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Improvement of Saccharification and Fermentation by Removal of Endogenous Chemicals from Pretreated Lignocellulosic Biomass (1). Effect of Ion-Exchange Resin Treatment

Tamotsu Hoshino[1,2*], **Masaharu Tsuji**[1], **Yuka Yajima**[1], **Kazuaki Yoshimune**[3] **and Akinori Matsushika**[1]

[1]*Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), Kagamiyama, Japan*

[2]*Graduate School of Advanced Sciences of Mater, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 739-8530, Japan*

[3]*College of Industrial Technology, Nihon University, Izumi-cho 1-2-1, Narashino 275-8575, Japan*

[*]**Corresponding author:** Tamotsu Hoshino, Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), Kagamiyama 3-11-32, Higashihiroshima, Hiroshima 739-0046, Japan, Tel: +81-11-857-8475; Fax: +81-82-420-8291, E-mail: tamotsu.hoshino@aist.go.jp

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Abstract

The effects of removal of enzyme inhibitors on saccharification of lignocellulosic biomass and fermentation of their hydrolysate were examined with the aim of improving bioethanol production. Hydrothermal-mechanochemical treated woody powders lost their cell structures and lignin was widely and randomly distributed in treated woody powders. Enzymatic reaction resulted in the formation of many cracks and pores on the woody powder surface, and these cavities extended to the interior of woody powders. The concentrations of 3 kinds of phenolic enzyme inhibitors (gallic, tannic and trimesic acids) in the supernatant of the reaction mixture gradually increased during the enzymatic hydrolysis. Cracks and pores of hydrolyzed woody powders were probably routes of inhibitor release to the reaction mixture. Enzymatic saccharification was improved 1.4-fold by ion-exchange resin treatment after 24 hours of hydrolysis, and inhibition of cellulase activity was decreased by 60-95% compared to the control. Ethanol yield was improved 2.2-fold and lactic acid by 1.3-fold compared to corresponding yields using normal hydrolysates.

Key words:

Cellulase inhibitor; Enzymatic hydrolysis; Ethanol fermentation; Lactic acid fermentation; Lignocellulosic biomass.

Abbreviations

IEEH: Ion-exchanged resin treated enzymatic hydrolysates; SEM: Scanning electron microscope; TEM: Transmission electron microscope.

Introduction

Lignocellulsic biomass, which is mainly composed of a heterogeneous complex of cellulose, hemicellulose and lignin is an attractive and enormously abundant renewable resource whose efficient utilization is required for bio refinery applications, including bioethanol production. The saccharides in linocellulosic biomass are of considerable interest for use as starting materials for sugar platform of microbial fermentation. Enzymatic hydrolysis from lignocellulosic biomass requires the addition of large amounts of cellulose for the reason that enzymatic products such as sugars and several unknown by-products (weak acids, furans and phenolic compounds) are thought to inhibit saccharification [1]. Glucose, cellobiose and ethanol also inhibit β-glucosidase or cellulase [2,3]. Furthermore, phenolic compounds from lignin degradation products are cellulase inhibitors and are toxic in fermentations [4]. More than 35 compounds have been identified as potential inhibitors of the fermentation activity of microorganisms [5]. The detoxification of hydrolysates for an effective fermentation has been studied using several methods such as alkaline treatment, biological processes, hydrophobic interaction chromatography, ion-exchange and polyethyleneimine adsorption [6,7].

Pretreatment studies have examined various chemical and physical processes which are known to increase the enzyme-accessible surface area by breaking up the wood into small pieces, improve reactivity by amorphization of highly crystalline cellulose, and break down and remove the lignin component. Our institution developed a hydrothermal-mechanochemical treatment; this combines a hydrothermal treatment at 135-180°C with wet-milling [8,9]. High temperature treatment at over 180°C produces enzyme inhibitors because over-decomposition; this increases the quantity of saccharification enzyme required [10]. Hydrothermal-methanochemical treated woody powders lost their cellular structure and were randomly rearranged cellular components (Figure 1). Therefore, enzymatic inhibitors were also probably random distributed in pretreated woody powers. There is no report of distribution and releasing process of enzyme inhibitors in hydrothermal-methanochemical treated woody powders.

In this study, we aim to observe inhibitor releasing process according to the hydrolysis of hydrothermal-methanochemical treated woody powders using electron and Raman microscopy. Along with the intermittently model experiments of cellulase inhibitor removal during the first half of hydrolysis (24 and 48 hours) by ion-exchange resin treatment, we tried improving the saccharification reaction and fermentation.

Materials and Methods

Chemicals

All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nisshin EM Co. (Tokyo, Japan). Eucalyptus wood chips (major axis, 25-50 mm; minor axis, 10-20 mm; thickness, 2-5 mm) were purchased from Oji Paper Co., Ltd. (Tokyo, Japan).

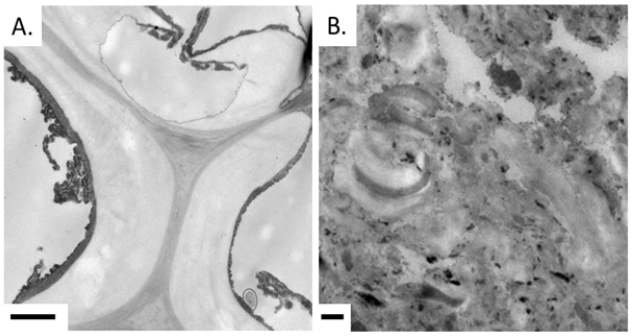

Figure 1: Morphological changes of eucalyptus powder by hydrothermal-mechanochemical treatment. A) 2 mm pore-size woody powder. B) hydrothermal-mechanochemical treated woody powder. Scale bars are 1 μm.

The eucalyptus chips consisted of materials from six species (main component: *Eucalyptus globulus*). The eucalyptus chips were cutter-milled to pass through 2 mm pore-size sieve. The initial composition of eucalyptus wood was determined to be 40.0% (w/w) glucan, 10.4% (w/w) xylan and 28.8% (w/w) acid-insoluble lignin, according to the analytical procedure recommended by NREL [11]. Hydrothermal and mechanochemical pre-treatment was carried out according to Lee et al. [9].

Saccharification

Saccharification condition: Pretreated woody powders (200 g) were added to 800 ml of 20 mM citrate buffer (pH 5.0), mixed with 9 FPU/g of the dry substrate *Trichoderma viride cellulase* (Amano Enzyme, Aichi, Japan) and 18 μl/g of the dry substrate Optimash BG enzyme (High activity betaglucanase, xylanase and cellulase enzyme complex, Genencor International, Rochester, New York, USA), and incubated a 37°C at 120 rpm. Reaction mixtures were collected every 24 hours, boiled for 10 min, and centrifuged at 20,000 × g for 10 min at 4°C. The supernatant was filtered through a 0.2 μm filter (Merck Millipore, Billerica, MA, USA). Filtered samples were used as hydrolysates, and glucose concentrations in the solutions were measured by high-performance liquid chromatography (HPLC) performed under the following conditions: detector, Model RI-8020 (Tosoh Bioscience Japan, Tokyo, Japan); columns, TSK-gel (G6000 PWXL+G3000 PWXL G2500 PWXL) connected in that order; mobile phase, deionized water; flow rate, 0.5 ml/min; column oven, Model CO8020C (Tosoh) at 60°C. The total phenol content was quantified according to the Folin-Ciocalteau method [12]. All experiments were performed in triplicate and standard deviation was calculated from obtained data.

Removal of endogenous phenolic chemicals by ion-exchange column

Chromatography

Enzymatic hydrolysis was carried out as described above. After 24 hours, slurries were divided into water-soluble and water-insoluble fractions by filtration under reduced pressure. Water-soluble fractions were passed through a column packed with Dowex MARATHON WBA ion-exchange resin (Wako Pure Chemical Industries) and was mixed with the water-insoluble fraction and incubated at 37°C for 96 hours, 120 rpm. We repeated same operation again (water-soluble fractions were pass through ion-exchange resin two times). The control reaction was performed under the same conditions as those for the normal enzymatic hydrolysis. Saccharification samples were collected every 24 hours, boiled for 10 min and centrifuged at 4°C for 10 min at 20,000 × g. The supernatant was then filtered through a 0.2 μm filter.

Filtered samples were used as hydrolysates, and concentrations of glucose and other endogenous chemicals in the solutions were measured by the HPLC method described above. Glucose formation rate was calculated from glucose concentration and saccharification time.

Determination of endogenous chemicals from eucalyptus chips in hydro lysates

Endogenous chemicals from eucalyptus powder were tentatively identified based on the retention time of normal reagents by HPLC, which was performed under the following conditions: detector: LaChrom 2420 UV-Vis detector (Hitachi High Tech, Japan); column: guard cartridge column Wakosil-II 5C18RS+Wakosil-II 5C18RC (Wako Pure Chemical Industries, Osaka, Japan); mobile phase: acetonitrile-0.05 50 mM phosphate buffer, pH 4.0 (10:90, v/v); flow rate: 1.0 ml/min; column oven: LaChrom L-2350 (Hitachi High Tech) at 40°C.

Effect of typical endogenous phenolic compounds in pretreated eucalyptus powders on saccharification reaction

The effects of candidate cellulase inhibitors were determined using the following procedure. First, 4 g Avicel cellulose (Sigma-Aldrich Japan, Tokyo, Japan) in 40 ml of 20 mM citrate buffer (pH 5.0) was heated at 121°C for 15 min, cooled to room temperature, and mixed with 4 FPU/ of substrate of T. *viride cellulase*. Then 7 μl/g of the substrate of Optimash BG was added to 7.6 g/L tannic acid, 1.2 g/L trimesic acid, 1.2 g//L gallic acid, respectively or mixture of phenolic compounds (7.6 g/L tannic acid, 1.2 g/L trimesic acid and 1.2 g/L gallic acid or 0.7 g/L tannic acid, 0.5 g/L trimesic acid and 0.06 g/L gallic acid), and the resulting mixture was incubated at 37°C for 120 hours at 120 rpm. The control was maintained as described above without the addition of phenolic compounds. Saccharification samples were collected, boiled for 5 min, and centrifuged at 4°C for 10 min at 20,000 × g. The supernatant was filtered through a 0.2 μm filter (Merck Millipore). Filtered samples were used as hydro lysates, and glucose concentrations in the solutions were measured by HPLC. Glucose concentrations were measured by the same method as that described above. Effects of cellulase inhibitors were determined by comparing glucose concentrations with the control.

Microscopic analyses

Raman microscopy

Dehydration was carried out in a graded ethanol series for 1 min each (25%, 50%, 75%, 90%, 95%, three times for 100%, v/v ethanol). Samples were infiltrated with Lowicryl HM20 resin in the Leica EM AFS2 and FSP (Leica Microsystems, Wetzlar, Germany) at -60°C, with increasing concentration of the resin (33%, 50%, 69%, 90%, six times for 100%, v/v). The resin was polymerized by UV light at -30°C for 48 hours. HM20-embedded samples were sectioned to 1 μm with a Diatome diamond knife on a Leica EM UC7 ultra microtome (Leica). The sectioned samples were collected on slide glasses. Images were taken with an inVia Reflex (RENISHAW, Gloucestershire, England).

Scanning electron microscopy (SEM)

Enzymatic hydrolysis was carried out as described above. The collected samples were boiled for 5 min and cooled to room temperature. Cooled samples were centrifuged at 4°C for 15 min at 3,500 × g. The precipitates were used as samples for SEM. Untreated and enzymatically hydrolyzed samples were prepared by freeze-drying, and these samples were coated with Pd–Au by a Hitachi E101 ion sputter (Hitachi Instruments, Tokyo, Japan). The morphology of untreated and enzymatically hydrolyzed samples was observed under a JSM-5310LV scanning electron microscope (JEOL, Tokyo, Japan) at 15 kV.

Transmission electron microscopy (TEM)

Untreated and enzymatically hydrolyzed woody powders were fixed in 4% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The samples were rinsed in three changes of 0.1 M sodium cacodylate buffer. Post-fixation was in 1% (w/v) OsO4 in 0.1 M sodium cacodylate buffer for 1 hour in the dark, and the samples were rinsed three times in 0.1 M sodium cacodylate buffer and deionized-distilled water. The samples were dehydrated in a graded ethanol series (25%, 50%, 75%, 90, 95% and twice for 100%, v/v) and 100% (v/v) acetone then and embedded in Eponate12/Araldite resin. Ultrathin sections were cut using a Leica EM UC7 ultramicrotome (Leica) and placed on slot grids. The grids were stained with uranyl acetate and lead citrate. Images were taken with on FEI Technai 12 (FEI Company, Oregon, USA) at 120 kV.

Fermentation

Ethanol fermentation

Ethanol fermentation experiments were performed in 100 mL flasks. Hydrolysates were prepared to a 200 g/L glucose concentration by freeze-concentration. Then 5 g/L yeast extract (Difco), 5 g/L malt extract and 3 g/L (dry weight) of *Saccharomyces cerevisiae* BY4743 were added to the prepared mixtures, which were incubated at 27°C for 72 hours at 120 rpm. As a control, instead of hydrolysates, 200 g/L glucose was used.

Fermented samples were collected and centrifuged at 4°C for 1 min at 20,000 × g. The supernatant was filtered through a 0.2 μm filter. Filtered samples were used as ethanol fermentation samples, and ethanol concentrations were measured by HPLC.

Measurement of ethanol and residual glucose concentration was carried out under the following HPLC conditions: detector: Model RI-8020 (Tosoh); columns: TSK-gel (G6000 PWXL+G3000 PWXL +G2500 PWXL) connected in that order; mobile phase: deionized water; flow rate: 0.5 mL/min; column oven: Model CO8020C (Tosoh) at 60°C. Effects of phenolic compounds on ethanol fermentation were determined by comparing ethanol concentrations with the control.

Lactic acid fermentation

Saccharification was performed under the same conditions as those for normal enzymatic hydrolysis with improvement of the saccharification reaction by removal of cellulase inhibitors. Lactic acid fermentations were performed in 1 L jar fermenters. Hydrolysates were prepared to 50 g/L glucose by freeze-concentration. Then 0.5 g/L yeast extract was added to the solutions with adjustment of the pH to 9.0 with 10 N sodium hydroxide using an alkali titration system with argon gas at 30°C. Precultured *Enterococcus casseliflavus* L-120 (0.1 g/L, dry weight) [13] was added to the solutions, which were adjusted to pH 9.0. The mixture was then incubated at 30°C for 72 hours at 180 rpm with argon gas, maintaining the pH at 9.0. As a control, instead of hydrolysates, 50 g/L glucose was used. Fermented samples (600 μl) were collected and centrifuged at 4°C for 1 min at 20,000 × g. The supernatant was passed through a 0.2 μm filter. Filtered samples were used for lactic acid fermentation, and the lactic acid concentrations in the samples were measured by HPLC using the following HPLC conditions: detector: LaChrom 2420 UV-Vis detector (440 nm); eluent: 3 mM chloric acid; flow rate: 0.5 mL/min; reaction solution: 0.1 mM bromothymol blue–15 mM disodium hydrogen phosphate; flow rate: 0.6 mL/min; column: GL-A180-S (Hitachi High-Technologies Corporation, Tokyo, Japan); column oven: LaChrom L-2350 at 40°C.

Ethanol fermentation with cellulase inhibitors

The basal ethanol fermentation mixtures were composed of 50 g/L glucose, 5 g/L yeast extract, 5 g/L Bacto peptone, 2 g/L NH_4Cl, 1 g/L KH_2PO_4 and 0.3 g/L $MgSO_47H_2O$ in 20 mM citrate buffer (pH 5.0) and were inoculated 3 g/L (dry weight) *S. cerevisiae* BY4743. We also added cellulase inhibitors (standard hydrolysate: 7.6 g/L tannic acid, 1.2 g/L trimesic acid and 1.2 g/L Gallic acid or IEEH: 0.7 g/L tannic acid, 0.5 g/L trimesic acid and 0.06 g/L Gallic acid) to the basal ethanol fermentation mixture, which was fermented at 120 rpm for 72 hours at 30°C. The control was maintained as described above without the addition of any of the compounds.

Results and discussion

Change of concentration of endogenous chemicals in hydrolysate

More than 30 chemical compounds were detected in normal hydrolysis solutions and inhibited or promoted cellulase activity [14]. Three phenolic compounds, tannic acid, Trimesic acid and Gallic acid had strong inhibitory effects among them (60-70% of enzymatic activity was reduced in the presence of final concentrations of these phenolic compounds (Figure 2).

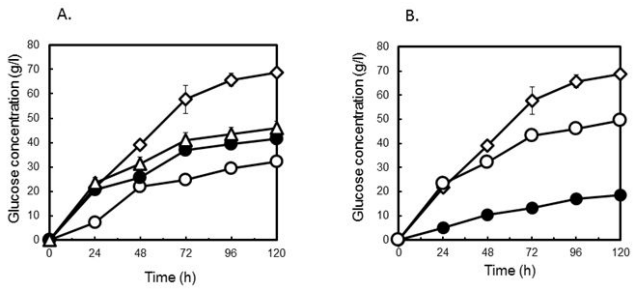

Figure 2: Effects of endogenous cellulase inhibitors on enzymatic hydrolysis of artificial cellulose. A) Individual phenolic compounds. Open diamonds: control (without inhibitors); open circles: addition of 7.6 g/L tannic acid; closed circles: addition of 1.2 g/L trimesic acid; opened triangles: addition of 1.2 g/L gallic acid. B) Mixed phenolic compounds. Opened diamonds: control (without inhibitors); opened circles: inhibitor concentration as same as IEEH solution (0.7 g/L tannic acid, 0.5 g/L trimesic acid and 0.06 g/L gallic acid); closed cycles: that as same as the standard hydrolysate (7.6 g/L tannic acid, 1.2 g/L trimesic acid and 1.2 g/L gallic acid). All experiments were performed in triplicate and error bars indicate standard deviations.

These phenolic compounds were showed non-competitive inhibition to cellulase with p-nitrophenyl-β-D- cellobioside as substrate [14]. Avicel cellulose was hydrolyzed in the presence of 7.5 g/L tannic acid, 1.2 g/L trimesic acid and 1.2 g/L gallic acid with roughly the same concentrations found in normal enzymatic hydrolysates, or with 0.7 g/L tannic acid, 0.5 g·m-3 trimesic acid and 0.06 g/L gallic acid, which were roughly the same concentrations found in IEEH (Ion-exchanged resin treated enzymatic hydrolysates). After an enzymatic hydrolysis, glucose concentration was 26.7% (w/w) of normal enzymatic hydrolysate condition and 71.8% (w/w) of IEEH condition from control (without typical endogenous enzyme inhibitors, Figure 2).

The concentration of these chemicals gradually increased during the saccharification process (Table 1). These values are approximately 2-fold to 25-fold higher than in the solution before hydrolysis. Other major endogenous chemicals in the normal hydrolysate were 0.6 g/L furfural, 0.11 g/L formic acid and 1.98 g/L acetic acid after an enzymatic hydrolysis. Concentrations of endogenous chemical compounds in the IEEH solutions were 0.73 g/L tannic acid, 0.06 g/L trimesic acid and 0.52 g/L gallic acid after 120 hours of hydrolysis (Table 1) with 0.12 g/L furfural, 0.05 g/L formic acid and 0.65 g/L acetic acid. Comparison of the concentration of cellulase inhibitors in IEEH and normal enzymatic hydrolysate indicated that the concentrations of these inhibitors decreased, by 90% (w/w) for tannic acid, 94.8% (w/w) for trimesic acid and 60% (w/w) for gallic acid. However, the concentration of these phenolic compounds also gradually increased with saccharification time.

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Cellulase inhibitors	Condition	Reaction period (hour)					
		0	24	48	72	96	120
Tannic acid (g/L)	Standard hydrolysate	0.65 ± 0.02	1.86 ± 0.10	3.60 ± 0.21	4.21 ± 0.24	6.21 ± 0.45	7.49 ± 0.88
	IEEH solution	–	–	0.32 ± 0.05	0.65 ± 0.13	0.69 ± 0.18	0.74 ± 0.25
Trimesic acid (g/L)	Standard hydrolysate	0.05 ± 0.01	0.70 ± 0.12	0.73 ± 0.08	0.53 ± 0.19	1.21 ± 0.24	1.23 ± 0.17
	IEEH solution	–	–	0.02 ± 0.01	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.02
Gallic acid (g/L)	Standard hydrolysate	0.57 ± 0.25	0.64 ± 0.25	0.72 ± 0.27	0.96 ± 0.24	1.02 ± 0.12	1.31 ± 0.31
	IEEH solution	–	–	0.33 ± 0.09	0.42 ± 0.14	0.48 ± 0.11	0.48 ± 0.15

Table 1: Effects of ion-exchange resin treatment on cellulase inhibitor concentration during the enzymatic hydrolysis.

Comparison of the concentration of cellulase inhibitors in IEEH and normal enzymatic hydrolysate indicated that the concentrations of these inhibitors decreased, by 90% (w/w) for tannic acid, 94.8% (w/w) for trimesic acid and 60% (w/w) for gallic acid. However, the concentration of these phenolic compounds also gradually increased with saccharification time.

Microscopic observations of eucalyptus wood powder

To determine lignin distribution in the enzymatically treated and untreated samples, unstained samples were observed by Raman microscopy. Compared to the untreated samples, both the internal region and the surface of enzymatically treated samples strongly reacted with lignin (Figures 3A and 3B).

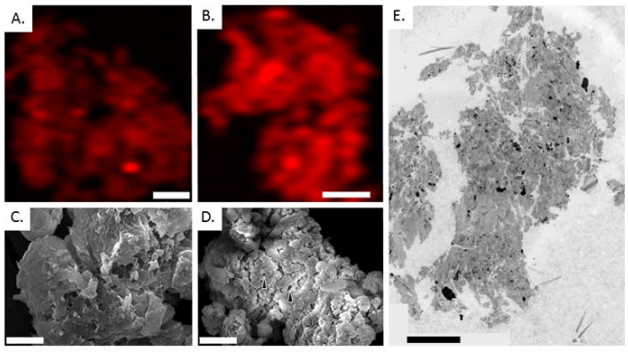

Figure 3: Morphological changes of hydrothermal-mechanochemical treated eucalyptus powders by enzymatic reaction. A, C: untreated eucalyptus woody powder; B, D, E: enzymatically hydrolyzed woody powder (24 h after). A, B: Raman microscopic images; C, D: SEM; E: TEM. Arrows (in D) indicate pores. Scale bars are 120 μm (A, B), 2 μm (C, E) and 5 μm (D).

SEMs indicated that untreated samples were clearly irregular and had rough surfaces. The sample surface was smooth and had small pores and cracks after 24 hours enzymatic hydrolysis (Figures 3C and 3D). Large hole was also formed though woody powders by enzyme treatment (Figure 3E). SEM and TEM micrographs of enzymatically treated and untreated samples indicated small pores derived from decomposition of cellulose and hemicellulose that are presumably formed by cellulase activity. These results suggested that cellulase inhibitors were eluted from lignocellulosic biomass due to decomposition of lignocellulose.

Effect of ion exchange on glucose concentration and glucose formation rate during enzymatic hydrolysis

About 50% (w/w) of lignocellulosic biomass can be enzymatically hydrolyzed within the first 24 hours [15]. When sugars are removed from reaction mixtures by ultrafiltration after 24 and 48 hours of hydrolysis, saccharification dramatically recovers [16]. Low molecular weight phenolic compounds are not only enzyme inhibitors but also most toxic to microorganisms capable of fermentation [17]. Nilvebrant et al. [18] demonstrated that phenolic compounds and uncharged inhibitors of fermentation microorganisms can be efficiently removed by ion exchange.

Thirty two g/L glucose was obtained after normal enzymatic hydrolysis (Figure 4A).

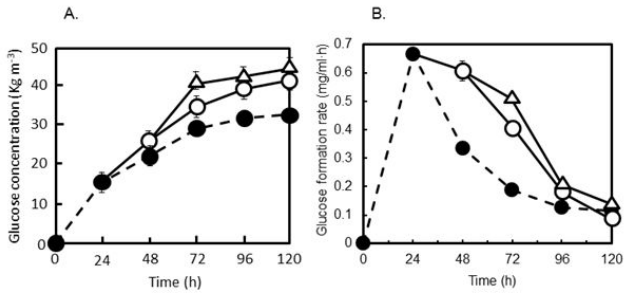

Figure 4: Effect of ion exchange treatment on enzymatic saccharification. A) Glucose concentration. B) Glucose formation rate. Closed cycles: control condition (without ion-exchange treatment). Open cycles: ion-exchange resin treatment after 24 hours enzymatic hydrolysis. Open triangles: ion-exchange resin treatment after 24 and 48 hours enzymatic hydrolyses. All experiments were performed in triplicate and error bars indicate standard deviations.

Approximately 40 or 45 g/L glucose was obtained from ion-exchanged resin treated enzymatic hydrolysates (IEEH 24 or 48 hours). The repetition of the ion-exchange resin treatment was not efficient for the saccharification. Comparison of glucose formation following further hydrolysis of IEEH and control enzymatic hydrolysates showed that the rate with IEEH was maintained even when hydrolysis proceeded for 48 to 72 hours according to the number of times of ion-exchanged resin treatment, while with normal enzymatic hydrolysates, it dramatically decreased after 48 hours of hydrolysis (Figure 4B). Consequently, an approximately 1.4-fold higher concentration of glucose was obtained using IEEH than normal enzymatic hydrolysates.

Improvement of ethanol and lactic acid fermentation by the use of IEEH

Ethanol concentrations were maximum after 48 hours of fermentation except for fermentation using normal hydrolysates. When normal hydrolysates were used, fermentation seemed to continue slowly after 72 hours. The maximum concentration of ethanol formed was 60.0 g/L using IEEH, 71.0 g/L using the control and 26.5 g/L using normal hydrolysates (Figure 5A).

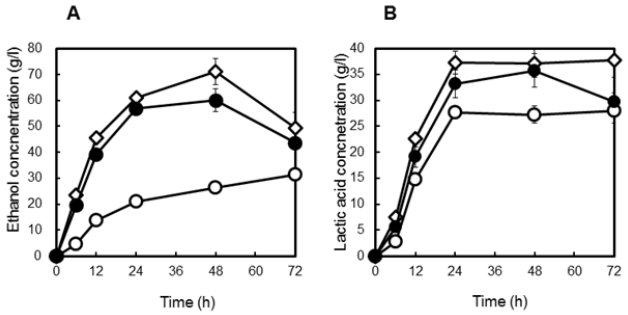

Figure 5: Effects of cellulase inhibitor removal on the fermentation of *Saccharomyces cerevisiae* and lactic acid bacterium, *Enterrococcus casseliflavus*. Ethanol. A) And lactic acid. B) Fermentation. Opened diamonds: control (glucose reagent was used); opened cycles: normal hydrolysate (without ion-exchange resin treatment); closed cycles: IEEH solution. All experiments were performed in triplicate and error bars indicate standard deviations.

After 48 hours of fermentation, 35.7 g/L lactic acid was formed using IEEH, 37.2 g/L lactic acid using the control and 27.2 g/L lactic acid using normal hydrolysates (Figure 5B).

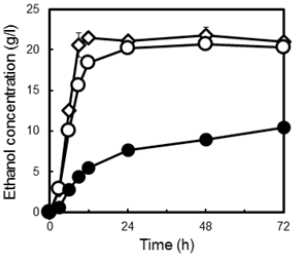

Figure 6: Effects of endogenous cellulase inhibitors on ethanol fermentation. Opened diamonds: control (without inhibitors); opened circles: inhibitor concentration as same as IEEH solution (0.7 g/L tannic acid, 0.5 g/L trimesic acid and 0.06 g/L gallic acid); closed cycles: that as same as the standard hydrolysate (7.6 g/L tannic acid, 1.2 g/L trimesic acid and 1.2 g/L gallic acid). All experiments were performed in triplicate and error bars indicate standard deviations.

Moreover, since ethanol fermentation in the presence of roughly the same concentration of normal hydrolysate was clearly inhibited (Figure 6). Compared in the presence with roughly the same concentration of IEEH solutions, cellulase inhibitors therefore also

inhibit fermentation. When IEEH solutions were used in the ethanol and lactic acid fermentation setup, the fermentation rate was improved compared to that using normal hydrolysates, which indicates that cellulase inhibitors also act as fermentation inhibitors.

Conclusion

In this study, in order to allow saccharification reactions to improve, an effort was made to remove cellulase and fermentation inhibitors by the use of an ion exchange resin after 24 hours of hydrolysis. As a result of treatment, the glucose formation rate was maintained after 48 hours of hydrolysis and the amount of glucose that finally accumulated was about 1.4-fold greater than that under normal hydrolysis conditions. When IEEH solutions were used for fermentation, approximately 2.2-fold more ethanol and 1.3-fold more lactic acid were produced. These inhibitors were gradually released from woody powers during enzymatic hydrolysis, therefore continuous removal of inhibitors were an effective process of the saccharification. We should improve the removal process of inhibitors during enzymatic hydrolysis.

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