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Bioethanol From Cellulose With Supercritical Water Treatment Followed by Enzymatic Hydrolysis

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Abstract

The water-soluble portion and precipitates obtained by supercritical (SC) water treatment of microcrystalline cellulose (Avicel) were enzymatically hydrolyzed. Glucose could be produced easily from both substrates, compared with the Avicel. Therefore, SC water treatment was found to be effective for enhancing the productivity of glucose from cellulose by the enzymatic hydrolysis. It is also found that alkaline treatment or wood charcoal treatment reduced inhibitory effects by various decomposed compounds of cellulose on the enzymatic hydrolysis to achieve higher glucose yields. Furthermore, glucose obtained by SC water treatment followed by the enzymatic hydrolysis of cellulose could be converted to ethanol by fermentation without any inhibition.

Index Entries: Lignocellulosics; supercritical water; enzymatic hydrolysis; inhibitor; ethanol.

Introduction

Global warming owing to the use of the fossil resources is becoming a progressively more serious issue. Much attention has been focused on ethanol from lignocellulosic biomass as an alternative to fossil fuels because of its low green house gas emission and overall environmental friendliness. To obtain sugars from lignocellulosics for ethanol production, numerous studies have been performed on the hydrolysis of lignocellulosics by acid catalysis *(1,2)*, steam explosion *(3)*, liquid hot water *(4)*, and enzymatic saccharification *(5)*.

Recently, on the other hand, supercritical (SC) fluid technology has been applied for the conversion of lignocellulosics to fuels and chemicals *(6–16)*. Ehara and Saka *(13)* reported that cellulose can be converted to sugars such as polysaccharides, oligosaccharides, and monosaccharides by SC water treatment of cellulose for 0.5 s in a flow-type system. They also reported that some other decomposition compounds of cellulose were obtained simultaneously such as levoglucosan, 5-hydroxymethyl furfural,

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erythrose, methylglyoxal, glycolaldehyde, and dihydroxyacetone. Sasaki et al. (17) reported that polysaccharides and oligosaccharides obtained by SC water treatment of cellulose can be effectively converted to cellobiose and glucose with cellulase. However, the ethanol fermentability of the obtained hydrolysate has not been studied. Furthermore, an influence of various decomposition compounds of cellulose obtained by SC water treatment on enzymatic hydrolysis and fermentation has not been studied.

The purpose of this study is, therefore, to establish the whole process for ethanol production from cellulose with SC water treatment followed by enzymatic hydrolysis. Thus, in this study, cellulase and β-glucosidase were applied after its SC water treatment to obtain glucose from cellulose for ethanol production. The effects of the various decomposition compounds of cellulose on enzyme activity and fermentation were also studied. In the previous studies, it is reported that alkaline treatment with wood ash (18) or wood charcoal treatment (19) is effective for removing inhibitors for ethanol fermentation in the wood hydrolysate obtained by dilute sulfuric acid hydrolysis of wood and improving its ethanol fermentability. Therefore, in this article, the treatment with alkali or wood charcoal was applied to SC water-treated decomposition compounds for enhancing enzyme activity as well as ethanol fermentability to attain high efficiency of ethanol production.

Materials and Methods

SC Water Treatment

Microcrystalline cellulose (Avicel PH-101) was treated by water at SC condition using the flow-type system described elsewhere (13). A volume of the slurried Avicel (2 wt%) at room temperature was mixed with 10-fold volume of the SC water at 380°C and 40 MPa pressure. The reaction time was set at 0.12 s. Furthermore, the reactant was immediately cooled down to 150°C by introducing a 2.5-hold volume of cold water at ambient temperature into the reaction mixture directly and further cooled below 50°C by an external cooler.

The treated samples were filtered into a SC water-soluble (WS) portion and a water-insoluble portion. After settling for 12 h, the former was separated into a WS portion and a water-insoluble portion (precipitates), which are precipitated resulting from the change of dielectric constant of water from the SC state to ambient conditions. The precipitates were separated from the WS portion by filtration. The WS portion obtained was condensed 20 times *in vacuo*. (This condensed WS portion is described as "WS portion" later on in this article.) The separated precipitates were added to the same amount of the ultra pure water (Nacalai Tesque, Inc., Kyoto, Japan) as the WS portion. The obtained WS portion and the medium contained with precipitates were used for the enzymatic hydrolysis.

Preparation of the Wood Charcoal

Western red cedar (*Thuja plicata* D. Don) flours were treated under nitrogen at a heating rate of 4°C/min and maintained at 700°C for 1 h in a rotary furnace to prepare the wood charcoal.

Alkaline Treatment or Wood Charcoal Treatment on the WS Portion

For alkaline treatment, the WS portion was adjusted at pH 12.0 with solid $Ca(OH)_2$, and then stirred for 30 min. For wood charcoal treatment, the wood charcoal was added to the WS portion at a loading of 1 wt% the WS portion. The WS portion with the wood charcoal was then stirred for 10 min. Subsequently, the wood charcoal was separated from the WS portion by filtration.

Enzymatic Hydrolysis for the WS Portion and Precipitates

Before enzymatic hydrolysis, the media used in this study was adjusted at pH 5.0 with HCl aq. or solid $Ca(OH)_2$. Subsequently, cellulase (Wako Pure Chemical Industries, Ltd.), β-glucosidase (Oriental Yeast Co., Ltd.), and $(NH_4)_2HPO_4$ were mixed at concentrations of 1, 0.5, and 0.5 g/L, respectively. Enzymatic hydrolysis was carried out in a 50-mL glass vial containing 4 mL of the media for 24 h at 37°C with gently magnetic stirring.

Enzymatic Hydrolysis Followed by Ethanol Fermentation

The enzymatic hydrolysis was also performed on the mixture of the WS portion and precipitates. After pH adjustment at 5.0, the mixtures untreated or treated with alkali were hydrolyzed enzymatically by the same procedure as described earlier.

For evaluating the fermentability of the media after enzymatic hydrolysis, the fermentation was carried out after enzymatic hydrolysis for 24 h. To prepare the inoculum for ethanol fermentation, a yeast, *Saccharomyces cerevisiae* (IFO 233), was grown in a 300-mL Erlenmeyer-flask capped with a silicone plug, which contained 50 mL of sterile medium. The medium composition was as follows; 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L glucose. The flask was set in a shaking water bath for 24 h at 28°C. Just after enzymatic hydrolysis of the mixture of the WS portion and precipitates, the inoculum, peptone, yeast extract, and $MgSO_4 \cdot 7H_2O$ were added at concentrations of 10, 10, 10, and 0.5 g/L, respectively. The fermentation medium was then incubated at 37°C for 24 h with gently magnetic stirring.

Enzymatic Hydrolysis of Cellohexaose With Decomposed Compounds of Cellulose

Medium was prepared with 1.0 g/L cellulase, 0.5 g/L β-glucosidase, and 0.5 g/L $(NH_4)_2HPO_4$. To this mixture, various compounds listed in Table 1 were added at a concentration of 0.01 g/L after pH adjustment

Table 1
Concentrations of Various Compounds Obtained in the WS Portion by Supercritical Water Treatment of Cellulose

		Concentration (mg/L)		
	Compounds	Untreated	Alkaline treatment	Wood charcoal treatment
Precipitates		3020	–	–
WS portion	Oligosaccharides	2247	2298.7	2188.2
	Glucose	157.5	156.4	140.4
	Fructose	30.6	29.3	26
	Methylglyoxal	9.4	13.3	7.9
	Glycolaldehyde	70.1	67.2	60.4
	Dihydroxyacetone	7.2	1.9	6.9
	Erythrose	4.7	1.9	3.1
	Levoglucosan	34.1	26.5	23.3
	5-Hydroxymethylfurfural	21.2	14.8	0
	Formic acid	18.5	0	17.3
	Glycolic acid	2.7	2.7	1.7
	Acetic acid	5.5	1.6	1.7
	Lactic acid	3.5	0	2.5

at 5.0 with HCl aq. Cellohexaose (Yaizu Suisankagaku Industry Co., Ltd., Shizuoka, Japan) was then mixed at a concentration of 1 g/L. Enzymatic hydrolysis on cellohexaose was carried out in a 50-mL glass vial containing 4 mL of the media for 24 h at 37°C with gently magnetic stirring.

Analytical Methods

Analysis of the WS portion was done by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan, LC-10A) equipped with an Ultron PS-80P column (Shinwa Chem. Ind. Co., Kyoto, Japan) and a refractive index detector (Shimadzu, RID-10A) or an ultraviolet detector (Shimadzu, SPD-10A) set at 280 nm. Ultra pure water (Nacalai Tesque, Inc., Kyoto, Japan) was used as mobile phase at a flow rate of 1.0 mL/min. The column oven temperature was set at 80°C.

For determining organic acids in the WS portion, capillary electrophoresis (CE) analysis was conducted with HP3D CE systems (Agilent Technologies, Palo Alto, CA). A fused-silica capillary (75 µm in inner diameter, 104 cm in total length, 95.5 cm in effective length) from Agilent Technologies was used. Indirect UV detection at 270 nm (reference wavelength at 350 nm) was performed using organic acid buffer (Agilent Technologies). Injection was carried out by pressure at the cathodic end at 50 mbar for 4 s. The applied voltage was set at –30 kV, with a capillary temperature at 15°C.

For determining glucose produced during enzymatic hydrolysis and ethanol produced during fermentation, the hydrolysis or fermentation medium were filtered through a 0.45-µm filter to separate the enzyme or yeast. The obtained filtrate was then analyzed by HPLC with the same conditions as in the analysis method for WS portion described earlier.

Results and Discussion

Chemical Composition of the Treated Cellulose With SC Water

After fractionation of treated cellulose samples with SC water, only the SC WS portion could be obtained without the SC water-insoluble residue. WS portion and precipitates were obtained from the SC water soluble portion. Oligosaccharides, glucose, fructose, methylglyoxal, glycolaldehyde, dihydroxyacetone, erythrose, levoglucosan, furfural, 5-hydroxymethylfurfural, acetic acid, formic acid, lactic acid, and glycolic acid could be identified by HPLC and CE analyses on the WS portion. The concentrations of these compounds in the WS portion and precipitates are quantified as shown in Table 1 (described as "Untreated"). In our previous study on the SC water treatment of cellulose, it was found that the oligosaccharides in the WS portion were made up of cello-oligosaccharides up to cello-dodecaose *(13)*. It was also reported that the precipitates had noncrystalline structure and contained polysaccharides whose degree of polymerization is in a range between 13 and 100. Therefore, these oligosaccharides in the WS portion and polysaccharides can be the substrates for enzymatic hydrolysis to obtain glucose.

Enzymatic Hydrolysis of the WS Portion and Precipitates

To elucidate the enzyme activity on the WS portion and precipitates, enzymatic hydrolysis for these substrates were carried out separately, and the results are shown in Fig. 1. For comparison, results for untreated Avicel are also shown. Both substrates were found to be hydrolyzed to glucose more easily than Avicel, consistent with previous findings *(17)*. Because of the lower degree of polymerization and noncrystalline structure of the oligosaccharide and precipitates, higher accessibility of enzymes must be obtained. A closer inspection indicated that glucose is produced from WS portion before the precipitates. The dissolved oligosaccharides in the WS portion are thought to be hydrolyzed easier, compared with the precipitates as the polysaccharides with the aggregated structure. These results clearly show that SC water treatment of cellulose can enhance enzyme activity for depolymerization and decrystallization of cellulose. However, the glucose concentration obtained after enzymatic hydrolysis was found to be lower than theoretical values of 2.2 g/L in the WS portion or 3 g/L in the precipitates approximately. Thus, it is assumed that various decomposed compounds of cellulose obtained by SC water treatment as shown in Table 1 affect on the enzymatic hydrolysis.

Effects of Decomposed Compounds of Cellulose on Enzymatic Hydrolysis

To evaluate the effects of various decomposed compounds of cellulose listed in Table 1 on the enzyme activity, a model media containing

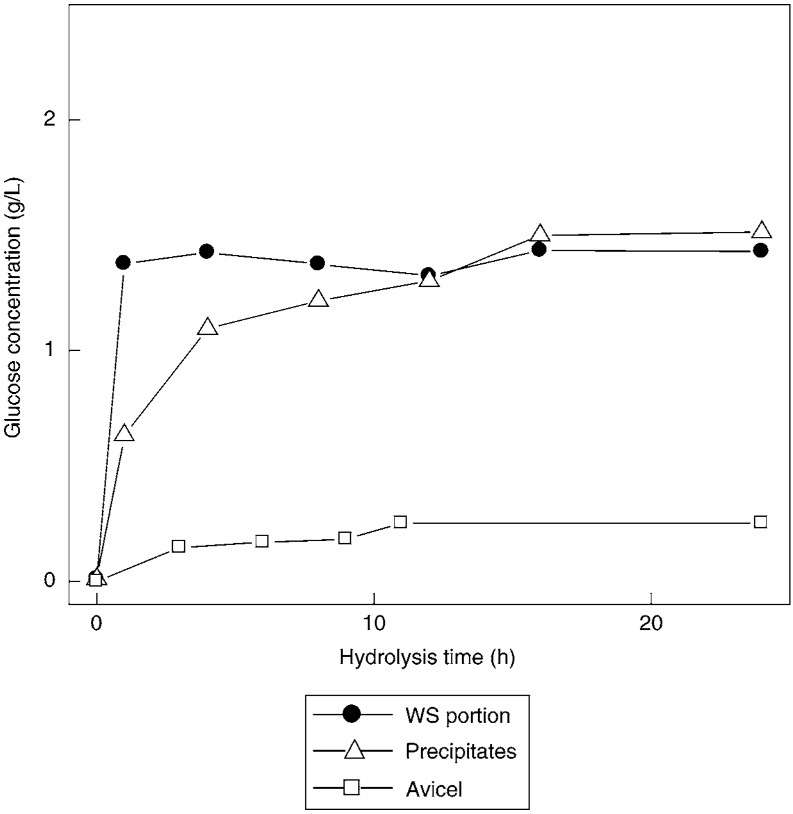

Fig. 1. Changes in glucose concentration during enzymatic hydrolysis of the WS portion and precipitates.

cellohexaose at 1 g/L with those compounds at 0.01 g/L was hydrolyzed enzymatically. Figure 2 shows the glucose concentrations obtained in the enzymatic hydrolysis of cellohexaose with various compounds after 9 h. Glucose concentrations were found to be lower in all media with the additives, compared with the medium with no additives. These results indicate that various compounds obtained by SC water treatment of cellulose have some inhibitory effects on enzymatic hydrolysis and these could account for the lower glucose yield after the enzymatic hydrolysis of the WS portion and precipitates in Fig. 1.

Alkaline or Wood Charcoal Treatments for Detoxification

To increase glucose yields, the enzymatic hydrolysis inhibitors in Fig. 2 should be detoxified or removed. In the previous studies, alkaline treatment with wood ash *(18)* or wood charcoal treatment *(19)* was found to be effective on improving ethanol fermentability of wood hydrolysate obtained by acid hydrolysis. In this study, therefore, we applied alkaline treatment or wood charcoal treatment to the enzymatic hydrolysis of the WS portion. Figure 3 shows the changes of the glucose concentrations for the WS portion

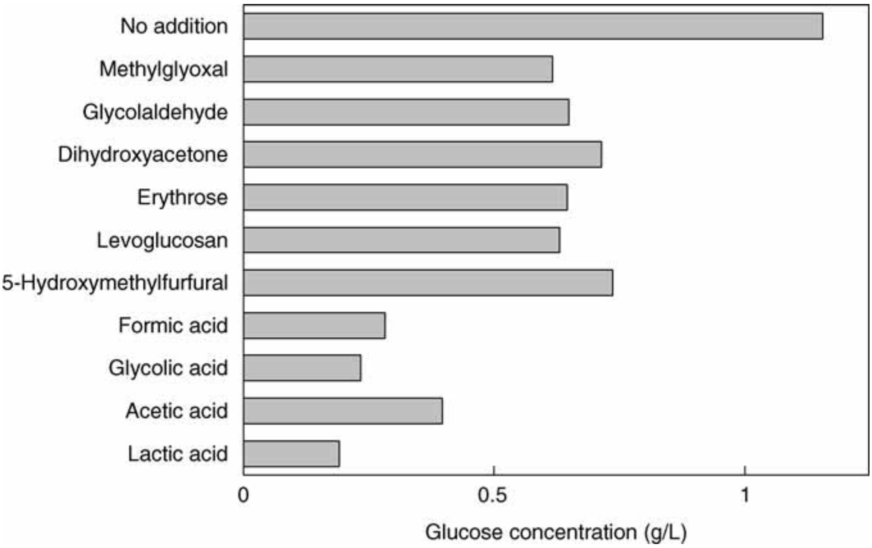

Fig. 2. The effect of various compounds on glucose release by enzymatic hydrolysis of cellohexaose for 9 h.

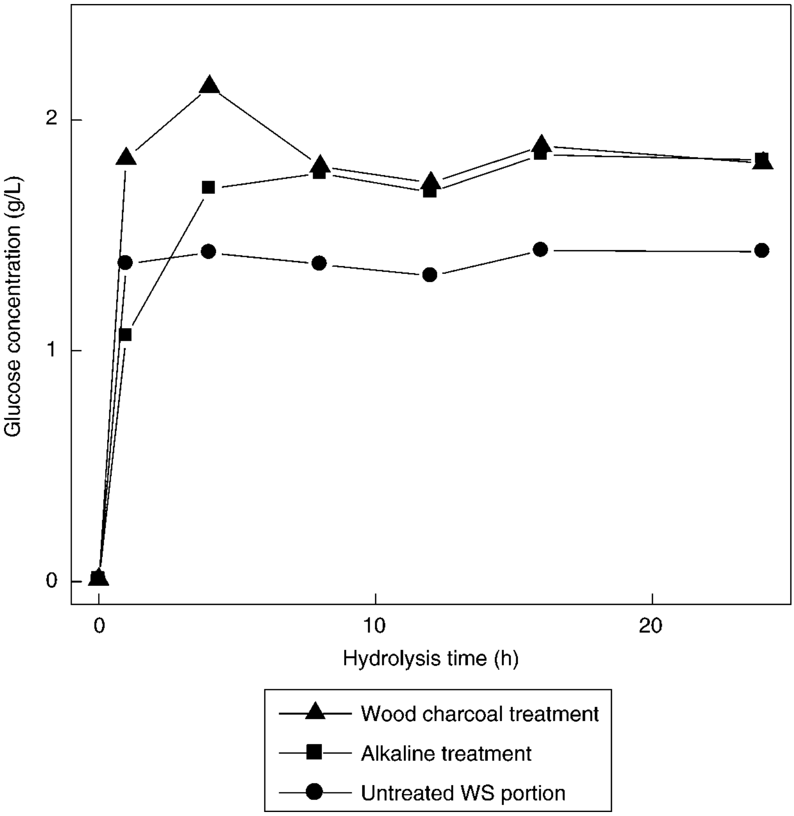

Fig. 3. Changes in glucose concentration for enzymatic hydrolysis of the WS portion without treatment and after treated with alkali or wood charcoal.

after the alkaline treatment or wood charcoal treatment in the enzymatic hydrolyses. Compared with the untreated WS portion, the glucose yields increased in the WS portion after the alkaline treatment or wood charcoal treatment. The concentrations of various compounds in the WS portion after alkaline or wood charcoal treatment are also shown in Table 1. For wood charcoal treatment, the concentrations of many compounds decreased probably owing to the adsorption by the wood charcoal, higher glucose yield were achieved as observed in Fig. 3. For alkaline treatment, on the other hand, the concentrations of many compounds listed in Table 1 also decreased and might be converted to other compounds which have less inhibitory effects. Although the details of the mechanism for reduction of inhibitors by alkaline treatment are unknown, these results showed that alkaline and wood charcoal treatment are effective in detoxifying the WS portion for the enzymatic hydrolysis.

Enzymatic Hydrolysis Followed by Ethanol Fermentation

For efficient glucose production from the WS portion and precipitates, it is preferred to hydrolyze both substrates together without separation. Furthermore, the medium after enzymatic hydrolysis which contains various decomposed compounds of cellulose should be evaluated on its fermentability to establish the process of ethanol production from cellulose with SC water treatment followed by enzymatic hydrolysis. Therefore, enzymatic hydrolysis of the mixture of the WS portion and precipitates was followed by ethanol fermentation as shown in Fig. 4. Alkaline treatment was applied to detoxify the mixture of the WS portion and precipitates. Unfortunately, it was difficult to apply wood charcoal treatment because the wood charcoal could not be separated selectively from the mixture after treatment. In the untreated medium some glucose was produced and fermented to ethanol. Considering that the maximum ethanol concentration was around half the consumed sugar concentration, all the produced glucose during enzymatic hydrolysis was thought to be converted to ethanol. Although the various inhibitory compounds in Fig. 2 were contained in the WS portion, inhibition of ethanol fermentation by those compounds was not observed. In the alkaline treatment, on the other hand, higher glucose yields could be attained in enzymatic hydrolysis, and the glucose obtained was fermented to ethanol without inhibition. These results reveal that SC water treatment of cellulose followed by alkaline treatment could achieve high ethanol yields from cellulose.

Conclusion

The oligosaccharides in the WS portion and polysaccharides as precipitates obtained by the SC water treatment of cellulose can be easily

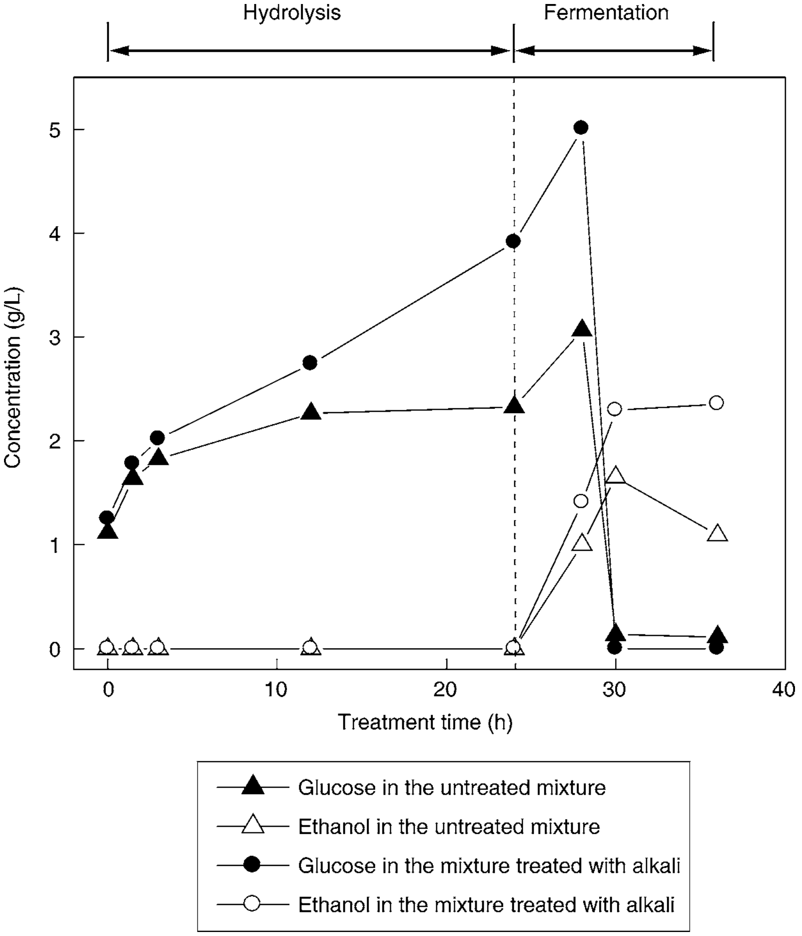

Fig. 4. Changes in glucose and ethanol concentration for enzymatic hydrolysis followed by fermentation of a mixture of WS portion and precipitates without treatment and after treated with alkali.

hydrolyzed to glucose by cellulose enzyme. Furthermore, the glucose obtained was fermented to ethanol without inhibition. Therefore, SC water treatment is a promising method for ethanol production from cellulose. Although various cellulose decomposition compounds from SC water treatment have some inhibitory effects on enzymatic hydrolysis, inhibition can be reduced by alkaline or wood charcoal treatment.

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