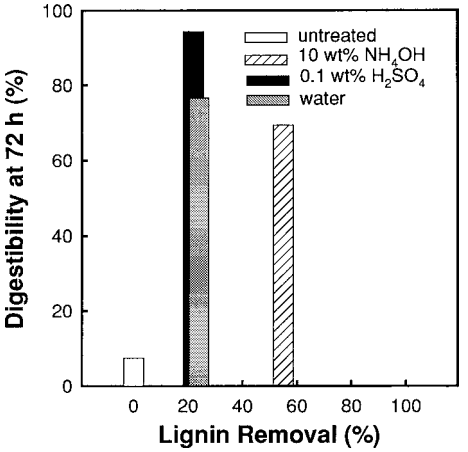

Fig. 7. Enzymatic digestibility vs percentage of lignin removal.

removal is increased. The hemicellulose removal and digestibility of each treatment were as follows: ammonia, 33% (as removal) and 65% (as digestibility); water, 81 and 77%; and acid, 90 and 92%. This means that digestibility is not linearly increased with hemicellulose removal. In the case of ammonia and water treatments, hemicellulose removal in water treatment was about 2.5 times higher than that in ammonia treatment, but the digestibility difference was only 1.2 times higher. Thus, it is assumed that digestibility can be affected by other factors, such as delignification. In spite of relatively low hemicellulose removal, it seems that high digestibility in ammonia treatment is attributed to relatively high delignification.

Digestibility was also affected by delignification, as shown in Fig. 7. In ammonia treatment, digestibility was 65% at 55% delignification. This was about 30% lower than the digestibility in the acid treatment case, whose delignification was only 22%. In the case of water treatment, digestibility was 77% at 24% delignification, which was 12% higher than that of ammonia treatment. This high digestibility at the low delignification can be explained by high hemicellulose removal in both water treatment and acid treatment.

Several studies have reported that hemicellulose *(17)* or lignin *(18,19)* hinders enzyme adsorption on cellulose. The results of the present study indicate that digestibility is more directly related to hemicellulose removal than delignification. However, it is not clear which component is more responsible for enzyme hydrolysis because neither hemicellulose nor lignin can be extracted separately without structural change of the other component. Therefore, in the pretreatment of lignocellulosic material, it is important to develop a proper pretreatment method suitable to a specific substrate because the mechanism of pretreatment has not been clearly illustrated and enzyme hydrolysis is affected by many diverse factors *(20)*.

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