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# Pretreatment of microcrystalline cellulose flakes with $CaCl_2$ increases the surface area, and thus improves enzymatic saccharification

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**Abstract**—Glucose production from cellulose flakes with cellulases was improved after pretreatment with saturated $CaCl_2$ at room temperature. When pretreated microcrystalline cellulose flakes (Funacel II, Funakoshi Co., Ltd, Tokyo, Japan) were saccharified with the cellulases, 76.8% of the substrate was converted into glucose within 5 h, whereas the corresponding conversion rate of water-pretreated cellulose flakes was 33.8%. To clarify the mechanism of the promotion, cellobiohydrolase I purified from *Trichoderma longibrachiatum* was used as the model cellulase, which degraded $CaCl_2$-pretreated cellulose more quickly than the water-pretreated cellulose under tested conditions. The maximum amount of the enzyme bound to $CaCl_2$-pretreated cellulose at 37 °C was estimated as 1.14 nmol/mg of cellulose, whereas that to water-pretreated cellulose was 0.527 nmol/mg of cellulose. The specific activity of the bound enzyme greatly decreased with the increase of the surface density ($\rho$) of the bound enzyme, and no significant positive effects of the $CaCl_2$-pretreatment on the specific activity could be observed at the same $\rho$ value, suggesting that the promotion was attributed mainly to the increase of the surface area of cellulose. The effect was also observed with dewaxed cotton or filter paper, but not with nata de coco cellulose or bagasse cellulose as the substrates. This suggests that the $CaCl_2$-pretreatment serves to increase the surface area of cellulose flakes via liberation of cellulose particles which were artificially aggregated during harsh drying processes of the flakes.


*Keywords:* Cellulose; Calcium chloride; Cellulase; Cellobiohydrolase

## 1. Introduction

Cellulose, a linear polysaccharide composed of β-(1→4)-linked D-glucose residues, is the main component of plant cell-wall biomass, which has attracted keen interest among biofuel and biorefinery industries as a glucose source alternative to starch or sucrose. In nature, cellulose partly forms a crystalline structure in which individual chains are compactly assembled via hydrogen bonding.[1] Enzymatic saccharification of cellulose has been considered an environmentally friendly method to replace conventional chemical saccharification methods with sulfuric acid treatments.[2] The enzymatic method, however, has had the fatal problem of a low saccharification rate for a highly crystalline cellulose substrate.[3,4]

From the aspect of enzymology, two types of cellobiohydrolases, CBH I and CBH II (Cel7A and Cel6A),[5] may primarily contribute to cellulose degradation in commercially available cellulase cocktails from *Trichoderma* sp. They exclusively bind to the hydrophobic faces of crystalline cellulose, for processive degradation of the single chains from the reducing or the non-reducing end.[6,7] Thus, enzymatic hydrolysis slowly advances in restricted areas on the surface of cellulose,[6] obstructing commercial utilization of cellulase systems for biomass conversion.

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Therefore, it is obvious that appropriate treatment for cellulose is a prerequisite for efficient saccharification. In 1978, cadoxen (cadmium triethylenediamine dihydroxide), an alkaline solvent for cellulose, was examined for pretreatment for 12 h at room temperature, and the solvent was substituted for water to regenerate cellulose with much higher susceptibility to cellulases than before.[8] This method, however, has not been commercialized yet, suggesting that it could involve such problems as long pretreatment time, high cost for the reagent, high toxicity of the reagent, and troublesome adjustment of the pH for enzymatic saccharification on a large scale. Accordingly, most of the known swelling/dissolving reagents for cellulose developed in the textile industry (e.g., expensive organic compounds, salts with heavy metals, and strong acids or bases) had to be excluded in the new screening for biomass pretreatment. This study demonstrates the possibility of a mild pretreatment of cellulose flakes for promoting saccharification: rapid pretreatment with $CaCl_2$ at room temperature can promote the following enzymatic hydrolysis.

## 2. Results and discussion

A preliminary screening was performed to establish a mild pretreatment system of cellulose for the subsequent enzymatic saccharification. Like other inorganic compounds (e.g., $Ca(SCN)_2$, NaSCN, $ZnCl_2$, $MgCl_2$, and $(NH_4)_2SO_4$) $CaCl_2$ promoted the increase in the rate of the saccharification of cellulose flakes. Considering the availability in bulk and the environmental friendliness of $CaCl_2$, this study began by characterizing the $CaCl_2$-pretreatment.

When the microcrystalline cellulose flakes were soaked in saturated $CaCl_2$ solution at room temperature, the treated flake apparently swelled in size. The saccharification rate became higher than that of water-pretreated cellulose as the substrate (Fig. 1). The $CaCl_2$-pretreatment resulted in the conversion of 76.8% of the substrate into glucose in 5 h, whereas the extent of the conversion in the water-pretreated cellulose was 33.8%.

The promotion of cellulose saccharification by $CaCl_2$-pretreatment at room temperature could be clearly observed at any pretreatment time exceeding 30 min and reached the maximum in 4 h (data not shown). A significant effect on cellulose degradation was observed for $CaCl_2$ concentrations exceeding 1.36 M, and the effect of pretreatment was prolonged for at least 3 h after washing the pretreated cellulose with water (data not shown).

With cellobiohydrolase I from *Trichoderma longibrachiatum* (TlCBHI) as the model enzyme, two experiments were performed to clarify the effects of the

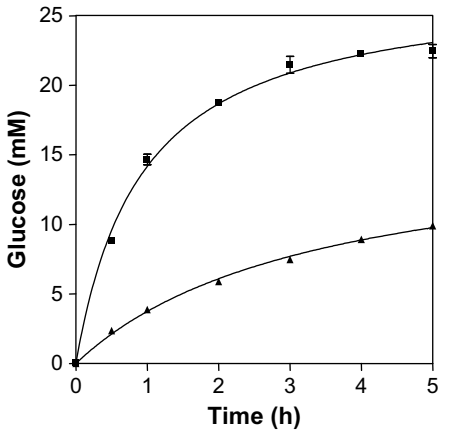


**Figure 1.** Effects of $CaCl_2$-pretreatment on enzymatic saccharification of cellulose flakes. Increase of D-glucose concentration during degradation of pretreated cellulose with commercially available cellulases was estimated. Squares denote cellulose pretreated with saturated $CaCl_2$ solution; triangles indicate cellulose pretreated with water. Average data with standard deviation are presented in the figure ($n = 3$).

$CaCl_2$-pretreatment on promoting enzymatic saccharification. TlCBHI has the same molecular mass (65 kDa) as cellobiohydrolase I from *Trichoderma reesei*, an enzyme with a carbohydrate-binding module (CBM)[9] that binds to the surface of crystalline cellulose.[10,11] Figure 2 depicts the binding isotherm of TlCBHI to either $CaCl_2$-pretreated or water-pretreated cellulose at 37 °C. The maximum amount of bound TlCBHI was calculated according to the Langmuir model, although it has been suggested that the binding isotherm of cellobiohydrolase fits well to a two-site binding model.[3,10] In this study, an approximation was performed because the binding property of the cellulase should be strongly affected by heterogeneous structures of the microcrystalline cellulose, composed of cellulose $I_\alpha$, $I_\beta$, and partially

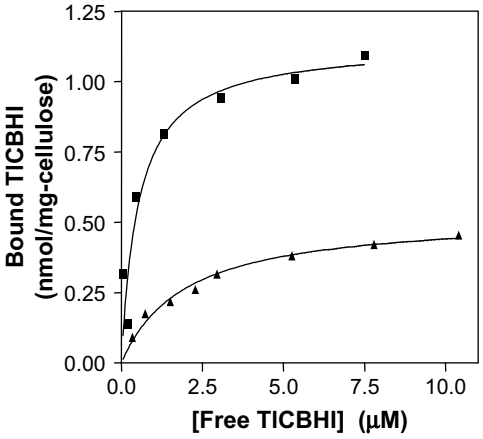


**Figure 2.** Binding isotherm of TlCBHI to $CaCl_2$-pretreated cellulose and water-pretreated cellulose. Squares denote cellulose pretreated with saturated $CaCl_2$ solution; triangles indicate cellulose pretreated with water.

amorphous regions. The maximum amount of enzyme bound to water-treated cellulose was estimated as 0.527 nmol/mg of cellulose, which was on the same order as that of *T. reesei* (0.8 nmol/mg of cellulose).[10] The discrepancy could be mainly due to the heterogeneous properties of microcrystalline cellulose preparations.[12] The maximum amount of bound TlCBHI after the $CaCl_2$-pretreatment was 1.14 nmol/mg of cellulose, which was twice that with water-pretreated cellulose. Thus, the pretreatment with $CaCl_2$ increased the surface area of cellulose available for TlCBHI.

The effects of the pretreatment on saccharification rate by TlCBHI were also investigated (Fig. 3). Figure 3A plots specific activity of the bound enzyme versus the amount of bound enzyme (nmol/mg of cellulose). When the amount of bound enzyme was 0.5 nmol/mg of cellulose, specific activities appeared to be higher in the $CaCl_2$-pretreated samples than in the water-pretreated ones. In both samples, specific activity decreased with increasing enzyme bound on the surface, suggesting that the overcrowding of enzymes on the surface lowered the specific activity. Next, the specific activity of TlCBHI versus the surface density ($\rho$) was plotted (Fig. 3B). The specific activity of the bound enzyme greatly decreased with the increase of the $\rho$ value, and no positive effects of the $CaCl_2$-pretreatment on the specific activity could be observed at the same $\rho$ value. This result suggested that the promotion was mainly attributed to the increase of the surface area of cellulose for the binding of cellulases after the $CaCl_2$-pretreatment.

Igarashi et al. examined the efficiency of enzymatic hydrolysis of cellulose in different crystal structures (cellulose $I_\alpha$,-rich, $I_\beta$, and $III_I$) and found that the degradation of cellulose $III_I$ advances ten times faster than that of the other cellulose crystals.[13] They speculated that the efficiency of processive hydrolysis of each cellulose crystal per molecule of the enzyme mainly affected the speed of its degradation because the maximum amount of enzyme bound to cellulose did not differ significantly. The $CaCl_2$-pretreatment in this study is distinct from the case of cellulose $III_I$ in that the pretreatment increased the surface area of the cellulose, but not the speed of the enzymatic degradation.

Then, the effects of the $CaCl_2$-pretreatment for other cellulose samples were evaluated as the amounts of D-glucose produced in 5 h (Fig. 4). The promotion of saccharification was observed with filter paper or dewaxed cotton as substrate, but not with bagasse cellulose or nata de coco cellulose. Dewaxed cotton and filter paper, as well as microcrystalline cellulose flakes such as Funacel II, could be manufactured with a harsh heat-drying process, which would promote the artificial reorganization of the cellulose particles into aggregated forms. Lee et al. reported that increase in surface area of microcrystalline cellulose flakes (Avicel PH102) after a long time of water swelling could be observed,[14] and it could be attributed to the collapse of the capillary structure of cellulose in a water-swollen state by air-drying.[15,16] Contrarily, the $CaCl_2$-pretreatment was not effective for bagasse cellulose and nata de coco cellulose, which are produced without harsh heat-drying processes to cause reorganization of cellulose particles. Any significant changes in particle size distribution of microcrystalline cellulose flakes could not be observed before and after

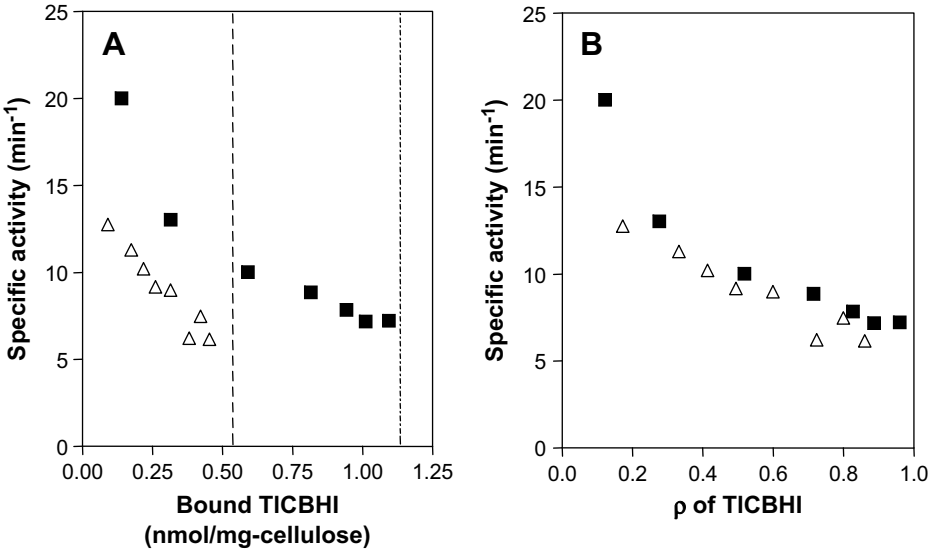


**Figure 3.** Effects of TlCBHI amount bound to cellulose on the specific activity. (A) Specific activity versus bound TlCBHI to 1 mg of cellulose. The dotted line indicates the maximum amount of bound TlCBHI on $CaCl_2$-pretreated cellulose, and the broken line indicates that on water-pretreated cellulose. (B) Specific activity versus the $\rho$ value of TlCBHI. Squares denote cellulose pretreated with saturated $CaCl_2$ solution; triangles indicate cellulose pretreated with water.

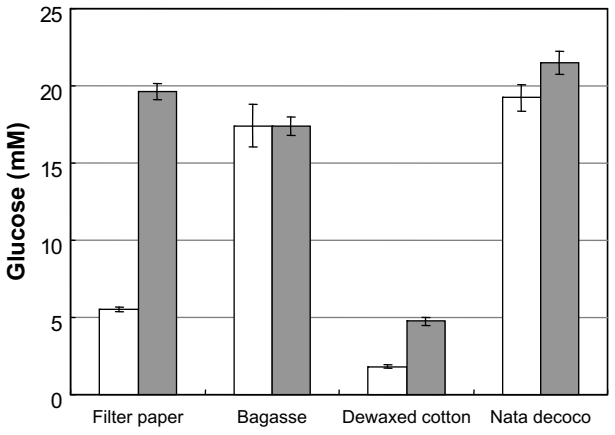


**Figure 4.** Effects of $CaCl_2$-pretreatment on enzymatic saccharification of various cellulose samples. Concentration of D-glucose in 5 h degradation of pretreated cellulose with commercially available cellulases was estimated. White bars denote cellulose pretreated with water; gray bars indicate cellulose pretreated with saturated $CaCl_2$ solution. Average data with standard deviation are presented in the figure ($n = 3$).

the $CaCl_2$-pretreatment, suggesting that the pretreatment is not effective enough to liberate smaller particles from microcrystalline cellulose flakes (data not shown). Microcrystalline cellulose flakes and filter paper have been used as artificial substrates for cellulase assays, and appropriate care must be taken for the evaluation of cellulase activities or pretreatments on enzymatic saccharification when using these substrates.

In viewpoint of application, the $CaCl_2$-pretreatment has merits that no equipment for heat/high-pressure pretreatments and pH neutralization is needed in the system, and most of the swelling reagent can be reused after separation. $CaCl_2$ can be readily obtained in bulk, and it exhibits high stability in acid, heat, and oxidants. $CaCl_2$ has been regarded as an environmentally friendly material to sprinkle on the ground as antifreeze; therefore, its treatment and carryover from the pretreatment/saccharification process to the following fermentation process would cause few problems. This pretreatment appears not to be suitable as a pretreatment for enzymatic saccharification of typical lignocellulosic biomass, as there is no need for a heat-drying processes of the biomass during pretreatment. Instead, this method would offer a new possibility in the cellulose industry for the facile recycling of used cellulosic materials such as paper and cloth.

## 3. Materials and methods

### 3.1. Materials

Funacel II (moisture content 4.0%; Funakoshi Co., Ltd, Tokyo, Japan) was used as the microcrystalline cellulose flakes. Nata de coco cellulose was partially purified from a food product with nata de coco (Miyama Shokuhin Co., Ltd, Gunma, Japan) via homogenization and filtration, and the solid material was washed with water for desyrupping. Sugarcane bagasse was kindly donated by Dr. M. Matsukoka, National Agricultural Research Center for Kyushu Okinawa Region, Japan. For bagasse cellulose preparation, sugarcane bagasse (45 g) was treated with 1.5 L of 5% KOH for 12 h at room temperature, and the solid material was washed with water. Then it was bleached with $NaClO_2$ in 100 mM NaOAc buffer (pH 4.9) for 4 h at 70 °C with stirring. The light-brown fibers of bagasse cellulose were rinsed with water and dried at 60 °C to give 18 g of bagasse cellulose. Filter paper flakes (Cellulose powder C) were purchased from Toyo Roshi Co., Ltd, Tokyo, Japan. A cellulase cocktail (Celluclast 1.5L FG, 165 mg/mL) was kindly donated by Novozymes Japan Ltd, Chiba, Japan; the other cellulase cocktail (Novozyme 188, 260 mg/mL) was purchased from Sigma–Aldrich Corporation, St. Louis, Missouri, USA. Cellobiohydrolase I from *T. longibrachiatum* (TlCBHI, purified to a single band on SDS–gel electrophoresis, 0.07 U/mg, according to the supplier's definition) and β-glucosidase from *Aspergillus niger* (purified to a single band on SDS–gel electrophoresis, 52 U/mg, according to the supplier's definition) were purchased from Megazyme International Ireland Ltd, Bray, Co., Wicklow, Ireland. Other chemicals were of analytical grade.

### 3.2. Pretreatment of cellulose with $CaCl_2$

A solution of saturated $CaCl_2$ was prepared by the addition of solid $CaCl_2{\cdot}2H_2O$ in water, and the insoluble salt was dissolved by heating. In a 1.5 mL plastic tube, 5 mg of cellulose and 1 mL of saturated $CaCl_2$ solution were added at room temperature and mixed using a vortex mixer. The tube was allowed to settle overnight at room temperature and was then centrifuged (20 600*g* for 30 min at 4 °C) to remove the supernatant. For washing the precipitate, 1 mL of water was added to the tube, and it was mixed using a vortex mixer with the following centrifugation (20,600*g* for 10 min at 4 °C) to remove the supernatant. This washing procedure was repeated three times, and the tube with pretreated cellulose was used for enzymatic digestion. For control experiments, 1 mL of water was used for pretreatment instead of saturated $CaCl_2$ solution.

### 3.3. Saccharification of cellulose by a cellulose cocktail

To each 1.5-mL plastic tube with pretreated cellulose, 1 mL of premixed cellulase cocktail composed of 1.65 mg/mL of Celluclast 1.5L FG, 1 mg/mL of Novozyme 188, and 20 mM NaOAc buffer (pH 5.5) was added. Enzymatic saccharification was performed at 37 °C by rotation (12 r.p.m., using an end-over-end

rotator). Aliquots (20 μL) were periodically taken from each tube. The amount of D-glucose liberated from cellulose was measured by the Glucose CII-Test Wako (Wako Pure Chemical Industries Ltd, Osaka, Japan). The protein concentration was estimated using a DC protein assay kit (Nippon Bio-Rad Laboratories KK, Tokyo, Japan). The curves were drawn by curve-fitting with a non-linear regression of a one-site binding model, using GraphPad Prism v. 3.00 for Windows (GraphPad Software, San Diego, CA, USA).

### 3.4. TlCBHI binding experiments

To a 1.5-mL plastic tube with pretreated cellulose flakes, 530 μL of 20 mM NaOAc buffer (pH 5.5) with TlCBHI (from 40 μg to 700 μg) was added at 37 °C. The sample was rotated for 1 h at 12 rpm using an end-over-end rotator. The tube was then centrifuged (20 600*g* for 3 min at 25 °C) to obtain the supernatant fraction. The amount of free TlCBHI in the supernatant was estimated by measuring the absorbance at 280 nm and with an absorption coefficient of 108 000/M/cm. The amount of TlCBHI bound to cellulose (nmol/mg of cellulose) was estimated by subtracting the amount of free enzyme from the amount of added enzyme. The amount of bound enzyme per milligram of cellulose was plotted versus free enzyme concentration. As the data for the binding isotherm did not fit the two-site binding model well, the maximum amount of bound TlCBHI (nmol/mg of cellulose) was estimated by non-linear regression of a one-site binding model (the Langmuir model), using GraphPad Prism (Fig. 3). The surface density ($\rho$) of TlCBHI was defined by the amount of bound enzyme over the maximum amount of bound enzyme.[3]

### 3.5. Degradation of cellulose by TlCBHI

The saccharification rate of pretreated cellulose by TlCBHI was estimated using an aliquot of the supernatant in the tube after the binding experiment. The production of soluble cellooligosaccharides was evaluated as the amount of D-glucose via their further degradation by β-glucosidase from *A. niger*. Three microliters of the β-glucosidase (40 units/mL) were added to 15 μL of the supernatant, and the reaction mixture was incubated for 40 min at 37 °C to convert the cellooligosaccharides into D-glucose. The amount of D-glucose liberated from the cellulose was measured by Glucose CII-Test Wako, and the rate of cellobiose production was estimated assuming that the enzyme could produce only cellobiose from cellulose. The saccharification rate for a reaction time of 1 h was approximated using the amount of cellobiose production after 1 h reaction. Specific activity of TlCBHI ($min^{-1}$) was defined as the saccharification rate per amount of enzyme bound to cellulose.

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