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Pretreatment of Wood by UCT-Solvent for the Enzymatic Hydrolysis

H. OOSHIMA,*[,1] M. KURAKAKE,[2]
J. KATO,[1] AND Y. HARANO[2]

[1]*Department of Bioapplied Chemistry, Osaka City University, Osaka 558, Japan; and* [2]*Department of Food & Technology, Fukuyama University, Fukuyama, Hiroshima 729-02, Japan*

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ABSTRACT

UCT-solvent pretreatment was carried out on woods (beech and akamatsu (pine)) for the enzymatic hydrolysis, in which pretreatment the ground woods were autoclaved with a mixture of water and cyclohexanol (37.5% vol% cyclohexanol) having upper critical temperature (UCT: 184°C) on the mutual solubility curve (named as UCT-solvent). Ninety-five and 92% of Klason lignin were removed from beech and akamatsu, respectively, whereas when the woods were autoclaved with water instead of UCT-solvent, only 43 and 18% of Klason lignin was removed from them, respectively. The excellent ability of UCT-solvent for the removal of Klason lignin is owing to that the solvent disturbs re-coupling between the degradation products. The enzymatic hydrolysis of wood was much improved by UCT-solvent pretreatment: the hydrolytic reactivity of akamatsu was enhanced by 2.8 times comparing with when akamatsu was pretreated with water instead of UCT-solvent.

Index Entries: Pretreatment; wood; upper critical temperature; cellulose; hydrolysis; cellulase; enzyme; lignocellulose.

INTRODUCTION

It has been strongly claimed that innovative technologies for resolution of the global environmental problems must be developed. Reduction

*Author to whom all correspondence and reprint requests should be addressed.

of carbon dioxide is one of the most important problems. An answer to the problem is to utilize biomass for production of chemicals and fuels. Plants fix carbon dioxide as cellulose. Cellulose is hydrolyzed to glucose, and glucose can be converted to more useful chemicals and fuels such as ethanol and hydrogen by biological processes. Even if carbon dioxide is newly produced during the biological process or after burning the fuel, it can be fixed by plants again.

One of important things in the utilization of lignocellulosic materials is how effectively cellulose is hydrolyzed to glucose. At present, two processes have been proposed for the hydrolysis: enzymatic hydrolysis and acid hydrolysis. The enzymatic hydrolysis is promising and may be better in certain features than acid hydrolysis: high recovery of sugar, mild reaction conditions, and so on. However, it also has problems. Cellulosic materials (biomass) must be pretreated for the effective attack of enzyme to cellulose before the hydrolysis. Purpose of the pretreatment is to increase the surface area of cellulose accessible to cellulase and to reduce surface area of lignin and hemicellulose on which cellulase adsorbs in waste for the hydrolysis of cellulose (*1–3*). This purpose can be attained by removing lignin and hemicellulose from lignocellulosic materials.

In our previous paper (*4*), we proposed a thermochemical pretreatment named "UCT-solvent pretreatment." UCT-solvent is binary solvent of water and organic solvent having upper critical temperature (UCT) in the mutual solubility curve: for example, cyclohexanol-water (named as UCT-solvent A; UCT: 184°C) and *n*-pentanol-water (UCT-solvent B; UCT: 187.5°C). The pretreatment by UCT-solvent A and B was applied to bagasse in success, where degradation of lignin and hemicellulose and removal of the degradation products from inside of cellulose fiber were well performed. As the result the enzymatic hydrolysis rate was significantly increased. The excellent capability of UCT-solvent for the removal of Klason lignin is caused by the large affinity of the mixed solvent for lignin and the high ability for the dissolution of the degradation product of lignin and hemicellulose. However, the sensitivity of lignin to the pretreatment may be different according as what its source is. Indeed, lignin of hardwood is more sensitive than lignin of softwood in the pretreatment of microwave (*5*).

In this work, we tried UCT-solvent pretreatment to woods, akamatsu (softwood) and beech (hardwood), other than bagasse. As UCT-solvent, we used UCT-solvent A (cyclohexanol-water).

MATERIALS AND METHODS

Materials

Cellulase from *Trichoderma viride*, Meicelase CEPB-5029 (Filter paper activity: 8000 u/mg), was supplied from Meiji Seika Kaisha Ltd., Japan and

used without any further purification. Bagasse was obtained from Prof. Toyama's Laboratory in University of the Ryukyus. Beech and akamatsu were obtained from Prof. Koshijima's Laboratory in Kyoto University. The wood samples were residues of extraction with alcohol and benzene. Cellulose content was determined to be 51.8 and 51.6% for akamatsu and beech, respectively, by JIS (Japanese Industrial Standard) -8007 method and Klason lignin content was determined to be 26.2 and 20.3% for akamatsu and beech, respectively, by JIS-8008 method. All other chemicals were of reagent grade.

Pretreatment

Four g of dry lignocellulose (beech, akamatsu, or bagasse) ground to less than 35 mesh and 40 mL of UCT-solvent A were autoclaved, where the temperature was raised from room temperature to a given high temperature of 170–230°C at the gradient of 1.5°C/min and the ratio of cyclohexanol in UCT-solvent A was 37.5% vol%. As soon as the high temperature was reached, the mixture was cooled down to room temperature leaving the autoclave sealed. Through a series of the process, the phase of UCT-solvent A changes as following: initially two phases at room temperature, then single phase at high temperature, and again two phases at room temperature. The mixture thus pretreated was centrifuged. The upper organic phase was replaced. The pretreated lignocellulose was washed with acetone and water to remove slightly remaining degradation products from lignin and hemicellulose before the enzymatic hydrolysis. Care was taken for drying through the pretreatment and storage. As a control, another pretreatment was carried out, which was the same except that water was used instead of UCT-solvent A.

Enzymatic Hydrolysis

Four g of untreated lignocellulose or all residue after the pretreatment of 4 g lignocellulose was placed in 50 mL screw capped vial with working volume of 42 mL and buffered to pH 4.8 with 0.1*M* acetate and the hydrolysis was started by adding cellulase solution. The working concentration of cellulase was 5 mg/mL. The vial was sealed and incubated in shaking bath at 40°C. At given intervals, 1 mL sample of the mixture was withdrawn and centrifuged. The supernatant was used for the determination of sugar and protein concentrations.

Determinations of Concentrations of Sugar and Protein

The concentration of the total reducing sugar (TRS) was determined by DNS method, and the concentration of glucose was separately determined by an enzymatic method using glucose oxidase/peroxidase reagent. TRS as cellobiose was determined from calibration curves for cellobiose

Table 1
Effects of Pretreatment at the Optimal Pretreatment Temperature[a] on Removal of Lignin from Lignocellulosic Materials and on Their Hydrolytic Reactivities

	Fractional conversion at 30h-hydrolysis (–)			Remaining lignin (–)		
	Pretreatment					
Lignocellulosics	Not carried out	Water[b]	UCT-solvent	Not carried out	Water[b]	UCT-solvent
Bagasse	0.20	0.62	0.69	1.0	0.39	0.22
Beech	0.08	0.53	0.61	1.0	0.57	0.05
Akamatsu	0.08	0.19	0.53	1.0	0.82	0.08

[a]Optimal pretreatment temperature (°C): 200 (bagasse) and 220 (beech and akamatsu).
[b]Water was used instead of UCT-solvent A.

and glucose. The concentration of protein in the supernatant was determined by the Bradford colorimetric assay from Bio-Rad Co., using bovine serum albumine as a standard.

Definition of Sugar Yield After the Pretreatment and the Hydrolysis

Sugar yield was defined as following, based on the total amount of cellulose contained in the three kinds of lignocelluloses before the pretreatment (0.47 g glucose/g bagasse, 0.56 g glucose/g akamatsu, and 0.57 g glucose/g beech), where unknown reducing sugar in the sample was evaluated as cellobiose.

RESULTS AND DISCUSSION

Table 1 presents the effect of UCT-solvent pretreatment on the reactivity of lignocellulosics in the hydrolysis, which was evaluated from the reaction conversion at 30 h hydrolysis, and also presents the removal of lignin from the lignocellulosic materials. The pretreatment was carried out at the optimal pretreatment temperature in the range of 170–230°C, which was determined from the hydrolysis rate at 40°C and pH 4.8 as the details are described later (Fig. 5 (b)). The reactivity of lignocellulose was much improved by the pretreatment, in particular by UCT-solvent pretreatment. The increase in the reactivity was caused by the removal of lignin. Largest effect of the pretreatment on the removal of lignin and the increase in the reactivity of lignocellulose were observed in akamatsu: 92% of lignin was removed and the reactivity of cellulose was enhanced by 2.8 times compared with the control pretreatment. Sensitivity of lignin

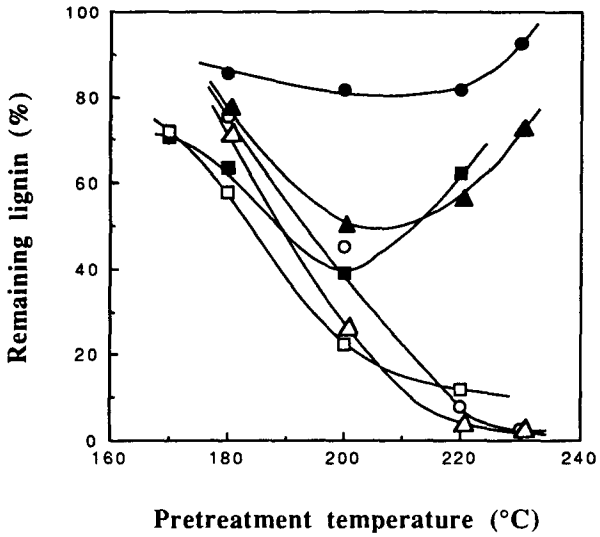
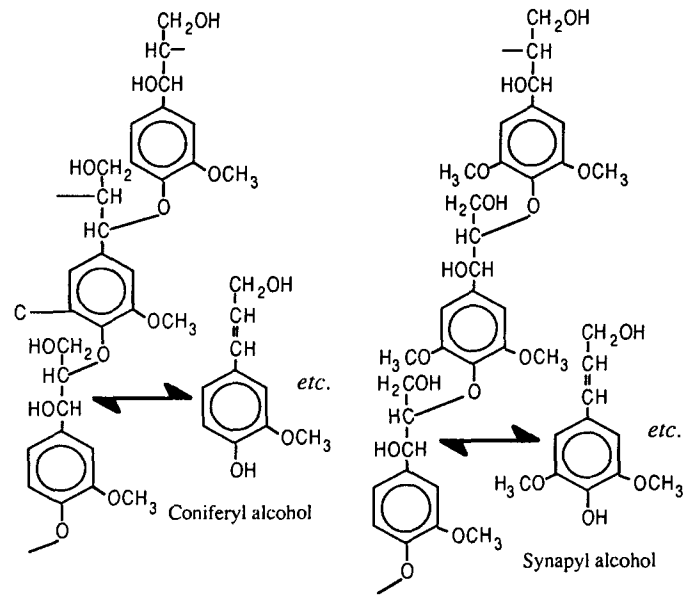


Fig. 1. Changes in remaining lignin after the pretreatment as a function of the pretreatment temperature. Pretreatment: control (■, ●, ▲) and UCT-solvent (□, ○, △). Lignocellulose: bagasse (■, □), beech (●, ○), and akamatsu (▲, △).

to a pretreatment is different according to what the source is: In a thermochemical pretreatment, it is often observed that softwood lignin is harder to be removed than hardwood lignin (5). However, as Table 1 shows, UCT-solvent pretreatment is able to remove softwood lignin as well as hardwood lignin. Almost all of lignin was removed from beech and akamatsu by UCT-solvent pretreatment.

As Figure 1 shows, the remaining lignin is a function of the pretreatment temperature. Lignins of bagasse, beech and akamatsu were removed at lower temperature in that order. The difference in the sensitivity of lignin to the pretreatment is caused by the difference of chemical structure of lignin in the three lignocellulosics. Hardwood lignin has a large content of synapyl alcohol residue of which aromatic ring is methoxylated at the two ortho positions as shown in Fig. 2. On the other hand, softwood lignin is composed of mainly coniferyl alcohol, of which aromatic ring is methoxylated only at the one ortho position. A part of coniferyl alcohol contained in sottwood lignin may form C-C linkage with other residue at the other ortho position. The presence of C-C linkage is one of the reasons why softwood lignin is hard to degrade. Figure 1 also shows that the degradation profile of lignin is different between the two pretreatments. In the control pretreatment, in which lignocellulose was autoclaved with water, the remaining lignin decreases as the temperature rises until the minimum and then increases again. In the UCT-solvent pretreatment, the remaining lignin monotonously decreases as the temperature rises. The difference in the degradation profile also can be explained



Model of softwood lignin (akamatsu)

Model of hardwood lignin (beech)

Fig. 2. Model of chemical structure of softwood lignin and hardwood lignin, and its degradation.

with the chemical structure of lignin. In a thermochemical pretreatment carried out in aqueous medium, lignin once decomposed is partially recombined through C-C and O-C coupling during the pretreatment at over 200°C (*5*). The coupling reaction also occurs between lignin and sugar from hemicellulose (*6*). The coupling often results in an increase in Klason lignin through the pretreatment, in spite of that lignin is once degraded thermochemically. Such the coupling is easy to occur in polar solvent and hard to occur in nonpolar solvent (*7*). Therefore, the coupling reaction is significant in the control pretreatment and not significant in the UCT-solvent pretreatment. In UCT-solvent pretreatment, the degradation products remain small and dissolved in nonpolar UCT-solvent at high temperature. This is the reason why in the UCT-solvent pretreatment the remaining lignin monotonously decreases as the pretreatment temperature rises and it does not do in the control pretreatment. Furthermore, the coupling reaction must be faster in softwood lignin rather than hardwood lignin, because in hardwood lignin the coupling is disturbed by steric hindrance by the two methoxyl groups: it means that softwood lignin is hard to degrade to small pieces. This is another reason why softwood lignin shows large resistance to the pretreatment. We conclude that the excellent effect of UCT-solvent pretreatment on removing lignin from wood as well as bagasse is caused by not only a high ability of UCT-solvent for the extraction of degradation products (*4*) and but more basically it is caused by that UCT-solvent disturbs the coupling of degradation products.

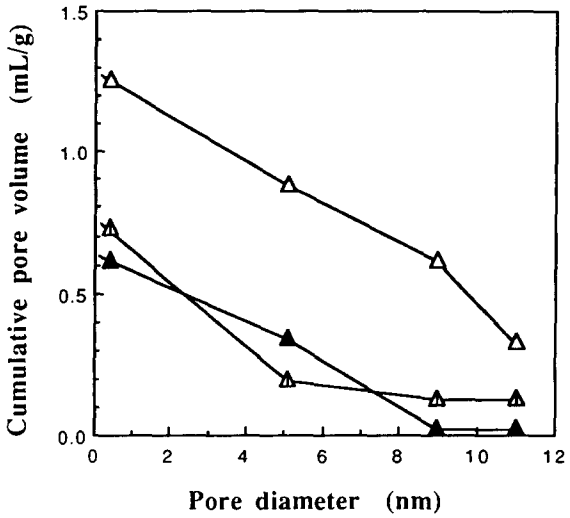


Fig. 3. Pore volume distribution of untreated and pretreated akamatsu. Pretreatment: untreated (△), control (▲), and UCT-solvent (△).

The pore volume of the pretreated wood was measured by solute exclusion method of Stone and Scallen (*8*) except for that concentration of dextran probe was determined from the dry weight of the sample solution instead of polarimetric assay. Figure 3 presents pore size distribution of akamatsu pretreated at 220°C. It is found that the removal of lignin resulted in an increase in the pore volume of cellulose accessible to cellulase, which has been estimated to be 5.1–9.0 nm in diameter (*9,10*). The large increase in the conversion of the hydrolysis of akamatsu pretreated by UCT-solvent in Table 1 was found to be caused by large increase in pore volume accessible to cellulase and hence by large increase in surface area accessible to cellulase. Figure 4 presents the relationship between the accessible surface area of wood and the initial hydrolysis rate, where each plot corresponds the untreated and the pretreated wood: The pretreatment was carried out with water at 180 and 220°C and with UCT-solvent A also. It shows that the initial hydrolysis rate becomes large with an increase in the accessible surface area. The difference in the specific hydrolysis rate at given surface area between beech and akamatsu probably is caused by the slight difference in the amount of remaining lignin.

UCT-solvent pretreatment enhances the reactivity of wood for the enzymatic hydrolysis by increasing the surface area accessible to cellulase. The increase in the accessible surface area corresponds an increase in the adsorption of cellulase on the substrate as shown in Fig. 5(a). However, the increase in the adsorption of enzyme on the substrate is not always increasing the hydrolysis rate as can be seen in the hydrolysis of akamatsu pretreated with water (the control pretreatment) in Fig. 5(b). That is because cellulase adsorbs on not only cellulose but also lignin and hemicellulose (*1–3*). Figure 6, which presents the specific enzymatic activity

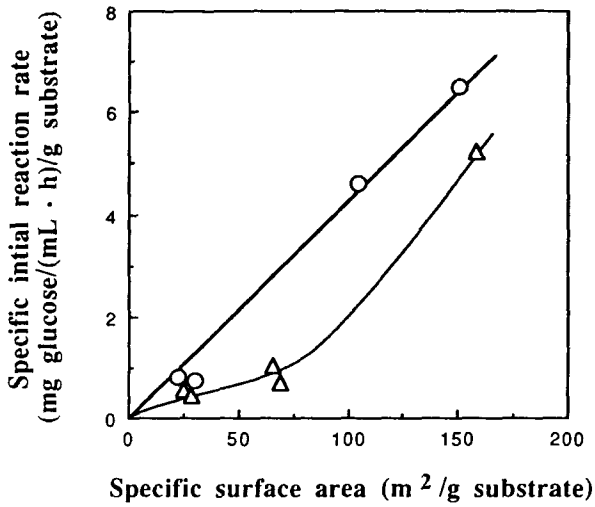


Fig. 4. Relationship between the initial hydrolysis rate and the specific surface area. Lignocellulose: beech (○), and akamatsu (△).

of cellulase adsorbed on akamatsu, shows that the fraction of enzyme truely used for the hydrolysis of cellulose in the adsorbed enzyme remarkably increase by the UCT-solvent pretreatment. These results mean that the removal of lignin also is very important from the point of view of the enhancement in the utilization efficiency of enzyme.

Figure 5(a) also shows that the adsorption of enzyme inversely decreases after reaching the maximum at around 210°C and the similar tendency is observed in the hydrolysis rate shown in Fig. 5(b). The decrease in the adsorption amount and the hydrolysis rate are probably caused by partial decomposition of cellulose, maybe by carbonization.

Figure 5(b) also shows that in case of bagasse there is a range of pretreatment temperature in which the control pretreatment is better than UCT-solvent pretreatment. This result is caused by that the degradation of hemicellulose is depressed in UCT-solvent at in particular below 190°C (*4*).

One purpose of the pretreatment is to raise the rate of the enzymatic hydrolysis. Another purpose is to raise the reaction conversion finally obtained by given reaction time. In the enzymatic hydrolysis of cellulose, it is well known that the reaction rate quickly decreases as the reaction proceeds, and the reaction reaches to almost stop even if a large amount of cellulose remains not hydrolyzed. In other words, there is a limit in the conversion actually obtained. The quick decrease in the hydrolysis rate has been explained by the quick decrease in the accessible surface area in the initial stage of the hydrolysis (*10,11*), the product inhibition, and the rapid decrease in the enzymatic activity of the adsorbed cellulase (*12,13*). We investigated how much lignin at least must be removed to raise the final conversion. Figure 7 presents the fractional conversions after the

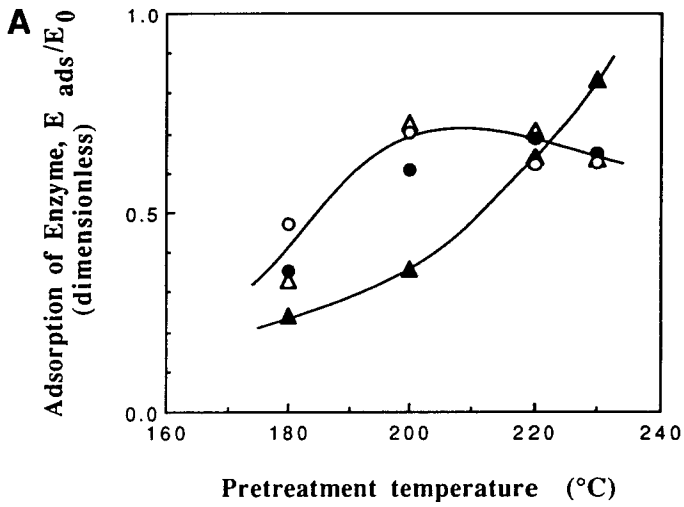


Fig. 5. (a) Adsorption of protein (cellulase) on the pretreated beech and akamatsu. Pretreatment: control (●, ▲) and UCT-solvent (○, △). Lignocellulose: beech (●, ○), and akamatsu (▲, △).

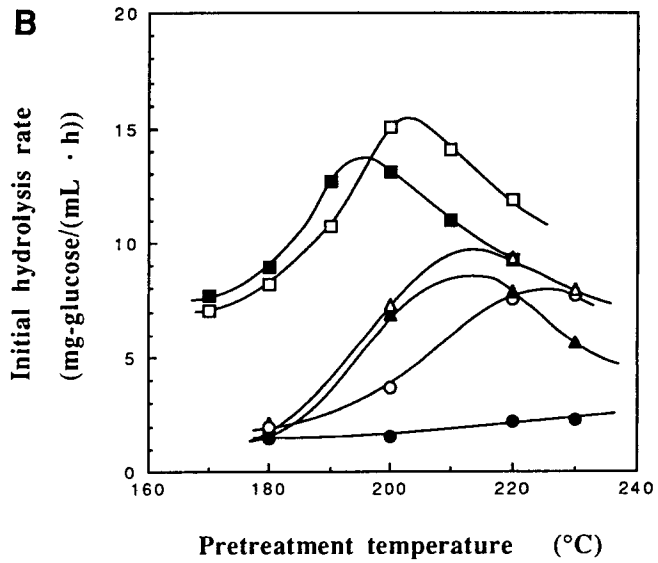


Fig. 5. (b) Initial hydrolysis rate of the pretreated lignocellulosics. Hydrolysis: see the text. Pretreatment: control (■, ●, ▲) and UCT-solvent (□, ○, △). Lignocellulose: bagasse (■, □), beech (●, ○), and akamatsu (▲, △).

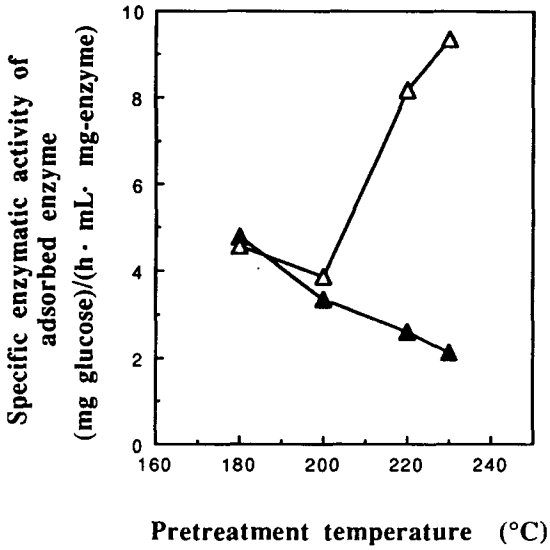


Fig. 6. Specific enzymatic activity of cellulase adsorbed on akamatsu pretreated at 180 to 230°C. Pretreatment: control (▲) and UCT-solvent (△).

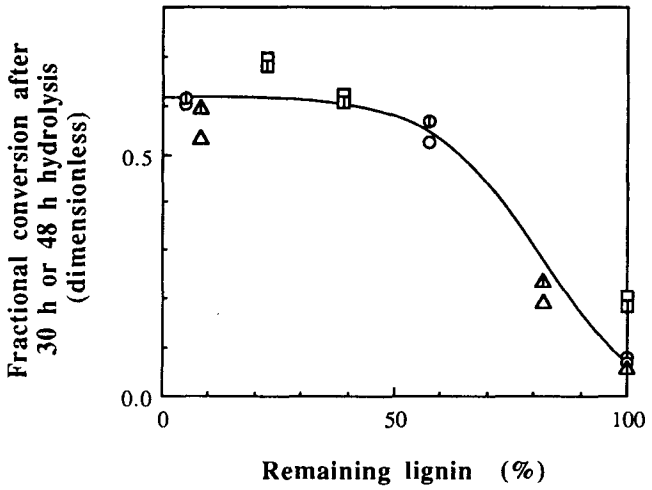


Fig. 7. Relationship between the fractional conversion after the 30 and 40 h hydrolysis of untreated and pretreated lignocellulosics and their fraction of lignin remaining after the pretreatment. Lignocellulose: bagasse (□, ▣), beech (○, ◑), and akamatsu (△, ◭). Hydrolysis time: 30 h (□, ○, △), 48 h (▣, ◑, ◭).

hydrolysis of 30 h and 48 h, which were obtained by using the untreated and the pretreated lignocellulases. The removal of 50–60% of lignin is enough to reach the limiting conversion, 62% by 30 h in this case. In order to raise the limiting conversion, unfavorable decomposition of cellulose during the pretreatment, such as the carbonization, has to be avoided,

because the decomposed cellulose may be not hydrolyzed by enzyme, and hence cellulose located under the decomposed cellulose also. In order to avoid the unfavorable decomposition, the pretreatment must be done quickly. However, our present apparatus for the UCT-solvent pretreatment is not best for the quick pretreatment because of the slow heating rate.

CONCLUSION

UCT-solvent pretreatment was carried out on woods (beech and akamatsu (pine)) for the enzymatic hydrolysis, in which pretreatment the ground woods were autoclaved with a mixture of water and cyclohexanol (37.5 vol% cyclohexanol) having upper critical temperature (UCT: 184°C) on the mutual solubility curve (named as UCT-solvent). Ninety-five and 92% of Klason lignin were removed from beech and akamatsu, respectively, whereas when the woods were autoclaved with water instead of UCT-solvent, only 43 and 18% of Klason lignin was removed from them, respectively. The removal of lignin resulted in the increase in the surface area of wood accessible to cellulase as shown in Fig. 3. As the result, the enzymatic hydrolysis of wood, especially in akamatsu, was much improved. The increase in the surface area of cellulose also raises the efficiency of cellulase by reducing the adsorption of enzyme on lignin. As we described in the previous paper (4) in which bagasse was pretreated by UCT-solvent method, the excellent ability of UCT-solvent for the removal of Klason lignin was owing to the large affinity of the solvent for lignin and the high dissolution ability for the degradation product of lignin. In the present work, we understood another role of UCT-solvent. It is that UCT-solvent disturbs the recoupling between the degradation products. As Figure 1 shows, water in the control pretreatment seems to have good ability to degrade lignin at least when the pretreatment was carried out below 200°C. A problem in the control pretreatment is that the degradation product is recoupled at higher temperature. The coupling reaction may take place not only between degradation products from lignin but also between lignin and degradation product from hemicellulose. In UCT-solvent the coupling reaction is hard to occur, and the mol wt of the degradation product remains small enough to dissolve in the solvent.

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